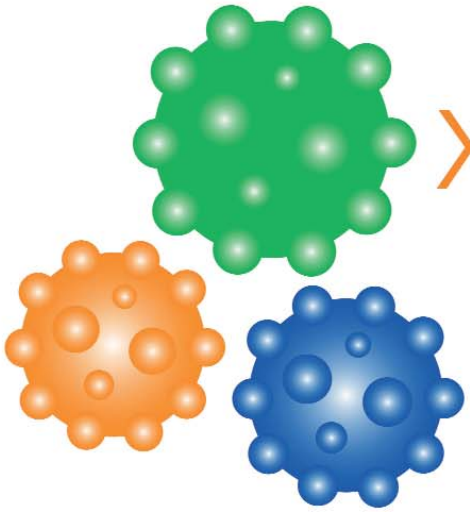


Xth International Congress for Veterinary Virology

9th Annual Meeting of EPIZONE

Changing Viruses in a Changing World

August 31st - September 3rd 2015

Le Corum, Montpellier, France

Table of Contents

INTRODUCTION

Welcome to Montpellier,
Welcome to ESVV2015-EPIZONE!

On behalf of the French Agricultural Research Centre for International Development (CIRAD), the European Society for Veterinary Virology (ESVV) and the European Research Group (ERG) EPIZONE, it is my great pleasure to welcome you in Montpellier for the **Xth International Congress of Veterinary Virology of ESVV**, combined with the **9th annual meeting of EPIZONE**.

The combined congress will take place at "Le Corum", downtown Montpellier, from August 31st afternoon to September 3rd morning. The ESVV congress will cover the first part, ending on September 2nd noon, followed by EPIZONE. Plenary and parallel oral sessions, and poster exhibition will cover all aspects of animal and zoonotic viruses, from basic virology to applied control measures. A large audience is expected with more than 280 accepted abstracts and 360 registered participants from more than 50 different countries.

The general theme of the congress is related to global changes: "**Changing Viruses in a Changing World**". In this context, several presentations dealing with virus evolution, arboviruses and new pathogen discovery were selected as keynote lectures or oral communications.

A new feature of this edition is the organization of a **special session on animal health in the Caribbean**, granted by the **European project EPIGENESIS**. The Caribbean region is considered as a hot spot for emerging diseases, particularly those caused by arboviruses. Top speakers from this region will illustrate the current situation and I am expecting fruitful interactions between the attendees and possibly, new collaborations.

Another important feature is the one-day parallel **meeting for young scientists** supported by EPIZONE. The content of the meeting is designed to provide our young colleagues with information, training and contacts that shall be useful for their future carrier.

The organization of such an international congress has required a strong multi-dimensional mobilization. I would like first to thank the **ESVV board, the EPIZONE coordination team and the Scientific and Local Organizing Committees** for their commitment and activities to set up this event. "**Montpellier event**" and "**Alpha Visa Congrès**" are the companies which provided a strong professional service to host and organize this meeting in the heart of our city. I want also to thank our **Public and Private Sponsors** for their essential support.

Finally, I wish for everybody, a successful and productive congress, a good stay in our beautiful city and some opportunities to visit a highly attractive region.

Enjoy the congress, enjoy Montpellier, and enjoy our Mediterranean lifestyle!

Emmanuel Albina
Chair Scientific and
Organizing Committees

Committees

Scientific Committee

Emmanuel Albina - *Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)*

Martin Beer - *Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health*

Anette Botner - *DTU VET National Veterinary Institute*

Emiliana Brocchi - *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER)*

Catherine Cêtre-Sossah - *Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)*

Trevor Drew - *Animal Health and Veterinary Laboratories Agency - (AHVLA)*

Serafin Gutierrez - *Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)*

Geneviève Libeau - *Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)*

Antonio Lavazza - *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER)*

Thierry Lefrançois - *Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)*

Marie-Frédérique Le Potier - *Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail - (ANSES)*

Thomas Mettenleiter - *Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health*

Hans Nauwynck - *Faculty of Veterinary Medicine, Ghent University*

Jose-Manuel Sanchez-Vizcaino - *Facultad de Veterinaria, Universidad Complutense*

Renata servan de Almeida - *Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)*

Thierry Van der Berg - *Veterinary and Agrochemical Research Center*

Wim Van der Poel - *Central Veterinary Institute, Wageningen University Research*

Organizing Committee

Emmanuel Albina
Denise Bastron
Nathalie Curiallet
Anne Dutour
Patricia Gil
Cécile Minet
Lysiane Parizot
Philippe Radigon
Nathalie Vachiery
Océane Wacrenier

Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)

Design

Delphine Guard

Communication

Nathalie Curiallet

Anne Hébert

Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)

Sponsors

Epigenesis
Domaine Duclos, Prise d'eau
17170 Petit-Bourg,
Guadeloupe

Région Languedoc-Roussillon
201 avenue de la Pompignane
34064 Montpellier cedex 02
France

IDEXX Europe B.V.
Scorpius 60 Building F
Hoofddorp, 2132 LR
The Netherlands

Merial
2 avenue Pont Pasteur
69367 Lyon Cedex 07
France

Thermo Fisher Scientific Inc.
Life Technologies Ltd.
3 Fountain Drive, Inchinnan Business Park,
Paisley, UK, PA4 9RF United Kingdom
United Kingdom

Montpellier Métropole Méditerranée
50 Place de Zeus
34000 Montpellier
France

Zoetis Manufacturing & Research Spain, S.L
Carretera Camprodon s/n Finca “La Riba”
17813 Vall de Bianya
Girona - Spain

MSD
Wim de Körverstraat 35
PO box 31, 5830 AA Boxmeer
The Netherlands

Pôle BioSanté Rabelais
c/o Institut de Génomique Fonctionnelle
141 rue de la cardonille
34094 Montpellier Cedex 05 - France

Organizers

ESVV european society for veterinary virology

cirad LA RECHERCHE AGRONOMIQUE POUR LE DÉVELOPPEMENT

EPIZONE

Advertisers

BioMed Central The Open Access Publisher

VETERINARY RESEARCH

VIROLOGY JOURNAL

Parasites & Vectors

BMC Veterinary Research

EPIGENESIS 2013 > 2016

EXPANDING RESEARCH CAPACITIES ON ANIMAL HEALTH IN GUADELOUPE

REINFORCE

SCIENTIFIC POTENTIAL

on emerging and vector-borne diseases in Guadeloupe

CONSOLIDATE

STRATEGIC PARTNERSHIPS WITH OUTSTANDING EUROPEAN RESEARCH ENTITIES

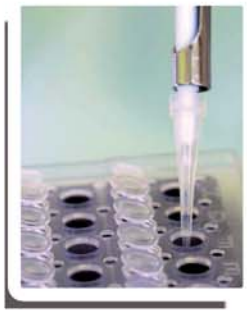
on animal health by the exchange of scientists between Europe and Guadeloupe and trainings

MAIN DISEASE MODELS

Tick borne diseases: Heartwater, Babesiosis, Anaplasmosis

Other arthropod-borne diseases: West Nile Fever and other arbovirus diseases

Other emerging diseases: Avian and Swine Influenza, Newcastle disease



INCREASE

INNOVATION STRENGTH IN GUADELOUPE

with the development of diagnostic tests, vaccines, tools to assist decision-making and alert system

IMPART

SCIENTIFIC INFORMATION AND KNOWLEDGE

to the scientific community, decision-makers, animal health stakeholders and the general public in Guadeloupe and in the Caribbean region

PARTNERS

- IN EUROPE:

Centre de Recerca en Sanitat Animal (CReSA), Spain ; Instituto de Biologia Experimental Tecnologica (IBET), Portugal ; Instituto de Tecnologia Química et Biologica (ITQB), Portugal ; UMR-INRA-Oniris, BioEpAR Institut National de la Recherche Agronomique (INRA), France ; École des Hautes Études de Santé Publique (EHESP), France ; Agriculture and Veterinary Information and Analysis (AviaGIS), Belgium

- IN GUADELOUPE:

INRA , Institut Pasteur, Université des Antilles, Agence Régionale de Santé, Centre Hospitalier Universitaire de Guadeloupe.

- IN THE CARIBBEAN:

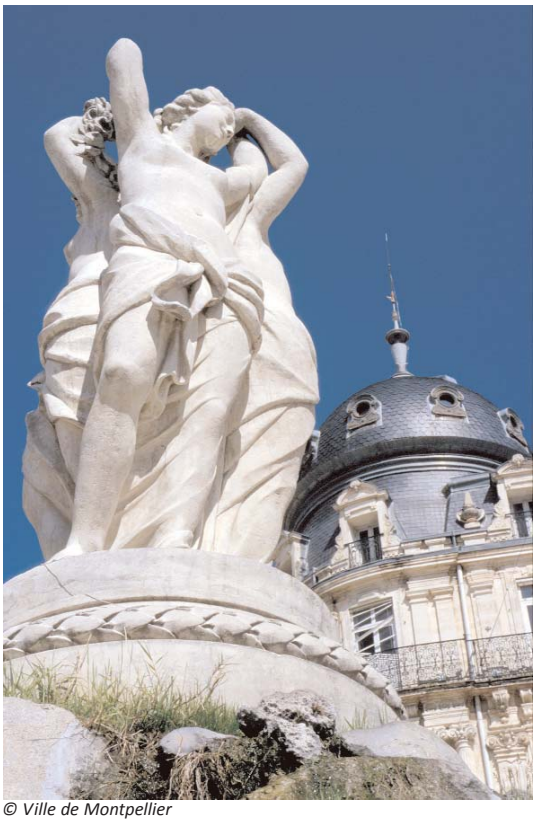
Members of CaribVET, the Caribbean Animal Health Network: Veterinary services of 33 countries and territories, research institutes (CENSA, CIRAD), Caribbean Universities (UWI, Saint Georges University, Guyana), regional (CARICOM) and international organizations (OIE, FAO, USDA, PAHO, IICA).

EPIGENESIS PROJECT IS FUNDED BY EUROPE (FRAMEWORK PROGRAMME 7) AND COORDINATED BY CIRAD

Conference venue

Montpellier and surroundings

Montpellier has become over the past decades a major hub for research on agriculture, environment and sustainable development issues. It is part of the "Global Partnership on Cities and Biodiversity" of the CBD, and hosts (among others) two of the National LabEx (Laboratory of Excellence) that are supporting PSP5: the LabEx AGRO-Agropolis Fondation, gathering a broad number of research groups in the fields of agricultural and plant sciences, and the LabEx CeMEB (Mediterranean Centre for Environment and Biodiversity), which brings together prominent research groups working in ecology and conservation issues. Montpellier, via Agropolis International, is one of the major agricultural science campus worldwide, with a large focus on Mediterranean and tropical agriculture. This is one of the reasons for Montpellier having been selected to host the secretary for the Consultative Group on International Agricultural Research (CGIAR).



© Ville de Montpellier

Montpellier is also one of the oldest University cities in France (XIIIth Century). It is located on hilly ground 10 kilometers inland from the Mediterranean Sea coast on the banks of the Lez River. The medieval centre, the so-called Ecusson, gives to the city its unique and intimate feeling.

Local transportation

Tramway & buses

The tramway Line 1, decorated in blue with white swallows, connects the northern part of the city with the Odysseum terminal on the southeast side. The Line 2, decorated in a flower-power theme, goes from east to west. The colorful Line 3, designed by the famous fashion designer Christian Lacroix, goes from west to east, arriving near the seaside at Perols. From there, you can rent a bike or take a short walk (around 20 minutes) to get to the beach. The golden Line 4, also designed by Christian Lacroix, is only for downtown. About 30 bus lines are connected to the tramway lines to offer a comprehensive network that will transport you in and around Montpellier.

© TAM Montpellier

TAM Ticket fares:

- One-way ticket: € 1.50
- 1-day pass: € 4.00
- 7-days pass: € 15.60
- 10-ride pass: € 10.00

Where can you buy tickets and top-off your transport card?

- At the TaM agencies
- At nearly 130 participating stores and shops
- At 120 automatic distributors

Vélomagg bicycle service

The Velomagg' service offers bicycles just the way you want them: available and inexpensive. For your riding pleasure, 50 automatic bike stations with over 2,000 bicycles are available in Montpellier and in the Metropole area. Service is open 24/7. All you need is personal identification and you can rent a bicycle to ride the streets of the city and outlying area. You can buy tickets at the Esplanade bike station (next to Montpellier Tourist Office).

Car Park

Le Corum Conference Centre has an underground car parks. Weekly special rates for delegates apply for registered participants. Information to be obtained at the Registration Desk, in the Joffre Space on level one.

Taxis in Montpellier

Taxi Tram - Tel: 04 67 58 10 10
Allo Taxi 34 - Tel: 04 67 81 42 74
Taxi Bleu - Tel: 04 67 03 20 00
Taxi Radio du Midi - Tel: 04 67 10 00 00

Restaurants

A large variety of restaurants, cafés and bars can be found all over Montpellier, with a very large selection available at walking distance from Le Corum Convention Centre. Some of them are open late at night. Prices for a menu usually start from 12 € at lunch and 18 € for dinner.

Getting around

Montpellier is the ideal place to stay and take advantage of both the seashore of Southern France and the many hidden treasures in the hinterlands of the Languedoc-Roussillon region. Discover major UNESCO World Heritage Sites, scenic villages and landscapes, and vast natural areas such as the Camargue marshland, and the Cévennes mountains.

Natural sites

Camargue marshes, with pink flamingos, ranches with black bulls and white horses.

Cévennes mountains, great for biking, mountain biking or bushwalking.

Pyrénées mountains, between Spain, Andorra and Ariège.

Mediterranean coastline, with many swamps near Montpellier, and rocks and cliffs when getting near the spanish boarder, in the scenic Côte Vermeille.

Historic cities

Nîmes, living Roman history (45 kms from Montpellier)

Sète, the fascinating birthplace of famous musician Brassens and poet Paul Valéry, situated between the Mediterranean sea and Thau lagoon, famous for its oyster farms (30 kms)

Pézenas, Molière's hometown (60 kms)

Aigues-Mortes, the medieval city of Saint Louis (30 kms)

Collioure, capital of Fauvism painting (190 kms)

Unesco World Heritage Sites

The colossal walled city of **Carcassonne**, a magnificent medieval city with its ramparts, the Basilica of St Nazaire and Château Comtal

The **Pont du Gard,** an impressive Roman aqueduct

The **Canal du Midi**, a beautiful canal with a succession of straits, locks and tunnels

The medieval **Gellone Abbey**, located in the beautiful village of Saint-Guilhem-le-Désert on the route to Saint Jacques de Compostelle at the edge of the Gorges of the Hérault river.

The **Causses** (elevated calcareous plateaux) and the unique Cirque de Navacelles where the Vis river has been carving the landscape and designing magnificent and peaceful gorges.

Insurance

French health care does not cover visitors to France. Please ensure that you have an adequate insurance coverage in the event of illness or accident. The organizing committee is not liable for personal injuries sustained by or for loss or damage to property belonging to the participants.

Emergency numbers

15: Emergency ambulance service
17: Police
18: Fire Brigade
04 67 72 22 15: Emergency Doctor

Xth International Congress ESVV 2015 for Veterinary Virology

CHANGING VIRUSES IN A CHANGING WORLD
31 August - 3 September 2015
Le Corum Montpellier France

EPIZONE 9th Annual Meeting of EPIZONE
2-3 September 2015 - Le Corum Montpellier France

PLACES OF INTEREST - MONTPELLIER

1. Mercure Montpellier Centre ****
2. Ibis Montpellier Centre ***
3. Ibis Montpellier Centre Comédie ***
4. Citadines Antigone Montpellier **
5. Best Western Hotel Eurociel ***
6. Océania Le Métropole ****

Food and drink areas

Practical information about Le Corum convention centre

Le Corum conference centre, Esplanade Charles De Gaulle, BP 2220, 34000 Montpellier
Tel: +33 0(4) 67 61 67 61

It is located in the city centre of Montpellier, a few minute's walk from Corum and Comedie tramway stations.

Registration

The Registration Desk is located at the Level 1 in Joffre space and will be open to delegates from 14:00 to 16:00 on Monday 31st August and all day long Tuesday 1st and Wednesday 2nd September 2015.

The telephone number for calling the Registration Desk is: 04 67 61 66 42

Badges are required for admission to all conference sessions, to the exhibition hall and the lunch area.

Speaker Support Centre

The Speaker Support Centre is located in the Joffre 4 Room at the first floor. There are facilities to upload and review your presentations on both PC and MacIntosh computers. Technical assistance is provided in this room.

All presentations will be projected from this room via Le Corum Palais des Congrès venue network.

Monday 31 August 2015: 14:00-18:00
Tuesday 1 September 2015: 08:30-18:30
Wednesday 2 September 2015: 08:00-18:00
Thursday 3 September 2015: 08:30

Presentations should be uploaded onto the conference system as early as possible, and at latest the day before.

It will not be possible to upload them directly in the conference lecture room (Pasteur Auditorium).

Abstracts

Abstracts for oral and poster presentations are available on the website and on the memory stick.

Poster exhibition

It is located in the Joffre space, Level 1. It will remain accessible throughout the conference.

Poster presenters should register at the Welcome desk at their arrival, where they will be allocated a display panel.

Internet access

A Cyber-Café equipped with a few computers will be available at the Eastern tip of the exhibition area in Joffre Room. Internet access will be accessible free of charge during the whole conference from 8:30 until 18:30.

The Cyber-Café is also equipped with WiFi wireless access to internet: ESVV2015

Catering information

A buffet will be served on Tuesday and Wednesday

Your badge is required to join the lunch.

Participants are kindly asked not to take food or beverages to the sessions rooms.

Cloakroom

A cloakroom, located nex to the registration desk, will be open to delegates throughout the symposium.

Lost Property

Please report any lost property to the Registration Desk in the main lounge at the first floor.

Messages

A message board is located next to the Registration Desk in Joffre Space. Messages may be left at the Registration Desk or pinned to the board. No responsibility will be taken to deliver messages personally, so please check this board at regular intervals.

Mobile Phones

As a courtesy to all other delegates, please ensure that mobile phones are switched off during all sessions (both poster and oral) and social functions. Public telephones may be found in the foyers within Le Corum Palais des Congrès. Phone cards or credit cards are needed for this purpose.

Smoking

Le Corum Conference Centre is a designated non-smoking venue.

Social Program

The **Welcome cocktail** will be held at Le Corum on Monday, 31st August, 18:45-21:00.

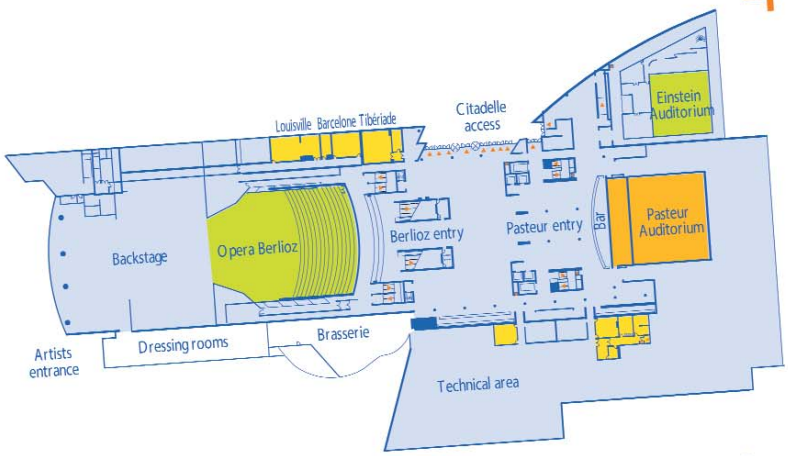
A one-hour pedestrian **city tour** is scheduled on Wednesday 2nd September. Meeting point in front of the tourist office at 19:15 (5mn walk from Corum conference centre adjacent to “Place de la Comédie”)

The **evening entertainment** will be held at Le Corum on Wednesday 2nd September, from 20:00 to 02:00. A jazz duo and DJ have been planned for our enjoyment.

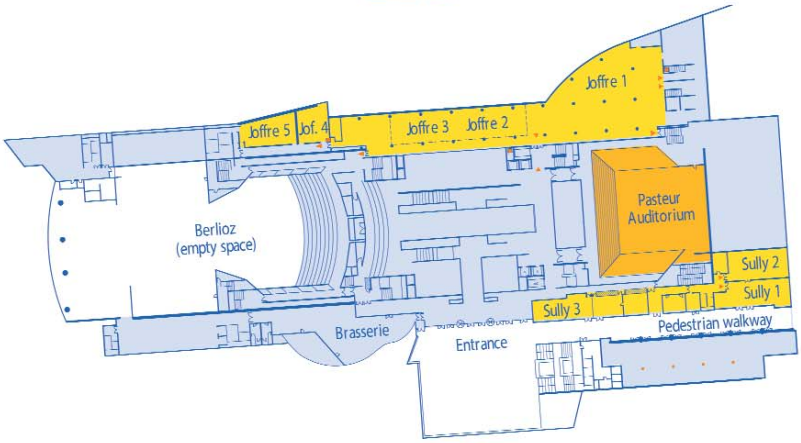
Dress code: "Dressy casual"

Le Corum's Plans
4 levels

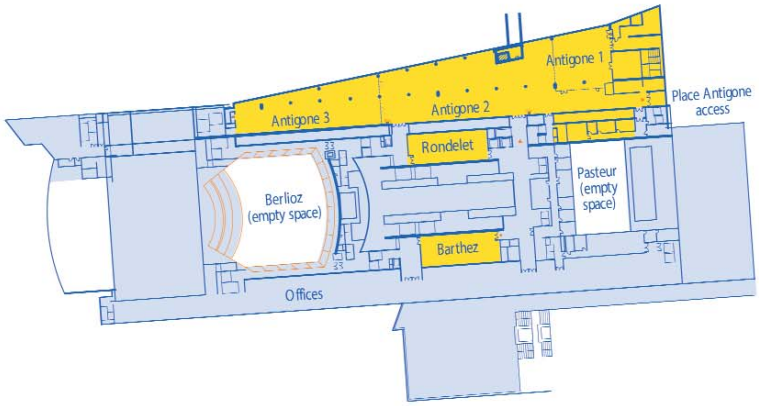
Level 0



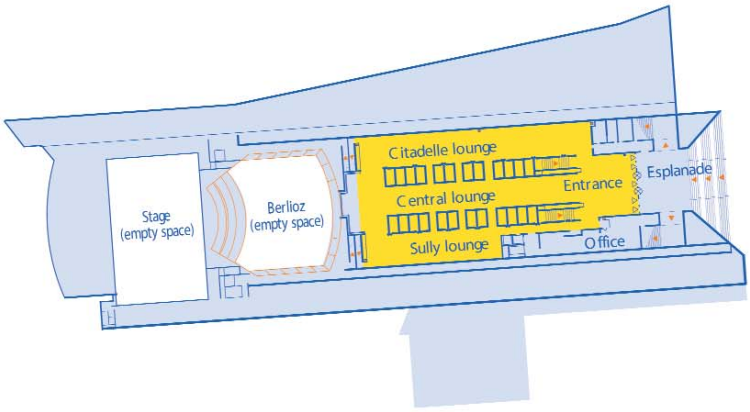
Level 1



Level 2



Level 3

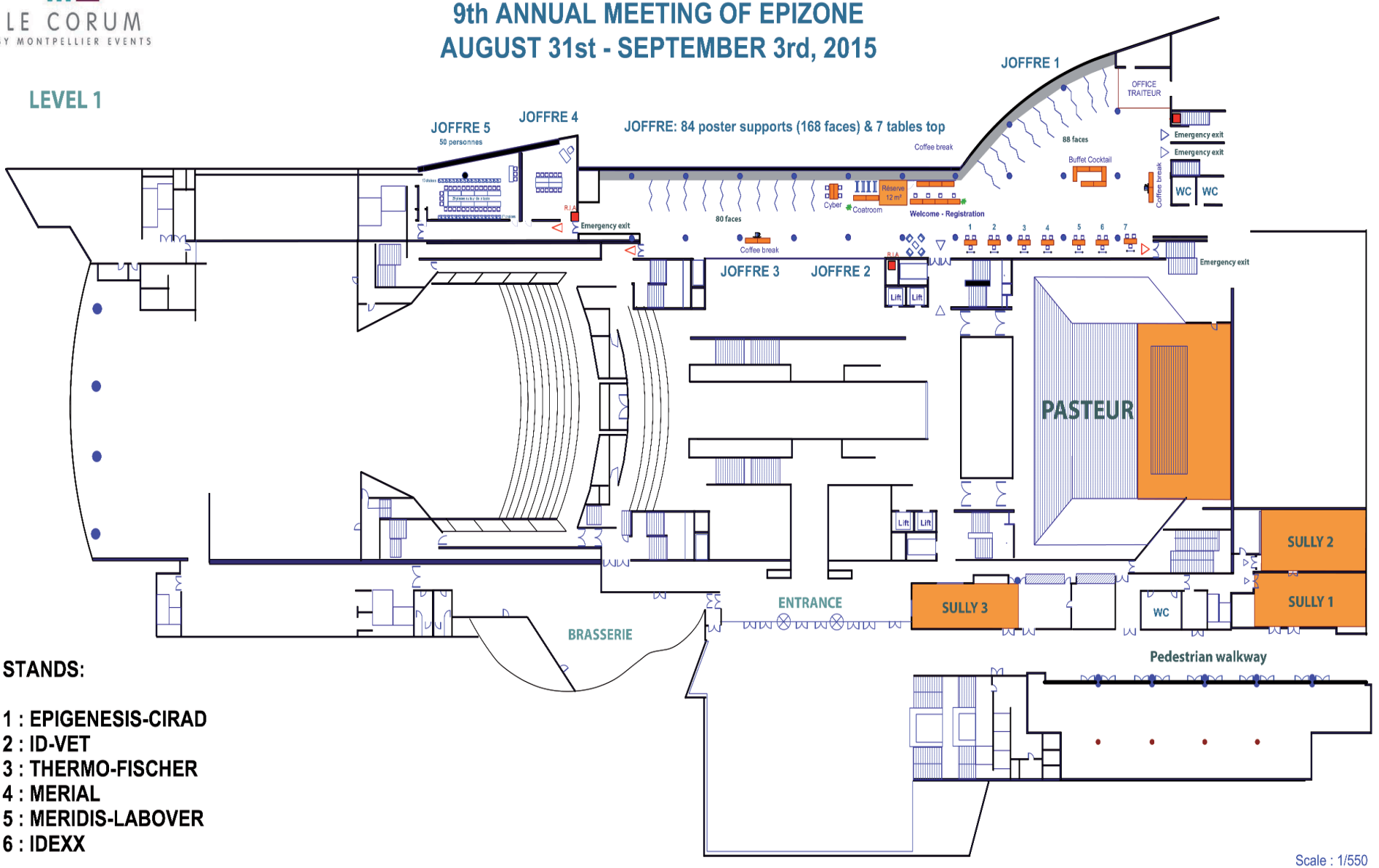


LE CORUM
BY MONTPELLIER EVENTS

LE CORUM
BY MONTPELLIER EVENTS

Xth INTERNATIONAL CONGRESS FOR VETERINARY VIROLOGY - ESVV2015
9th ANNUAL MEETING OF EPIZONE
AUGUST 31st - SEPTEMBER 3rd, 2015

LEVEL 1



STANDS:

1 : EPIGENESIS-CIRAD
2 : ID-VET
3 : THERMO-FISCHER
4 : MERIAL
5 : MERIDIS-LABOVER
6 : IDEXX

Scale : 1/550
10 August 2015

ESVV 2015-Epizone - Program at a glance
"Le Corum", Montpellier, 31st August - 3rd September 2015

Date	Time	Main Hall Pasteur (745 seats)	Room Joffre 2 & 3	Room Joffre 5 (60 seats)	Room Barthez (140 seats)	Room Rondelet (140 seats)
Monday 31 August	14h00 – 16h00		Registration			
	16h00 – 16h30	ESVV Welcome, Opening Session	Registration			
	16h45 – 18h30	ESVV Plenary session 1, Keynote lectures 1 & 2 **Prof P. PALESE** "Pan-influenza vaccines" **Prof S. WEAVER** "Evolution of arboviruses"	Registration			
	18h45 – 21h00		Welcome aperitif			
Tuesday 1 September	9h00 – 10h30	ESVV Plenary session 2, Keynote lectures 3 & 4 **Prof M. ELOIT** "Virus discovery" **Dr G. ANDRES** "*Asfivirus* assembly"	Registration			
	10h30 – 11h00		Coffee break, Poster & Stand Viewing			
	11h00 – 12h30	Parallel ESVV Session 1: "Virus evolution"	Poster & Stand Viewing		Parallel ESVV Session 2: "Influenza"	Parallel ESVV Session 3: « Arboviruses »
	12h30 – 13h45		Lunch, Poster & Stand Viewing			
	13h45 -14h30	ESVV Plenary session 3, Keynote lecture 5 **Dr S. GANDON** "Virulence evolution during epidemics"				
	14h30 – 16h30	Parallel ESVV Session 4: "Vaccines and antivirals"	Poster & Stand Viewing		Parallel ESVV Session 5: "Host/virus interactions & viral immunity 1"	Parallel ESVV Session 6: "Emerging virus & diseases 1"
	16h30 – 17h00		Coffee break and Drinks, Poster & Stand Viewing			
	17h00 – 18h30	ESVV Poster Presentation Session 1 (+ poster award)	Poster & Stand Viewing	Parallel ESVV board meeting	ESVV Poster Presentation Session 2	ESVV Poster Presentation Session 3
	19h30	Special event: Public debate, Salle Rabelais: « Viruses: old enemies, new friends » (1h00) in French				

Date	Time	Main Hall Pasteur (745 seats)	Room Joffre 2 & 3	Room Joffre 5 (60 seats)	Room Sully 3 (80 seats)	Room Barthez (140 seats)	Room Rondelet (140 seats)
Wednesday 2 September	8h30 – 09h15	ESVV Plenary session 4, Keynote lecture 6 **Dr B. MARTINEZ LOPEZ** "Real-time surveillance and risk assessment"	Registration				
	9h15 – 10h30	Parallel ESVV Session 7: « Enteric viruses »	Poster & Stand Viewing	Parallel Young Scientists Meeting	Parallel ESVV Session 8: "General virology 1"	Parallel ESVV Session 9: "General virology 2"	Parallel ESVV Session 10: "Emerging virus & diseases 2"
	10h30 – 11h00		Coffee break, Poster & Stand Viewing		LinkTADs Dissemination Event		
	11h00 – 12h00	Parallel ESVV General Assembly	Poster & Stand Viewing	Parallel Young Scientists Meeting			
	12h00 – 13h15		Lunch, Poster & Stand Viewing				
	13h15 - 15h00	EPIZONE opening session & Keynote lectures 7 & 8 **Dr P. MURCIA** "Impact of evolution and ecology on host species jumps" **Prof L. SAIF** "Elusive coronavirus vaccines: strategies for PEDV vaccines"	Registration				
	15h00 – 16h30	Parallel EPIZONE Session 1 "Epidemiology, surveillance & risk assessment 1"	Poster & Stand Viewing	Parallel Young Scientists Meeting	Parallel EPIZONE Session 2: "Intervention strategies"	Parallel EPIZONE Special Session "Host/virus interactions & viral immunity 2"	Parallel EPIGENESIS Session "Animal health in the Caribbean"
	16h30 – 17h00		Coffee break, Poster & Stand Viewing				
	17h00 – 18h00	EPIZONE Poster Presentation Session 1 (+ poster award)	Poster & Stand Viewing	Parallel EPIZONE Coordinating Forum meeting (17h00-18h30)	EPIZONE Poster Presentation Session 2	EPIZONE Poster Presentation Session 3	Parallel EPIGENESIS Session "Animal health in the Caribbean"
	18h30	Guided City tour					
	20h00	Gala Diner					
Thursday 3 September	9h00 – 9h45	EPIZONE plenary session, Keynote lecture 9 **Dr J-C. MANUGUERRA** "New development in diagnosis of viral infections"					
	9h45 – 10h10		Coffee break, Poster & Stand Viewing				
	10h10 – 12h10	Parallel EPIZONE Session 3 "Diagnosis"	Poster & Stand Viewing	Parallel EPIZONE Executive Committee meeting		Parallel EPIZONE Session 4 "Epidemiology, surveillance & risk assessment 2"	Parallel EPIZONE Special Session: "Focus on PED & SBV"
	12h10 – 12h30	ESVV & EPIZONE Closing ceremony					

Detailed Oral final program

		Parallel ESVV session 1	Parallel ESVV session 2	Parallel ESVV session 3
01/09/2015, Morning	Topic	Virus evolution	Influenza	Arboviruses
	Duration	90 min	90 min	90 min
		Chair: T. Drew & E. Albina	Chair: T. Van den Berg & M. Beer	Chair: T. Mettenleiter & S. Gutierrez
	11h00-11h15	Genetic characterization of a distinct worldwide-spread genetic lineage of Infectious Bursal Disease virus Ruben Pérez, Martín Hernández, Gonzalo Tomás, Ana Marandino, Gregorio Iraola, Diego Hernández, Pedro Villegas, Alejandro Banda, Yanina Panzera	Codon deletions and substitutions in the PA linker of the RNA-polymerase allow generation of temperature-sensitive and attenuated influenza viruses Léa Meyer, Bruno Da Costa, Alix Sausset, Ronan Le Goffic, Bernard Delmas	Knockout of non-structural protein NS3/NS3a abolishes propagation of bluetongue virus in Culicoides sonorensis Femke Feenstra, Barbara Drolet, René van Gennip, Jan Boonstra, Piet van Rijn
	11h15-11h30	Characterization and phylogenetic analysis of cetacean morbillivirus infection in eastern Atlantic-based cetacean populations Carolina Bento, C. Eira, J. Vingada, M. Ferreira, A. Lopez, L. Tavares, A. Duarte	Detection of a new Influenza D Virus in Cattle, France, 2011-2014 Elias Salem, Gilles Meyer, Claire Pelletier, Hervé Cassard, Mariette Ducatez	Genetic diversity of West Nile virus in Europe Norbert Nowotny, Tamás Bakonyi, Jolanta Kolodziejek, Ivo Rudolf, Zdenek Hubálek, Karin Pachler, Karin Sekulin, Bernhard Seidel
	11h30-11h45	Development of a bioinformatics pipeline to identify and characterise minor variants in Foot-and-mouth disease virus populations using data generated from the Illumina MiSeq David King, Graham Freimanis, Richard Orton, Donald King, Daniel Haydon	Anti-nucleocapsid protein antibody is sufficient to confer resistance to lethal infection with influenza A viruses of several subtypes in transgenic mice Yoshikazu Fujimoto, Kinuyo Ozaki, Gen-Ichiro Uechi, Hiroki Takakuwa, Yukiko Tomioka, Toshiyo Yabuta, Haruka Suyama, Sayo Yamamoto, Masami Morimatsu, Toshihiro Ito, Koichi Otsuki, Mai Q. Le, Tetsu Yamashiro, Etsuro Ono	The entomological surveillance of West Nile virus in Lombardia and Emilia-Romagna regions, Northern Italy, 2014 Mattia Calzolari, Davide Lelli, Romeo Bellini, Paolo Bonilauri, Francesco Defilippo, Giulia Maioli, Ana Moreno Martin, Marco Tamba, Mario Chiari, Paola Angelini, Silvano Natalini, Marco Farioli, Antonio Lavazza, Alice Prosperi, Francesca Faccin, Mariagrazia Zanoni, Michele Dottori
	11h45-12h00	Comparative molecular epidemiology of the two closely related bovine coronavirus (BCoV) and human coronavirus OC43 (HCoV-OC43) revealed different evolution dynamic pattern Nathalie Kin, Meriadeg Le Gouil, Fabien Miszczak, Laure Diancourt, Valérie Caro, Astrid Vabret	Mutation of Serpine1 in mice results in increased susceptibility to influenza A virus infection due to increased vascular leakage and virus dissemination Dai-Lun Shin, Bastian Hatesuer, Ruth L.O. Stricker, Klaus Schughart	Broad Molecular Detection of Flaviviruses Alia Yacoub, Giorgi Matreveli, Valeria Ieskova, Frederik Widén, Kerstin Falk, Sándor Belák, Mikael Leijon
	12h00-12h15	Fitness evaluation and molecular characterization of a recombinant murine norovirus during serial passages in cell culture Edmilson Ferreira de Oliveira Filho, Elisabetta Di Felice, Barbara Toffoli, William Zonta, Ana Carolina Terzian, Elizabeth Mathijs, Etienne Thiry, Axel Mauroy	Antigenic mapping of the avian influenza virus M2 protein extracellular domain using overlapping short peptides-based ELISA Noor Haliza Hasan, Jagoda Ignjatovic, Simson Tarigan, Anne Peaston, Farhid Hemmatzadeh	Demographics of natural oral infection of mosquitos by Venezuelan equine encephalitis virus Serafin Gutierrez
	12h15-12h30		Streptococcus suis affects the replication of swine influenza virus in porcine tracheal cells Nai-Huei Wu, Fandan Meng, Maren Seitz, Peter Valentin-Weigand, Georg Herrler	Simbu sero-group virus serum neutralizing antibodies in cattle in Tanzania Coletha Mathew, Siv Klevar, Armin Elbers, Wim van der Poel, Peter Kirkland, Jacques Godfroid, Robinson Mdegela, George Mwamengele, Maria Stokstad

		Parallel ESVV session 4	Parallel ESVV session 5	Parallel ESVV session 6
01/09/2015, Afternon	Topic	Vaccines and antivirals	Host/virus interactions & viral immunity 1	Emerging virus & diseases 1
	Duration	120 min	120 min	120 min
		Chair: M-F. Le Potier & R. Servan de Almeida	Chair: H. Nauwynck & C. Cêtre-Sossah	Chair: W. van der Poel & N. Nowotny
	14h30-14h45	Assessment of efficacy and immune responses in mice vaccinated with recombinant MVAs expressing single RVFV glycoproteins Elena López-Gil, Gema Lorenzo, Alejandro Marín-López, Sandra Moreno, Javier Ortego, Alejandro Brun	Postnatal persistent infection with classical swine fever virus: Hitherto unreported strategy of the virus to persist in domestic pigs and wild boars. Llilianne Ganges, Sara Muñoz-Gonzalez, Oscar Cabezón, Nicolas Ruggli, Marta Perez-Simó, José Bohórquez, Rosa Rosell, Ignasi Marco, Santiago Lavín, Arthur Summerfield, Mariano Domingo	Identification of a novel HCV-like virus in German domestic cattle Christine Baechlein, Nicole Fischer, Adam Grundhoff, Malik Alawi, Daniela Indenbirken, Alexander Postel, Jennifer Offinger, Kathrin Becker, Andreas Beineke, Juergen Rehage, Paul Becher
	14h45-15h00	Salmonid alphavirus glycoprotein E2 requires low temperature and E1 for virion formation and induction of protective immunity Mia Hikke, Stine Braaen, Stephane Villoing, Kjartan Hodneland, Corinne Geertsema, Lisa Verhagen, Petter Frost, Just Vlak, Espen Rimstad, Gorben Pijlman	Inefficacy of a CSFV live attenuated vaccine when postnatal persistent infection takes the stage Sara Muñoz-Gonzalez, Marta Perez-Simó, Marta Muñoz, José Bohórquez, Rosa Rosell, Arthur Summerfield, Mariano Domingo, Nicolas Ruggli, Llilianne Ganges	Detection of a novel bovine astrovirus in a cow with encephalitis Kore Schlottau, Christoph Schulze, Sabine Bilk, Dennis Hanke, Dirk Höper, Martin Beer, Bernd Hoffmann
	15h00-15h15	Potential edible vaccine against influenza virus Tomasz Łęga, Paulina Weiher, Dawid Nidzworski	Transcriptome analysis reveals the host response to Schmallenberg virus in bovine cells and antagonistic effects of the NSs protein Anne-Lie Blomström, Quan Gu, Gerald Barry, Gavin Wilkie, Jessica Skelton, Margaret Baird, Melanie McFarlane, Esther Schnettler, Richard Elliott, Massimo Palmarini, Alain Kohl	Peste des petits ruminants in Spanish sheep breed: protection study and experimental infection using NIG75/1 vaccine strain and MOR/08 field isolate Cristina Cano-Gómez, Francisco Llorente, Paloma Fernandez-Pacheco, Ana Robles, Amalia Villalba, Mª Carmen Barbero, Geneviève Libeau, Miguel Ángel Jiménez-Clavero, Jovita Fernández-Pinero
	15h15-15h30	Antiviral activity of type I, II and III porcine interferons against classical swine fever virus Bentley Crudgington, Helen Everett, Mike Skinner, Helen Crooke	Early events of canine herpesvirus 1 infections in canine respiratory and genital mucosae by the use of ex vivo models Yewei Li	Novel insights in experimental peste des petits ruminants virus infection, transmission, pathogenesis and propagation Claudia Schulz, Christine Fast, Martin Beer, Bernd Hoffmann
	15h30-15h45	RNA interference as antiviral therapy against Peste des Petits Ruminants: proof of concept of in vivo efficiency using a small animal model Cécile Minet, Renata Servan de Almeida, Carine Holz, Renaud Lancelot, Geneviève Libeau, Mattias Hällbrink, Ülo Langel, Emmanuel Albina	Epigenetic regulation of gga-microRNA-126 during lymphoproliferative disease in chicken Isabelle Gennart, Lore Parisse, Srdan Pejakovic, Benoît Muylkens	Serological Evidence of Peste des Petits Ruminants in Yak, Pakistan Muhammad Abubakar, Shumaila Manzoor, Ehtisham-ul haq Khan, Hussain Manzoor, Muhammad Afzal, Qurban Ali, Jonas Wensman, Muhammad munir
	15h45-16h00	Use of in silico prediction models to predict T-cell epitopes for the development of vaccines against African swine fever virus Eefke Weesendorp, Ben Peeters, Peter Willemsen	A equine herpesvirus type 1 (EHV-1) abortion outbreak caused by a neuropathogenic strain in vaccinated mares Ljubo Barbic, Vladimir Stevanovic, Snjezana Kovac, Madic Josip	Outbreak of henipavirus in the southern Philippines, 2014 Debbie Eagles, A. Ruth Foxwell, Fedelino Malbas, Sam McCullough, Glenn Marsh
	16h00-16h15	Generation a new Newcastle Disease vaccine by reverse genetics based on a recently genotype XI virus Haijin Liu, Patricia Gil, Cécile Minet, Renata Servan de Almeida, Emmanuel Albina	Effect of inoculation dose and age of the pigs on clinical, virological and serological parameters of an African swine fever infection Phaedra Eblé, Eefke Weesendorp, Bernie Moonen-Leusen, sjaak quak, Willie Loeffen	Kobuvirus in brains of piglets diagnosed with congenital tremor type A-II Fredrik Granberg, Maja Malmberg, Oscar Cabezón, Juliette Hayer, Rosa Rosell, Sándor Belák, Joaquim Segalés
	16h15-16h30	A domain located at the amino terminus of the envelope glycoprotein (Gc) of an orthobunyavirus is targeted by neutralizing antibodies Gleyder Roman-Sosa, Emiliana Brocchi, Horst Schirrmeier, Kerstin Wernike, Christian Schelp, Martin Beer	Tubulins interact with porcine and human S proteins of the genus Alphacoronavirus and influence the viral replication cycle of the porcine coronavirus TGEV Anna-Theresa Ruediger, Peter Mayrhofer, Yue Ma-Lauer, G. Pohlentz, J. Muething, Albrecht von Brunn, Christel Schwegmann-Weßels	Identification of a new non-pathogenic lagovirus in Lepus europeaus Patrizia Cavadini, Stefano Molinari, Giulia Pezzoni, Mario Chiari, Emiliana Brocchi, Antonio Lavazza, Lorenzo Capucci

02/09/2015, Morning		Parallel ESVV session 7	Parallel ESVV session 8	Parallel ESVV session 9	Parallel ESVV session 10
	Topic	Enteric viruses	General Virology 2	General Virology 1	Emerging virus & diseases 2
	Duration	75 min	75 min	75 min	75 min
		Chair: J-M. Sánchez-Vizcaíno & T. Lefrançois	Chair: L. Dixon & T. Mettenleiter	Chair: T. Van den Berg & S. Bertagnoli	Chair: T. Drew & A. Lavazza
	9h15-9h30	Comparative analysis of the faecal virome of dogs with various inflammatory intestinal diseases Paloma Moreno, Josef Wagner, James Gilkerson, Matthew Stevens, Carl Kirkwood, Caroline Mansfield	Next generation sequencing of British outbreak cases of Equine Infectious Anaemia Virus: sequence variation and phylogeny among European outbreaks Daniel Robinson, Nicolas Locker, Falko Steinbach, Bhudipa Choudhury	Molecular analysis of canine parvovirus type 2 in New Zealand Magdalena Dunowska, Sylvia Ohneiser, Simon Hills, Nick Cave, Doug Passmore	A devastating outbreak of orthopoxvirus infection in Macaca Tonkeana Giusy Cardeti, Claudia Eleni, Maria Teresa Scicluna, Fabrizio Carletti, Stefania Sittinieri, Roberta Giordani, Marina Cittadini, Lorenzo De Marco, Gian Luca Autorino
	9h30-9h45	Clinical, virological, and immunological parameters during experimental feline enteric coronavirus infection Lowiese Desmarets, Sebastiaan Theuns, Nádia Conceição-Neto, Inge Roukaerts, Delphine Acar, Jelle Matthijnssens, Hans Nauwynck	MetLab: a bioinformatics platform for viral metagenomics experiments Martin Norling, Oskar Karlsson, Hadrien Gourlé, Erik Bongcam-Rudloff, Juliette Hayer	Canine infectious tracheobronchitis in Swedish dogs & what does a metagenomics approach reveal? Maja Malmberg, Juliette Hayer, Oskar Karlsson, Anne-Lie Blomström, Bodil Ström Holst, Jonas Johansson Wensman	New bornavirus discovered in variegated squirrels: Potential link to fatal human infections Bernd Hoffmann, Dennis Tappe, Kore Schlottau, Dirk Höper, Christiane Herden, Maria Jenckel, Donata Hoffmann, Jens Teifke, Christine Fast, Kerstin Tauscher, Daniel Cadar, Christian Mawrin, Jonas Schmidt-Chanasit, Rainer Ulrich, Martin Beer
	9h45-10h00	Factors affecting the occurrence of canine parvovirus disease in dogs Carla Miranda, Júlio Carvalheira, Colin Parrish, Gertrude Thompson	Capsid proteins of porcine circoviruses interact differentially with the cellular protein gC1qR Guy Kouokam, Claire de Boisséson, Cecilia Bernard, Lionel Bigault, Annette Mankertz, Yannick Blanchard, André Jestin, Beatrice Grasland	Staining and expression kinetics of the feline immunodeficiency virus envelope glycoprotein Inge Roukaerts, Chris Grant, Lowiese Desmarets, Sebastiaan Theuns, Isaura Christiaens, Delphine Acar, Hans Nauwynck	Wobbly possum disease: the proof of causation Magdalena Dunowska, Julia Giles, Matthew Perrott
	10h00-10h15	Complete genome characterization of recent and ancient Belgian pig group A rotaviruses and assessment of their evolutionary relationship with human rotaviruses Sebastiaan Theuns, Elisabeth Heylen, Mark Zeller, Inge Roukaerts, Lowiese Desmarets, Marc van Ranst, Hans Nauwynck, Jelle Matthijnssens	Superinfection exclusion during infection with African swine fever virus Raquel Portugal, Gunther Keil	Evaluation of genetic differences between the avirulent and virulent pathotypes of feline coronaviruses Ruisong Yu, Herman Egberink, Hui-Wen Chang, Peter Rottier	Detection and characterization of a novel reassortant Mammalian Orthoreovirus of bats in Europe Davide Lelli, Ana Moreno, Andrej Steyer, Tina Naglič, Alice Prosperi, Francesca Faccin, Antonio Lavazza
	10h15-10h30	First detection and phylogenetic analysis of porcine bocaviruses identified in Slovenia Ivan Toplak, Zoran Žlabravec, Petra Lainšček, Danijela Rihtarič	Epigenetic regulation of the viral RNA telomerase subunit over-expressed in lymphoma induced by Marek's Disease Virus Srđan Pejaković, André Claude Mbouombouo Mfossa, Carole Mignion, Isabelle Gennart, Damien Coupeau, Sylvie Laurent, Denis Rasschaert, Benoît Muylkens	Molecular epidemiology, prevalence and risk factors of FCV infection in European domestic felines Maria Afonso, Gina Pinchbeck, Shirley Bonner, Rosalind Gaskell, Janet Daly, Susan Dawson, Alan Radford	Genetic characterization of a novel adenovirus detected in captive bottlenose dolphin (Tursiops truncates) suffering from self-limiting gastroenteritis Maja Malmberg, Consuelo Rubio-Guerri, Daniel García-Párraga, Elvira Nieto-Pelegrín, Mar Melero, Teresa Álvaro, Mónica Valls, Jose Luis Crespo, Jose Manuel Sánchez-Vizcaíno, Sándor Belák, Fredrik Granberg

02/09/2015, Afternoon		Parallel EPIZONE session 1	Parallel EPIZONE session 2	Parallel EPIZONE special session 1	Parallel EPIGENESIS session
	Topic	Epidemiology, surveillance and risk assessment 1	Intervention strategies	Host/virus interactions & viral immunity 2	"Animal health in the Caribbean"
	Duration	90 min	90 min	90 min	210 min
		Chair: B. Martínez-López & T. Lefrançois	Chair: L. Dixon & M-F. Le Potier	Chair: A. Brun & S. Zientara	Chair: C. Lazarus & N. Vachiery
	15h00-15h15	Development of a Pipeline for the High-Throughput Sequencing of FMDV: An Application to a Large Outbreak Graham Freimanis, David King, Richard Orton, Donald King	Classical swine fever virus marker vaccine strain CP7_E2alf: shedding and dissemination studies in boars Carolin Dräger, Anja Petrov, Martin Beer, Jens Teifke, Sandra Blome	Cowpox virus: virulence studies in different animal species Annika Franke, Donata Hoffmann, Rainer G. Ulrich, Maria Jenckel, Bernd Hoffmann, Martin Beer	Research and surveillance on Animal Health in the Caribbean Cedric Lazarus
	15h15-15h30	Classical Swine fever in wild boar: Surveillance strategies under the microscope Katja Schulz, Jana Sonnenburg, Birgit Schauer, Timothée Vergne, Marisa Peyre, Christoph Staubach, Franz Conraths	First expression in baculovirus of major capsid proteins belonging to two new lagoviruses Giulia Pezzoni, Lidia Stercoli, Patrizia Cavadini, Antonio Lavazza, Emiliana Brocchi, Lorenzo Capucci	Equine monocytic cells as a 'Trojan horse' for equine herpesvirus type 1 (EHV-1) dissemination within the host Kathlyn Laval, Herman Favoreel, Katrien Poelaert, Jolien van Cleemput, Hans Nauwynck	
	15h30-15h45	Quantification of African swine fever virus transmission parameters in carriers and the possible role of indirect virus transmission Willie Loeffen, Eefke Weesendorp, Bernie Moonen-Leusen, Thomas Hagenaars, Phaedra Eblé	In vivo testing of deletion mutants as candidate vaccines for African swine fever in vaccination/challenge models in pigs Carmina Gallardo, Alejandro Soler, Angel Carrascosa, Elena Sanchez, Raquel Nieto, Alicia Simon, Miguel Sanchez, Carlos Martins, Victor Briones, Yolanda Revilla, Marisa Arias	Systemic spread of wild boar hepatitis E virus in pigs Damien Thiry, Nicolas Rose, Axel Mauroy, Frédéric Paboeuf, Stefan Roels, Nicole Pavio, Etienne Thiry	
	15h45-16h00	Lessons learnt from a cross-sectional field survey: how to implement a serological monitoring of BVD-free herds in the Belgian BVDV eradication programme? Jean-Baptiste Hanon, Miet De Baere, Camille De la Ferté, Sophie Roelandt, Brigitte Cay, Yves Van der Stede	Comparative study of protection in pigs immunised by different routes with attenuated African swine fever virus isolate OUR T88/3 and evaluation of the role of immunomodulatory cytokines. Pedro Sanchez-Cordon, Dave Chapman, Lynnette Goatley, Linda Dixon	A cell culture-adapted Classical swine fever virus phenotype does not require the 476Arg Erns mutation Carolin Dräger, Sandra Blome, Martin Beer, Ilona Reimann, Thomas Rasmussen	Orbiviruses in the Caribbean – should we worry about them? Christopher Oura, Tamiko Brown-Joseph, Lorraine Frost, Carrie Batten
	16h00-16h15	One World One Health: The STAR-IDAZ Global Network for Coordination of Animal Disease Research Alex Morrow, Luke Dalton	Deoptimization of codon pair usage of the major capsid protein VP72 of African swine fever virus: effect on protein expression in vitro Raquel Portugal, Axel Karger, Richard Bishop, Günther Keil	Next-generation sequencing fails to identify viral miRNAs encoded by PCV2 in subclinically infected pigs Fernando Núñez, Lester Pérez, Gonzalo Vera, Sarai Córdoba, Joaquim Segalés, Armand Sánchez, José Núñez	
	16h15-16h30		Efficacy of foot-and-mouth disease vaccines A22 Iraq 64 and A Malaysia 97 against challenge with a recent South East Asian serotype A field strain in cattle and sheep Jacquelyn Horsington, Nagendrakumar Singanallur, Aldo Dekker, Charles Nfon, Wilna Vosloo	Studying CSFV specific immune response using dendrimeric peptides José Bohórquez, Sara Muñoz-Gonzalez, Sira Defaus, Beatriz De la Torre, Marta Perez-Simó, Rosa Rosell, Mariano Domingo, David Andreu, Lorenzo Fraile, Llilianne Ganges	Molecular epidemiology of two important viral diseases of pigs in Cuba: classical swine fever and swine influenza Lester Perez
	16h30-16h45				
	17h00 -17h30				Situation of the Porcine encephalomyelitis with teschovirus in the Republic of Haiti Max F. Millien
	17h30-17h45				Rabies in the Caribbean: A Situation Analysis Janine F.R. Seetahal, Jennifer Pradel, Christine V.F. Carrington, Bowen Louison, Rohini Roopnarine, Astrid Van Sauers, Colin James, Max F. Millien, Charles E. Rupprecht, Alexandra Vokaty
	17h45-18h00				West-Nile in the Caribbean Nonito Pages, Nathalie Vachiery, Thierry Lefrançois, Ken Giraud-Girard, Emmanuel Albina, Jennifer Pradel
	18h00-18h15				Avian influenza in Belize Victor Gongora, Miguel Depaz, Orlando Habet
	18h15-18h30				Avian diseases in Cuba and the Caribbean María T. Frias Lepoureau, Ana M. Acevedo Beiras, Abdulahi Alfonso Morales, Damaris Relova Vento, A. Vega Redondo, E. Ferrer Mitanda, P. Alfonso Zamora, Heidy Díaz de Arce Landa, L. J. Pérez Rodriguez, L. Perera González
	18h30-18h45				Identification of viruses causing respiratory disease of poultry flocks in Trinidad and Tobago Arianne Brown Jordan, Lemar Blake, Judy Bisnath, Zul Mohammed, Chad Ramgattie, Jamie Sookhoo, Gabriel Brown, Christine Carrington, Christopher Oura

03/09/2015, Morning		Parallel EPIZONE session 3	Parallel EPIZONE session 4	Parrallel EPIZONE special session 2
	Topic	Diagnosis	Epidemiology, surveillance & risk assessment 2	Focus on PED & SBV
	Duration	120 min	120 min	120 min
		Chair: A. Bøtner & A. Lavazza	Chair: B. Martínez-López & C. Saegerman	Chair: B. Hoffman & C. Oura
	10h10-10h25	Applicability of faeces samples for detecting antibodies against African swinve fever Elvira Nieto Pelegrín, Belén Rivera-Arroyo, Antonio Sanz, Jose Manuel Sánchez-Vizcaíno	MINTRISK, a Method for INTegrated RISK assessment of vector-borne livestock infections. Aline de Koeijer, Wil Hennen, Sofie Dhollander, Clazien De Vos	Study of the nuclear targeting of the NSs protein of Schmallenberg virus Julie Gouzil, Aurore Fablet, Grégory Caignard, Cindy Kundlacz, Pierre-Olivier Vidalain, Frédéric Tangy, Stéphan Zientara, Damien Vitour
	10h25-10h40	Towards serological surveillance in the Belgian BVDV eradication programme: evaluation of commercial ELISA kits for the detection of antibodies against BVDV in serum and milk using a large collection of field samples Miet De Baere, Jean-Baptiste Hanon, Camille De la Ferté, Sophie Roelandt, Yves Van der Stede, Ann-Brigitte Caij	Assessment of African swine fever diagnostic tehniques as a response to the epidemic outbreaks in eastern European Union countries: how to improve surveillance and control programmes Carmina Gallardo, Jovita Fernández-Pinero, Raquel Nieto, Alejandro Soler, Virginia Pelayo, Iwona Markowska-Daniel, Gediminas Pridotkas, Imbi Nurmoja, Rita Granta, Alicia Simon, Covadonga Perez, Elena Martin, Paloma Fernandez-Pacheco, Marisa Arias	Schmallenberg virus incursion into Great Britain: identification of mutations and a large deletion in the M segment of British field samples Sarah McGowan, Anna La Rocca, Sylvia Grierson, Falko Steinbach, Bhudipa Choudhury
	10h40-10h55	Cartridge-based real-time molecular diagnostic assays for the rapid and simple detection of African swine fever and foot-and-mouth disease virus Katja Goller, Veronika Dill, Paul Martin, Sandra Blome, Martin Beer, Bernd Haas	Prevalence of some potentially zoonotic pathogens in the Dutch equine population Kees van Maanen, Martine Bloemer, Joke van der Giessen, Inge Santman-Berends, Miriam Koene	Reoccurrence of Schmallenberg virus 2014/2015 in Germany Kerstin Wernike, Bernd Hoffmann, Franz Conraths, Martin Beer
	10h55-11h10	PCR detectability and stability of Aujeszky's disease virus in porcine oral fluid Sara Verpoest, Rodolphe Michiels, Ann Brigitte Cay, Nick de Regge	Spatio-temporal network analysis of pig movements in Great Britain: implications for disease transmission and control strategies Claire Guinat, Anne Relun, Aaron Morris, Linda Dixon, Dirk Udo Pfeiffer	Is flock renewal a risk for novel Schmallenberg virus episode in sheep flocks? Francois Claine, Damien Coupeau, Laetitia Wiggers, Benoît Muylkens, Nathalie Kirschvink
	11h10-11h25	Approaches to DIVA assays for West-Nile virus Belen Rebollo, Sylvie Lecollinet, Ana Camuñas, Elena Soria, Antonio J. Sanz, Angel Venteo	Risk of transmission of Coxiella burnetii during the caprine in-vitro produced embryo transfer Jean-Louis Pellerin	Evolution of Schmallenberg virus seropositivity over years among sheep naturally infected at different ages Francois Claine, Damien Coupeau, Laetitia Wiggers, Benoît Muylkens, Nathalie Kirschvink
	11h25-11h40	Optimized antigen expression and presentation for diagnostic purposes – a new potential for Equine Herpesvirus 1 as a vector Susanne Koethe, Walid Azab, Nicole Bürger, Patricia König, Benedikt Kaufer, Nikolaus Osterrieder, Claudia Engemann, Martin Beer	First Isolation of Canine Parvovirus in Morocco Nadia Amrani, Khalil Zro, Chafiqa Loutfi, Jamal Malik, Jaouad Berrada, Ahlam Kadiri	Re-emergence of Porcine epidemic diarrhea virus in Germany Dennis Hanke, Anne Pohlmann, Dirk Höper, Carola Sauter-Louis, Mathias Ritzmann, Julia Stadler, Valeriy Akimkin, Bernd Schwarz, Martin Beer, Günter Strebelow, Sandra Blome
	11h40-11h55	Tracking of African swine fever outbreaks through variation of intergenic I73R/I329L region. Ilya Titov, Alexander Malogolovkin, Galina Burmakina, Irina Syndryakova, Sergey Katorkin, Ksenya Mima, Dmitriy Kudryashov, Andrey Gogin, Denis Kolbasov	African Swine Fever in Sardinia: evidence of several persistent clusters of infection during the re-epidemic wave 2012-2014 Francesco Feliziani, Sandro Rolesu, Andrea Felici, Stefano Cappai, Carmen Iscaro, Carmen Maresca	Description of the first case of porcine epidemic diarrhea in France in December 2014 and the duration of viral shedding in the herd Beatrice Grasland, Cécilia Bernard, Lionel Bigault, Aurélie Leroux, Hélène Quenault, Olivier Toulouse, Christelle Fablet, Nicolas Rose, Fabrice Touzain, Yannick Blanchard
	11h55-12h10	Serology and molecular diagnostics of epizootic hemorrhagic disease virus (EHDV) Stephane Daly, Sandrine Moine, Flavia Tisserant, Alex Raeber	Modelling economic impacts of an epidemic spread of West Nile virus in Belgium Marie-France Humblet, Sébastien Vandeputte, Fabienne Fecher-Bourgeois, Philippe Léonard, Christiane Gosset, Thomas Balenghien, Benoît Durand, Claude Saegerman	Genetic characterization of porcine epidemic diarrhea virus strains isolated from novel outbreaks in the European Union, Belgium. Isaura Christiaens, Nádia Conceição-Neto, Sebastiaan Theuns, Mark Zeller, Lowiese Desmarets, Inge Roukaerts, Elisabeth Heylen, Jelle Matthijnssens, Hans Nauwynck

Detailed Poster presentations program

01/09/2015, Afternoon	Parallel ESVV Poster Presentation Session 1	Parallel ESVV Poster Presentation Session 2	Parallel ESVV Poster Presentation Session 3
Room	**Main Hall Pasteur**	**Sully 2**	**Sully 3**
Duration	90 min	90 min	90 min
	Chair: S. Zientara & R. Servan de Almeida	Chair: A. Bøtner & G. Libeau	Chair: C. Gallardo & S. Gutierrez
17h00-17h05	The characterization of avian influenza viruses isolated from wild birds in Vietnam from 2010 to 2014 Hiroki Takakuwa, Toshiyo Yabuta, yoshiki Kinoshita, takafumi amemori, Kosuke Soda, Tatsufumi Usui, Kozue Hotta, Le Mai, Tetsu Yamashiro, Hiroichi Ozaki, Hiroshi Ito, Tsuyoshi Yamaguchi, Toshiyuki Murase, Toshihiro Ito, Etsuro Ono, Koichi Otsuki	Emerging pestiviruses and impact on animal production Nicola Decaro, Leonardo Occhiogrosso, Viviana Mari, Maria Stella Lucente, Gabriella Elia, Eleonora Lorusso, Michele Losurdo, Vittorio Larocca, Canio Buonavoglia	Infectivity of CBPV major RNAs Ibrahim Youssef, Frank Schurr, Magali Ribiere-Chabert, Richard Thiery, Eric Dubois
17h05-17h10	The effect of Streptococcus suis co-infection on the infection of well-differentiated porcine respiratory epithelial cells by swine influenza viruses Meng Fandan, Nai-Huei Wu, Maren Seitz, Peter Valentin-Weigand, Xiaofeng Ren, Georg Herrler	Emergence of a virulent BVDV type 2C strain in the Netherlands Jet Mars, Kees van Maanen, Linda van Duijn	Rare recombination events and occurence of superinfection exclusion during synchronous and asynchronous infection with homologous murine norovirus strains Elisabetta Di Felice, Louisa Ludwig, Barbara Toffoli, Chiara Ceci, William Zonta, Barbara Di Martino, Fulvio Marsilio, Etienne Thiry, Axel Mauroy
17h10-17h15	Investigation of a possible link between vaccination and the 2010 Sheep pox epizootic in Morocco Andy Haegeman, Khalil Zro, Frank vandenbussche, Donal Sammin, MM Ennaji, Kris De Clercq Annebel De Vleeschauwer	Commercial vaccines may not protect against local strains of bovine viral diarrhea virus Kadir Yesilbag, Gizem Alpay	Feline Immunodeficiency Virus (FIV) in free ranging Leopards (Panthera pardus), from the Kruger National Park, South Africa Tanya Kerr, Susan Engelbrecht, Conrad Matthee, Danny Govender, Sonja Matthee
17h15-17h20	Prevalence of antibodies to selected viral pathogens in wild boars (Sus scrofa) in Serbia Tamas Petrovic, Diana Lupulovic, Gospava Lazic, Jasna Prodanov Radulovic, Vladimir Polacek, Radoslav Dosen, Sava Lazic	Experimental infection of bluetongue virus serotype 4 MOR2009/09 strain in IFNAR (-/-) mice Alejandro Marin-Lopez, Roberto Bermudez, Eva Calvo-Pinilla, Alejandro Brun, Javier Ortego	Intra-host phylodynamic analysis of canine distemper virus Yanina Panzera, N. Sarute, G. Iraola, M. Vignuzzi, R. Pérez
17h20-17h25	Is Ornithodoros erraticus able to transmit the Georgia2007/1 African Swine Fever virus isolate to domestic pigs? Jennifer Bernard, Laurence Vial, Evelyne Hutet, Frédéric Paboeuf, Vincent Michaud, Fernando Boinas, Marie-Frédérique Le Potier	Bluetongue and epizootic haemorrhagic disease viruses in Reunion Island Catherine Cêtre-Sossah, Aurélie Pedarrieu, Lorène Rieau, Corinne Sailleau, Emmanuel Breard, Cyril Viarouge, Stéphan Zientara, Olivier Esnault, Eric Cardinale	Molecular phylogeography of canine distemper virus Yanina Panzera, N. Sarute, G. Iraola, M. Hernández, R. Pérez
17h25-17h30	A multidisciplinary approach to combat wildlife diseases: Vaccination with hematophagous arthropods as "living syringes" Anne Darries, Yi Li	First detection of porcine epidemic diarrhea virus in Slovenia, 2015 Ivan Toplak, Marina Štukelj, Danijela Rihtarič, Peter Hostnik, Jože Grom	Genome evolution of canine parvovirus in South America Ruben Pérez, Sofía Grecco, Ana Paula Silva, Jaime Aldaz, Marina Gallo-Calderón, Alice Alfieri, Lucía Calleros, Ana Marandino, Gregorio Iraola, Lourdes Francia, Rodrigo Martino, Yanina Panzera
17h30-17h35	BLV experimental infection in buffalo species (Bubalus bubalis): preliminary data Francesco Feliziani, Alessandra Martucciello, Carmen Iscaro, Domenico Vecchio, Stefano Petrini, Ester De Carlo	Identification and genetic characterization of Aichivirus (porcine kobuvirus) in pig farms in Slovakia Stefan Vilcek, Ivan Sliz, Michaela Vlasakova, Rene Mandelik, Anna Jackova	Molecular characterization of Rift Valley fever virus isolates from Mozambique and phylogenetic comparison with selected other isolates Benjamin Mubemba, Peter Thompson, Peter Coetzee, Estelle Venter, Jose Fafetine
17h35-17h40	Coronavirus diversity and ecology in South African bat populations Nadine Sampson, Ndapewa Ithete, Corrie Schoeman, Leigh Richards, Samantha Stoffberg, Wolfgang Preiser	Rotavirus A and C infections in Belgian Diarrheic suckling pigs Sebastiaan Theuns, Philip Vyt, Lowiese Desmarets, Inge Roukaerts, Elisabeth Heylen, Mark Zeller, Marc Van Ranst, Jelle Matthijnssens, Hans Nauwynck	Experimental infection of domestic pigeons (Columba livia L.) with lineage 2 West Nile virus Petra Forgách, Károly Erdélyi, Bernadett Pályi, Norbert Nowotny, Tamás Bakonyi

01/09/2015, Afternoon	Parallel ESVV Poster Presentation Session 1	Parallel ESVV Poster Presentation Session 2	Parallel ESVV Poster Presentation Session 3
Room	**Main Hall Pasteur**	**Sully 2**	**Sully 3**
Duration	90 min	90 min	90 min
	Chair: S. Zientara & R. Servan de Almeida	Chair: A. Bøtner & G. Libeau	Chair: C. Gallardo & S. Gutierrez
17h40-17h45	Ecology of small mammal-borne viruses with zoonotic potential in South Africa Ndapewa Ithete, Nadine Sampson, Corrie Schoeman, Leigh Richards, Samantha Stoffberg, Sonja Matthee, Peter Witkowski, Boris Klempa,	Novel astroviruses in the gastrointestinal complex of Suidae - Characterisation of the pathobiome by metagenomics Oskar Karlsson, Maja Malmberg, Ádám Bálint, János Benyeda, Sándor Belák, Fredrik Granberg	Long range RNA-RNA interactions within the genome of classical swine fever virus; influence on viral RNA replication Johanne Hadsbjerg, Thomas Bruun Rasmussen, Graham J. Belsham
17h45-17h50	Orbivirus screening on dried blood spots from captive oryx in United Arab Emirates stresses the importance of pre-import measures Ludovic Martinelle, Andy Haegeman, Louis Lignereux, Anne-Lise Chaber, Fabiana Dal Pozzo, Ilse de Leeuw, Kris de Clercq, Claude Saegerman	New astroviruses detected in wild birds Ivan Toplak, Danijela Rihtarič, Petra Bandelj, Modest Vengušt, Tomi Trilar	Expression and serological reactivity of Hemorrhagic enteritis virus hexon protein Dana Lobova
17h50-17h55	Near-universal contamination of commercial equine serum pools with hepaci- and pegiviruses Alexander Postel, Jessika Cavalleri, Stephanie Pfaender, Walter Stephanie, Eike Steinmann, Karsten Feige, Ludwig Haas, Paul Becher	Update on rabies situation in Serbia Diana Lupulovic, Jelena Maksimovic Zoric, Nikola Vaskovic, Budimir Plavsic, Tamas Petrovic, Zivoslav Grgic, Sava Lazic	Genetic diversity and pathological findings of Small Ruminant Lentiviruses Virus in sheep and goats in the European bordering region (Marmara) of Turkey Eda Altan, Gulay Yuzbasioglu Ozturk, Utku Cizmecigil, Ozge Erdogan Bamac, Aydın Gurel, Nuri Turan, Huseyin Yilmaz
17h55-18h00	Introduction of the three-tiered diagnostic system in Equine Infectious Anaemia surveillance in Croatia Vladimir Stevanovic, Ljubo Barbic, Snjezana Kovac, Matko Perharic, Vilim Staresina	Characterisation in France of non-pathogenic lagoviruses closely related to the Australian Rabbit calicivirus RCV-A1: confirmation of the European origin of RCV-A1 Ghislaine Le Gall-Reculé, Evelyne Lemaitre, François-Xavier Briand, Stéphane Marchandeau	Immunization of Day-Old Chickens with Recombinant Viruses Expressing Chicken Parvovirus VP2 Protein Laszlo Zsak, Stephen Spatz, Qingzhong Yu
18h00-18h05	Luminex technology for group and serotype specific Bluetongue antibodies detection Emmanuel BREARD, Annabelle Garnier, Sandra Blaise-Boisseau, Philippe Despres, Stéphan Zientara, Philippe Pourquier, Corinne SAILLEAU, Damien Vitour, Cyril Viarouge	Serological and entomological studies on Schmallenberg virus in Poland, 2013-2014 Julia Kęsik-Maliszewska, Magdalena Larska, Jan Żmudziński	Replication characteristics of respiratory and nephrophathogenic infectious bronchitis virus (IBV) strains M41 and B1648 in respiratory mucosa and monocytes Vishwanatha Reddy, Ivan Trus, Hans Nauwynck
18h05-18h10	Large-Scale Nucleotide Sequence Alignment for Universal Screening PCR Assay Design Alexander Nagy, Tomáš Jiřinec, Lenka Černíková, Helena Jiřincová, Martina Havlíčková	Detection of neuropathogenic variant of equine herpesvirus 1 associated with abortions in mares in Poland Jerzy Rola, Karol Stasiak, Wojciech Socha, Jan Żmudzinski	Dissecting respiratory viral co-infections in poultry using a nanofluidic PCR screening assay Guillaume Croville, Alexis Senet, Charlotte Foret, Mariette Ducatez, Faouzi Kichou, Mohammed Mouahid, Jean-Luc Guérin
18h10-18h20	Poster awards		

02/09/2015, Afternoon	Parallel EPIZONE Poster Presentation Session 1	Parallel EPIZONE Poster Presentation Session 2	Parallel EPIZONE Poster Presentation Session 3
Room	**Main Hall Pasteur**	**Sully 1**	**Sully 2**
Duration	60 min	60 min	60 min
	Chair: C. Saegerman & G. Libeau	Chair: J-F. Valarcher & Karl Stahl	Chair: J-L. Guérin & C. Cêtre-Sossah
17h00-17h05	The detection of West Nile virus in veterinary surveillance: how to overcome the flavivirus similarity and control the absence of inhibition in RNA extracted Paolo Bonilauri, Deborah Torri, Francesco Defilippo, Mattia Calzolari, Andrea Luppi, Michele Dottori	Molecular typing of Bluetongue Viruses using nCounter Analysis System platform Alfreda Tonelli, Stefano Gottardi, Cesare Cammà, Alessio Lorusso, Giovanni Savini	Development of a duplex real-time qRT-PCR method for detection of flaviviruses belonging to Japanese encephalitis and Ntaya serogroups Maia Elizalde, Francisco Llorente, Elisa Perez-Ramirez, Ana Robles, Amalia Villalba, Mª Carmen Barbero, Miguel Ángel Jiménez-Clavero, Jovita Fernandez-Pinero
17h05-17h10	Mosquito species involved in West Nile and Usutu viruses transmission in Italy between 2008 and 2014 Giuseppe Mancini, Mattia Calzolari, Gioia Capelli, Davide Lelli, Fabrizio Montarsi, Mario Chiari, Adriana Santilli, Michela Quaglia, Valentina Federici, Monica Catalani, Federica Monaco, Maria Goffredo, Giovanni Savini	Detection of a divergent Alpha Coronavirus in bats in Piedmont (Italy) Francesca Rizzo, Luigi Bertolotti, Serena Robetto, Carla Lo Vecchio, Cristina Guidetti, Simona Zoppi, Alessandro Dondo, Sergio Rosati, Paola Culasso, Mara Calvini, Roberto Toffoli, Riccardo Orusa, Maria Lucia Mandola	Validation of the ID SCREEN-FMD NSP competitive ELISA Philippe Pourquier, Loic Comtet, Fabien Donnet, Mickael Roche
17h10-17h15	Vector Competence of European Culex pipiens (Diptera: Culicidae) mosquitoes for Rift Valley fever Virus Marco Brustolin, Sandra Talavera, Raquel Rivas, Núria Pujol, Marta Valle, Nonito Pagès, Núria Busquets Marco Brustolin, Sandra Talavera, Raquel Rivas, Núria Pujol, Marta Valle, Nonito Pagès, Núria Busquets	Experimental infection of domestic pigs with African swine fever virus Lithuania 2014 Genotype II field isolate Carmina Gallardo, Alejandro Soler, Raquel Nieto, Cristina Cano, Virginia Pelayo, Miguel Sanchez, Gediminas Pridotkas, Jovita Fernández-Pinero, Victor Briones, Marisa Arias	Validation of the ID SCREEN-African swine fever indirect ELISA Philippe Pourquier, Loic Comtet, Stéphanie Vérité, Mickael Roche, Fabien Donnet
17h15-17h20	What is metadata and why is such a good idea? Matteo Mazzucato, Matteo Trolese, Nicola Ferre, Paolo Mulatti, Monica Lorenzetto, Dona Trimarchi, Grazia Manca, Stefano Marangon	The complex serological monitoring of an ongoing Small Ruminant Lentiviruses eradication campaign in Italy Maria Serena Beato, G. Bertoni, S. Nardelli, E. Stifter, A. Tavella	Preliminary validation of the ID SCREEN® PEDV indirect ELISA Loic Comtet, Mickael Roche, Stéphanie Vérité, Fabien Donnet, Muriel Malzac, Philippe Pourquier
17h20-17h25	ANIHWA PROJECT "TRANSCRIPTOVAC": Host response gene signatures associated with FMDV infection, vaccination and persistence Martin Beer, Kris De Clercq, David Lefebvre, Annebel De Vleeschauwer, Isabelle Schwartz-Cornil, Jean-François Valarcher, Sara Hägglund, Bernd Haas, Pascal Hudelet, Jenifer Richardson, Bernard Klonjkowski, Sandra Blaise-Boisseau, Bakkali-Kassimi Labib, Stéphan zientara	Diagnosis of respiratory outbreak in swine caused by a H1N1 pandemic virus Maria Serena Beato, M. Ustulin, L. Tassoni, F. Zuliani, M. Giorgiutti, D. Vio	Development of a multi-check rRT-PCR method for panFMDV detection Lliliam Rios, Carmen Perea, Liani Coronado, Damarys Relova, Ana Álvarez, Llilianne Ganges, Heidy Díaz de Arce, Lester Pérez, José Núñez
17h25-17h30	An unexpectedly major BTV4 epidemic in Greece, 2014 Konstantia Tasioudi, Peristera Iliadou, Dimitrios Dilaveris, Spiridon Doudounakis, Eleni Chondrokouki, Olga Mangana-Vougiouka	H9N2 Avian Influenza virus serological study among poultry workers in Iran Alireza Heidari	Development and validation of a new ASFV real time PCR Elodie Coulon, Sandrine Moine, Immanuel Leifer, Flavia Tisserant
17h30-17h35	Baculovirus mediated generation of rabbit haemorrhagic disease virus variant 2 VLPs in SF9 insect cells and RK13 rabbit cells from codon modified VP60b open reading frames Günther Keil, Claudia Müller, Horst Schirrmeier	H5N8 highly pathogenic avian influenza in Italy: control measures applied to live decoy birds Tiziano Dorotea, Paolo Mulatti, Laura Amato, Calogero Terregino, Laura Bortolotti, Pietro Di Bianca, Claudia Cesarotto, Stefano Marangon, Lebana Bonfanti	Evaluation of commercial ELISA kits for the detection of BVDV-specific antibodies in serum and milk using well-characterized sample panels Miet De Baere, Jean-Baptiste Hanon, Camille De la Fertéa, Sophie Roelandt, Yves Van der Stede, Ann-Brigitte Caij

02/09/2015, Afternoon	Parallel EPIZONE Poster Presentation Session 1	Parallel EPIZONE Poster Presentation Session 2	Parallel EPIZONE Poster Presentation Session 3
Room	Main Hall Pasteur	Sully 1	Sully 2
Duration	60 min	60 min	60 min
	Chair: C. Saegerman & G. Libeau	Chair: J-F. Valarcher & Karl Stahl	Chair: J-L. Guérin & C. Cêtre-Sossah
17h35-17h40	The prevalence of ten pathogens detected by real-time PCR method in lung tissue samples collected from dead cattle after respiratory disease Ivan Toplak, Peter Hostnik, Milan Pogačnik, Tomislav Paller	Modelling the transmission tree of the highly pathogenic avian influenza H5N1 epidemic in Israel, 2015 Timothee Vergne, Guillaume Fournié, Michal Perry Markovitch, Ram Katz, Avishai Lublin, Shimon Perk, Dirk Pfeiffer	Interlaboratory comparison of assays for detection of antibodies against Porcine Epidemic Diarrhea virus Anette Bøtner, Bertel Strandbygaard, Wim van der Poel, Renate Hakze-Van der Honing, Falko Steinbach, Akbar Dastjerdi, Beatrice Grasland, Lionel Bigault, Frederik Widén, Gunnel Svedlund
17h40-17h45	Malignant catarrhal fever in the Czech Republic: four outbreaks and seroprevalence Vlastimil Křivda, Alexander Nagy, Lenka Černíková, Roman Vodička, Jiří Váhala, Ivan Nágl, Kamil Sedlák	Reassortant Swine Influenza A detected in a pig-farm in Piedmont (Italy) Carla Lo Vecchio, Francesca Rizzo, Chiara Chiapponi, Bruno Sona, Silvia Origlia, Simona Zoppi, Silvia Bertolini, Giuseppe Ru, Emanuela Foni, Maria Lucia Mandola	Evaluation of a newly developed Classical swine fever DIVA ELISA Denise Meyer, Stefanie Fritsche, Claudia Engemann, Carsten Schroeder, Paul Becher, Alexander Postel
17h45-17h50	Survey of pestiviruses in ovine abortions by different detection methods Maria Guelbenzu-Gonzalo, Joe Clarke, Lynsey Cooper, Craig Brown	Generation of engineered BTVs with chimeric structural proteins using reverse genetics - study of functional domain Marc Guimerà Busquets, Gillian Pullinger, Massimo Palmarini, Eva Veronesi, Peter Mertens	Development and evaluation of a multiplex classical RT-PCR for simultaneous detection and typing of FMDV in West Africa Kamila Gorna, Aude Allemandou, Camille Roublot, Aurore Romey, Anthony Relmy, Stephan Zientara, Sandra Blaise-Boisseau, Labib Bakkali-Kassimi
17h50-17h55	Suvaxyn-CSF Marker, the first live marker vaccine against Classical Swine Fever Disease authorized at European level Sandra Juanola, Alicia Urniza, Ase Uttenthal, Marie-Frédérique Le Potier, Gábor Kulcsár, Willie Loeffen, Gian Mario De Mia, Martin Hofmann, Sophie Rossi, Rong Gao, Sándor Belák, Volker Moennig, Jose-Manuel Sanchez-Vizcaíno, Thulke Hans-Hermann, Paolo Calistri, Niels Delater, Frank Koenen, Martin Beer	A real-time PCR test for the detection and differentiation of porcine epidemic diarrhea virus and porcine deltacoronavirus Martina Kahila, Christa Goodell, Lori Plourde, Kathy Velek, Lisa Gow, Valerie Leathers, Michael Angelichio	Development of a Luminex assay for the serological diagnosis of Vesicular Stomatitis virus Anthony Relmy, Aurore Romey, Kamila Gorna, Stefan Zientara, Sandra Blaise-Boisseau, Labib Bakkali-Kassimi
17h55-18h10	Poster award		

PLENARY SESSIONS

ESVV Plenary Session 1

Monday 31st August 2015
16:45-18:30
Auditorium Pasteur

Plenary keynote 1:

Toward a universal influenza virus vaccine

Prof Peter Palese

Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, United States

Influenza remains a major public health concern and causes significant morbidity and mortality in humans and animals worldwide. Current influenza virus vaccines in humans are an effective countermeasure against infection but need to be reformulated almost every year owing to antigenic drift. Furthermore, these vaccines do not protect against novel pandemic strains, and the timely production of pandemic vaccines remains problematic because of the limitations of current technology. Several improvements have been made recently to enhance immune protection induced by seasonal and pandemic vaccines, and to speed up production in case of a pandemic. Specifically, we developed vaccines with chimeric hemagglutinins which redirect the immune response toward the conserved stalk domain of the hemagglutinin as well as to the conserved neuraminidase. Importantly, these vaccine constructs which promise to induce broad or even universal influenza virus protection are currently in preclinical and clinical development.

Plenary keynote 2:

Evolution of arboviruses

Prof Scott Weaver

Institute for Human Infections and Immunity and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, United States

Arboviruses are transmitted biologically by arthropods among vertebrate hosts, with obligatory infection and replication in each. The term arbovirus does not represent a single taxonomic group but a mode of transmission that has evolved in several groups of RNA viruses and a single DNA virus. Understanding of the evolution of arboviruses has been driven for the past 30 years by genome sequencing and phylogenetic studies complemented by experimental approaches to understand the constraints and tradeoffs imposed by the alternating host transmission cycle. Recently, next generation sequencing has resulted in the identification and characterization of large numbers of insect-specific viruses, which are typically unable to infect any vertebrates or vertebrate cells. Many of these insect-specific viruses are closely related to virus taxa traditionally occupied mainly by arboviruses, resulting in major changes in our understanding of arbovirus evolution. Together with the identification of arbovirus-derived sequences in the genomes of several hosts, we now believe that many arbovirus groups are ancient and that the arbovirus mode of transmission may have evolved on many occasions by the acquisition of vertebrate infection competence. Once arboviruses evolved, they diversified greatly by exploiting different groups of vectors and vertebrate hosts, and recently several have increased as pathogens by adapting for amplification by domesticated animals and humans, sometimes with complementary changes in their vector host range. Examples of these mechanisms of disease emergence will be presented for the alphaviruses Venezuelan equine encephalitis and chikungunya, and the flavivirus dengue.

ESVV Plenary Session 2

Tuesday 1st September 2015
9:00-10:30
Auditorium Pasteur

Plenary keynote 3:

Virus discovery by metagenomics: Increasing the knowledge of the human virome at homeostasis and disease by deep sequencing of clinical samples

Prof Marc Eloit

Institut Pasteur, Biology of Infection Unit, Inserm U1117, Pathogen Discovery Laboratory, Paris, France

The composition, interindividual and temporal variability of the human virome are not precisely known but new questions arise in the context of the rapidly increasing knowledge regarding its composition and function. Its impact on human health has received less attention than that of the bacterial microbiome, but is likely to be as important, both in homeostasis and disease. In fact, it is increasingly apparent that viral pathogens are only the salient members of a larger group of viruses associated with humans that are not directly linked to disease, analogous to the bacterial component of the microbiome. In particular, viruses abound at the body interfaces with the external environment, on the skin and in the mucosa. In contrast to this, the presence of viruses in the blood and organ parenchyma of healthy people is rather the exception than the rule, although a limited number of viral species establish persistent, albeit mostly silent, systemic infections, more often in immunocompromized individuals. With the ever-extending use of next generation sequencing, rapid progress on its impact on human health is to be expected in the coming years including the identification of viruses with roles in chronic diseases. We will show how deep sequencing of human clinical cases samples reveals, rather than new viral species, an unexpected role of known viruses in human diseases particularly in immunocompromised patients. On the other hand, whilst cross transmission from animals is preeminent in emerging viral diseases, close or identical new viral species are found in human and animals at homeostasis, which suggests more circulation between species than anticipated.

Plenary keynote 4:

Assembly and disassembly of African Swine Fever virus

Dr Germán Andrés

Center of Molecular Biology Severo Ochoa (CBMSO), Madrid, Spain

African swine fever virus (ASFV), the sole member of the Asfarviridae family, is the causative agent of a highly lethal hemorrhagic disease of domestic pigs. The disease is endemic in sub-Saharan Africa and recent outbreaks have been reported in many countries of Eastern Europe and the Caucasus. Currently, there is no vaccine or antiviral strategy available.

ASFV belongs to the superfamily of nucleocytoplasmic large DNA viruses (NCLDV), which are among the most complex viruses known. Its genome is a double-stranded DNA molecule containing more than 150 genes and its extracellular virus particle, with a diameter of 200 nm, includes more than 50 structural proteins. Like other NCLDVs, ASFV possesses a multilayered structure consisting of a genome-containing nucleoid successively wrapped by a thick protein core shell, an inner lipid membrane, an icosahedral protein capsid and an outer lipid envelope. Such a multilayered structure suggests intricate and unconventional mechanisms of virus morphogenesis and uncoating.

By using inducible ASFV recombinants and a combination of advanced light and electron microscopy techniques, we have dissected the cytoplasmic assembly of the infectious virus particle as well as the endocytic uncoating of the incoming virus. Our results indicate that the inner lipid envelope, which is formed by rupture of the endoplasmic reticulum, plays a pivotal role in both the virus morphogenesis and the penetration of the viral genome into the cytoplasm. The current working models for these complex processes of virus assembly and disassembly will be presented and discussed.

ESVV Plenary Session 3

Tuesday 1st September 2015
13:45-14:30
Auditorium Pasteur

Plenary keynote 5:

Virulence evolution during epidemics

Dr Sylvain Gandon

Ecology and evolutive epidemiology unit, CNRS, Montpellier, France

Why are some pathogens more virulent than others? Theory predicts that pathogens that 'keep their host alive' can sometimes outcompete virulent and more transmissible pathogens in times when transmission to new susceptible hosts is unlikely. Yet, the prospect of finding a new susceptible host changes throughout an epidemic. In the early stage of an epidemic susceptible hosts are abundant and virulent pathogens that invest more into horizontal transmission should win the competition. Later on, the spread of the infection reduces the pool of susceptible hosts and may reverse the selection on virulence. This may favor benign pathogens after the acute phase of the epidemic. We model this transient benefit for virulence and predict both the epidemiology and the evolution of pathogens during an epidemic. To put these predictions to the test we monitor the competition of the temperate bacterial virus λ and its virulent mutant λcI857 in experimental epidemics. In a second experiment we used the same experimental system to study the effect of spatial structure on virulence evolution. Theory predicts that spatial structure may limit access to susceptible hosts and favor less virulent strategies. Our experimental results agree remarkably well with our theoretical predictions. This demonstrates the ability of evolutionary epidemiology to predict selection for virulence in ongoing epidemics.

ESVV Plenary Session 4

Wednesday 2nd September 2015
08:30-09:15
Auditorium Pasteur

Plenary keynote 6:

Realtime surveillance and risk assessment

Prof Beatriz Martínez-López

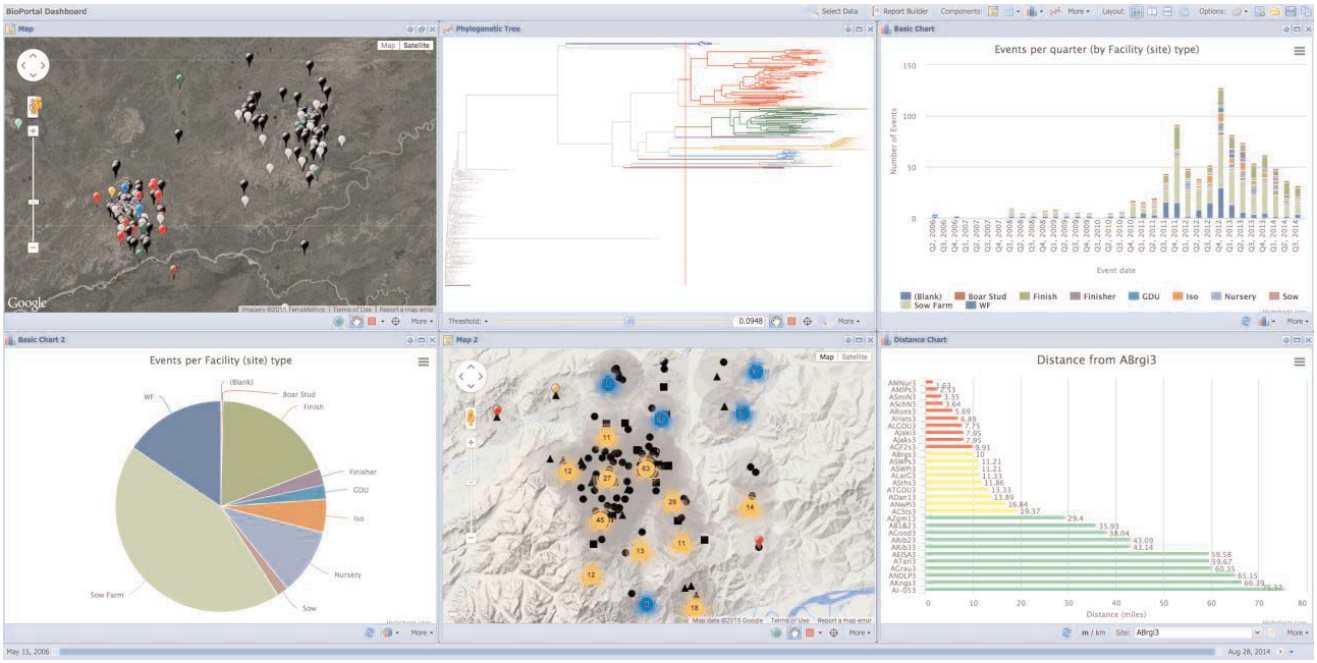
Center for Animal Disease Modeling and Surveillance, Department of Medicine and Epidemiology, School of Veterinary Medecine, University of California (CADMS), Davies, California, United States

Infectious diseases of animal health importance, such as foot-and-mouth disease (FMD), African swine fever (ASF), avian influenza (AI), Rift valley fever (RVF) or porcine reproductive and respiratory syndrome (PRRS) can have devastating economic implications for the affected regions or countries. To prevent or minimize the disease impact, timely decisions need to be made for the rapid prevention, management, control and eradication of such diseases. Ideally, those decisions need to be informed by reliable data collected by effective surveillance systems as well as by scientific-based epidemiological analyses, risk assessments and models based on the most reliable and current data. However, most of the epidemiological analyses, risk assessments and models conducted so far need substantial amount of time and effort, mainly to gather and integrate the needed information, and are difficult and time consuming to update if the epidemiological scenario changes (e.g. introduction of the disease to new areas, implementation of zoning, vaccination or trade restrictions, changes in policies, etc.), which limits their potential use to inform decision making in a timely manner. Therefore, if decisions about animal health need to be made continuously and rapidly (particularly, in crisis time), why there are no tools that allow to inform and support those decisions with the same velocity? Here we aimed to fill this gap by develop-

ing innovative, accessible and user-friendly tools for the Big data analytics and real-time risk assessment and modeling of infectious diseases within the web-based platform known as Disease BioPortal® to support timely decisions in animal health.

Big data analytics may be defined as the process of examining large data sets that are characterized by the three Vs (i.e., their variety of data types, their volume –*big* data- and their velocity of change) with the objective to uncover hidden patterns, recognize unknown correlations or trends, identify risks or improve efficiency of an industry, organization or company. While in areas such as marketing, medical insurance or financial transactions, the use of Big data analytics is becoming increasingly attractive and widely used to assess risks, their use in animal health and veterinary epidemiology remains circumstantial. Real-time surveillance, risk assessment and modeling systems allow the early detection of a specific disease or prediction of increments in the risk for a given disease in a targeted susceptible population (Thurmond, 2003). The cost-efficiency of those systems is defined by its maximum temporal sensitivity (i.e. ability to detect an infectious agent in real-time or near real-time), while using minimal personal and financial resources. However, the main challenges to achieve these goals are 1) to access and connect all information necessary in a timely manner and 2) being able to properly handle and analyze such information in near real-time. In other words, we need integration with Big Data analytics.

Disease BioPortal®, is a user-friendly, web-based information technology (IT) system for near real-time and secure access, sharing and analysis of local, regional and global disease information for animal disease surveillance and modeling (Perez et al., 2009). Since 2007, CADMS team, in collaboration with multiple researchers and collaborators of national and international institutions have been continuously improving and expanding the analytical capabilities Disease BioPortal® for the integration, secure access, sharing, visualization, analysis and modeling of multiple streams of information (e.g., field, lab, formal and informal disease notifications, environmental and climatic information, news, etc.). Currently the system includes multilevel (e.g., animal, site, county, state, country, regional, global-level) and space-time-genomic surveillance capabilities of more than 120 diseases and syndromes. The platform can be freely accessed and used after registration at: http://bioportal.ucdavis.edu/. Publicly available databases are currently accessible to all registered users. Additionally, access to confidential databases is individually granted with different levels of secure-access and visualization for participating producers, labs, veterinary practitioners and other stakeholders. It also incorporates automation of diagnostic data transfer, including pathogen's molecular/genetic data, from national and international reference laboratories for diseases such as FMD, PRRS, PED, AI, RVF, ASF or finfish diseases, and links to diverse national and global databases (i.e., OIE-WAHID, EMPRES-i, NAHLN, PANAFTOSA, WHO, GenBank, FMD News, PED News, ASF News, IRD, etc.). It also incorporates advanced analytical methods very useful for infectious disease epidemiology such as cluster analysis, social network analysis, phylogenetic models, time-series analysis, and space-time-genomic visualization methods. Depending on the level of access, the user can visualize and download data in different formats: 1) tabular, which includes: events (e.g., test results of the reference labs, animal movements, production information, etc.), date, farm location (i.e. coordinates), type of facility, disease, strains, address, etc.; 2) maps (linked to Google maps and Google Earth); 3) graphs (columns, pie, bars, phylogenetic trees, contact networks, homology and distance pareto charts for genetic and geographic distance visualization, etc.). Parameters and variation over time can be dynamically evaluated using different criteria cut-off and the temporal and genetic slide bar. All the aforementioned information can be combined for the exploration of the spatial and temporal distribution of cases, genetic and geographic distance and evaluation of high risk contacts among farms. The user can select and save different displays of one or more datasets, using maps, phylogenetic trees, graphs, distance pareto charts (i.e. proximity analysis), homology tables, etc. (**Figure 1**). All this information can be summarized in customized reports using the Report Builder tool that allows to easily generate reports to inform producers and other stakeholders periodically.



Fi***gure 1****: Example of Disease BioPortal® Dashboard: Customized visualization of spatial, temporal and genetic properties of PRRS virus isolated into three productions systems in the US using maps, dynamic phylogenetic trees, graphs and distance pareto charts.*

Here, we will illustrate the characteristics and performance of the new Big data analytics and real-time risk assessment and modeling capabilities within Disease BioPortal® using ASF in Europe and PRRS and PED in the US. We will also discuss the limitations, challenges and future directions for the implementation of real-time risk assessment and modeling tools to support animal health policies locally and globally.

References

Thurmond, M.C., 2003. Conceptual foundations for infectious disease surveillance. J Vet Diagn Invest 15, 501-514

Perez A, Alkhamis M, Carlsson U, Brito B, Carrasco-Medanic R, Whedbee Z, Willeberg P. Global animal disease surveillance. Spat Spatiotemporal Epidemiol. 2011 Sep;2(3):135-45

EPIZONE Opening Session

Wednesday 2nd September 2015
13:15-15:00
Auditorium Pasteur

Plenary keynote 7:

The impact of evolution and ecology on host species jumps

Dr Pablo R. Murcia

University of Glasgow, Centre for Virus Research, Glasgow, United Kingdom

RNA viruses change continuously by mutation, recombination and reassortment. They often cause spillover infections (single infections with no onward transmission) in humans or other animals (such as influenza H5N1 and H7N9 in humans), and occasionally they cause pandemics (e.g. human immunodeficiency virus, influenza A virus) and panzootics.

Understanding the rules that govern viral emergence is extremely challenging due to the multifactorial nature of cross-species jumps. Molecular, evolutionary and ecological factors are central players during viral emergence and they are likely to vary depending on the viruses, species and ecosystems involved.

The horse is a natural host to several different RNA viruses that have arisen as a result of cross-species transmission. Furthermore, its genome -together with the genome of other equid species- has been sequenced and annotated. Therefore, by studying equine viruses and equid genomes we can gain important insight on the mechanisms that underpin viral emergence.

I will discuss how equine influenza viruses (EIVs) evolve at different levels (from the individual to the global scale) and how evolution might have played a role in the interspecies transmission of an EIV into dogs and its further establishment as a canine influenza virus. Second, I will show results that support the hypothesis that cross species jumps are indeed very rare events, as spillover infections of avian influenza viruses into horses seem to be common in some areas of Asia despite the lack of detection of novel avian-like EIVs, highlighting the importance of host species barriers to avoid viral emergence. Third, I will discuss preliminary data on the analysis of endogenous retroviruses (ERVs) within equid genomes that shows how viral invasions have shaped the genome of the modern horse. This will illustrate the dynamic coevolution of horses and their viruses as well as the importance of ecological transitions on the epidemiology of viruses due to changes in population structure.

Plenary keynote 8:

Elusive Coronavirus Vaccines: Strategies for PEDV Vaccines and Lessons from TGEV Vaccines

Prof Linda J. Saif

Food Animal Health Research Program, CFAES, Ohio Agricultural Research and Development Center, Dept of Vet Prev Med, The Ohio State University Wooster, OH 44691 - United States

Introduction. The alphacoronavirus porcine epidemic diarrhea virus (PEDV) emerged in the US in April 2013 as a devastating diarrheal disease in swine (1). It spread rapidly, infecting swine of all ages, but with highest mortality in newborn pigs. At least two PEDV strains have been identified since its emergence in US swine: 1) The original highly virulent PEDV strains, causing 50-100% mortality in nursing piglets; and 2) a variant that has insertions and deletions (INDEL) in the spike (S) protein, designated as "S-INDEL" PEDV (2-4). The latter reportedly causes milder disease in the field and in some experimental challenge studies (2, 3, 5-7, Lin et al, submitted, 2015). Recently the milder S-INDEL strains have also been reported in fattening swine in Europe (6,7). Genetically, the highly virulent US PEDV strains resemble recent virulent strains from China (China AH2012) (4,8). For unknown reasons, vaccines based on historic European and Asian PEDV strains failed to control the recent virulent PEDV outbreaks in Asia (9).

Maternal vaccines to prime lactogenic immunity to enteric infections. Like the alphacoronavirus transmissible gastroenteritis virus (TGEV), PEDV also infects intestinal villus enterocytes causing severe villous atrophy and dehydration, resulting in fatal (80-100% mortality) diarrheal disease in seronegative suckling piglets (2,10). The focus of vaccination strategies for both diseases is on induction of mucosal immunity to protect the target intestinal enterocytes. This requires protective levels of intestinal immunity in the sow and in her suckling piglets. Maternal vaccination strategies against TGEV to achieve intestinal immunity in the sow and to induce lactogenic immunity (passive transfer of antibodies via colostrum and milk) for nursing piglets will be reviewed (10,11). These vaccination principles are also applicable against emerging enteric infections such as PEDV. TGEV research has provided both a basic understanding of the concept of lactogenic immunity, as well as vaccination strategies for its induction in seronegative pregnant swine. Our discovery of the gut-mammary SIgA axis (trafficking of IgA immunocytes from the gut to the mammary gland) in swine was a predecessor for the concept of a common mucosal immune system (12, 13). It provided an explanation for why sows that recovered from TGEV infection, or ones orally inoculated with live TGEV, had high persisting levels of IgA antibodies in milk that protected their piglets from TGEV. However sows systemically immunized with inactivated or subunit TGEV vaccines had mainly IgG antibodies that declined rapidly in milk and provided little passive immunity to piglets (10).

Vaccines to boost intestinal and lactogenic immunity. Subsequent studies of enteric virus vaccines (TGEV, rotavirus in swine; polio in children) suggested that after effective priming of the gut by natural infection or oral live vaccines, parenteral booster vaccines, including subunit or inactivated vaccines, could enhance and maintain

mucosal or lactogenic immunity (10,11,14). The oral prime/parenteral booster approach may explain why such parenteral vaccines are effective in some senarios in sows recovered from TGEV or rotavirus infections or after use of oral live vaccines.

PEDV vaccine development. The need for oral live vaccines to prime gut immunity in sows requires development of attenuated strains of PEDV. This is accomplished by serial passage of PEDV in cell culture as was done for prior Korean and Japanese PEDV vaccines (9). However adapting the highly virulent PEDV strains to growth in cell culture remains challenging. Alternatively the use of infectious clones of PEDV and reverse genetics will allow targeted mutations in virulence genes and the engineering of genomic changes that limit PEDV replication in vivo, to rationally attenuate PEDV (15,16, Beall, Baric, Wang, Saif, 2015, submitted).

In spite of such advances, many unanswered questions remain about development of TGEV or PEDV vaccines to induce lactogenic immunity. They include: 1) Level of attenuation of the vaccine required to infect the gut of the sow, but not induce disease and still evoke active (sow) and passive (piglets) immunity; and 2) Role of parity, sexual maturation (gilts), stage of pregnancy and virus dose in stimulation of the gut-mammary SIgA axis and induction of SIgA antibodies in milk. The devastating effects of PEDV should provide the incentive to address these fundamental questions related to enteric viral vaccines.

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EPIZONE Plenary Session

Thursday 3rd September 2015
09:00-09:45
Auditorium Pasteur

Plenary keynote 9: **New development in diagnosis of viral infections**

Dr Jean-Claude Manuguerra

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The detection and identification of viruses or of the trace they leave on the adaptative immune system are pivotal in most viral infections, in particular in chronic or severe illnesses, for patient care or for outbreak control in humans and in animals. The qualities of the ideal diagnostic assay vary according to what is expected and where it is performed. However, diagnostic assays should anywhere be Affordable, Sensitive, Specific, User-friendly and Rapid (ASSUR). The first criterion to set is the level of information required, which is different for clinical or for epidemiological interventions. For the former, virus species identification is generally sufficient although it

may sometimes be useful and important to determine drug resistance levels. For the latter, it is often necessary to determine partial viral genomic sequences in order to address the origin of the outbreak and to analyse chains of transmissions. Except in outbreak situations, an array of possible aetiologies have to be investigated at once, either by a number of parallel single target assays or by multiparametric tests. Although ELISA based virus detection assays remain interesting, nucleic acid based assays have established themselves as leaders in multiplex detection, mainly under the form of multiplex (RT)-qPCR but also to a lesser extent DNA resequencing micro-arrays and soon Next Generation Sequencing (NGS). These are expensive and sophisticated technologies requiring specialised reading devices and are limited to central reference laboratories. On the contrary, field laboratories need not only ASSUR assays but also robust assays compatible with degraded conditions of work such as heat, dust, electrical instability and these assays must be independent of any chain of cold. Among technologies gathering these extra necessary qualities are (RT)-LAMP and (RT)-RPA.

In this presentation, examples will illustrate the spectrum of virological and serological diagnostics available in a wide variety of settings and circumstances, including points of care.

PARALLEL SESSIONS

Parallel ESVV Session 1: Virus evolution

Tuesday 1st September 2015
11:00-12:30
Auditorium Pasteur

Chair: Trevor Drew & Emmanuel Albina

Genetic characterization of a distinct worldwide-spread genetic lineage of Infectious Bursal Disease virus

Ruben Pérez [1],Martín Hernández [1], Gonzalo Tomás [1], Ana Marandino [1], Gregorio Iraola [1], Diego Hernández [1], Pedro Villegas [2], Alejandro Banda [3], Yanina Panzera [1].

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Infectious bursal disease virus (IBDV) is an avian virus (Avibirnavirus, Birnaviridae) that constitutes one of the most concerning sanitary problems to the world poultry production. The viral genome is a double- stranded RNA molecule of two segments, A and B, of 3.3 and 2.9 kb, respectively. IBDVs comprise four well-defined evolutionary lineages known as classic (c), classic attenuated (ca), variant (va) and very virulent (vv) strains. Here, we analyze IBDVs from South America (Uruguay) by the genetic analysis of both segments of the viral genome. Comparative phylogenetic and a discriminant analysis of principal components (DAPC) were performed with global strains. Viruses belonging to c, ca, and vv strains were unambiguously classified by the presence of molecular markers and phylogenetic analysis of the hypervariable region of the vp2 gene (segment A).Most characterized viruses could not be accurately assigned to any of the previously described strains, and were then denoted as distinct (d) IBDVs. These dIBDVs constitute an independent evolutionary lineage that also comprises field IBDVs from America, Europe and Asia. The hypervariable VP2 sequence of dIBDVs has a unique and conserved molecular signature (272T, 289P, 290I and 296F) that is a diagnostic character for classification. DAPC also identified the dIBDVs as a cluster of genetically related viruses that is highly divergent separated from the typical strains. The vp1 gene (segment B) of the dIBDVs has non-vvIBDV markers and unique nucleotide and amino acids features that support their divergence in both genomic segments. The analysis of complete genomes provides further evidences of the divergence of this lineage, suggesting that the dIBDVs comprise a neglected, highly divergent lineage that has been circulating in the world poultry production since the early time of IBDV emergence.

Characterization and phylogenetic analysis of cetacean morbillivirus infection in eastern Atlantic-based cetacean populations

Carolina Bento [1,5], C. Eira [2,5], J. Vingada [3,5], M. Ferreira [3,5], A. Lopez, [2,4], L. Tavares [1], A. Duarte [1]

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5: Portuguese Wildlife Society, Department of Biology, Minho University, 4710-057 Braga, Portugal

Objective: The objective of the present study was to determine the prevalence of morbillivirus in cetacean populations from the eastern Atlantic and to investigate the relationship between morbillivirus strains circulating in the eastern Atlantic and elsewhere in the world, especially in the Mediterranean. For this purpose, tissue samples from 325 animals stranded in Continental Portugal and Galicia (Spain) were tested by reverse transcription-quantitative PCR (rt-qPCR) for morbillivirus RNA. Positive samples were used to amplify longer genomic regions by conventional one step rt-PCR for phylogenetic analysis.

Methods: Tissue samples from 325 cetaceans stranded in the Portuguese and Galician coasts were collected during necropsies and stored at -20°C in RNAlater®. Samples were collected between 2007 and 2014, and common dolphins (Delphinus delphis; n=193), striped dolphins (Stenella coeruleoalba; n=69), harbor porpoises (Phocoena phocoena; n=47) and bottlenose dolphins (tursiops truncatus; n=7), pilot whale (Globicephala melas; n=5) were tested, among other species that occur less frequently (Fin whale n=2; True's beaked whale n=1; Pigmy sperm whale n=1). Total RNA was extracted from a pool of tissue homogenates including lung, brain, pulmonary lymph node and mesenteric lymph node, using RNeasy mini kit (Qiagen, GmbH, Germany). RNA quantification was performed using the Nanodrop 2000C (ThermoScientific). The detection of antigen was performed by rt-qPCR in a StepOnePlus thermocycler (Applied Biosystems). The primers and probes used were previously described by other authors. For positive samples, total RNA was extracted individually from each of the available organ and the infection was mapped in the different organs. Additional sequences were amplified from the positive samples by conventional one step rt-PCR using previously described primers, targeting the F, H, N and P gene. The amplicons obtained were sequenced (Stab Vida) and the nucleotide sequences identity compared in a Blast analysis (NCBI). Available CeMV sequences were retrieved and phylogenetic analysis was performed.

The concatenated nucleotide and amino-acidic sequences of the Portuguese and Galicia isolates were compared together with selected DMV sequences and outgroup taxa (Measles Virus; CDV) retrieved from GenBank (table 3), according to their primary structure similarity using the multiple alignment ClustalW program. Only viral sequences including the same genomic range were used. Phylogenetic trees were inferred by Maximum Likelihood and Bayesian methods, PhyML 3.0 and MrBayes v.3.2.1 programs respectively. A phylogenetic tree based on the P gene was also constructed, with a higher number of available CMV sequences, covering a wider temporal and geographical sampling.

Results: A total of 16 positive samples were identified by rt-qPCR (prevalence of 4.9%). From the Portuguese coastline 8 positive animals were detected (6 striped dolphins and 2 common dolphins): SC/15/2007, SC/257/2011, SC/221/2012, DD/302/2012, SC/11/2013, DD/191/2013, SC/193/2014 and SC/290/2014. From Galicia 8 positive striped dolphins were detected: SC/21/2007, SC/24/2008, SC/31/2009, SC/42/2010, SC/49/2011, SC/51/2012, SC/53/2012 and SC/55/2012. Three of the positive striped dolphins from Portugal were positive only in the brain, while for animal SC/15/2007 only lung was tested and for animal SC/221/2012 all the organs available tested positive (lung, brain, pulmonary and mesenteric lymph nodes, kidney and liver). Animal SC/193/2014 was positive in the tissue pool but it was not possible to map the infection in the available organs. Regarding the common dolphins, animal dd/191/2013 tested positive in all the tissue samples tested (lung, pulmonary lymph node, mesenteric lymphnode, brain, kidney and liver) and dd/302/2012 was positive in the lung and pulmonary and mesenteric lymph nodes but not in the brain, liver or kidney. The prevalence among the striped dolphins tested (n=69) was the highest among all species reaching 20.3%. In common dolphins prevalence was 1% with a significant difference in prevalence between striped and common dolphins [P=0.039 for a significance level of 0.05]. Positive animals were detected every year, from 2007 to 2014 in striped dolphins and only in 2012 and 2013 in common dolphins. Striped dolphins stranded in Galicia had a detected prevalence of 24.2% against 16.7% in Portugal. A concatenated phylogenetic tree was constructed using the nucleotide and amino acidic sequences of SC/15/2007 (Portugal), SC/257/2011 (Portugal), DD/302/2012 (Portugal), DD/191/2013 (Portugal), SC/53/2012 (Galicia) and SC/55/2012 (Galicia), together with available DMV viral sequences, with the same genomic range.

In the nucleotide concatenated tree, sequences from Portugal and Galicia obtained in 2011 and afterwards were included in the same phylogenetic branch; samples from the Mediterranean and Portugal from 2007 and prior were grouped in a different branch. This temporal assemblage was supported high bootstrap (BS) (954) and posterior probability values (PP) (1). One sample from the Mediterranean from the 90's outbreak was separated from the three samples from 2007 (SC/15/2007 from Portugal and two DMV samples from the Mediterranean) with BS/PP value of 753/0.63. Looking at the more recent samples (≥2011), one sample (SC/257/2011) was sepa-

rated from the others with BS/PP values of 962/0.97, corresponding to an animal stranded in Portugal in which viral antigen was only detected in the brain.

Looking at the nucleotide tree for the P gene the temporal assemblage is still present, with samples from 2007 and prior from Portugal and the Mediterranean grouping in a separate branch from those from 2011 and after (BS/PP 755/0.69). The only exceptions were two animals from 2011 (one from Portugal) that were included in the 2007 samples branch. Both of these samples were obtained from brain tissue, being the only positive tissue sample from the Portuguese animal.

Conclusion: In this study we surveyed 326 animals and found that the prevalence of DMV (viral RNA) is higher among striped dolphins (20.3%) than common dolphins (1%) in the Atlantic based populations. Prevalence among striped dolphins from Galicia was 24.2% and this prevalence is probably underestimated because the only available organ to test was the lung. Prevalence in striped dolphins stranded in Portugal was 16.7%.

The most important feature observed in the phylogenetic trees was the grouping of viral sequences according to their collection date: this temporal arrangement appears to be independent of the geographic origin of the samples (Atlantic or Mediterranean) and may suggest a pattern of temporal variation. In the P gene tree the same temporal arrangement was observed, although a smaller genomic region was analyzed and a higher number of sequences was included, covering a wider temporal and geographical sampling. However, animal sc/257/2011 assembled within the same branch as the 2007 samples, contradicting the overall arrangement. This repositioning may be due to the fact that this animal was positive only in the brain, suggesting a chronic infection and explaining why it clusters with samples from previous years, further away from more recent samples.

Cetacean morbillivirus (dolphin morbillivirus) seems to be endemic in cetacean populations from the eastern Atlantic, with positive animals detected annually since 2007 without being associated with a rise in stranding rates. Prevalence among striped dolphins is significantly higher than in common dolphins, suggesting a higher susceptibility in these animals, similarly to what was observed previously in the Mediterranean. The phylogenetic analysis suggested a temporal evolutionary pattern, supported by high BS/PP values. This phylogenetic arrangement potentially implies striped and common dolphin populations from the eastern Atlantic as a source of CMV infection for the Mediterranean cetacean populations.

Development of a bioinformatics pipeline to identify and characterise minor variants in Foot-and-mouth disease virus populations using data generated from the Illumina MiSeq

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Objective: RNA viruses, such as Foot-and-mouth disease virus (FMDV), exist as heterogeneous populations known as 'quasispecies', comprising of related but non-identical genomes. Such complexity is due to their high replication rate, large population size and error-prone replication. Whole genome consensus level sequencing identifies the most dominate viral species however traditional sanger sequencing cannot identify minor variants which exist within the viral swarm population, the importance of which remains unclear in relation to FMDV evolution and transmission. Using sequence data generated from the Illumina MiSeq platform from a panel of different FMDV serotype viruses, a bioinformatics pipeline was developed, which was designed to identify and remove host reads, generate consensus sequence, coverage plots and variant data.

The aim of the project was to develop a bioinformatics pipeline which generates accurate consensus sequences and able to identify minority variants of FMDV (and indeed other Picornaviruses). The pipeline was optimised from data produced from a panel of different FMDV serotypes; variant data generated were used to develop a snapshot of population diversity.

Methods: Raw read data generated from the MiSeq from a panel of FMDV viruses was passed through a bioinformatics pipeline. A read aligner called fastq_screen was used to identify and remove host and contaminating

reads, low quality reads were removed using sickle. The performance of different de-novo assembler methodologies was compared (IDBA_hybrid, Velvet and Lasergene Ngen) to generate a closest reference consensus sequence. Reads were aligned to this reference to obtain the most complete genome sequence and consensus sequences and coverage plots were generated. Finally the performances of a number of low frequency variant callers were compared, including Lofreq, VICUNA and ViQuas, to identify minor genomic changes.

Results: Using this pipeline, host reads were identified and viral consensus sequences and variant data generated. Comparison of different de-novo assembler methods determined that IDBA_hybrid produced the longest and most accurate FMDV contigs on average and was the least computational intensive. Variant analysis against a panel of different FMDV viruses allowed us to develop an estimate of processed introduced biases. Analysis was performed and compared using different variant callers on sequence data characterising polymorphism frequency. When applied to a dataset generated from 7 animals from a single herd during an FMDV outbreak in the UK in 2007 we identified 109 variant sites, 45 sites contained non-synonymous nucleotide changes to the viral protein coding regions. In total 29 of these sites were found to be shared between 2 or more different viral samples.

Conclusion: The importance of accurate assemblies is critical to generate reliable consensus sequence and variant data. Host reads can be characterised, which (if not known) allows for the identification of the infected host species. Identifying variant data will provide important insights to the strategies for reconstructing patterns of transmission and monitoring future outbreaks.

Keywords: Next Generation Sequencing, Beyond the Consensus, Food-and-Mouth Disease Virus, Bioinformatics.

Comparative molecular epidemiology of the two closely related bovine coronavirus (BCoV) and human coronavirus OC43 (HCoV-OC43) revealed different evolution dynamic pattern

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Objective: Bovine coronaviruses (BCoVs) are widespread around the world. These highly contagious viruses cause enteric infections such as winter dysentery and neonatal calf diarrhea. BCoVs are also responsible for respiratory infections. BCoVs are closely related to the Human Coronaviruses OC43 (HCoV-OC43s). Genomes of these two coronaviruses share more than 96% of nucleotide identity. The time to Most Recent Common Ancestor (tMRCA) of these two coronaviruses is estimated around 1900. Previously, we conducted a phylogenetic study based on the complete sequencing of the three nsp12, S and N genes from 15 HCoV-OC43 collected during a 12-year period (2001 to 2013) in Lower-Normandy, France. We showed the co-circulation of non-recombinant and recombinant genotypes among the species of HCoV-OC43. We also observed a 12-nucleotide deletion among S genes of some HCoV-OC43s, which is also observed in S genes of all available BCoV sequences. S gene sequences of HCoV-OC43 containing this deletion are grouped in a cluster that is closer to BCoV group than other HCoV-OC43s. Due to the high overall similarity of HCoV-OC43 and BCoV genomes, we wondered if a similar evolution dynamic pattern could be observed between these two coronaviruses. We then conducted a phylogenetic analysis similar to those previously conducted on HCoV-OC43, with fecal samples collected from bovine during a same period, at the same place, with a similar methodology.

Methods: Nine BCoV positive fecal samples collected from diarrheic cows in Lower-Normandy during a 9-year period (2005 to 2014) were included in this analysis. A BCoV isolated in France in 1997 and cultivated on HRT18 cells was used as control. The entire sequence of nsp12, S, and N genes of the 10 selected BCoVs were determined. Three alignment based on nsp12, S and N genes are constructed from 59 sequences, including the 10 sequences obtained in this study, 16 HCoV-OC43s from Lower-Normandy (France) previously sequenced by our team, and 33 sequences available in GenBank as follow: 16 BCoVs from USA, Quebec and Japan, 11 bovine-like

coronaviruses from USA, three dromedary camel coronaviruses (DcCoV) from Qatar, two Porcine hemagglutinating Encephalomyelitis Virus (PHEV) from USA, and one Human Enteric Coronavirus (HEC) from Germany. The phylogenetic trees corresponding to nsp12, S and N genes were constructed using the neighbor joining method. An additional neighbor joining S tree was constructed; including 124 sequences of BCoV and bovine-like coronaviruses from Asia (China, Japan, Korea), America (USA, Quebec, Brazil) and Europe (France, Swedish, Denmark, Italy, Germany). Sixteen sequences of HCoV-OC43 from Lower-Normandy were also included in this tree.

Results: Among the 59 sequences of the three nsp12, S and N alignments, we focused on nine BCoVs and nine HCoV-OC43s sequences collected in Lower-Normandy from 2005 to 2014. The lowest percentages of homology from the nine field BCoVs collected from 2005 to 2014 are 99.18%, 98.54% and 99.12%, for nsp12, S and N genes, respectively. The numbers of polymorphic sites for these 3 genes are respectively 18, 51 and 10. The lowest percentage of homology from the nine clinical HCoV-OC43s collected during the same period, at the same location are 99.11%, 96.94%, and 97.9% for nsp12, S, and N genes, respectively. The numbers of polymorphic site associated to these 3 genes are respectively 15, 248, and 32. Focusing on the 16 HCoV-OC43s sequences of the S alignments, we observed the occurrence of 7 deletions from 3 to 15 nucleotides leading to a variation of S gene length from 4062 to 4092 nucleotides. No such deletion was observed when we compared the 27 sequences of BCoV S gene, which has a unique length of 4092 nucleotides. No deletion neither length variation was observed among nsp12 and N genes of BCoV and HCoV-OC43 sequences. The three trees constituted from alignment of nsp12, S and N genes sequences are characterized by a high similar topology. Three clusters could be observed among the 38 BCoVs and bovine-like coronaviruses. The cluster 1 contains the reference strains and roots the remaining BCoVs and bovine-like coronavirus. These last are distributed into two clusters: the cluster 2 includes BCoV and bovine-like sequences from USA; and the cluster 3 includes the BCoV sequences from Lower-Normandy (France). The 3 DcCoVs are located on the cluster 3 on nsp12 and N trees. They constitute an outlier group that roots all BCoVs on the S tree. To obtain more information, we constructed an additional S tree including 140 sequences of BCoV, bovine like coronavirus and HCoV-OC43 from different geographic area in Asia, America and Europe. The topology of this additional S tree revealed three clusters among BCoV sequences that were supported by high bootstrap values. The cluster 1 incudes 9 references, avirulents and vaccinals strains. The cluster 2 includes 61 BCoVs and bovine-like sequences from Asia (Japan, Korea, China) and America (USA, Canada, Brazil). The cluster 3 includes 49 sequences from Europe (Swedish, Denmark, Italy, France). A fourth cluster including only two French sequences is observed, but not reliable due to a low bootstrap value.

Conclusion: HCoV-OC43 and BCoV are two closely related coronaviruses with an overall percentage of homology of more than 96%. They both emerged from a tMRCA estimated around 1900. We recently identified a cluster of HCoV-OC43 more closely related to BCoV than those constitute by other HCoV-OC43s. This cluster seems to have emerged earlier than other HCoV-OC43. Based on all this observation, we supposed than a molecular epidemiological study of BCoV would reveal to us a similar intra-specific variability among the species of BCoV than those of HCoV-OC43. However, our sequences analysis based on nsp12, S and N gene sequences from BCoVs and HCoV-OC43s collected during a same period (2005 to 2013) in Lower-Normandy revealed that BCoV sequences are more conserved than HCoV-OC43 sequences, particularly on S gene. The intraspecific variability is then less important among BCoVs than among HCoV-OC43s. The phylogenetic analysis does not reveal any recombination event among BCoVs, in comparison to HCoV-OC43s. The topologies of the four neighbor joining trees clearly show that field BCoVs are distributed into two clusters: the cluster 2 including sequences from America and Asia; and the cluster 3 including sequences from Europe. The three DcCoV collected in Dubai (Qatar) in 2013 are located on the cluster 2 on nsp12 and N trees. This gives us insight of a potential emergence of DcCoV from European BCoVs. The results of this study revealed us that a geographical intraspecific diversity seems to exist among BCoVs. No such geographic distribution was observed during a similar study conducted on HCoV-OC43. Asian and America BCoVs seem to have a common origin. The low variability of BCoVs sequences could be linked to the environment of bovines, particularly at modality farmed of herds.

Fitness evaluation and molecular characterization of a recombinant murine norovirus during serial passages in cell culture

Edmilson Ferreira De Oliveira Filho [1], Elisabetta Di Felice [1,2], Barbara Toffoli [1], William Zonta [1], Ana Carolina Terzian [1], Elizabeth Mathijs [1], Etienne Thiry [1], Axel Mauroy [1]

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Objective: Viral recombination can dramatically change virulence properties of the viruses and has been evidenced in silico for different human NoV strains isolated from clinical cases. Previously, a recombinant Wu20/CW1 strain was obtained after in vitro coinfection of RAW264.7 cells with parental MuNoV strains CW1 and Wu20 (Mathijs et al 2010). The recombinant strain showed reduced plaque size compared to the parental strains and it was suggested that this was due to modified virulence properties in vitro. The aim of this study was to observe and molecularly characterize the natural genetic evolution of the recombinant MuNoV strain across in vitro replications.

Methods: MNV strains used in this study were CW1, WU20 (Thackray et al., 2007, kindly provided by prof. H. Virgin) and Rec MNV (Mathijs et al., 2010). RAW 264.7 cells (ATCC TIB-71) grown in Dulbecco's modified Eagle's medium (Invitrogen) complemented (DMEMc) with 10 % heat inactivated FCS (BioWhittaker), 2 % penicillin (5000 U /ml) and streptomycin (5000 mg/ml) (PS; Invitrogen) and 1 % HEPES buffer (1 M; Invitrogen).

The recombinant strain was serially replicated in vitro in RAW264.7 cells (up to 14 passages). RAW 264.7 (Mouse leukaemic monocyte macrophage) cells were infected with MNV for 72 hours and afterwards lysed by freeze and thaw and viruses purified by ultracentrifugation of both cells and supernatant. Viral plaque sizes of early and late progenies (30 for each virus) were compared with the Image J software. The experiment was repeated two times. RNA was extracted from 140 ml purified suspension 1:5 diluted using the QIAamp Viral RNA Mini KitTM (Qiagen) according to the manufacturer's instructions. cDNA was generated using a poly-A primer tagged GCCAACGACCGGGAGGCCAGC(T)20 previously described (Müller et al 2007) using superscript ii reverse transcriptase kit (Invitrogen®) treated with RNase H or with other antisense primers using iScript select kit (Bio-Rad®). For the genetic characterization two different studies were conducted. The first study aimed to develop a sequencing strategy in order to obtain the complete genome of the recombinant MNV. Then, in the second study, sequences obtained from different viral passages into RAW cells (e.g. P5 and P14) were compared in order to study the viral adaptation. Primers were designed using the Primer Express® software and netprimer® (Premier biosoft). PCR was performed using taq polymerase with thermopol buffer (new England biolabs) as per manufacturer's instructions. Afterwards, fragments were excised from agarose gel and DNA purified using the QIAquick Gel Extraction KitTM (Qiagen) and cloning using the PGEM T easy cloning kit (Promega) plasmid DNA was transferred to sequencing by GATC Biotech (Koblenz, Germany).

Results: The size of the lysis plaque surface of P2 and P14 showed a considerable divergence. The average plaque size increased from the earlier to the later progenies (from 0.1 mm2 to around 0.5 mm2). A significant difference was demonstrated between them with the Mann and Whitney non parametric statistical test. The genetic characterization of the recombinant strain obtained in vitro was previously based on partial genomic sequences, which provided limited information. Accordingly to our initial molecular analysis of 1.5 kb partial genomic sequence comprising the part of the RdRp and the part of the VP1 did not show any genetic modifications between passage 4 (accession number HM044221) and passage 14 recMNV. Therefore, a strategy for sequencing the complete genome of the different MNV strains was established. The genome of the recombinant MNV was divided into seven regions and the amplification was performed using either new designed or previous published primers. Molecular analysis using the nearly complete genome of the recombinant MNV passage 14 and the two parental strains (CW1 and WU20) showed nine modifications in the genome, comprising three aminoacid changes. Accordingly, two modification were in the RdRp region aa position 1384 Glycine (G) instead of Aspartic acid (D) and aa position 1393 Serine (S) instead of Asparagine (N) and one modification was in the capsid region one modification on aa position 296 Glutamic Acid (E) instead of Lysine.

Conclusion: Even preliminary, our data provide evidence of virus adaptation to a new environment (here a cell culture system) after a recombination event. In order to specify whether these hints of genetic mutations could explain fitness modifications during in vitro evolution we need to compare the sequences of passage 14 and the

previous viral cellular passages. In addition, two other parameters of in vitro virulence modification will be investigated: (i) virus production and (ii) growth kinetics. The data should provide interesting information about genetic evolution in the genus Norovirus, especially regarding recombination events and explain how a recombinant strain, first disadvantaged compared to its parental strains, could regain fitness by genetic evolution.

Parallel ESVV Session 2: Influenza

Tuesday 1st September 2015
11:00-12:30
Room Sully 2

Chair: Thierry Van den Berg & Martin Beer

Codon deletions and substitutions in the PA linker of the RNA-polymerase allow generation of temperature-sensitive and attenuated influenza viruses

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The influenza virus RNA-dependent RNA polymerase catalyses genome replication and transcription within the cell nucleus. Efficient nuclear import and assembly of the polymerase subunits, PB1, PB2, and PA are critical steps in virus cycle. We investigated the function of the PA linker (residues 197-256), located between its N-terminal endonuclease domain and its C-terminal structured domain that binds PB1, the polymerase core. A large series of PA linker mutants engineered by codon substitutions and deletions exhibited a temperature- sensitive (ts) phenotype (reduced viral growth at 39.5°C vs. 37°C/33°C). The ts- phenotype was associated to a reduced efficiency of replication/transcription of a pseudo-viral reporter RNA in a minireplicon assay. Using a fluorescent-tagged PB1, we observed that ts- and lethal PA mutants do not efficiently recruit PB1 to reach the nucleus at 39.5°C. For substitution mutants, the selection of revertant viruses (able to grow at restrictive temperature) allowed the identification of different types of compensatory mutations located in one or the other of the three polymerase subunits. No revertant was selected for deletion mutants. Several substitution and deletion ts-mutants were shown to be attenuated and able to induce antibodies in mice. Taken together, our results identified a PA domain as being critical of PB1-PA nuclear import and as a “hot spot” to engineer ts-mutants that could be used to design novel attenuated genetically stable vaccines.

Detection of a new Influenza D Virus in Cattle, France, 2011-2014

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Objectives: Recent studies in the USA have identified a new Genus of the Orthomyxoviridae. The new pathogen, C/swine/Oklahoma/1334/2011 (C/OK), was first identified in pigs with influenza- like illness and was only moderately related to previously characterized influenza C viruses (+/- 50% overall homology between C/OK virus sequence and its closest related sequences). C/OK virus genome was more distant from influenza C virus genomes than influenza A are from influenza B virus genomes. In hemagglutination-inhibition assays, cross-reactivity between antibodies against C/OK virus and human influenza C virus was lacking, which again suggests a new genus in the Orthomyxoviridae family, tentatively named Influenza virus D. C/OK-like viruses also were

isolated from cattle in the United States in 2013 (1). This novel virus was shown to efficiently replicate and transmit in ferrets, the animal model of choice for the study of influenza in human, suggesting that humans could be infected. Because cattle were hypothesized to represent the reservoir for this novel influenza virus, we screened bovine samples in France for influenza D virus and characterized the virus from positive specimens.

Methods: Field specimens were obtained from 134 acute respiratory outbreaks in young cattle, from animals that did not receive antibiotics and sometimes not vaccinated against Bovine Respiratory Syncytial Virus and Parainfluenza 3 virus. These specimens included nasal swabs, trans-tracheal aspiration, broncho-alveolar lavages or post mortem lung tissue samples. They were firstly screened by quantitative RT-PCR or quantitative PCR for well- known respiratory pathogens including bovine respiratory syncytial virus, parainfluenza 3 virus, bovine coronavirus, Pasteurella multocida, Mannheimia haemolytica, Histophilus somni and Mycoplasma bovis, by the Laboratoire Départemental d'Analyses de Saone et Loire (Macon, France). In addition we tested the samples by real-time reverse transcription-PCR (rRT-PCR) for influenza D virus as previously described (Hause BM, Ducatez M, Collin EA, Ran Z, Liu R, Sheng Z, et al., PLoS pathogens. 2013;9(2):e1003176.). Twenty-five archive samples per year for 2010 through 2013 and 34 samples collected from January through March 2014 were tested. The specimen with the lowest cycle threshold (CT value) was selected for further molecular characterization and its full genome was amplified by PCR and sequenced on a 3130XL Applied Biosystems capillary sequencer at the Plateau de Génomique GeT-Purpan, UDEAR UMR 5165 CNRS/UPS, CHU PURPAN, Toulouse, France.

Results: Influenza virus D was detected in 6 samples (4.5%), 5 lung tissue and one nasal swab samples, out of the 134 field isolates collected between 2010-2014 with cycle threshold (CT) values ranging from 15 to 35: one in 2011, one in 2012, and 4 in 2014.Co-infections were detected with Pasteurella multocida, Mannheimia haemolytica, Histophilus somni, bovine respiratory syncytial virus, and/or bovine herpesvirus 1 in 4 of the influenza D–positive specimens. Two samples collected in 2014 were negative for all tested respiratory pathogens, despite reports of clinical signs in the animals.

The French virus complete genome (D/bovine/France/2986/2012) of the sequenced specimen was 94-99 % identical to its American and Chinese counterparts suggesting a common origin of these new influenza viruses. We found no evidence of reassortment between influenza C and D (C/OK-like) viruses. The estimated ranges of evolutionary distances (in number of substitutions per site using the maximum composite likelihood model) between D/bovine/France/2986/2012 and US influenza D viruses ranged between 0.8 and 5.7% and were as follows: 1.9%–2.1%, 0.8%–0.9%, 2.1%–2.5%, 2.3%–2.7%, 1.8%–3.8%, 3.6%–4.2%, and 5.1%–5.7% for polymerase basic (PB) 2, PB1, polymerase 3/polymerase acidic, nucleoprotein, M, nonstructural protein, and HE gene segments, respectively. We also identified unique features in D/bovine/France/2956/2012 genome. In addition, although the hemaglutinin-esterase HE proteins of human influenza C and C/OK viruses contain 7 and 6 potential glycosylation sites, respectively, D/bovine/France/2986/2012 just had 5: at positions 28, 54, 146, 346, and 613 (ATG numbering), identical to 5 of the 6 identified for C/OK virus. The missing potential glycosylation site in D/bovine/France/2986/2012 was located at position 513, probably likely in the F3 = HE2 fusion domain of the protein and not in the globular head of the protein.

Conclusions: We have identified the novel influenza D virus in French cattle with respiratory symptoms from 2011 through 2014. The genome of a representative specimen was closely related to North American swine and bovine influenza D viruses. Further studies are warranted to assess the emergence threat associated with the circulation of influenza D viruses in Europe, and at providing essential information about host range, origin, diversity, and evolution of the emerging pathogen throughout the continent.

Anti-nucleocapsid protein antibody is sufficient to confer resistance to lethal infection with influenza A viruses of several subtypes in transgenic mice

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Objective: The nucleoprotein (NP) of influenza A virus possesses highly conserved amino acid regions among all influenza A viruses, and therefore, it has been attractive targets for developing a universal influenza virus vaccine to induce cellular immunity. However, it is still unclear whether anti- NP antibody can confer the resistance to infections with influenza viruses showing high fatality rates in mammals. In this study, to investigate the antiviral effects of anti-NP human monoclonal antibodies (mAb) in vivo, we generated the transgenic mice expressing anti-NP mAbs derived from lymphocytes of a patient infected with H5N1 HPAI virus, and experimental infections with H5N1 highly pathogenic avian influenza (HPAI) viruses and H1N1 human influenza virus were conducted.

Methods: Peripheral blood from a volunteer who had recovered from H5N1 HPAIV infection in northern Vietnam in 2007 was collected at 12 weeks after the onset of severe respiratory symptoms with high fever. Lymphocytes from the blood were separated on a Ficoll-paque gradient by centrifugation, then, total RNA was extracted. Following that, each cDNA of the whole heavy and light chains amplified by RT-PCR and Fab clones against influenza virus were selected by the phage display method. The heavy chain of each Fab clone was fused with human IgG1 Fc portion. Using the heavy and light chain genes, a plasmid containing the heavy and light chain genes under the control of the CAG promoter in parallel was constructed to express whole human IgG. The constructed plasmid was transfected into 293T cells and then, expression and specificity of whole human IgG were confirmed by Western blot and ELISA. The transgene fragment containing the heavy chain and the light chain of human IgG genes was microinjected into the nuclei of C57BL/6 mouse eggs to generate transgenic mice. Expression levels of human IgG in sera of the transgenic mice were determined by human IgG ready-set-go kit (eBioscience) according to the manufacturer's instructions.

For intranasal viral infections, mice at 6-8 weeks of age were anesthetized by intraperitoneal injection of a mixture of medetomidine (0.3 mg/kg), midazolam (4 mg/kg) and butorphanol (5 mg/kg), and then intranasally inoculated with 40 µl of each virus. Survival and body weight of mice infected with lethal doses (10LD50) of H5N1 HPAI viruses A/mountain hawk-eagle/Kumamoto/1/07 (MHE/Kumamoto/07) and A/duck/Vietnam/G12/08 (G12), and mouse-adapted H1N1 strain PR8 were recorded for 10 days. MHE/Kumamoto/07 belongs to clade 2.2 and possessed PB2-627 Lys identified as the virulence marker to mammals. On the other hand, G12 belongs to clade 2.3.4 and did not possess the virulence marker in PB2-627. Experimental infections with PR8 were carried out in the BSL-2 facility at Center of Biomedical Research, Research Center for Human Disease Modeling, Graduate School of Medical Sciences, Kyushu University and those with H5N1 strains G12 and MHE/Kumamoto/07 were carried out in the BSL-3 facility at Kyoto Sangyo University, respectively. The ethics committees of Kyushu University and Kyoto Sangyo University approved all animal experiment protocols. This study was approved by the Institutional Review Board of National Institute of Hygiene and Epidemiology, Vietnam.

Results: Two Fab clones against influenza virus, named as 5C and 6C, were selected and used in this study, because of their high affinities to the virus, although they were estimated to recognize anepitope by sequencing analyses. To examine the specificity of human IgG (5C and 6C), an immunoprecipitation assay was performed using the lysates of MDCK cells infected with influenza A viruses. Only a band with a molecular mass of approximately 50 kDa corresponding to the size of intact NP protein (53 kDa) was detected by two human mAbs and a mouse anti-NP mAb (C43) used as a control, demonstrating that 5C and 6C antibodies recognized NP. To assess the antiviral potential of anti-NP mAb in vivo, cDNAs coding heavy and light chains of two clones of human IgG (5C and 6C) were injected into mouse eggs and 4 transgenic mouse lines, 5C(2495), 5C(2497), 6C(2647) and 6C(2660) were generated. Western blot analysis revealed that the heavy and the light chain of human IgG were expressed in sera of all founder transgenic mice. Concentrations of human IgG in sera from transgenic mice were 0.2 ± 0.1 for line 5C(2495), 0.4 ± 0.1 for line 5C(2497), 1.6 ± 0.2 for line 6C(2647) and 1.2 ± 0.2 mg/ml for line 6C(2660), respectively. All the antibodies expressed in transgenic mouse lines were confirmed to bind to viral NP of all subtypes (H1-H13) by immunofluorescence assay. In experimental infections with MHE/Kumamoto/07

of H5N1 HPAI virus, survival rate of line 5C(2495), 5C(2497), 6C(2647) and 6C(2660) were 0, 30, 80 and 80%, respectively. In contrast, all of the control littermates died within 10 dpi. These results demonstrated that anti-NP mAb conferred resistance to lethal H5N1 viral infections in transgenic mice although the expression level of antibodies in the mice was of considerable significance to exert antiviral effects. Next, to confirm the resistance to infection with H5N1 HPAI virus of clade 2.3.4 that is predominantly in northern Vietnam in 2007, transgenic mice of line 6C(2660) were inoculated with strain G12. All the transgenic mice survived, on the other hand 64% of control mice succumbed to the infection within 10 dpi. In addition, to assess the resistance of transgenic mice to infection with influenza A virus other than H5 subtype, a mouse-adapted strain PR8 of H1N1 subtype was inoculated into transgenic mice of line 6C(2660). All of transgenic mice survived for 10 days, in contrast, all the control littermate died at 7 dpi. Considering all the results described above, the anti-NP human mAbs could confer the resistance to lethal infections with influenza A viruses of both H5N1 and H1N1 subtypes, indicating that the mAbs possess the potential of heterosubtypic immunity against influenza A viruses.

Conclusion: The present results demonstrate that anti-NP antibodies expressed in transgenic mice exert antiviral effects against infection with influenza A viruses of different subtypes in vivo.

Mutation of Serpine1 in mice results in increased susceptibility to influenza A virus infection due to increased vascular leakage and virus dissemination

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Objective: Influenza A viruses (IAV) cause a contagious respiratory disease in human and animals. Besides intensive studies on the virus and its virulence factors, the biological mechanisms of the host-pathogen interactions are still only partially understood. Recent studies showed that IAV alter the plasminogen conversion pathway after infection, which induces hyperfibrinolysis and results in lung injury. Serpine1 (PAI-1) is a member of the serine protease inhibitor (serpine) family that inhibits plasminogen activators, which reduce the fibrosis in lung. Deficiency of PAI-1 can lead to bleeding due to unstable hemostatic plug formation. Here, we studied virus dissemination and host susceptibility to IAV infection and compare the differences between H1N1 virus strains.

Methods: We performed IAV infection in Serpine1-/-mutant mice with intranasal infection. A/Puerto Rico/8/34 (PR8, H1N1) and A/WSN/33 (WSN, H1N1) were used. WSN carry a unique neuraminidase gene that binds and converts plasminogen into plasmin. Activated plasmin can then cleave viral hemagglutinin (HA) in the absence of serine proteases. We determined the histopathological changes and measured the protein leakage level with in vivo permeability assay. Additionally, qRT-PCR was used to detect viral RNA in the extrapulmonary organs.

Results: Our results showed that Serpine1-/- mutant mice were susceptible to PR8 infections compared to wild type mice. Knock-out animals exhibited increased body weight loss, enhanced lethality and higher viral loads on day 5 post infection (p.i.). On the other hand, the knock-out mice lost more body weight and showed higher mortality compared to wild type mice although virus titers were similar in WSN infection. Furthermore, red blood cells leaked into the alveolar space in Serpine1-/- mutant mice after infection with both PR8 and WSN which was associated with increased vascular permeability. Finally, we detected viral RNA in kidneys of infected Serpine1-/- mice indicating viral dissemination from the lung into the blood stream.

Conclusion: In summary, our results suggest that due to the loss of PAI-1, PR8 can replicate to higher titers and cause a more severe outcome. WSN by itself can convert plasminogen into plasmin which results in an even more severe phenotype than observed by PR8. Thus, the plasminogen activation pathway plays an important role in IAV induced lung injury and viral dissemination.

Antigenic mapping of the avian influenza virus M2 protein extracellular domain using overlapping short peptides-based ELISA

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The extracellular domain of the avian influenza virus (AIV) M2 (M2e) protein has been proposed as the universal detecting antigen for differentiating infected from vaccinated animals (DIVA) strategy. This owes to the highly conserved nature of the protein across all AIV subtypes and its characterized differential immune responses which are indicative of a prior AIV infection in a host. This study attempted to characterize the antibody responses of eight chicken sera experimentally exposed to two liveH5N1isolates and identify the most reactive regions of the M2e protein. Eight overlapping short peptides of 9 – 10 amino acid (aa) spanning the full length M2e protein 24 aa were used in ELISA to map the M2e antigenic sites. It was identified that sequence ETPTRNEWEC(8-18aa) is the most reactive region for chicken sera raised against the A/chicken/West Java/Sbg-29/2007. The identified region holds an important value as a refined target antigen for DIVA strategy or for designing effective vaccine against AIV.

Keywords: avian influenza virus, M2e protein, antigenic mapping, short overlapping peptides, ELISA

Streptococcus suis affects the replication of swine influenza virus in porcine tracheal cells

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Objective: Swine influenza viruses (SIV) are important pathogens affecting pigs of all ages. Secondary infection by Streptococcus suis may enhance the severity of disease in piglets infected by SIV resulting in substantial economic losses. To date, the molecular basis of the interplay between SIV and S. suis still remains unclear. In order to understand the interaction between SIV and S. suis, we established an in vitro co-infection model based on newborn pig trachea cells (NPTr).

Methods: Two SIV variants A/sw/Bad Griesbach/IDT5604/2006 H1N1 and A/sw/Herford/IDT5932/2007 H3N2 were used to compare subtype differences. Our previous studies showed that the H3N2 strain had a higher replication rate and induced a stronger ciliostatic effect in pig precision-cut lung slices compared to H1N1 strain. Wild type S. suis serotype 2 strain 10 (wt) and an noncapsulated mutant strain (Δcps) were used as secondary infectious agents in this study. NPTr cells were first inoculated with SIV, followed by bacterial inoculation. The course of infection was monitored by immunofluorescence microscopy and by determining the virus titers at different time points.

Results: Our results show that the viral hemagglutinin expressed on the surface of virus-infected cells interacted with a2,6-linked sialic acids of the capsular polysaccharide and thus enhanced the binding of S. suis and facilitated bacterial infection. On the other hand, the release of H1N1 and H3N2 SIV were delayed when NPTr cells were co-infected with S. suis. Depending on the affinity to sialic acid, gradual differences in the interaction between SIV and S. suis were detected. Moreover, most of the wt bacteria adhered to SIV infected cells.

Conclusion: These findings indicated that S. suis and SIV affect each other in the infectious behavior in swine respiratory epithelial cell. Furthermore, this interaction is mediated by hemagglutinin of influenza viruses that recognizes a2,6-linked sialic acid on the capsular polysaccharide of S. suis.

Parallel ESVV Session 3: Arboviruses

Tuesday 1st September 2015
11:00-12:30
Room Sully 3

Chair: Thomas Mettenleiter & Serafin Gutierrez

Knockout of non-structural protein NS3/NS3a abolishes propagation of bluetongue virus in Culicoides sonorensis

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4: Department of Biochemistry, Centre for Human Metabonomics, North-West University - South africa

Objective: The bluetongue virus (BTV) causes Bluetongue in ruminants and is transmitted by Culicoides biting midges. BTV is non-enveloped but has a triple-layered capsid containing the ten-segmented dsRNA genome and the replication complex. In addition to structural proteins, BTV encodes four non- structural proteins of which NS3/NS3a is not essential for in vitro virus replication. However, NS3/NS3a is functional in virus release as NS3/NS3a knockout leads to delayed virus release in mammalian cells and largely inhibits release in insect cells. BTV release from infected cells occurs via both a lytic and non-lytic pathway. Non-lytic release is more prominent in insect cells, which suggests an important role for NS3/NS3a proteins in the competent Culicoides insect vector.

Recently, our group has developed a novel vaccine candidate for Bluetongue, named the Disabled Infectious Single Animal (DISA) vaccine, which is based on live-attenuated BTV without NS3/NS3a expression. DISA vaccine is highly protective, is not virulent, and enables DIVA (Differentiation of Infected from Vaccinated Animals). DISA vaccine does not cause viremia in sheep, and therefore uptake of DISA vaccine by biting midges is hardly possible. Furthermore, due to the important role of NS3/NS3a in virus release from cultured Culicoides cells, we hypothesised that DISA vaccine is unable to propagate in the competent Culicoides vector.

Methods: Colonized Culicoides sonorensis midges were orally infected with an American BTV11 field strain as a control or with reverse genetics generated BTV1 containing VP2 of serotype 2 (BTV1 VP2 2). Colonized Culicoides sonorensis midges were also injected intrathoracically with BTV1 VP2 2 or with the same virus without NS3/NS3a expression (DISA 2). Groups of 25 or 50 midges were decapitated at different days post infection/injection. Heads and bodies were individually tested for viral RNA using semi-quantitative real time PCR.

Results: In contrast to BTV11, no increase in viral RNA was found in both bodies and heads of midges fed with BTV1 VP2 2. Even more, BTV1 VP2 2 was hardly detectable in heads of fed individuals. This clearly indicated the absence of replication and subsequent dissemination of after oral infection of colonized competent C. sonorensis. However, after intrathoracical injection, BTV1 VP2 2 was able to propagate as was clearly observed by increase of viral RNA in both bodies and heads. Apparently, injection has circumvented the mesenteron infection barrier. In contrast, DISA 2 (BTV1 VP2 2 without NS3/NS3a expression) was not able to propagate in injected midges. The amount of viral RNA was not increasing in both bodies and heads at several days post injection. Apparently, DISA 2 is unable to replicate in secondary organs, including salivary glands.

Conclusion: It has been shown that uptake of DISA vaccine by midges is highly unlikely due to absence of viremia in vaccinated hosts, such as sheep. Here, we showed that oral infection by DISA vaccine of colonized competent Culicoides midges is abolished in at least two ways. The ancestor of DISA 2 which is still expressing NS3/NS3a (BTV1 VP2 2) is abolished in an early stage of oral infection. We assume that the mesenteron barrier cannot be passed by this virus, and likely DISA 2 is also unable to pass this barrier. More importantly, we showed that propagation of DISA vaccine in colonized competent Culicoides midges is abolished by the NS3/NS3a knockout mutation. Taken together, these results demonstrate that onward transmission of DISA vaccine is hardly possible, since uptake, infection, propagation, and likely release of DISA vaccine virus to susceptible hosts

by biting midges are all abolished by the NS3/NS3a knockout mutation. Thus, deletion of NS3/NS3a expression in BT vaccine prevents viremia in the host and propagation in the insect vector, which is a powerful safety element of replicating BT DISA vaccine.

Genetic diversity of West Nile virus in Europe

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Background: West Nile virus (WNV) has been circulating in Europe since long time, as evidenced by anecdotal, mainly serological reports. Rarely larger outbreaks were observed, such as the 1996 Romanian WNV lineage 1 outbreak with more than 500 human cases of West Nile neuroinvasive disease (WNND). This epidemiological situation changed dramatically with the independent introduction of two different WNV lineage 2 strains, one to Hungary, identified in 2004, and one to the Volgograd region of Russia, identified in 2007.

Objective: To investigate the genetic diversity of WNV strains currently circulating in Europe.

Methods: WNV-positive samples from all over Europe and beyond were collected from humans, animals and arthropods by our group and by collaborating colleagues. WNV was either isolated from the samples or the samples were directly subjected to nucleic acid extraction and subsequent specific PCR analyses. From positive samples the complete nucleic acid sequences were determined, genetically analysed and compared to WNV genomes which had been deposited in GenBank database. The genetic relatedness of the WNV strains was determined by phylogenetic analyses.

Results: The central/southern European lineage 2 WNV strain, which emerged in Hungary in or before 2004, spread – after a few years of adaptation – in 2008 all over Hungary and to the eastern part of Austria. Most likely the strain dispersed already at this time to neighbouring Balkan states. In 2010, this WNV strain caused a devastating outbreak of human WNND in Greece, and in 2011 this virus strain was also identified in Italy. In 2012, Serbia and other Balkan states experienced large WNND outbreaks due to this WNV strain. And finally in 2013, this strain was identified in mosquitoes in the southern Czech Republic, which constitutes its current northernmost range of distribution. A different lineage 2 WNV strain, designated eastern European lineage 2 WNV, emerged in the Volgograd region of Russia and has been causing WNND there since 2007. Interestingly, this strain was also responsible for outbreaks of WNND in Romania since 2010. Besides lineage 2 WNV strains, lineage 1 strains are still circulating and evolving in Europe, mainly in Italy. Further genetically different lineage 1 and 2 WNV strains are circulating in Turkey and in Israel, respectively, having the potential to jump to the European continent easily. Besides these pathogenic WNV lineages, at least three further WNV lineages or sublineages without known human pathogenicity were identified in different species of mosquitoes in Europe: lineage 3 (Rabensburg virus) in the southern Czech Republic, lineage 4a in Russia and Romania, 4b in Spain, and 4c in Austria and Hungary.

Conclusion: The cocirculation of various WNV lineages and genetic variants in Europe is not only a threat for

public health and animal health but also a diagnostic challenge due to the known cross- reactivity of flaviviruses including Usutu virus and tick-borne encephalitis virus. Continuous WNV surveillance activities in mammals, birds and arthropods all over Europe and subsequent genetic characterisation of the identified viruses will be essential for a potential control of WNV in Europe.

Acknowledgment: The authors wish to acknowledge the valuable contributions of several other colleagues to this review on WNV in Europe. The study was funded by the FP7 grants 'EuroWestNile' and 'EDENext' of the European Commission.

The entomological surveillance of West Nile virus in Lombardia and Emilia-Romagna regions, Northern Italy, 2014

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Objective: West Nile virus (WNV) is an arbovirus which raises public health and veterinary concerns for its capability to infect horses and humans, which are considered as dead end hosts due to their inability to develop a sufficient viremia to infect mosquitoes. Besides the direct risk to human health, infected but asymptomatic blood donors represent a recognized problem for safety of blood transfusions in affected areas, as well as infected solid organ donors.

In Italy, outbreaks of West Nile disease, with occurrence of human cases, have been increasingly reported since 2008. This is particularly true in Northern Italy, where entomological surveillance systems were implemented at a regional level to monitor the presence and spread of the virus. The main tasks of this activity are: (I) the detection of virus circulation before the occurrence of disease in human, (II) the identification of the geographic area in which the virus circulate. Other important data on epidemiology and ecology of the virus were also produced by the surveillance activity.

Methods: Lombardia and Emilia-Romagna cover a large part of Pianura Padana (Po Valley) that, with an extension of about 46.000 km2, is the widest plain of Italy. The mosquito-based surveillance system was based on the collection of insects at a total 106 stations, fortnightly from June to October, by using self-made carbon dioxide baited attractive traps; other 16 sites were surveyed by gravid traps in Emilia-Romagna (John W. Hock Company). Stations were evenly located on surveyed territory, each station was geo-referenced and traps worked for one night, from 17:00 to 9:00 the next day. Sampled mosquitoes were identified at the species level by morphological keys and then pooled according to station and date of sampling, with a maximum of 200 specimens per pool, only mosquitoes of the Culex genus were then tested. Ochlerotatus and Stegomya taxons were considered as an Aedes subgenus.

Pools were ground, then RNA was extracted from pools and reverse transcribed (RT) using random primers. After RT reactions, the samples were subjected to real-time PCR protocols for the detection of both WNV (Tang et al. 2006) and Usutu virus (USUV), and to a screening PCR for the presence of flaviviruses (Scaramozzino et al 2001). The obtained amplicons were sequenced for virus identification. Then the obtained sequences were aligned with sequences of those strains identified in past years of surveillance and with sequences available in GenBank, by the ClustalW algorithm implemented in MEGA 6 software, and the alignment was refined manually. To infer the phylogenic history of the WNV strains E gene sequence obtained by strains isolated in both regions since 2008 were utilized.

To attempt viral isolation, PCR-positive samples were inoculated in confluent monolayers of VERO cells and obs-

erved daily for viral cytopathic effect(CPE). In the absence of CPE, the cryolysates were sub-cultured twice into fresh monolayers and checked for WNV and USUV by sandwich-ELISA using monoclonal antibodies (MAbs).

Results: A total of 371,941 mosquitoes, belonging to 11 species, were sampled in the 122 surveyed sites. About 95% were identified as Culex pipiens L., followed by Aedes (Ochlerotatus) caspius (Pallas) (2.9%) specimens. Other frequently recorded mosquitoes, with abundance <1%, were Aedes vexans (Meigen), Culex modestus Ficalbi, the species of the Anopheles maculipennis complex and the invasive Aedes (Stegomya) albopictus (Skuse) (Table 1).

More than 96% of the collected mosquitoes were tested (359,378 specimens sorted in 3,637 pools) and WNV was detected in 118 pools (3.2%), while USUV was found in 95 pools from Emilia-Romagna (Table 1). In 57 sites, on the 122 surveyed, a WNV-positive pool was sampled, while USUV were detected in 47 on 93 sites in Emilia-Romagna. In Emilia-Romagna the first WNV-positive pool was sampled on July 4, and the last on September 23, while in Lombardia on the July 3 and on the September 10 respectively.

WNV partial sequences obtained from field samples have a high rate of identity between them, and identity ranging from complete to 98.5% with sequences obtained in 2013 in both regions trough the surveillance system. Also the obtained sequences from USUV show an high rate of identity with the sequences obtained in 2013.

The obtained tree of the E gene of viruses isolated in Emilia-Romagna and Lombardia from 2008 clearly show that the strain that sustain the 2013-14 outbreak in Northern Italy was a lineage II strain, similar to other lineage II WNV strains detected in Europe in recent years. The 2013-14 WNV strains differ from other strains isolated from surveyed area in 2008-2009, these viruses belonged to lineage I and were more similar to Israeli strains.

Conclusion: The surveillance system detects a wide circulation on WNV in collected Culex pipiens (117/3605 pools, 3.2%) confirming this mosquitoes as the main vector of the virus in northern Italy. The probable involvement of the species Cx. modestus in WNV cycle as secondary vector can be also hypothesized, as one pool out of 32 tested (3.1%) was positive to the virus. Cx. pipiens mosquito represents also the principal vector of USUV in northern Italy.

All the WNV detected sequences belonged to lineage II, and had a high identity with the virus detected in 2013 and linked with human disease in surveyed area. The lineage II detected virus was closely related to the strains circulating in Europe, from Hungary to Greece. The lineage I WNV isolates in the same area in 2009-10 are genetically distant from these isolates, demonstrating a new introduction of the virus in surveyed territory. This picture is consistent with a progressive expansion of this WNV strain from Eastern European countries to neighboring countries, as far as Italy, while the lineage I strains responsible to the previous outbreak are closely related with strains isolated from Israel from 2008 to 2010.

The surveillance system was also able to detect the non-target USUV. USUV and WNV are closely related, and a differentiation of the two viruses is important in a surveillance plan to avoid false positive results. The circulation areas of the two viruses largely overlapped, confirming their co- circulation in Pianura Padana, as already reported. Moreover these viruses show ecological similarities, they circulate between wild birds and mosquitoes, particularly Culex pipiens. Despite this the two viruses showed different temporal patterns of circulation in the surveyed area: WNV had been circulating discontinuously, with different strains, and in different locations, over the years, while USUV was persistently detected at similar levels since 2009 showing a continuous circulation. These differences raise the question of which epidemiologie characteristic could be able to produce these different patterns of spread and abundance of the two viruses.

Broad Molecular Detection of Flaviviruses

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Objectives: To provide an easy, simple and rapid assay for general molecular detection of flaviviruses.

Methods: The PCR assay presented here is developed using similar techniques as previously described (1). Primers were designed from an alignment containing 1159 flavivirus NS5 gene sequences. Two PCRs are involved in a semi-nested format. The first PCR (pre-amplification) include a single degenerate primer pair. The second PCR include 19 tagged primers at low concentration and one labeled detection primer corresponding to the tag-sequence. The reverse primer is the same for both steps. RNA from isolates of West Nile Virus (WNV), Yellow Fever Virus (YFV), Japanese Encephalitis Virus (JEV), Dengue Viruses (DENV1-4) and Tick borne Encephalitis Virus (TBEV) as well as the Usutu virus and Bagaza virus were used to evaluate the broad detection capacity of the assay. In addition, 65 clinical samples were used to evaluate the assay. They included human sera and urine, brain samples from birds and horses and mosquito and tick vectors.

Results: All isolates were successfully detected. The analytical sensitivity of the assay was evaluated using two different panels (WNV and DENV) from the Quality Control in Molecular Diagnostics organization (QCMD; http://www.qcmd.org/) from 2011. The detection limit was 104 copies/ml of WNV and 103 copies/ml of Dengue virus. Several tested pestiviruses, which also belong to the Flaviviridae family, and other RNA viruses such as the Chikungunya virus, which gives similar clinical symptoms as DENV, and the avian viruses APMV 2, 3 & 6, H5N1 & H7N1 and NDV gave no appreciable signal. To benchmark the present broad detection assay, flavivirus detection from the 65 clinical samples were compared with the detection obtained with dedicated TaqMan PCR assays (2-4). This showed 92% were detected with both methods. Only 68% of samples that were detected by the present method were detected by the benchmarking TaqMan systems (2-4) while three samples were negative by the former but detected by latter.

Conclusion: A simple broad detection method for flaviviruses was developed. It show excellent sensitivity as evaluated using the QCMD panels for WNV and Dengue virus and also high specificity using a broad range of RNA viruses including pestiviruses. The assay was successful with clinical materials varying from brain tissue to insect vectors. Compared to dedicated standard TaqMan based assays the sensitivity was superior. However, this is largely explained by one of the TaqMan system used (2) failed to detect a set of twelve samples from a recent WNV outbreak in Europe in 2008/2009. Otherwise it is likely that the presently described assay has a comparable sensitivity to TaqMan PCR and the outstanding virtue of this novel method is the broad detection of this highly variable virus genus.

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4-Unpublished

Demographics of natural oral infection of mosquitos by Venezuelan equine encephalitis virus

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RNA viral infections generally generate large and diverse populations within the infected host. This diversity plays a key role in important epidemiological and evolutionary processes. However, several steps during transmission can constrain the demographics and genetics of the virus population, with host primary infection being

one of the main barriers. During primary infection, the number of initially infected cells is not infinite, potentially lowering the size and genetic diversity of the colonizing population compared to that present in the donor host. The multiplicity of cellular infection (MOI) in those cells is thus a fundamental parameter determining the demographics and genetics of the colonizing population. The MOI is the number of genomes of a virus that enter and replicate in a cell. This parameter impacts the size of population bottlenecks during primary infection because, for a given number of primary infected cells, the higher the MOI, the larger the colonizing population. Furthermore, the MOI also influences genetic diversity as it largely defines the intensity of genetic exchange and complementation among genotypes during cell co-infection. Despite the importance of the MOI and population bottlenecks, there is a striking lack of formal estimates of these parameters, not only during primary infections but throughout the virus transmission cycle. Here, we use available datasets to estimate the demographics of Venezuelan equine encephalitisvirus (VEEV), a mosquito-borne arbovirus, during the primary oral infection (i.e. the midgut infection) of its mosquito vectors. We estimated the MOI during primary infection in the two transmission cycles using the method developed in Gutiérrez et al. (2010), and we use the results to model the potential for within-cell interactions among viral genotypes during primary infection. We also estimated the population bottleneck Ne endured by VEEV during bloodmeal ingestion and midgut infection. The methodology used is based on Fst statistics and uses genetic variance within and between populations (i.e. the virus populations in the inoculum and midguts) to estimate the effective population size. Despite of the fact that severe bottlenecks, in the order of single digits, are the rule during primary infection in the few virus models analyzed so far, VEEV population sizes were between one and two orders of magnitude higher. Larger populations at primary infection could be crucial during the arboviral cycle of VEEV because they can preserve diversity and facilitate adaptation during the compulsory alternation between arthropod and vertebrate hosts.

Simbu sero-group virus serum neutralizing antibodies in cattle in Tanzania

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Objective: Simbu sero-group viruses occur worldwide and include the newly recognized Schmallenberg virus (SBV) in Europe. These viruses cause in utero congenital malformations and reproductive losses in ruminants. Information on presence of these viruses in Africa is scarce and the origin of SBV is unknown. The aim of the present study was to investigate the presence of antibodies against SBV and closely related viruses in cattle in Tanzania, and their possible association with reproductive disorders.

Methods: Serum samples from 659 cattle in 202 herds collected in 2012/2013 were analyzed using a commercial SBV ELISA. The possible association between ELISA positivity and reproductive disorders were investigated by use of univariable logistic regression. Sera from 130 animals from the same area collected in 2008/2009, before the SBV epidemic in Europe, were also included and tested in the same ELISA. To interpret the ELISA results, SBV virus neutralization test (VNT) was performed on 110 sera from 2012/2013 and 71 from 2008/2009. In order to investigate the potential cross-reactivity with related viruses, 45 sera from 2012/2013 were analyzed in a series of VNTs, including viruses were Aino virus, Akabane virus, Douglas virus, Peaton virus, SBV and Tinaroo viruses.

Results: In the SBV ELISA test, 61% of the sera were positive. A significant association was found between ELISA seropositivity and reproductive disorders (OR= 1.9). Out of the samples from 2008/2009, 55% were positive. When analysed in SBV VNT, 51% of the samples from 2012/2013 and 21% of the samples from 2008/2009 were positive.

When analyzed in series of VNTs, antibodies against all six simbu sero-group viruses were detected. All sera

were positive for at least one, most for two or more of the tested viruses. This time, 64% were positive for SBV whereby four had the highest titer for this virus. 91% had antibodies against Aino virus, 75% against Tinaroo virus, 73% against Douglas virus and 71% against Peaton virus. Few sera were positive for Akabane virus, and they had low titers.

Conclusion: This is the first serological indication of Simbu sero-group viruses including SBV in cattle in Tanzania. All the tested viruses may circulate in the surveyed area. The results indicate that SBV was present in this area before the European epidemic. However, potential cross reactivity between the viruses used in this study, and possibly others that circulate in the area, complicate the interpretation of such serological studies. The results also indicate that this group of closely related viruses has some negative effect on reproductive disorders. Isolation and further genetic characterization of the viruses will be essential for understanding molecular epidemiology and evolution of SBV related viruses. Virus detection, although difficult since the viremic period is short and clinical signs usually mild in cattle, will be needed to further identify the viruses circulating in this region.

Parallel ESVV Session 4: Vaccines and antivirals

Tuesday 1st September 2015
14:30-16:30
Auditorium Pasteur

Chair: Marie-Frédérique Le Potier & Renata Servan de Almeida

Assessment of efficacy and immune responses in mice vaccinated with recombinant MVAs expressing single RVFV glycoproteins

Elena López-Gil, Gema Lorenzo, Alejandro Marín-López, Sandra Moreno, Javier Ortego, Alejandro Brun

Centro de Investigación en Sanidad Animal (INIA-CISA) - Spain

Objective: In this work we have assessed the efficacy and immune responses of recombinant MVA viruses expressing RVFV glycoproteins Gn and Gc. Previous data obtained in our laboratory showed that a single inoculation of MVA expressing both Gn and Gc was sufficient to induce a protective immune response in mice after a lethal challenge with RVFV. The protection elicited by the MVA vaccination was related to the presence of glycoprotein specific CD8+ cells, in the absence of a consistent detection of neutralizing antibodies in vitro. To study the contribution of each glycoprotein antigen to protection a similar approach was extended to vaccines expressing only a single RVFV glycoprotein (either Gn or Gc). Rift Valley Fever virus (RVFV), a mosquito-borne bunyavirus widely distributed in Sub-Saharan countries, Egypt and the Arabian Peninsula, causes disease in both human and livestock and is now considered an emerging threat for non-endemic countries due to the movement of infected animals and insect vectors including mosquitoes. The ample range of competent mosquito vectors for RVFV in many areas of the Mediterranean basin suggests that RVF outbreaks in non-endemic areas could potentially end-up in establishment of enzootic infection cycles. If this happen it would cause serious concern for both public and animal health. It is therefore desirable to develop control tools as well as enhance our knowledge about the immune mechanisms that correlate with the protection elicited by RVFV vaccines.

Methods: Generation of replication-deficient MVA expressing RVFV glycoproteins.

MVA recombinants were constructed by cloning the complete ORF of the mature Gn/Gc, the Gn or the Gc ORFs from the M segment of the MP-12 RVF virus strain (GenBank accession number DQ380208) into the shuttle vector MVA-GFP, which places the ORF under the control of the vaccinia p7.5 early/late promoter, and also includes GFP as a marker gene under the control of the vaccinia p11 late promoter. The shuttle vector was transfected into chick embryo fibroblasts (CEF) infected with MVA and homologous recombination allowed the shuttle vector to recombine with the MVA, inserting the RVFV ORFs and GFP marker gene at the TK locus of MVA. The

recombinant viruses (rMVAGnGc, rMVAGn and rMVAGc) were plaque purified and then expanded in CEF cells.

The N terminus of each recombinant polypeptide contained an in-frame fusion of the human tissue plasminogen activator leader sequence (tpa), known to enhance transgene expression and immunogenicity. The C terminus of the polyprotein contained a H-2Kd restricted CD8+ T cell epitope (SYIPSAEKI) from Plasmodium berghei circumsporozoite protein and an anti-V5 monoclonal antibody recognition sequence IPNPLLGLD.

Immunization, sampling for immunological assays and RFVF challenge.

Groups of 10 BALB/c mice (Harlan) were immunized intraperitoneally with 1x10e7 pfu of MVA vectors in phosphate-buffered saline (PBS). One or two weeks post-vaccination, blood samples were taken either for neutralization assays (serum) or IFN-g ELISpot (PBLs), and splenocytes for ex vivo IFN-g ELISpot and ICS assays. The remaining mice (n=5), together with additional groups of unvaccinated BALB/c mice and immunized with non-recombinant MVA, were all challenged intraperitoneally with 103 plaque-forming units (pfu) of the South African RVF virus strain 56/74. The immunization and challenge studies were also performed in a similar manner using 129SvEv mice. Efficacy estimation was evaluated in terms of morbidity and mortality monitoring daily over three weeks. All surviving mice were culled after 21 days of follow-up.

Assessment of RVF virus neutralizing antibodies

Serum neutralizing antibody titers were measured in Vero cell monolayers by serial dilutions of serum mixed with an equal volume of medium containing MP-12 RVF virus strain and incubated for 1 hour at 37ºC. After 72 hours the cells were fixed and stained in a solution containing 10% formaldehyde and 2% crystal violet in PBS. Plaque formation was then scored and neutralization titer defined as the highest serum dilution at which plaque formation was reduced by 50% relative to that in cells incubated with RVF virus only. The assays were performed in triplicate and scored by an operator blinded to vaccination regimen.

Assessment of T-cell responses to RVF viral glycoproteins.

RVF viral glycoprotein-specific T cells were measured by ex vivo IFN-g ELISpot assay on splenocytes and pooled PBLs. Gn, Gc-specific and non-specific peptides were used for re- stimulation at a final concentration of 5ug/ml in all assays for 18 hours. For ICS assay, peptide re-stimulation of cells was done for 5 hours and the frequency of cells staining positive for IFNg, measured by flow cytometry. IL-2, IL-6, IL-4, and IL-5 cytokine capture ELISAs were also performed using supernatants from peptide re-stimulated cell cultures.

Statistical analysis.

The log rank (Mantel-Cox) test was used for survival analysis following RVFV challenge. Individual ELISPOT values were determined by subtracting background values obtained after stimulation with media only and log10 transformed for analysis. Data from each vaccination group were analyzed using a randomized block analysis of variance. Dunn's and Tukey's post hoc tests were used for multiple comparisons among groups. All analysis were done using the GraphPad 5.0 software (San Diego, CA). Differences were significant when p values <0.05

Results: Expression of recombinant glycoproteins in MVA infected cells.

Western blot and immunoblotting, as well as immunofluorescence staining, confirmed expression of the glycoprotein antigens encoded by each recombinant MVA. Immune detection of the expressed antigens was performed with antibodies specific against Gn or Gc as well as with the anti-V5 tag antibody that allowed quantifying the levels of expression of each glycoprotein upon infection.

Efficacy assessment in mice.

Balb/c mice immunized with MVAGc virus showed an 80% of survival after challenge. At 11 dpi only one animal showed signs of delayed-onset neurological disease dying at day 14 pi. In contrast, two mice vaccinated with MVAGn showed earlier clinical disease dying at day 4 and 6 post challenge. As expected, in the group of mice vaccinated with the MVAGnGc construct the survival after challenge was 100%, with only one animal showing mild clinical signs between 3 and 4 dpi. In mice from both control groups (non-recombinant MVA and unvaccinated) the mortality was 80 and 100% respectively with an early onset of disease in both groups. On the other hand, the survival rates in the 129SvEv mice immunized with MVAGnGc reached 80% with one animal dying at 5 dpi. Contrarily to what was observed in the BALB/c experiments all of 129 mice vaccinated with MVAGc or MVAGn died upon challenge with a slight delay in mortality in the MVAGc group with respect to MVA control group.

Humoral and cellular immune responses

Low or no detectable levels of neutralizing antibodies were found in the serum from immunized BALB/c mice with titers below the established detection threshold (1,3log10). On the other hand in the 129 mice two mice from the MVAGnGc group showed titers slightly above the threshold limit (1,6log10). The rest of the mice from all groups did not show titers above the established limit. Upon challenge all surviving animals showed titers reaching 3 logs.

ELIspot assay using 14dpi pooled PBLs from BALB/c mice immunized with MVAGc showed a high number of IFN-g secreting cells upon re-stimulation with Gc-specific, MHC-I- restricted peptides, with lower numbers upon stimulation with Gn peptide or irrelevant control peptide. The rest of the groups showed much lower levels of IFN-g secreting cells, with the only exception of PBLs re-stimulated with the pb9 control peptide from the MVAGn group. Similar data were obtained in ELISpot using splenocytes collected at 7 or 14dpi although the numbers of IFN-g secreting cells were higher for the MVAGnGc and MVAGn upon re- stimulation with Gn specific peptide. Higher responses were observed upon stimulation with pb9 control peptide in the MVAGn group in comparison with MVAGc or MVAGnGc groups. With respect to the phenotype of IFN-g secreting cells it was possible to detect the highest percentages of IFN-g+ CD8+ T-cells in splenocytes from mice immunized with MVAGc. These data correlated with the higher secretion of IL-2 and IL-6 detected by ELISA in supernatants of re-stimulated cultures. In contrast no IL-4 or IL-5 was detected in the same supernatants.

Conclusions: Our data suggest that protection of BALB/c mice upon RVFV challenge can be mediated by the activation of a strong cellular response (mainly against Gc epitopes) in the absence of a clear induction of neutralizing antibodies. However, this protection may be restricted to specific genetic backgrounds determining susceptibility to infection as shown by the lack of survival upon challenge of 129SvEv mice immunized with the same vaccines (MVAGn or MVAGc). Our data also point out that the expression of both glycoproteins enhances humoral immunogenicity perhaps explaining the higher protection rates in MVAGnGc vaccinated 129 SvEv mice. The detection of IL-2 and IL-6 supports the induction of cellular responses since both cytokines play a role in T-cell survival and activation. Thus, the identified Gc specific CD8+ T-cell population may act as a key component in the protection after challenge observed in the MVA immunized mice, contributing to the elimination of infected cells and reducing morbidity and mortality.

Salmonid alphavirus glycoprotein E2 requires low temperature and E1 for virion formation and induction of protective immunity

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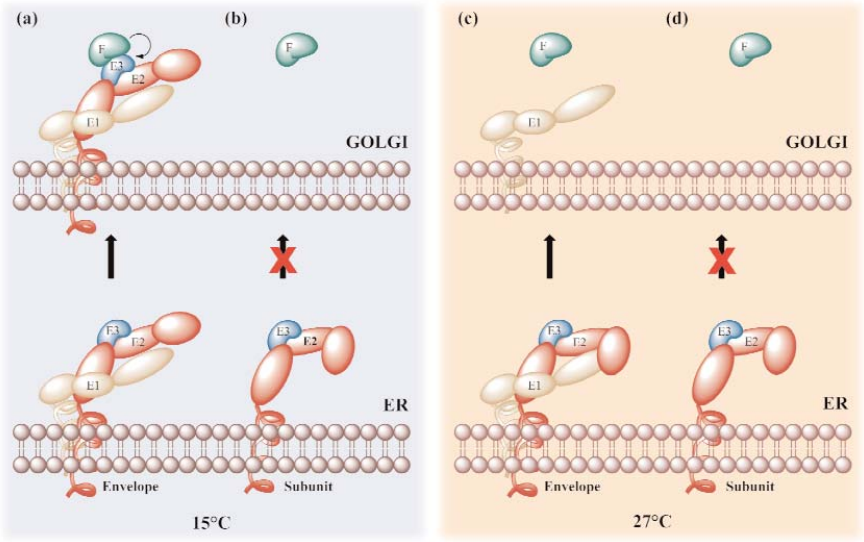
Objective: Salmonid alphavirus (SAV; also known as Salmon pancreas disease virus; family Togaviridae) causes pancreas disease and sleeping disease in Atlantic salmon and rainbow trout, and poses a major burden to the aquaculture industry. It is an outlier amongst other, mostly mosquito-borne, alphaviruses as it has no known arthropod vector. In addition, SAV infection in vivo is temperature- restricted and progeny virus is only produced at low temperatures (10-15°C). Also, SAV virus-like particles (VLP) could only be produced at low-temperature (at 15°C but not >18°C) in Sf9 insect cells. The aim of this study was to understand the low temperature-dependent virion formation of SAV at the molecular level.

Methods: Using engineered SAV replicons, we examined viral RNA replication and viral spread at low and high temperature in salmon cells. The processing/trafficking of SAV glycoproteins E1 and E2 as a function of temperature was investigated via baculovirus vectors in Sf9 insect cells and by transfection of CHSE-214 fish cells with DNA constructs expressing E1 and E2. A vaccination-challenge model in Atlantic salmon demonstrated the biological significance of our findings.



Results: We showed that SAV viral RNA replication is not temperature-restricted, suggesting that the viral structural proteins determine low-temperature dependency. We identified SAV E2 as the temperature determinant by demonstrating that membrane trafficking and surface expression of E2 occurs only at low temperature and only in the presence of E1. At higher temperatures, E1 was still able to travel to the cell surface, while E2 was retained in the ER. Finally, the vaccination-challenge trial in Atlantic salmon showed that SAV replicon DNA vaccines encoding E2 elicit protective immunity only when E1 is co-expressed.

Conclusion: This is the first study that identifies the structural glycoprotein E2 as the critical determinant of SAV low-temperature dependent virion formation and defines the prerequisites for induction of a potent immune response in Atlantic salmon by DNA vaccination.

Potential edible vaccine against influenza virus

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Background: Effective vaccination against influenza virus infection is a serious problem mainly due to antigenic variability of the virus. Among the many of investigated antigens, the extracellular domain of the M2 protein (M2e) features high homology in all strains of influenza A viruses and antibodies against M2e are protective in animal models what makes it a potential candidate for generation of a universal influenza vaccine. However, due to the low immunogenicity of the M2e, formulation of a vaccine based on this antigen requires some modification to induce effective immune responses.

Objective: In this work we evaluated the possible use of Bacillus subtilis 168 spores as a carrier of the Influenza A M2e antigen in the mucosal vaccination.

Methods: A tandem repeat of 4 consensus sequence coding for human – avian – swine - human M2e (M2eH-A-S-H) peptide was fused to spore coat proteins and were stably exposed on the spore surface as demonstrated by the immunostaining of intact, recombinant spores.

Results: Oral immunization of animal with recombinant endospores carrying M2eH-A-S-H elicited specific antibody production without the addition of adjuvants.

Conclusion: The approach described in our work is a contribution to the development of an alternative and universal vaccine against influenza virus omitting time-consuming and cost-intensive immunogen purification process and use of adjuvants.

Antiviral activity of type I, II and III porcine interferons against classical swine fever virus

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Classical swine fever (CSF), caused by classical swine fever virus (CSFV), substantially impacts swine production industries worldwide. An important consideration for CSF control strategies in free areas is the ability to demonstrate freedom from disease serologically, thus allowing rapid resumption of trade after disease outbreaks. Biotherapeutics that stimulate the host's immune response to restrict infection, but without impeding serological detection of disease, represent a novel disease control option. Interferons (IFN) are integral components of the host's innate defense against viral infections and are able to protect against virus infections. Three types of IFN have been described which vary in their specific mode of action and target cell populations. Type I interferons (eg IFNα, β) induce a protective antiviral state in diverse cell types, whilst type II interferon (IFNλ) stimulates cell mediated responses and amplifies the effects of IFNα,and β. Type III IFNs (IFN λdemonstrate antiviral properties on a variety of cells, although the distribution of the IFN receptor (IFNLR) is mostly limited to cells of epithelial origin. Porcine IFNλs may thus provide a useful protection against CSFV at the primary sites of infection.

Objective: To assess the antiviral activity of type III, as well as type I and II, porcine interferons, when expressed as both recombinant proteins and via an adenoviral delivery vector, against CSFV.

Methods: Porcine cell lines were screened for expression of the interferon λ receptor, IFNLR, by RT-PCR. Genes encoding porcine IFNs (PoIFN) (type I (α12, β), type II (γ) and type III (λ1, 3)) were cloned into the pcDNA3.1 V5-HisB mammalian expression vector and the proteins expressed by transfection into newborn porcine tracheal (NpTr) cells. The antiviral activity of the recombinant proteins, within the transfected cell supernatants, was assessed by treatment of NPTR cells prior to infection with vesicular stomatitis virus (VSV) or CSFV. Antiviral activity, compared to treatment with mock transfected cell supernatants, was assessed in a CPE reduction assay (VSV) or by assessing the percentage of cells expressing the E2 viral envelope protein (CSFV) by flow cytometry. The five poIFNs genes were subsequently cloned into an adenovirus delivery/expression vector. The anti-VSV and CSFV activity was similarly assessed after inoculation of NpTr cells with the IFN expressing adenovirus constructs, or control constructs expressing GFP alone.

Results: Screening of a range of porcine cell lines identified that NpTr cells express the IFNLR. All of the recombinant porcine interferons had antiviral activity against VSV in this cell line. At the concentrations tested, recombinant PoIFN λ1, as well as poIFN α12 and β, significantly reduced the percentage of NPTR cells from subsequent infection with CSFV. When delivered via the adenovirus vectors all five porcine interferons significantly protected NPTR cells against CSFV infection compared to controls inoculated with adenovirus vectors expressing GFP alone.

Conclusion: Both interferon λ1 and λ3, as well as type I (IFNα12, β) and type II (IFNγ) interferon have antiviral activity against CSFV in-vitro. The use of adenovirus vectors to deliver interferons to pigs has potential as an intervention to assist in control of CSFV.

RNA interference as antiviral therapy against Peste des Petits Ruminants: proof of concept of in vivo efficiency using a small animal model

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Objective: Morbilliviruses are responsible of important diseases in human beings and animals with economical impact in affected countries. “Peste des petits ruminants” is one of these diseases affecting goats and sheep with high mortality and morbidity. Efficient vaccines exist but they are often used in emergency situation in animals and ten days are necessary to induce a sufficient immune protection.

Co-administration of an antiviral treatment with the vaccine could limit the disease impact while conferring a long-lasting protection. CIRAD has explored a biological antiviral therapy based on RNA interference. The identification of siRNA against morbilliviruses has been previously published and patented. The objective of this work was to validate the in vivo efficiency of these siRNAs.

Methods: The proof of concept for an efficient in vivo delivery of anti-PPRV siRNAs was developed in a mouse model. Briefly, it is based on the siRNA dynamic extinction of a luciferase reporter gene in mice measured by bioimaging. The originality is also based on the use of a double-reporter expression plasmid allowing standardization within and between the trials. The plasmid is made of a firefly gene placed downstream of one of our morbillivirus siRNA target sequence and a renilla gene used as a constant gene-expression system. In the initial phase, mice received a co-injection (double reporter plasmid + relevant or irrelevant siRNA-PPRV) in the tibialis muscle, followed by an electroporation to promote cellular uptake of DNA. The firefly and renilla signals were measured daily using a bio- imager. The firefly expression was normalized using renilla signal. The specificity of RNA interference was checked by comparison with an unrelated siRNA. Once this initial phase validated, a second phase consisted in testing a delivery system for siRNA based on a cell membrane penetrating peptide.

Results: The model was validated. In absence of any siRNA treatment, a good correlation was observed between the firefly and renilla luminescence activities. When the irrelevant siRNA was co- administrated, no incidence on these activities was detected. In contrast, mice treated with siRNA-PPRV showed a strong inhibition of about 99% of the firefly signal. This mouse model system is a proof of concept of in vivo siRNA efficiency and a very useful tool to assess in vivo siRNA delivery systems. Several candidates for in vivo delivery systems were investigated in our laboratory. Preliminary results showed that a cell membrane penetrating peptide could efficiently deliver a siRNA and inhibit the expression of firefly when tested in this mouse model.

Conclusion: This mouse model system is a very useful tool that can be applied to test siRNA delivery systems in vivo. In this model, a cell penetrating peptide showed encouraging performances for systemic delivery of siRNA but extensive confirmation will be necessary. The model is now available for the screening of alternative delivery systems, including viral expression vectors that might represent a better cost-effective strategy for small ruminant’s treatments in emerging countries.

Use of in silico prediction models to predict T-cell epitopes for the development of vaccines against African swine fever virus

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Objective: African swine fever virus (ASFV) causes a haemorrhagic and often lethal viral disease in pigs. With the exception of Sardinia, Europe was free of ASFV since the disease was eradicated from the Iberian Peninsula in 1995. In 2007, ASFV was introduced in Georgia, and spread from there to East-European countries where it has become endemic in several regions. The control of ASFV outbreaks in commercial pigs has been accomplished thus far by stamping out-methods. In regions where ASFV is endemic in wildlife, the use of a vaccine could make an important contribution to control of this disease. There have been several attempts to develop a vaccine for ASF, but until now this has not resulted in an efficient and safe vaccine. Studies have shown that effi-

cient protection against ASFV is probably dependent on both humoral as well as cellular immunological defence mechanisms, with especially the latter being most crucial for protection. In this project we addressed the question if it is possible to use current techniques of computational prediction of T-cell epitopes to develop a vaccine that is protective against ASFV. The ultimate goal is a broadly protective vaccine that is independent of the ASFV strain and is widely applicable for all pigs.

Methods: On the basis of the complete genome sequences of known ASFV isolates and the different swine leukocyte antigen (SLA) gene classes, the epitope prediction program NetMHCpan was used to predict a list of T-cell epitopes that are independent of virus strain or pig SLA class. Based on the prediction of the best twenty T-cell epitopes, a poly-epitope DNA vaccine was generated. The DNA sequence encoded 20 nonapeptide epitopes, separated by spacers of 2 to 5 amino acids that contain signals for proteasome cleavage and further processing by the intracellular antigen transport (TAP) machinery. In addition a universal PADRE B-cell epitope and an immunostimulatory CpG DNA motif were included to improve presentation of the epitopes to host T-cells. Groups of 6 pigs were vaccinated either three times with DNA vaccine (group 1), or two times with DNA vaccine followed by a combination of DNA vaccine and a peptide booster consisting of a cocktail of the twenty nonapeptides (group 2). A control group of 6 pigs was vaccinated three times with empty plasmid (group 3). Pigs were vaccinated with intervals of 2 weeks. The vaccine was applied intramuscularly followed by immuno-electroporationto improve the uptake of DNA by surrounding tissue. The peptides were applied by intramuscular vaccination with Stimune as an adjuvant. Two weeks after the final vaccination, pigs were challenged with the Netherlands ’86 strain and followed for 2 more weeks for clinical symptoms, virus levels in blood, antibody responses and levels of IFN-y secreting cells after in vitro stimulation with virus or the cocktail of twenty nonapeptides.

Results: ASFV infection of the non-vaccinated animals in control group 3 resulted in 40% survival, whereas infection of vaccinated animals in both groups 1 and 2 resulted in 83% survival. Pigs in group 2 had significant lower total clinical scores than pigs in the other groups. Pigs in this group also had a significant IFN-y secreting cell response against the cocktail of nonapeptides from day 42 post first vaccination (p.v.) (day 0 of challenge [p.c.]) until the end of the experiment. Pigs in group 1 had a significant IFN-y secreting cell response against the cocktail of nonapeptides from day 49 p.v.(day 7 p.c.). No IFN-y secreting cell response against the cocktail of nonapeptides was observed in the control group. All groups showed a significant IFN-y secreting cell response against the virus on day 56 p.v. (day 14 p.c.) There were no significant differences in levels of viral DNA in blood between groups. No significant differences were observed in levels of blocking percentages of the ASFV ELISA. The levels of infectious virus in blood and antibody levels detected by IPMA (immunoperoxidase monolayer assay) will be presented at the EPIZONE conference.

Conclusion: Promising results were obtained in this study by using the T-cell epitope prediction program NetMHCpan for development of an ASFV vaccine. Expression of predicted T-cell epitopes using a DNA vaccine provided at least partial protection against mortality and clinical disease. The immunological response could be further increased by using a peptide cocktail as booster. Based on these results, different vaccination strategies (e.g. using viral vectors) will be tested in future experiments.

Generation of a new Newcastle Disease vaccine by reverse genetics based on a recently genotype XI virus

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Objective: Newcastle disease (ND) is the major viral infection of poultry inducing high morbidity, mortality, and significant economic impacts on the poultry industry. ND is caused by virulent strains of avian paramyxovirus serotype 1 (aPMV-1) which have the capacity to spread over long distances by animal and human movements. All strains of aPMV-1 belong to a single serotype and current vaccines have been used worldwide for a long time and demonstrated their efficacy in terms of clinical protection. Up to date vaccines have been made of old genotypes that emerged several decades ago. However, recent studies have shown that the virus has undergone significant evolution which has led to the progressive emergence of new genotypes with potential antigenic drifts. The recently described genotype XI in Madagascar contains original amino acid substitutions on F and HN

proteins, some of them clustered in the head of the proteins, presumably exposed to the host immune system, suggesting that these substitutions may account for antigenic drifts. Indeed, we showed in vivo, under controlled conditions, that current vaccines conferred clinical protection against both the homologous genotype and genotype XI, but were unable to prevent virus shedding of heterologous genotype. Our objective was to produce genotype adapted vaccines, using reverse genetics to replace the F and HN of a live attenuated old-genotype virus (the genotype II Lasota strain) by the corresponding proteins of the more recent Madagascar genotype XI. This chimeric genotype XI-II will be characterized in vivo and evaluated in immunization/challenge trials.

Methods: NDV minigenomes expressing eGFP under two promoters (pT7 polymerase and pCMV) have been constructed and compared in vitro. Subsequently, segments of the full genome of the genotype XI MG-725 strain and LaSota strain have been generated by reverse transcription, cloned and assembled under the control of CMV promoter. Rescue of the virus is done by co-transfection of the full length genome and plasmids expressing NP, P and L proteins in BHK21 cells.

Results: The expression of the NP, P and L proteins of NDV MG725-08 strain has been confirmed on BHK 21 and by conventional RT- PCR on mRNA with specific NP, P and L primers.

Then, the NDV rescue system has been validated using eGFP minigenomes in antisens position to demonstrate the function of the NDV ribonucleoprotein. These results have been confirmed by extraction of mRNA and RT-qPCR or RT-PCR with specific eGFP primers. The NDV rescue system is now available to rescue the virus from an assembled full genome of NDV. Two genomes have been assembled from eight cloned segments and will be shortly rescued.

Conclusion: Full length genome of NDV MG725_08 strain is now available and the NDV rescue system is available in vitro. The rescue of the LaSota strain will be achieved soon and the corresponding infectious clones characterized in vitro and in vivo on embryonnated SPF chicken eggs. The next step will be to replace the F and HN protein of the LaSota strain (live attenuated vaccine strain) by the corresponding proteins of the MG725-08 strain (the more recent genotype XI with a modification of the F protein to introduce an avirulent cleavage site) and to evaluate the protection and viral shedding in chicken experiments.

A domain located at the amino terminus of the envelope glycoprotein (Gc) of an orthobunyavirus is targeted by neutralizing antibodies

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Objective: The Schmallenberg virus (SBV) is an arthropod-borne virus of the genus *Orthobunyavirus* of the *Bunyaviridae* family. SBV is enveloped and possesses a tripartite genome composed of three single-stranded RNA molecules of negative sense of different sizes, designated as small (S), medium (M) and large (L) respectively. The single ORF of the M segment encodes for the surface proteins, which according to their order in the precursor are referred to as Gn and Gc, and in between the NSm.

The humoral immune response against the orthobunyaviral glycoproteins in the course of a natural infection has not been analyzed so far. Therefore, the present study aimed at identifying the domains of the SBV-glycoproteins targeted by antibodies from naturally and experimentally infected animals as well as monoclonal antibodies (mAbs).

Methods: The fragments encoding for the ectodomains of the glycoproteins Gn and Gc were cloned into an eukaryotic expression vector with a twin-strep tag at the carboxyl terminus. The Gc protein was either expressed as full-length fusion protein with the Gn (*Gn-L-Gc*) or in two truncated forms, including its amino terminal third (*Gc Amino*) or its two carboxyl terminal thirds (*Gc Amino* Δ). The reactivity pattern of bovine and mouse sera from negative or infected animals was analyzed with each antigen in an indirect ELISA. The reactivity of anti-SBV Gc murine monoclonal antibodies was investigated with the three antigens, and the effect of disulfide bond-reduction on the antibody-antigen interaction was tested.

Results: The proteins “Gn-L-Gc” and “Gc AminoΔ” were isolated from cell lysates whereas the protein “Gc Amino” was recovered from the supernatant. The protein preparations of “Gn-L-Gc” and “Gc Amino” had a purity of over 90%. When cattle sera from infected animals were tested, the sera reacted clearly with the three antigens. In addition, the humoral immune response of experimentally infected mice resembled the pattern observed in the cattle. Furthermore, a panel of monoclonal antibodies against SBV-virions has been recently characterized (Wernike, Brocchi et al. 2015 Vet Res). We could show, that all anti-Gc antibodies recognized conformational epitopes of linear discontinuous nature that were disrupted upon reduction of disulfide bonds. The majority of the mAbs reacted with “Gc Amino”, whereas the epitope of the mAb 5F8 is located in the “Gc Amino?”. Interestingly, with the exception of one mAb, all those with a neutralizing activity against SBV recognized their epitopes in the “Gc Amino”.

Conclusion: In the present study, we isolated the SBV Gc-protein in its full-length form covalently linked to the Gn as well as in two subdomains that seem to fold properly in an independent manner. When applied as antigen in an indirect ELISA assay, these proteins were functional to characterize the humoral immune response against the Gc protein of the orthobunyavirus SBV in two animal species. The fact that monoclonal antibodies with virus neutralizing activity target the amino-terminal domain of the Gc protein of SBV underlines the importance of such a domain as an immunogen. Considering the similarities within the orthobunyavirus genus at a structural level, our study could be helpful for the development of diagnostics and prophylactic strategies also against other orthobunyaviruses.

Parallel ESVV Session 5:
Host / virus interactions & viral immunity 1

Tuesday 1st September 2015
14:30-16:30
Room Sully 2

Chair: Hans Nauwynck & Catherine Cêtre-Sossah

Postnatal persistent infection with classical swine fever virus: Hitherto unreported strategy of the virus to persist in domestic pigs and wild boars.

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It is well established that trans-placental transmission of classical swine fever virus (CSFV) during mid-gestation can lead to persistently infected offspring. The aim of the present study was to evaluate the ability of CSFV to induce viral persistence upon early postnatal infection in wild boars and domestic pigs. Ten new-born domestic piglets and fifteen new-born wild boars were infected intranasally within the first 10 hours after birth with the Catalonia 01 strain (Cat01, CSFV of moderate virulence). Viral replication, innate and specific immune responses were evaluated. During six weeks after postnatal infection (duration of the experiment), most of the piglets remained clinically healthy, despite persistent high virus titres in the serum, organs and body secretions. The levels of viral RNA detected were similar in both, domestic pigs and wild boar. The clinical signs recorded were also similar, with some temperature peaks above 40°C mainly during the first 14 days post infection. Approximately 50 percent of infected animals (pigs and wild boar) were persistently infected without any clini-

cal signs at the end of the experiment. Importantly, these animals were unable to mount any detectable CSFV-specific humoral immune response. Four weeks after infection, PBMCs from the persistently infected seronegative piglets were unresponsive to both, specific CSFV and non-specific PHA stimulation in terms of IFN-γ-producing cells. In the case of wild boar, heterogeneous results were observed in the INF-γ-producing cells after CSFV and PHA stimulations. Although scarce, IFN-γ- producing cells were detected upon CSFV stimulation in three out of nine inoculated wild boars at week 4. Furthermore, high level of IFN-γ-producing cells was detected after PHA stimulation in four of the infected animals. However, a decrease or lack of IFN-γ-producing cells from week 4 to 6 against Cat01 CSFV or PHA was observed. These results suggested the development of a state of immunosuppression in these postnatally persistently infected animals. Taken together, we provided the first data demonstrating the feasibility of generating a postnatal persistent CSFV infection, which has not been shown for other members of the Pestivirus genus yet. Since serological methods are routinely used in CSFV surveillance, persistently infected animals might go unnoticed. In addition, the induction of persistent infection in wild boar provides new insights towards understanding possible mechanism of maintenance of CSFV in the European countries. These experiments were approved by the Ethics Committee for Animal Experiments of the Autonomous University of Barcelona (UAB) according to existing national and European regulations.

Inefficacy of a CSFV live attenuated vaccine when postnatal persistent infection takes the stage

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Classical swine fever (CSF) is a devastating disease for the pig industry worldwide. The endemic countries implement vaccination programs against classical swine fever virus (CSFV) in their health policies for prevention and control. Live attenuated vaccines are efficacious and are the most extensively used, regardless of their inability to differentiate vaccinated from infected animals. However, even with intensive vaccination programs in endemic areas for more than 20 years, CSF has not been eradicated yet. Currently, moderate-virulence and low-virulence strains have become the most predominant strains prowling in endemic areas, related to a milder manifestation of the disease. Molecular studies conducted in some of these countries strongly suggest that the viral variants circulating in the field have evolved under positive selection pressure exerted partly by the immune response to the vaccine strain. Recent work by our group demonstrated that a high proportion of persistently infected piglets can be generated by early postnatal infection with a low and a moderate virulent CSFV strains.

Considering the CSF epidemiologic situation in endemic zones and the epidemiologic implication that persistent infected animals may exert in the eradication of the disease, we studied the immune response to a live attenuated CSFV vaccine (lapinized C-strain) in 6-week-old piglets persistently infected with the Catalonia 01 strain (Cat01). CSFV-negative pigs were vaccinated as control. The humoral and interferon (IFN)-gamma responses, as well as the IFN-alpha levels and the CSFV RNA load in sera (Cat01 and C-strain) were monitored during 21 days post vaccination. None of the persistently infected piglets developed a detectable immune response against the vaccine virus, in contrast to the control group. Interestingly, despite high levels of CSFV RNA load of the Cat01 strain, vaccine viral RNA remained undetectable in the serum samples and in the tonsils of the CSFV postnatal persistently infected pigs during the trial. To our knowledge, this is the first report demonstrating the absence of response to vaccination in CSFV persistently infected pigs. Furthermore, these findings provide insights towards better understanding of CSFV persistent infection. The experiments were approved by the Ethics Committee for Animal Experiments of the Autonomous University of Barcelona (UAB), according to existing national and European regulations.

Transcriptome analysis reveals the host response to Schmallenberg virus in bovine cells and antagonistic effects of the NSs protein

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Objective: Schmallenberg virus (SBV) belongs to the *Orthobunyavirus* genus within the *Bunyaviridae* family. The virus emerged in European ruminants in 2011. It causes mainly mild symptoms in adults but can also cause foetal malformations and abortions. As for other viruses within this family/genus the non-structural protein NSs has been identified as a major virulence factor. But despite of this little is known of the overall inhibitory effect and targets of NSs of orthobunyaviruses. In this study, we use RNA-seq to investigate the host response to SBV as well to a mutant Schmallenberg virus lacking NSs (SBVdelNSs) in bovine primary cells.

Methods: SBV and SBVdelNSs were used to infect bovine primary cells (three replicates each, and uninfected controls). At 16h post infection total RNA was extracted. Cell lysates were analysed by WB and IF to confirm viral infection prior to RNA-seq using Miseq. Reads of high quality was referenced back to the bovine genome and differential analysis was performed to identify up- and down-regulated genes in the SBV and SBVdelNSs infected cells. The differential expressed genes (DE) were annotated and IPA was used for functional and pathway analysis.

Results: Almost 80% of the reads that passed the quality filtering assembled to the bovine reference genome. Few genes were differentially expressed in the SBV infected cells compared uninfected cells but over 600 hundred genes were differentially expressed in the SBVdelNSs infected cells. The majority of the identified genes are known to have antiviral functions. This was also confirmed by further pathway analysis, as the major pathways identified involved interferon signalling, activation of IRF by cytosolic pattern recognition receptors and NF-kb signalling etc.

Conclusion: This study shows in detail the effect of SBV NSs on the host transcriptome upon infection. It also shows how efficient NSs is in shutting down the immune response of the host, however, few antiviral genes are still up-regulated during SBV infection indicating that the virus can not completely shut down the immune response. The data from this study can thus serve as a base for more mechanistic studies on antiviral responses and NSs actions in orthobunyavirus infection.

Early events of canine herpesvirus 1 infections in canine respiratory and genital mucosae by the use of ex vivo models

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Objective: Canine herpesvirus 1 (CaHV-1) is a major pathogen in dogs. It causes a systemic disease in newborn puppies (fading puppy syndrome) and is associated with kennel cough and genital disorders in juvenile and adult dogs. The aim of this study was to examine the viral behavior during the early stages of infection in respiratory and genital mucosae, the portals of CaHV-1 entry.

Methods: Three CaHV-1 seronegative female dogs without any respiratory or genital problems were used in this study. Equal pieces (25mm2) of tracheal and vaginal mucosa explants were maintained in an air-liquid culture at 37°C and 5% CO2, and its viability was examined during 96 h of cultivation. All tracheal tissues were observed under a stereomicroscope for cilia beating, and only tracheal tissues in which cilia beating was present all over the explants were used. Tracheal and vaginal explants were inoculated with the CaHV-1 F205 strain at 24 hours after cultivation, and sampled at 0, 24, 48 and 72 hours post inoculation (hpi). Viral antigen positi-

ve cells were stained by a direct immunofluorescence staining and series of stained cryosections were acquired with a Leica TCS SP2 confocal microscope. A quantitative morphological analysis of viral replication and invasion in the mucosae was performed by measuring plaque latitudes and penetration depths underneath the basement membrane (BM).

Results: The tracheal and vaginal mucosa explants showed only a little decrease in tissue viability during 96 h of cultivation. Upon inoculation, CaHV-1 replicated in tracheal and vaginal mucosae in a plaque-wise manner as a result of direct cell to cell spread. The plaques were observed starting from 24 hpi and their latitude increased over time. At 48 hpi, the average plaque latitude in tracheal explants was smaller than that in vaginal explants. CaHV-1 started to penetrate the BM only after 48 hpi in tracheal explants but already after 24 hpi in vaginal explants. The plaque penetration depth increased over time and was larger in vaginal mucosa explants compared to tracheal mucosa explants.

Conclusion: The canine respiratory and genital explants were established in this study, enabling research on CaHV-1 replication and invasion in the mucosae. Both explants were successfully infected with CaHV-1. The virus spread in a plaque wise manner, as shown by immunofluorescence analysis. CaHV-1 showed a better capacity to replicate and invade vaginal mucosa compared to tracheal mucosa, based on the latitude and penetration depth of the plaques of viral antigen positive cells.

Epigenetic regulation of gga-microRNA-126 during lymphoproliferative disease in chicken

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Objective: Both in human and animal, some of the herpesvirus latent infections progress to cancer. Gallid herpesvirus-2 (GaHV-2), an oncogenic α-herpesvirus, modulates viral and cellular gene expression and triggers transformation of latently infected cells. This avian pathogen, responsible of the Marek's disease (MD), naturally infects chickens and provides a unique model for studying virus induced lymphoma development. This study focus on a microRNA (gga-miR-126) downregulated during the viral life cycle. Originally described as a miRNA mediating proper angiogenesis and vascular integrity, gga-miR-126 has been reported to impair cancer progression through signaling pathways that control tumor cell proliferation, migration and survival. The two aims of this study is to confirm the downregulation of this cellular miR during viral infection and to characterize this repression by studying epigenetic modifications as DNA methylation.

Methods: First, in order to confirm the downregulation of gga-miR-126, it was quantified by Q-RT-PCR, in tumoral latently infected cell line (MSB-1) and nine organs of three uninfected chicken (testicle, kidney, thymus gland, brain, cerebellum, lung, heart, liver and spleen). Then, to characterize this low expression level, DNA methylation pattern was assessed in MSB-1 cell line and in the nine organs through Bisulfite Genomic Sequencing Assay (BGSA).

Results: Gga-miR-126 was undetectable in the infected cell lines while in the nine organs it was expressed. Gga-miR-126 expression was more abundant in the lung, the heart, the liver and the kidney. It can be concluded that gga-miR-126 is not detectable in tumor-induced latently infected cells.

Gga-miR-126 is located on chromosome 17 into the 6th intron of the cellular gene EGFL-7 (Epidermal Growth Factor Like-7). By an *in silico* analysis, two CpG islands were found in this gene. One CpG island (CpG1) is located in the first intron and the other one (CpG2) is located around the precursor of gga-miR-126 (gga-pre-miR-126). The DNA methylation pattern was assessed in these two CpG islands. In MSB-1 cell line, the level of DNA methylation was very high at the two CpG islands (CpG1, 85 % and CpG2, 95 %). In the nine organs, DNA methylation was also observed in CpG1 and CpG2 with a different percentage. Indeed, a lower level of methylation was observed in CpG1 (from 7 % to 31 %) while CpG2 presented a high level of methylation (from 53 % to 85 %). The repression of gga-miR-126 in latently infected cells (MSB-1) might be explained by the high percentage of methylation at the two CpG islands. In human, these two CpG islands are also present in EGLF-7 and DNA methylation was assessed at CpG1 site in normal and tumoral cells (Watanabe et al., 2012). In tumoral cells CpG1 site presented a high percentage of methylation while in normal cells a low level of DNA methylation was

observed. Watanabe et al., quantified then hsa-miR-126 in tumoral and normal cells and observed that a low and a high expression were observed, respectively. These results showed that there is a relation between CpG1 site and the regulation of hsa-miR-126 in cancerous and normal cells.

Conclusion: For now in the chicken, it was observed a low level of gga-miR-126 in tumoral cells (MSB-1). This repression of gga-miR-126 could be explained by the high level of DNA methylation in the two CpG islands (CpG1 and 2) present into EGFL-7. It would be interesting to identify transcriptional start site(s) to assess if its expression is independent or dependent expression from EGFL-7 and to determine the exact impact of DNA methylation on gga-miR-126 expression.

Reference:

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A equine herpesvirus type 1 (EHV-1) abortion outbreak caused by a neuropathogenic strain in vaccinated mares

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Objective: Equine herpesvirus type 1 and type 4 (EHV-1 and EHV-4) cause infections of horses worldwide. While both EHV-1 and EHV-4 cause respiratory disease, abortion and myeloencephalopathy are more often observed after infection with EHV-1. Single nucleotide polymorphism in the EHV-1 polymerase gene (ORF 30) is significant for the determination viral strains as neuropathogenic or non-neuropathogenic (1). Odds of the neurological disease when infected with the neuropathogenic EHV-1 genotype versus the non-neuropathogenic genotype are 162 times greater (2). Even though neuropathogenic strains of EHV-1 can induce abortions cases of abortion without neurological disease are rare (3, 4). In Croatia during 2009 there was a significant difference in incidence of abortion and EHM in two reported outbreaks caused by the same neuropathogenic EHV-1. Same virus type caused different clinical signs with high fatal EHM rate in Quarter horse mares and abortion storm in Lipizzaner mares raising possibility of a breed as a risk factor in pathogenesis of EHV-1 infection (5).

Like the other herpesviruses, EHV-1 establishes a life-long infection in a high percentage of animals following exposure to the virus, and outbreaks could be initiated by viral reactivation. Reactivation is generally asymptomatic but sometimes abortion or neurologic disease can be recorded.

Vaccination had been widely used to reduce clinical signs of respiratory disease and the occurrence of abortion. Now days the only vaccine with a claim against EHV-1 induced abortions is an inactivated EHV-1/EHV-4 combination vaccine that is to be applied in months 5, 7 and 9 of gestation. Protection of vaccination is doubtful because evidence of the continuing cycle of EHV-1 and EHV-4 and clinical signs in vaccinated population was described (6).

In this article we describe an EHV-1 abortion outbreak caused by a neuropathogenic strain in vaccinated mares.

Methods: Abortion outbreak occurred in a group of Lipizzaner mares at the same breeding farm where abortion outbreak caused by a neuropathogenic EHV-1 strain in at that time naive mares was described in 2009. From February to July 2014 five out of 10 mares aborted in the last third of pregnancy (274-285 day). Additionally three mares gave birth to weak non-viable foals that died in the first 24 hours. Only two mares gave birth to healthy foals with no complications during foaling recorded. There was no evidence of other clinical signs that could be attributed to EHV-1/EHV-4 infection in the mares or any other animal on the breeding farm. Mares were aged from 5 to 16 year and all except one were resident in the same breeding farm during outbreak 2009. In six month period before first abortion and during outbreak 2014 no new arrival or transport of resident animals were documented. Since outbreak 2009 all horses were yearly vaccinated with an inactivated EHV-1/EHV-4 vaccine. In addition all pregnant mares were routinely vaccinated in months 5, 7 and 9 of gestation.

Sera samples from mares that have aborted were serologically tested to EHV-1 and equine viral arteritis virus

(EVAV) using virus neutralization-test (VN-test) and to leptospirosis using microscopic agglutination test (MAT). Lung, liver and spleen tissue samples of aborted foetuses and deceased foals were pooled and used for PCR testing for mentioned pathogens.

To specify the EHV-1 isolates, DNA polymerase catalytic subunit gene specific PCR was performed to find out if abortion outbreak is associated with neuropathogenic or non-neuropathogenic virus strain. Obtained nucleotide sequences were compared with those of other EHV-1, including former Croatian isolates isolated on the same breeding farm in 2009.

Table 1. Epidemiological data, serological results and genomic detection during the EHV-1 outbreak.

Outbreak data				Serology (paired sera)						Genom detection and/or isolation from aborted foetus		
Mare	Age (years)	Day of outbreak	Lenght of pregnacy	EHV-1 (VN-test)		EVA (VN-test)		Leptospirosis (MAT)		EHV	EVA	Leptospira sp.
1	16	1	274	1:23	1:91	<1:4	<1:4	<1:50	<1:50	+	-	-
2	13	19	285	1:91	1:128	<1:4	<1:4	<1:50	<1:50	+	-	-
3	16	24	278	1:91	-	1:32	1:45	<1:50	<1:50	+	-	-
4	15	28	274	1:128	-	<1:4	<1:4	<1:50	<1:50	+	-	-
5[b]	17	45	310	1:64	-	<1:4	<1:4	<1:50	<1:50	-	-	-
6[a]	5	47	325	-	-	-	-	-	-	-	-	-
7	15	52	277	1:128	-	1:91	1:91	<1:50	<1:50	+	-	-
8[a]	16	58	322	-	-	-	-	-	-	-	-	-
9[b]	15	86	341	1:128	-	1:32	-	<1:50	<1:50	+	-	-
10[b]	15	148	318	1:128	1:128	1:64	1:91	<1:50	<1:50	-	-	-

- , not sampled
a – foaled viable foals
b – gave birth to weak non-viable foals
Mares with confirmed abortion caused by EHV-1 are given in bold-faced letters.

Results: All eight mares that have aborted were serologically negative for leptospirosis, four were positive to EVA and all 8 were positive to EHV-1 with titre range from 1:23 to 1:128. There was no significant antibody titre rise in paired serum samples for any of those pathogens (Table 1).

Using PCR in pooled tissue samples none of them gave positive result for neither leptospirosis nor EVA. EHV-1 DNA was confirmed in tissue samples of all five aborted foetuses and one of three weak non-viable foals (Table 1).

Sequence analysis of the obtained partial ORF30 nucleotide sequences showed that abortion outbreak was caused by a neuropathogenic strain of EHV-1. All obtained nucleotide sequences were identical as well they were 100% identical with the sequences from EHV-1 isolated during outbreak on the same farm in 2009.

Conclusion: From February to July 2014 neuropathogenic strain of EHV-1 was confirmed as a cause of abortion on the same breeding farm were in 2009 EHV-1 abortion storme was described.

All aborted mares with confirmed EHV-1 abortion 2014 were aged between 13 and 16 years and were resident on breeding farm during outbreak 2009. Since no entry of new animals and no transport of resident horses abortion outbreak is probably caused by viral reactivation of the same virus that is present on the breeding farm since 2009. This thesis is supported by 100% identically nucleotide sequences of neuropathogenic EHV-1 isolated from aborted foetus with isolates from outbreak 2009 on the same breeding farm. Absence of clinical signs other than abortion is in accordance with significant influence of breed on clinical manifestation of neuropathogenic EHV-1 infection described during outbreak 2009 (5).

Since outbreak in 2009 all horses on breeding farm were annually vaccinated and pregnant mares, including six that have aborted during 2014 outbreak, were vaccinated in months 5, 7 and 9 of gestation. Cases of abortion outbreaks as a consequence of EHV-1 infection in regularly vaccinated mares are described as well as abortion in single animals as a consequence of local reactivation of infection. In this outbreak, abortions were consequence of local reactivation of virus in six animals five years after primal infection despite regularly vaccination during this period. Reactivation of latent EHV-1 in six animals in the same season must include some other risk factors in pathogenesis of EHV-1 infection. Even though risk factors for reactivation of the EHV-1 infection are still not clarified little is known about the potential adverse effects of administering a potent vaccine too frequently, which may attenuate the immune response.

Further studies must be done to validate existing vaccination recommendations and influence of frequent vaccination to reactivation of latent EHV-1 infection.

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Effect of inoculation dose and age of the pigs on clinical, virological and serological parameters of an African swine fever infection

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Objective: Until recently, the occurrence of African Swine fever (ASF) was restricted to countries in Africa and Sardinia. In 2007 the disease was introduced in Georgia and Russia and it reached the EU (Poland and the Baltic states) in 2014. This resulted in renewed interest for ASF in Europe. In Europe, transmission of the virus will predominantly be caused by direct contact between infected and non-infected pigs or through indirect contact with ASFV contaminated materials. An ASFV infection can result in a peracute, acute or sub-acute disease with a high mortality rate. Animals that survive an ASF infection usually become persistently infected (carriers). Detailed virus excretion dynamics of ASFV infected animals were investigated previously, but in young piglets only and with inoculation doses close to the minimum infectious dose (1, 2). Under field conditions many different age categories are present (either at a farm or in a wild population) that can become infected. Also, individual pigs may be exposed to very different doses of virus. For the implementation of efficient control measures, more detailed knowledge about virus excretion dynamics and survival in different age groups after an ASF infection is needed. The aim of this study was to investigate under controlled circumstances the possible effect of inoculation dose and age of the pigs on clinical, virological and serological parameters of an ASFV infection.

Methods: Four groups of five pigs each were used. Two groups (A and B) were 12 weeks old. The other two groups (C and D) were 18 weeks old. All pigs were inoculated with ASFV (Netherlands ’86, a moderately virulent strain) intranasally, 1ml in each nostril. Groups A and C were inoculated with a relatively low dose (2ml of 103.5TCID50/ml) while groups B and D were inoculated with a relatively high dose (2ml of 106 TCID50/ml).

Post infection, body temperatures and clinical signs of the pigs were monitored daily. Occurrence and severity of clinical signs were recorded on score lists (list of Mittelholzer (3), adapted for ASF).

Blood samples and OPF samples were taken at days 0, 3, 5, 7, 10, 13, 17, 20, 24, 27, 31, 34, 38, 41,

45, 48, 52 and 55 post infection (dpi). Serum samples were taken at 0, 7, 10, 17, 27 and 55 dpi. Air samples were taken at 0, 7, 10, 13, 17, 20, 24 and 27 dpi (MD8, Sartorius). From the blood samples, leucocyte and platelet counts were performed. The blood samples, OPF samples and air samples were tested by qPCR for the presence of virus. The serum samples were tested by ELISA and IPMA for the presence of antibodies against ASFV.

Results: After inoculation, all animals developed fever (> 40.5) and clinical signs of an ASF infection. A remarkable drop in both the number of leucocytes and platelets was observed in all animals, resulting in leucocytopenia (< 10 x 10^9 cells/L) and thrombocytopenia (< 200 x 10^9 cells/L) in almost all animals. In group A, all animals died with maximum clinical scores (Max CS) of 15-12-13-12-13 respectively. In group B, four animals died (Max CS 5-19-8-13) and one animal survived the infection (Max CS 9). In group C, two animals died (Max CS 9-14) and

three animals survived (Max CS 5-5-5). In group D, three animals died (Max CS 17-5-15) and two survived (Max CS 5-9). Thus, more survivors were observed in the older pigs, and survivors had a significantly lower maximum CS than the pigs that died. None of the surviving pigs showed clinical signs of ASF at the end of the experiment. All animals shed virus in their OPF after infection, with peak shedding around 7 dpi, and all survivors became carriers, still shedding virus in their OPF at the end of the experiment at 55 dpi. Also all animals developed a viraemia that lasted in the carriers until 48-55 dpi. All animals that survived after 7 dpi developed detectable levels of antibodies against ASFV. Air samples from all groups tested positive during several days in the period from 7-17 dpi.

Conclusion: For many parameters (cell count, viraemia, virus excretion) only minor differences between groups were observed. Age and infection dose seem to have no or only a very limited effect on these parameters. However, more of the older piglets survived the ASF infection (as compared to the younger piglets), with a lower maximum CS in the survivors. The carriers still shed virus and showed a viraemia after 48-55 dpi, without showing clinical signs of ASF. The different investigated virus doses (the lowest dose slightly above the 50% infectious dose for intranasal/oral infection (1) and the higher dose more than 300 times the lower dose) did not seem to have a significant effect on the progress of the infection. Once the infection actually catches on, the exposure dose doesn't seem to make a difference for the progress of the disease. Increased survival times will likely result in more virus shedding into the environment by older pigs. Older pigs may therefore play a more important role in transmission of the virus in both in the acute and chronic phase of the disease. Carriers can shed virus for a long time, while showing no clinical signs. The extent to which carriers were able to transmit the virus in the carrier phase was investigated in a separate study

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Tubulins interact with porcine and human S proteins of the genus Alphacoronavirus and influence the viral replication cycle of the porcine coronavirus TGEV

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Objective: Coronaviruses (CoV) are single-stranded RNA viruses with positive orientation. Three to four structural proteins are embedded in their lipid bilayer – the spike (S) protein, the membrane (M) protein, the envelope (E) protein, and in some cases the hemagglutinin esterase (HE) protein. Within the virion the nucleocapsid (N) protein is associated to the viral RNA. Coronaviruses mainly cause infections of the respiratory and/or the intestinal tract of animals and humans. In recent years, CoVs like the Middle East respiratory syndrome coronavirus (MERS-CoV) and the severe acute respiratory syndrome coronavirus (SARS-CoV) in humans emerged. Since 2012, the porcine epidemic diarrhea coronavirus (PEDV) leads to high economic losses in the North American pig industry. Transmissible gastroenteritis virus (TGEV) is a porcine CoV, which belongs like PEDV to the genus *Alphacoronavirus*. We use TGEV as a representative of the genus *Alphacoronavirus* for our analysis of the biology of coronavirus-host interaction. The coronavirus S protein is responsible for receptor binding as well as membrane fusion. It contains a large ectodomain, a transmembrane domain and a cytoplasmic domain. Within the cytoplasmic tail of the TGEV S protein, a tyrosine-based sorting signal as well as a cysteine-rich domain is present. Both are important for the proper incorporation of S proteins into viral particles and the subsequent release of infectious virions (Gelhaus et al., 2014; Schwegmann-Wessels et al., 2004).

The interaction between coronaviruses and their host cells during the viral replication cycle are quite complex and not fully understood. We hypothesize that an interaction of the S protein cytoplasmic domain with cellular

proteins during assembly may play a crucial role for the generation of infectious virions. Therefore, we work on the identification of cellular interaction partners and analyze their role in the assembly process of porcine and human CoVs.

Methods: By using a GFP pull-down method, potential interaction candidates were precipitated with the last C-terminal 39 amino acids of the TGEV S protein and identified by mass spectrometry. Candidates interacting with the S protein of TGEV and two human CoVs (NL63 and HCoV 229E) were confirmed by co-immunoprecipitation studies. The role of tubulin during TGEV infection was further analyzed by nocodazole treatment to inhibit tubulin polymerization. Viral infectivity titers were quantified by a plaque-assay. Co-localization studies of viral proteins with endogenous tubulin were performed in infected as well as in transfected cells and analyzed by confocal microscopy.

Results: Several tubulins were found to interact with the 39 amino acid stretch of the TGEV S protein or the cytoplasmic domain of the two human CoVs tested so far (NL63 and HCoV 229E). Nevertheless, these two human proteins differed in their intensity of interaction with specific tubulins. After nocodazole treatment the infectivity of TGEV was significantly reduced. By confocal microscopy a partly co-localization of the TGEV S full length protein fused to GFP with endogenous beta-tubulin was observed. Inhibition of tubulin polymerization led to a redistribution of TGEV S protein from the perinuclear region 6 hours post infection.

Conclusion: Our results indicate that tubulins can interact with the cytoplasmic domain of CoV S proteins of the genus *Alphacoronavirus* and may help the S protein during the viral assembly process.

In further studies, we will analyze if the cytoplasmic domain of the SARS S and MERS S protein can interact with tubulins as well. These S proteins have no retention signal compared to the other tested S constructs. We are interested in exploring the role of the tyrosine-based retention signal of alphacoronaviruses for tubulin binding. The S protein of PEDV will be included in our future studies.

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Parallel ESVV Session 6: Emerging virus & diseases 1

Tuesday 1st September 2015
14:30-16:30
Room Sully 3

Chair: Wim van der Poel & Norbert Nowotny

Identification of a novel HCV-like virus in German domestic cattle

Christine Baechlein [1], Nicole Fischer [2], Adam Grundhoff [3], Malik Alawi [3], Daniela Indenbirken [3], Alexander Postel [1], Jennifer Offinger [4], Kathrin Becker [5], Andreas Beineke [5], Juergen Rehage [6], Paul Becher [1]

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Evolution of animal viruses may force the emergence of novel pathogens with the potential to switch hosts and to cause disease in human individuals. Thus, it is of major importance to search for possible virus reservoirs and to describe the risks arising from such animal hosts for public health.

By applying unbiased high-throughput sequencing methods, a so far undescribed virus of the *Flaviviridae* family was discovered in a bovine serum sample. *De novo* assembly yielded a near full length genome of 8,841 nucleotides harboring a single open reading frame which codes for a polyprotein of 2,779 amino acids. Comparative genome analyses showed that the virus belongs to the genus *Hepacivirus*. For analysis of the hepacivirus prevalence, bovine serum samples from different regions of Germany were investigated by RT-PCR. Our results showed that five out of 158 cattle herds held at least one virus positive animal. Phylogenetic analyses demonstrated that the bovine hepacivirus sequences were only distantly related to respective sequences from dogs, horses, rodents, and bats. Repeated RT-PCR analyses of animals from two independent dairy herds proved that a substantial percentage of cows were infected. Individual animals were chronically infected as demonstrated by the presence of viral RNA in the blood for over eight months. Furthermore, a liver tropism for bovine hepacivirus was revealed by quantitative PCR analyses and the identification of a putative miR-122 binding site.

Future steps will include the establishment of a serological assay and will concentrate on the (sero-) prevalence of the bovine hepacivirus in the life stock population in Germany and other countries. Investigations will focus on potential transmission routes via milk and meat products and will assess the zoonotic potential of this newly described virus.

Detection of a novel bovine astrovirus in a cow with encephalitis

Kore Schlottau [1], Christoph Schulze[2], Sabine Bilk [2], Dennis Hanke [1], Dirk Höper [1], Martin Beer [1], Bernd Hoffmann [1]

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Encephalitis can be caused by many infectious agents, including bacteria, fungi, parasites and viruses. Often the causative agent cannot be identified, due to the fact, that the diagnostic methods are not suitable, or because it is a matter of new or unexpected agents. In June 2014 a 15 month old cow developed central nervous disor-

ders and died within 6 days. The histopathology revealed an acute rhombencephalitis and ganglionitis of the trigeminal ganglion with massive neuronal necrosis in brain and ganglion. The relevant bacterial and viral infections of cattle were routinely excluded.

A brain sample from the cow was analyzed using metagenomic next generation sequencing and a bovine astrovirus (BoAstV) was identified. To confirm these findings a specific BoAstV RT- qPCR as well as a pan-reactive Mamastrovirus (MAstV) RT-qPCR for screening purposes were developed.

The genome of the novel bovine astrovirus (BoAstV), belonging to the family of *Astroviridae* in the genus *Mamastrovirus*, had a length of 6443 bp and showed only 71% sequence identity to a sheep astrovirus and 69% sequence identity to two newly described bovine astroviruses from the USA and Switzerland, which also caused encephalitis in cattle. The new virus was found in different brain sections using a specific quantitative BoAstV RT-PCR. Screening of the goat herd, in which the cow had been kept, revealed that no further animals were infected.

Astroviruses are mainly connected to gastroenteritis and are common in many animals and humans. Although, recently astrovirus caused encephalitis was described in a few human cases and also twice in cattle. However, the source of infection remains obscure and work on this newly discovered bovine astrovirus will be extended.

Peste des petits ruminants in Spanish sheep breed: protection study and experimental infection using NIG75/1 vaccine strain and MOR/08 field isolate

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Introduction and objectives: Peste des petits ruminants virus (PPRV), classified in the genus Morbillivirus, within the family *Paramyxoviridae*, causes an acute transboundary animal disease that affects mainly small ruminants, whereas cattle undergo a subclinical infection. PPR is considered to be highly contagious and spreads rapidly by direct contact through excretions/secretions from sick animals. This ability to spread, together with its socio-economic importance in developing countries made it one of the notifiable diseases listed by the OIE[1].

The severity of the disease depends on diverse factors such as host species and/or breed as well as on the virus strain involved. The acute clinical pattern is characterized by high fever, nasal and ocular discharge, oral lesions, bronchopneumonia, evidenced by coughing, dyspnoea and often diarrhoea. Affected animals may die within 5–10 days after the onset of clinical signs, reaching up to 90 % of mortality and morbidity in naïve populations. A subacute form is known in which clinical signs are very mild, the animals usually recovering within a week of the onset of symptoms.

PPR is endemic in most of Africa, the Middle East, South Asia and China. The most effective way of controlling PPR is by vaccination. Attenuated vaccines, mainly Nigeria 75/1 strain, have been commonly used in different endemic zones, inducing a reported life-long protective immunity in sheep and goats against all genetically defined lineages. Due to the epidemiological and economic significance, the international animal health organizations have given priority to the establishment of effective vaccination programs, control of animal movements and availability of efficient and rapid diagnosis.

Recently, PPRV strains belonging to genetic lineage IV have undergone a major expansion in close proximity to the Southern European borders, increasing the perception of risk associated with its introduction in this continent. Consequently, preparedness plans were implemented in Southern European countries to enable an earlier response in case of PPR emergence in their territories. In the framework of these plans, a project was implemented in Spain on the effect of PPRV in local sheep breeds, which included an evaluation of the efficacy of the vaccines available. The study presented here aimed at evaluating the protection conferred by the immunization of a Spanish native sheep breed with the most widely used live attenuated PPRV vaccine (Nigeria75/1 vaccine, lineage II) against a challenge with a pathogenic strain of PPRV isolated in Morocco in 2008 (Mor/08), belonging

to lineage IV. A second objective was to study the disease pattern produced in this Spanish sheep breed by the same pathogenic PPRV strain.

Methodology: PPRV Nigeria75/1 attenuated vaccine was inoculated subcutaneously to Spanish "Colmenareña" sheep breed (n=6) at a dose of 10^3 TCID50. At day 21 post-vaccination, four out of the six vaccinated sheep were challenged intravenously with a virulent PPRV Moroccan strain (Mor/08) at a dose of 10^4TCID50 and housed in contact with two naïve sheep (Group A). In parallel, in a different housing physically separated from the above group, another group of naïve sheep (n: 4) were infected intravenously with identical dose and strain, and housed together in contact with two naïve sheep and two previously vaccinated sheep (Group B).

Samples consisting of whole blood, serum, faeces, ocular, pharyngeal and nasal swabs, were collected at different days post-infection (dpi) and/or post-vaccination (dpv). Clinical signs using a clinical score system and rectal temperature (ºC) were recorded daily. Selected tissues were collected at post-mortem examination from all sheep used in the experiment.

Total RNA was extracted using QIAamp®cador Pathogen Kit (Qiagen®), adding an exogenous internal positive control (IPC). A real-time qRT-PCR reference protocol2 was adapted as a duplex method in order to incorporate IPC primers and probe. In order to detect specifically the vaccine RNA (Nig75/1), a DIVA qRT-PCR was developed. Duplicates of serum samples were tested for detecting PPRV-specific antibodies with a competitive ELISA[3], according to the manufacturer´s instructions.

Results:

Clinical follow-up: Sheep remained healthy during the vaccination stage. Group A (vaccinated and in-contact control sheep) remained asymptomatic after intravenous challenge. However, Group B presented a varying degree of symptoms: while the naïve infected sheep and in-contact sheep developed mild and discontinuous clinical signs consistent with the milder form of PPR disease, in- contact vaccinated sheep did not show any symptomatology. Necropsies displayed unspecific lesions in most of the animals. Only mild lesions were observed in symptomatically affected sheep.

Viral load estimated by qRT-PCR: Neither viral load in blood nor fecal virus shedding was detected by qRT-PCR in vaccinated animals prior to challenge. Only low levels of vaccine RNA were occasionally detected in swabs in 3 out of 6 individuals, gradually disappearing.

After intravenous challenge with the virulent strain, Group A remained RT-PCR-negative at any time in all samples examined, evidencing a sterilizing protective response on immunized sheep, and showing that no cross-infection between challenged and contact sheep occurred, which demonstrated an absence of PPRV circulation in the animal setting hosting Group A.

Regarding Group B, PPRV RNA was detectable by qRT-PCR as early as 2 dpi in whole blood in intravenously inoculated sheep before the onset of the first clinical signs, peaking at 7 dpi on average, and rapidly decreased at 10 dpi, but persisting for up to 21 dpi in all sheep and later showing up intermittently in some sheep up to the end of the experiment (up to 37 dpi). Virus excretion/secretion in collected swabs and faeces samples was first detected at 4 dpi, with a peak observed at 7 dpi on average, and then gradually declined. On the other hand, transmission of PPRV from infected to in-contact animals, including both naïve control and vaccinated sheep, was observed, with a kinetics of viral load in blood (peaking at 17 dpi) and virus shedding (peaking at 21 dpi) in the unvaccinated animals consistent with the acquisition of the infection from the experimentally infected sheep housed together. Lower virus loads in swabs and faeces and lack of viremia throughout the experiment were observed in the vaccinated contact sheep.

Serology: Vaccinated sheep developed detectable antibodies by c-ELISA at 7 dpv peaking at 10 dpv on average, remaining positive until the end of the vaccination phase.

During the second stage of the study, vaccinated sheep of Group A remained seropositive after challenge up to the end of the experiment, while the absence of antibodies in naïve contact controls confirmed that there was no cross-infection in this setting.

Similarly, in Group B, animals infected with MOR/08 strain showed antibodies from 4-7 dpi, rising on 7-10 dpi and remaining high during the period of the study. Within Group B, in-contact naïve and in-contact vaccinated

sheep showed different results: the first seroconverted between 14 and 21 dpi, while in the vaccinated group, one individual maintained the antibody titre acquired in the vaccination phase, but the other one showed a gradual decrease in its humoral response raised by vaccination to become undetectable after 10 dpi until the end of the experiment (30 dpi).

Vaccine genome detection by DIVA qRT-PCR: Tested at necropsy, in Group A, PPRV vaccine genome was present mainly in lymph nodes and spleen in vaccinated sheep but not detectable in unvaccinated control sheep. Notably, only vaccine RNA was detected in vaccinated sheep from Group B (kept in contact with infected sheep), but to a much lesser extent than in vaccinated sheep in Group A. Naïve control and infected sheep from Group B showed no vaccine RNA but a high level of challenge virus RNA in almost all selected tissues.

Conclusions: Spanish sheep breed "Colmenareña" inoculated with the pathogenic strain Mor/08 showed generally mild clinical signs, suggesting that this sheep breed is not particularly susceptible to PPR disease, at least in the conditions of this study. Vaccination with live attenuated vaccine PPRV Nigeria75/1 strain protected these animals against direct intravenous challenge with the pathogenic strain PPRV Mor/08. Conversely, the results evidenced the ability of Moroccan PPRV to spread by direct contact from excretion/secretion sites to contact sheep, including vaccinated ones. Absence of viremia and clinical signs in these vaccinated sheep that became infected, probably through contact, suggests the activation of a kind of immune response conferring partial protection. The role this process plays in the epidemiology of PPR as generator of potential asymptomatic carriers is as yet unknown. Interestingly, the loss of antibodies observed in one of these sheep upon challenge might indicate a weakness of the humoral antibody response elicited by vaccination in this particular sheep. Whether this loss is permanent or transitory and could be recovered after 30 dpi (monitoring period in the experiment) is not known.

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Novel insights in experimental peste des petits ruminants virus infection, transmission, pathogenesis and propagation

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The recent rapid spread of PPRV lineage 4 (L4) to Northern African and in Asian countries bordering Europe poses a high risk of PPRV emergence to the naïve population of susceptible European animal species. PPRV-pathogenesis in goats that are highly susceptible to PPRV infection has been thoroughly investigated. However, the maximal duration of excretion of infectious PPRV after experimental infection and the possibility of oral PPRV-infection remain to be investigated. Whether other Artiodactyla species might contribute to PPRV-spread is scarcely understood, but is important for the design of effective vaccination strategies with regard to the discussed plans about the eradication of PPRV.

Therefore, various animal trials were conducted to further illuminate these gaps in knowledge:

1) The minimum infectious dose (MID) of a highly virulent PPRV-lineage 4 strain from Kurdistan (2011) was investigated in 4 groups of 4 goats and compared to virus isolation in a Vero cell line expressing CD150 (SLAM). Four goats in each group were intranasally infected with any of the undiluted or tenfold diluted (10^{-1} to 10^{-3}) PPRV preparations. In four goats that showed mild clinical signs the maximum duration of PPRV excretion was monitored until PPRV-RNA excretion has ceased according to real-time RT-PCR analysis at 90 dpi.

2) Three cattle and three pigs were intranasally infected with PPRV Kurdistan/2011 in two independent experiments. Two days after infection (dpi), two goats were housed together with the cattle and pigs, respectively.

3) Four goats were orally infected with cell culture supernatant of PPRV Kurdistan/2011.

Oronasal, conjunctival and faecal swabs, blood and urine were collected from the animals in regular intervals and tested for PPRV antibodies, PPRV-RNA and infectious PPRV. Various pathological samples were collected from PPRV-infected animals at post-mortem examination. Furthermore, the performance characteristics of different serological and virological test systems were compared using reference materials collected during the animal trials.

In summary, the animal trials revealed important differences in the excretion dynamics and pathogenesis of various Artiodactyla species and in PPRV infection depending on the inoculation route in goats. Furthermore, PPRV detection and propagation considerably differed between different virological test systems. The final results will be presented and discussed in comparison to published data.

Serological Evidence of Peste des Petits Ruminants in Yak, Pakistan

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Peste des Petits Ruminants (PPR) is highly contagious and acute viral disease of mainly small ruminants such as goats and sheep but can also affect wild animals infrequently. The yak (Bos grunniens and Bos mutus) is a long-haired mammal found throughout the Himalayan region of south Central Asia, the Tibetan Plateau and as far north as Mongolia and Russia. In Pakistan, yaks are confined to the high plateau of the Northern Areas, from Gilgit to the valley of Ladakh. There is no current information on the presence of PPR in the hilly areas of Pakistan. There is quite high number of animal population including yaks which share the wild as well as domestic habitat. In this study, a total of 250 serum samples of healthy yaks were collected from four different localities of Gilgit Bultistan province. The sampling was done with two different age groups of animals. The first group comprised of 2 years or less of animals, while other was more than two years of age. N-cELISA was used to detect the antibodies against PPRV. Twenty three out of 250 serum samples were found positive for PPRV antibodies' giving an overall seroprevalence was around 9.20 % but has significance as considering that there was no current active clinical outbreak reported in area. There were more positives in animals with age more than two years depicting the old presence of PPR in these populations. The presence of positive in animals with less than two years of age shows that the disease episode was occur even in the near past. Presence of positive animals in all four localities showed that the PPR infection could be wide spread in the area. So the study provides the serological evidence of PPR in yaks and signifies the need to include the wild and companion animals in the campaign towards progressive control and eventual eradication of PPR from this region.

Outbreak of henipavirus in the southern Philippines, 2014

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Objective: This paper describes the investigation of an outbreak of neurological disease with high case fatality amongst people and horses in southern Philippines in 2014. Clinical case presentation, epidemiologic findings and laboratory results suggest that the causative virus was a henipavirus, most likely Nipah virus or a virus closely related antigenically and genetically. The outbreak occurred in an area where consumption of horsemeat is customary, and there appeared to be a strong epidemiological link between involvement in the slaughter of horses and human illness.

Methods: From March-May 2014, in two small villages in Sultan Kudarat province in the southern Philippines, at least 11 people suffered acute encephalitis and 9 horses developed neurological signs. Post-outbreak investigation by a joint World Health Organisation and Philippines Department of Health team included interviews of villagers and collection of sera from convalescent human cases and in-contact individuals and animals. These samples and the limited number of clinical human samples available were tested for a range of possible aetological agents. Both agent detection and serological assays were used. As all horses had been consumed, no clinical samples were available for testing.

Results: Eleven cases of acute encephalitis in people were considered to be outbreak related. Of these, 9 individuals died. Additionally, 5 cases of severe influenza-like illness (ILI) and one with meningitis were also thought to be outbreak related.

Epidemiological investigation showed a strong link between clinical disease in people and involvement of those individuals in the slaughter of horses and/or the handling of raw horse meat for preparation for consumption. However, for at least 5 cases, clinical and epidemiologic evidence suggested direct human-to-human virus transmission. No protective equipment was used by those who cared for case-patients in the home, and health care workers used gloves and a face mask but not eye protection.

All horses were either found dead or were slaughtered after acute onset, rapidly progressive neurological signs including ataxia, circling and headpressing. Four deaths in cats were also thought to be outbreak related.

All samples were negative for all neurological aetiologies for which they were tested, except for henipaviruses. Three recovering individuals (two recovering from encephalitis and one from ILI) had antibodies to Nipah virus in a range of serological assays. Some of the same sera also tested positive to Hendra virus, but to significantly lower titres than to Nipah virus. Only one sample, a serum sample from an individual that subsequently developed Nipah antibodies, tested positive to Nipah virus in a real-time PCR assay. Laboratory testing was limited by the small number of samples available from clinical cases and sample degradation due to the remote area of the outbreak andconsequent lack of appropriate storage facilities. No virus could be isolated from any samples and no significant sequence data could be obtained.

Subsequent testing of sera from a range of bat species showed antibodies to Nipah virus in Pteropus vampyrus bats sampled near the outbreak region.

Conclusion: Based on case presentation in people and horses, epidemiologic findings and laboratory results the outbreak was a result of a henipavirus. The causative virus was either Nipah virus or a virus that is antigenically and genetically closely related to Nipah virus.

Epidemiologic data suggest that the most common route of virus transmission to humans was direct exposure to infected horses, contact with contaminated body fluids during slaughter of sick horses, and/or consumption of undercooked meat from infected horses. The evidence of human-to-human transmission in this outbreak confirms the need for preventative measures in home care and health care settings.

As with all henipaviruses, it is expected that the reservoir host is a flying fox (one or many species). Preliminary serological testing confirms the presence of antibodies in at least one species of flying fox within the area.

Rapid response to new outbreaks, collection of appropriate samples for further characterization of the virus and investigation of risk factors associated with spillover and virus transmission are all required for prevention of future outbreaks.

Kobuvirus in brains of piglets diagnosed with congenital tremor type A-II

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Objective: Congenital Tremor (CT) is a sporadic disease seen in neonatal pigs. It causes symptoms such as muscle tremor, shaking of the body and incoordination. These symptoms can disable the piglet from finding a teat, potentially leading to its death. CT has been linked with inheritance, viruses and toxins. It is classified as type A (displaying histopathological lesions in the central nervous system) and B (with no evident microscopic findings). The latter one is considered of unknown cause, while type A has been subdivided in sub-types I to V. The most common form worldwide is considered to be subtype A-II, which is transmissible and likely to be caused by an unknown virus. A number of efforts have been done to clarify the etiology of CT A-II, but so far, no definitive conclusions have been drawn. The aim of this study was to identify possible viral causes for CT A-II using a high-throughput sequencing (HTS) approach.

Methods: Initially, a total of 13 piglets showing clinical signs of CT were submitted to the Servei de Diagnòstic de Patologia Veterinària at the Universitat Autònoma de Barcelona (Spain). Causes of CT subtypes A-I, A-III, A-IV and A-V were ruled out by means of clinical history and epidemiological findings. In consequence, CT subtype A-II was established. Brain samples from these 13 pigs were transferred to Swedish University of Agricultural Sciences and prepared for HTS.

In brief, sample preparation was performed as earlier described [1] with the exception that homogenization was performed using Omni Tissue Homogenizer (OMNI International) and freeze thawing. Libraries were prepared by standard Nextera XT protocol and sequencing was performed on MiSeq (Illumina). Sequence data was pr ocessed with a previously described method [1] and analyzed using a new methodology involving data cleaning performed with PRINSEQ and a taxonomic classification of the sequences using the Kraken software [2]. Computing resources at UPPNEX and SLU Global Bioinformatics Centre, Uppsala, Sweden, were used.

The initial study was recently extend, using the same methodology to investigate several new cases of piglets with CT from Spain. In addition to brain biopsies, feces samples were also collected for a few individuals. Samples from healthy control animals were also include to allow better assessment of correlation.

Results: For the initial 13 cases, the sequencing generated 16.4 GB of data, varying between 0.9-1.6 GB per sample. On average 36% of the sequences, i.e. 33.9 million, were of good quality and used for classification. One of the most notable findings was that porcine kobuvirus, a positive-sense ssRNA virus of the picornaviridae family, was identified in 5 out of 13 samples (38%). The sequencing of the new cases and the healthy controls is ongoing.

Conclusion: Porcine kobuvirus (Aichivirus C) was first identified in 2007 in stool samples from healthy piglets in Hungary [3]. It has thereafter been found in many other countries, in serum samples and been inconclusively associated with piglet diarrhea. Close relatives to Kobuviruses within the Picornaviridae family are known to cause neurological symptoms. However, to date, no Kobuvirus have been identified in the brain. The relatively high prevalence of Kobuvirus identified in CT A-II pigs could be a step closer to clarifying the etiology of this devastating disease. Work to validate the findings in larger sample sets including healthy animals is ongoing.

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Identification of a new non-pathogenic lagovirus in Lepus europeaus

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Objectives: European Brown Hare Syndrome (EBHS) is a viral disease mainly observed in European brown hares (*Lepus europeaus*), characterized by mild nervous symptoms, severe necrotic hepatitis, and circulatory dysfunction. The causative agent of EBHS (EBHSV) is a calicivirus belonging to the *Lagovirus* genus, highly related to Rabbit Hemorrhagic Disease Virus (RHDV) (1). A third type of *Lagovirus*, named rabbit calicivirus (RCV) (2), has been detected in Italy in 1996 in domestic rabbit. This virus causes a silent infection of the intestinal tract without inducing clinical signs and relevant pathological lesions. The existence of a non-pathogenic EBHSV-like virus in hares has been put forward for explaining some "unexpected" positive serological results obtained either from brown hares and other lepus species individuals living in areas where the disease has never been detected (i.e. Australia, South American and Central Africa), but until now no direct evidences of its presence was reported.

The aim of this work is to report the results of investigations performed to seek this putative virus eventually resulted in the first identification of a non-pathogenic *Lagovirus* in hares.

Methods:

Sampling

Fecal (including rectal swabs) and blood samples from 30 young healthy hares (1-2 months of age) born and reared in cages in a breeding hare farm of North of Italy (Brescia province), were collected during the summer of 2014.

Extraction, detection and sequencing of viral RNA

Total RNA was extracted from feces of hares, analyzed by RT-PCR using the universal primers for lagovirus able to amplify a conserved region of the *vp60* gene. The 300bp amplification product was sequenced. To perform the phylogenetic analysis the entire *vp60* gene was completely amplified and sequenced and the sequences were aligned by ClustalW with the vp60 gene sequences available in GenBank. The Neighbor-Joining phylogenetic tree was generated, by using the Kimura 2 parameters evolutionary model implemented in MEGA 6.

ELISA assay

Serological analysis was performed using two ELISAs methods. A competition ELISA specific for Abs induced by the antigens on the outer shell of EBHSV (EBHSV specific) and an IgG ELISA able to detect also Abs reactive with antigenic determinants of the internal shell of EBHSV (lagovirus specific) (3). In both the ELISAs the antigen used was an homogenate of hare liver containing EBHSV.

Virus like particles production

Details on baculovirus expression of VP60 protein of this lagovirus is reported in the communication of Pezzoni et al. at this meeting (4).

Results: The fecal samples of three out of the 30 young hares sampled in the Brescia hares farm resulted RT-PCR positive. The sequence analysis of the entire capsid protein *vp60* gene showed an average of nucleotide identity of approximately 73% and aminoacidic identity of 82%, compared to the EBHSV sequences and respectively 68% and 76.3% compared to the RHDV sequences present in GenBank. Phylogenetic analyses based on the vp60 gene nucleotide sequences of the 3 studied strains and the available sequences of RHDV, EBHSV and RCV in the databases showed that the new isolates form a separate group in the clade of EBHSV.

ELISA tests performed on sera collected from the hares showed contrasting results. In the case of competition ELISA most of the sera resulted negative with just few of them positive at very low titers. Differently, most of the sera resulted positive in IgG ELISA with medium-high titers.

The VP60 protein, expressed in the baculovirus (4), correctly folded in a capsid calicivirus-like structure as sta-

ted by EM observation. Thus, it was adsorbed to an ELISA plates and tested with a panel of anti EBHSV MAbs. All the EBHSV specific MAbs (i.e. those recognizing epitopes on the surface of the EBHSV) resulted negative while 3 MAbs directed towards epitopes buried in the internal shell of the virus were positive.

Conclusion: In this study we identified a new member of the *Lagovirus* genus within the *Caliciviridae* family. The virus was quite easily identified from fecal materials of few hares but most of the rest resulted seropositive with the ELISA test that detects cross-specific antibodies. This suggests that the virus was probably present since some time in the farm. This aspect and the lack of any sign of disease among the hares, for sure not typical of EBHS, show that the viral infection has a subclinical course and thus the virus could be considered non pathogenic.

The genetic relationships determined on the complete capsid gene sequences show that the identified virus is phylogenetically distinct from all previously described members of the genus *Lagovirus* and forms a new genetic group.

Both serological data and the antigenic analysis performed on the baculovirus expressed VP60 demonstrate that the antigenic profile of the identified hare virus is largely distinct from that of EBHSV. From a strict serological point of view, this indicates that EBHSV and the new lagovirus constitute two distinct serotypes. At the light of the results obtained and on the basis of the present lagovirus nomenclature, we propose to preliminary name the new virus as “Hare Calicivirus” (HaCV).

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Parallel ESVV Session 7: Enteric viruses

Wednesday 2nd September 2015
09:15-10:30
Auditorium Pasteur

Chair: Jose-Manuel Sánchez-Vizcaíno & Thierry Lefrançois

Comparative analysis of the faecal virome of dogs with various inflammatory intestinal diseases

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Objective: The role of the intestinal virome in health and disease is gaining increased attention in human medicine, by the use of next generation sequencing which has allowed identification of diversity and distribution of the virome. These approaches can equally be applied to dogs. Thus this study aimed to identify and characterize the virome present in faeces collected from dogs with acute diarrhoea (AD) and chronic enteropathy (CE), and compare these with the virome present in healthy dogs.

Methods: Faecal samples from 8 healthy control dogs (CD), 8 dogs with AD and 8 dogs with CE were analyzed using a next generation sequence approach. A viral enrichment protocol, using a series of centrifugation, endonuclease treatments and bacterial filtration steps was performed. The enriched viral DNA and RNA was extracted using QIamp® Viral RNA mini kit and amplified using a sequence-independent single-primer amplification (SISPA) protocol. The amplified DNA/cDNA was sequenced using the Illumina MiSeq platform at The

Australian Genome Research Facility. Two bioinformatic pipelines were used to analyze the viral population. After selecting high quality reads (HQRs) and removing canine and bacterial sequences, sequence information was compared against the CAMERA viral reference database as well as the NCBI non-redundant nucleotide database. In order to better understand the viral diversity within this virome, whole genome sequences of selected viral species were characterized and phylogenetic analyses performed.

Results: We identified a total of 32,667 viral contigs, with 31,226 DNA viral sequences and 1,332 RNA sequences across all 24 dog samples.

The majority of viral hits from all faecal samples were bacteriophage from several families mainly from the Caudovirales order (73.8% in CD, 97.9% in AD and 99.7% in CE).

There were eight eukaryotic viral families identified, five of which represented RNA virus and three were DNA virus families.

Within the RNA virus families, sequences with high similarity to viruses within the *Astroviridae* (overall 28 contigs) and *Caliciviridae* (8 contigs) families were identified only in samples from dogs with AD (5/8 dogs and 1/8 dogs, respectively). While sequences similar to *Picornaviridae* (12 contigs) were identified in the faeces from one dog with CE and one with AD. Only sequences similar to *Reoviridae* (59 contigs) were identified in all three groups (1/8 in CD, 4/8 in AD and 2/8 in CE) and sequences similar to *Coronaviridae* (overall 1123 contigs) family were identified in CD and dogs with CE (1/8 in each group).

Sequences similar to three DNA families were identified. Sequences similar to the *Papillomaviridae* (overall 4 contigs) family were found in CD and dogs with CE (1/8 and 2/8 respectively). Sequences similar to members of *Parvoviridae* (overall 47 contigs) family were found in CD and dogs with AD (1/8 and 2/8). Sequences similar to *Adenoviridae* (1 contig) family were identified in one CD. The average size of all contigs was 241bp.

Further phylogenetic analysis and genomic characterisation was undertaken on 2 viruses. The 11 genome segments of a Rotavirus (Reoviridae) isolate were determined for a single faecal sample. Similarly, the sequence of the entire coding region of a Kubovirus (*Picornaviridae*) isolate was determined.

Preliminary analyses indicated that all rotavirus gene segments exhibited between 55% -98% nt homology to previously reported canine rotaviruses. The kubovirus sequence exhibited moderate nt homology (55%) to previously described genomes and clustered with other canine kobuvirus sequences available in GenBank.

Conclusion: The eukaryotic virome present in healthy dogs, and dogs with AD and CE was determined. Viral sequences from a range of different virus families, including both RNA and DNA families, and known pathogens implicated in enteric diseases were identified and characterized. Results from these studies indicate that metagenomic analyses are useful for the investigation of viral populations in the faeces of dogs with various inflammatory intestinal diseases. Studies to elucidate the epidemiologic and biologic relevance of these findings are warranted.

Clinical, virological, and immunological parameters during experimental feline enteric coronavirus infection

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Objective: Feline infectious peritonitis (FIP) has remained one of the few insurmountable and highly feared cat diseases to date. It is widely accepted that the majority of all FIP cases result from mutations occurring in the viral genome during common intestinal feline enteric coronavirus (FECV) infections. FECV is enzootic in virtually all multi-cat environments worldwide, but due to its low pathogenic character, FECV has not received much attention in the fight against FIP. Given that FECV infection precedes the development of FIP, the aim of this study was to contribute to the current understanding of the pathogenesis by performing experimental FECV infections, thereby filling some of the missing links, such as viral infectivity of oral and faecal excretions, the generation of neutralizing antibodies, the dynamics of several leukocyte subsets, and the evolution of the viral genome constellation during infection.

Methods: Three specific pathogen free cats were orally inoculated with the serotype I FECV strain UCD and monitored for 3 months. Virus shedding was quantified in faeces and oral fluid by 2 different RT-qPCRs (one recognising the 3' of all genomic and subgenomic mRNAs (3' qPCR), and the other recognising the ORF1b of the genomic RNA (5' qPCR)), and by virus titration in feline enterocyte cultures. Blood samples were used to assess viraemia (analysed by RT-qPCR), neutralizing antibodies (analysed by seroneutralization assay in enterocyte cultures), and different subsets of leukocytes (analysed by flow cytometry). Next generation sequencing was performed to reveal the viral genome constellation in faeces during an early and late time point of the infection.

Results: The three cats remained clinically healthy during the course of the experiment, although some loss of appetite and slight weight loss was noticed in 2 cats during the first week of the experiment. In these 2 cats, viral RNA was detected in faeces from day 2 (3' qPCR) or day 4 (5' qPCR) post inoculation (p.i.), and remained to be shed at high levels until 28 days p.i. Thereafter, viral RNA shedding dropped and both cats had ceased shedding by day 84 p.i. By using 2 RT-qPCRs, it was shown that the widely used 3' qPCR gave a viral genome overestimation of 3-4.3 log10. Infectious viruses were found from day 4 until day 21 or 28 p.i. It was remarkable that, whereas qPCR- and infectivity titres correlated well during the first week of the infection, infectivity titres rapidly declined to undetectable levels by 4 weeks p.i., while qPCR titres remained high until day 28 p.i. For both cats, comparison of the viral genome constellation during an acute (day 6 or 9 p.i.) and a later time point (day 28 p.i.) revealed some major changes in the spike protein, and some other amino acid changes in polyprotein 1a, nucleocapsid and/or 7b protein. In these two cats, neutralizing antibodies were detectable from day 9 p.i,

and a cell-associated viraemia was detected at infrequent time points after the onset of faecal shedding. No abnormal leukocyte numbers were noticed, except for a granulocytopenia in cat 1. Interestingly, the third cat showed a deviating infection pattern, characterized by absence of clinical signs, a delayed faecal shedding (from day 14 p.i.) that lasted for 6 months but was not infectious in enterocyte cultures, a delayed rise in antibody titres (from day 21 p.i.), and a viraemia that was detected far before any intestinal replication. Next generation sequencing of the virus shed by this cat at day 21 p.i. revealed that 83.8% of all faecally shed viruses had a deletion of 101 bp in the gene encoding for the 7b protein, but this deletion was completely restored at day 84. However, in accordance with the other cats, the viruses shed during this more chronic phase of the infection showed major amino acid changes scattered over entire spike protein, and some other amino acid changes in polyprotein 1a and the membrane protein. No abnormalities or differences were seen in leukocyte numbers compared to the other two cats, with the exception of CD8+ regulatory T cells, but if and how these cells played a role remains unknown. In all cats, oral shedding occurred at very low levels and was only detectable with RT-qPCR at inconsistent time points.

Conclusion: The simultaneous assessment of different clinical, virological, and immunological parameters during experimental FECV infection revealed 2 different infection/excretion patterns. Two cats showed a temporary, but highly infectious excretion, mild clinical signs, a cell-associated viraemia detectable at inconsistent time points from day 5 p.i., a highly neutralizing antibody response from day 9 p.i., and no abnormalities in leukocyte subsets. In contrast, a deviating infection pattern was noticed in another cat, characterized by a delayed, long-lasting faecal shedding (starting from day 14 p.i. and detectable up to 6 months) that was not infectious in enterocyte cultures, absence of clinical signs, a delayed antibody response (detectable from day 21 p.i.), and a cell-associated viraemia that was detectable far before any signs of intestinal replication. No differences could be seen in leukocyte numbers compared to the other cats, with the exception of CD8+ regulatory T cells, but their role remains elusive. Mutational variants were detected in the cat with the aberrant excretion pattern and, for all cats, in the viruses that were shed in the more chronic phase of the infection, but the reason for the onset and the impact of these mutations requires further investigation.

Factors affecting the occurrence of canine parvovirus disease in dogs

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Objective: The purpose of this study were to detect CPV in naturally infected dogs with clinical signs, admitted to several Veterinary Clinics distributed throughout the country, for a period of approximately 2.5 years (2012-2014) and to identify risk factors associated with CPV disease.

Methods: During the anamnesis of 209 dogs, general information was collected, which comprised breed composition, breed name, body weight, age, sex, lifestyle and housing conditions. The presence of the following clinical signs, attitude, hydration status, body condition, gastroenteritis (GI) signs, mucous membrane color, rectal temperature, were examined, as well as duration of infection, vaccination status and survival data were collected. Fecal samples of these dogs were screened for CPV by PCR assay and the positive samples were confirmed by sequence analysis. Logistic regression and chi-square test were used to investigate associations between each of the predisposing factors and CPV.

Results: Of the samples collected, 77.5% were tested positive. CPV infection was significantly associated with some general information variables: body weight (large body weight), age (>12 months of age), lifestyle (both lifestyles) that decreased of the risk to occur disease. Anthelminthic treatment (non-treated), age (>3 to 6 months of age) and season (summer) were associated to increase of the risk to develop the CPV disease. Clinical signs recorded at the physical examination such as attitude (depression or stupor), hydration status (1 to 9% of dehydration), GI signs (vomiting and diarrhea) and the duration of infection (5 to 9 days) were significantly associated with the presence of the virus. On the other hand, dogs in ideal body condition had less risk to get the

CPV disease than those in lower condition. The survival of CPV infected dogs was positively associated with breed (other purebreds than Labrador and mixed breed), age (up to 3 months) and duration of infection (<5 days).

Conclusion: The results indicate that although having a high morbidity, the Portuguese dog population has showed a high rate of survival (84%) to CPV disease. The variables associated for survival potential suggested a better prognosis for infected animals. The risk factors identified as significant in this study, are not usually reported. They are easy to obtain and can be used as prognostic indicators in the veterinary practice.

Complete genome characterization of recent and ancient Belgian pig group A rotaviruses and assessment of their evolutionary relationship with human rotaviruses

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Objective: Group A rotaviruses (RVAs) are an important cause of diarrhea in young pigs and children. Remarkably, pig RVAs can harbor a wide diversity of genotypes for their outer capsid proteins VP7 and VP4. However, our knowledge on the composition of the other 9 genes of pig RVAs is scarce, which hampers our full understanding of their evolutionary relationship with human RVAs. In the present study, it was aimed to reveal the complete genomes of a selection of Belgian pig RVA strains in order to assess their evolutionary relationship with human Wa-like RVAs. Furthermore, it was aimed to consider the risks for interspecies transmission events of pig RVAs to the human population.

Methods: The complete genomes of six recent (G2P[27], G3P[6], G4P[7], G5P[7], G9P[13], and G9P[23]) and one historic (G1P[7]) Belgian pig RVA strains were revealed using Sanger sequencing. The 5'- and 3'-terminal sequences were obtained using a modified version of the single-primer amplification method. Sequence analysis was performed using 4Peaks software, and multiple-sequence alignments were executed in MEGA 5.2.2. Substitution models were determined for each gene segment separately. Maximum likelihood trees were constructed, and pairwise distances calculated, in order to investigate the relationship between pig and human RVAs.

Results: In contrast to the large diversity of genotypes found for the outer capsid proteins VP4 and VP7, a relatively conserved genotype constellation (I5-R1-C1-M1-A8-N1-T7-E1-H1) was found for the other 9 genes in most pig RVA strains. However, the ancient strain possessed an I1 genotype for VP6, whereas T1 genotypes were found for NSP3 genes of 3 strains. One strain beared the rare E9 genotype for the enterotoxin NSP4. VP1, VP2, VP3, NSP2, NSP4, and NSP5 genes of porcine RVAs belonged to genotype 1, which is shared with human Wa-like RVAs. However, for most of these gene segments, pig strains clustered distantly from human Wa-like RVAs, indicating that viruses from both species have entered different evolutionary paths. However, VP1, VP2, and NSP3 genes of some archival human strains were moderately related to pig strains. Phylogenetic and amino acid analysis of the VP6, NSP1, and NSP3 genes and proteins, as well as amino acid analysis of the antigenic regions of VP7, further confirmed this evolutionary segregation. Most contemporary human strains that clustered in between pig RVA strains were suspected interspecies transmission events from pigs to humans, and most of them beared the P[6] genotype for VP4. Interestingly, 5 of the Belgian strains carried a gene duplication behind the stop codon of gene segment 11 (NSP5).

Conclusion: These results confirm the existence of a clear evolutionary relationship between pig and human RVA strains, but also indicate that viruses from both host species have entered different evolutionary paths. It seems that the species barrier is less strict for pig P[6] strains, but that chances for successful spread in the human population are hampered by the better adaptation of pig RVAs to pig enterocytes. However, future surveillance of pig and human RVA strains is warranted.

First detection and phylogenetic analysis of porcine bocaviruses identified in Slovenia

Ivan Toplak, Zoran Zlabravec, Petra Lainšcek, Danijela Rihtaric

University of Ljubljana, Veterinary Faculty - Slovenia

Objective: Porcine bocavirus (PBoV) has been first discovered and associated with postweaning multisystemic wasting syndrome and pneumonia. Later, several porcine bocaviruses have been detected worldwide and this report is the first detection and phylogenetic analysis of PBoV in Slovenian domestic swine herds.

Methods: 50 pig faeces samples were collected from randomly selected healthy pigs in slaughterhouse and 86 faeces samples from dead pigs with respiratory illness or diarrhoea. After extraction of DNA, samples were tested by real-time PCR targeting NS1 gene of PBoV.

Results: Among the total of 136 tested samples 40% were positive for PBoV in slaughterhouse and 33,7% in dead pigs. A representative number of positive samples (n=11) were amplified by conventional PCR and sequenced for phylogenetic analysis. Based on phylogenetic comparison of 636 nucleotides of the NS1, the detected viruses were clustered within PBoV3, into two previously determined genetic groups, Group I and Group IV. Into Group I were clustered 10 Slovenian sequences with 90,3 to 100% nucleotide identity to each other and between 92 and 97% identity to the closest sequences in GenBank. New strain of PBoV, clustered into Group IV, was identified with 79 to 81% nucleotide identity to other Slovenian PBoV and only with 84% nucleotide identity to the closest strains of PBoV from China and USA.

Conclusion: The detected high prevalence and genetic heterogeneity of PBoV in Slovenia is probably the result of importation of live positive pigs from different countries. Careful evaluation of potential economic impact, effective prevention and control strategies for this new virus are of essential importance.

Parallel ESVV Session 8: General virology 1

Wednesday 2nd September 2015
09:15-10:30
Room Sully 1
Chair: Linda Dixon & Thomas Mettenleiter

Next generation sequencing of British outbreak cases of Equine Infectious Anaemia Virus: sequence variation and phylogeny among European outbreaks

Daniel Robinson [1, 2], Nicolas Locker [2], Falko Steinbach [1], Bhudipa Choudhury [1]

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Objective: Equine Infectious Anaemia Virus (EIAV) is the aetiological agent of equine infectious anaemia (EIA) otherwise known as swamp fever. EIAV is a member of the *Retroviridae* family, genus lentivirus, which comprises viruses such as HIV, SIV, FIV and Maedi Visna virus. A trait all lentiviruses share is a low fidelity polymerase and integrase which results in error prone reverse transcription followed by integration into the host genome. Although EIAV has been much studied, this has mostly involved vaccine work or study as a model for HIV. Consequently, there is a gap in sequence data of EIAV strains. Despite the virus being present on every continent and outbreaks occasionally occurring across Europe, published full genomes only represent a range of American and Chinese molecular clones as well as selected American, Irish, Chinese and Japanese field strains. Wider geographical representation is available, but only in the form of individual genes or polyproteins (mostly the gag gene) which represent a number of European field strains. The lack of sequences makes phylogeny difficult and the extent of sequence variation difficult to establish.

In Europe EIAV is present mainly in Eastern and Southern Europe where it is consideredendemic in Romania and has a high incidence in Italy as well as being present in many surrounding countries. In Western and Northern Europe sporadic outbreaks have occurred in Germany, France and Belgium. Over the last decade there have been four outbreaks on mainland Britain. There were three outbreaks in 2010: one involving two asymptomatic cases in Wiltshire, one in Northumberland which involved a single asymptomatic case and one in Devon comprising a single symptomatic case. Then in 2012 there was a single outbreak in Cornwall with two cases, one was asymptomatic and the other symptomatic.

As currently there are no published EIAV sequences from British cases, this project aimed to carry out full genome sequencing of the six British cases from 2010 and 2012 using PCR and next generation sequencing (NGS) techniques to increase the diversity of full genome sequences in the public domain and shed light on the variation between geographically separate cases. This could also allow phylogenetic analysis to be carried out to allow some basic epidemiological examination.

Methods: Thirty six published or in house designed PCRs were initially used for full genome sequencing. Initial sequencing targeted the gag gene. In house primers were designed based on a gag alignment of the Irish, American, Chinese and Japanese full genome sequences as well as gag sequences of Italian and Romanian origin. Subsequent sequencing attempts targeted the pol and LTR genes and primers were based on the full genome sequences mentioned above. This was followed by next generation sequencing using the illumina MiSeq platform. Mapping projects initially utilised multiple reference sequences to compensate for sequence variability, and the areas that mapped reads were cut and pasted together to create a hybrid sequence which were sequentially run through mapping projects each time extending reads and adjusting the consensus to match the viral reads. The sequences were then aligned with sequences from multiple geographical origins in MEGA 5 to allow phylogenetic analysis and evaluation of sequence diversity.

Results: Of the 36 PCRs used only 8 returned sequence for the Devon case. Fewer PCRs worked with the other British cases, and so NGS was pursued to negate sequence variability as a barrier. Using this process the full genomes of the 2010 Devon and a 2012 Cornish case (both symptomatic cases) were sequenced. Phylogenetic analysis of the gag gene of American, Chinese, Irish, Japanese, Italian, Romanian, Belgian and British viruses showed both British cases clustering independently with different Romanian and Belgian strains. Nucleic acid identity of the full genome sequences varies between individual genes and ranges from 46% (gag P9, British Devon vs British Cornish) to 98% (gag protease, British Cornish vs Chinese).

Conclusion: The highly variable nature of EIAV makes the use of primer walking strategies for sequencing lengthy and laborious whereas NGS provides an easy way of attaining full genome in a single sequencing run. However a significant viral load is required to overcome the high host background typical of clinical extractions making sequencing of asymptomatic cases problematic. The phylogenetic analysis of the 2 British symptomatic cases was consistent with current epidemiological reports that they originated from Romania and transited through Belgium but revealed that they may have diverged before their arrival in Britain or Belgium. The variation and different clustering of the two British cases highlight the ability of horse and horse product movement, both legal and otherwise, in transporting the disease quickly across continents. Currently EIAV diagnosis relies solely on serological testing, provision of additional genetic information from diverse sources should enable the design of PCRs that reliably target wider varieties of strains thus facilitating better detection and more detailed epidemiological analysis of outbreaks.

MetLab: a bioinformatics platform for viral metagenomics experiments

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The advent of metagenomics has provided investigators with a powerful tool for monitoring emerging infectious diseases of both enzootic and zoonotic potential. While it can be an effective method for pathogen detection and discovery, these studies are only as accurate as the quality of the bioinformatics data processing and ana-

lysis allows. In addition to bioinformatics, any successful project requires proper experimental design as well.

Objective: We are developing a bioinformatics platform, MetLab, to perform the *in silico* analysis of a viral metagenomics project, as well as support with the experimental design. MetLab will provide the users with:

- an experimental design module for helping to decide the NGS platform to use and the sequencing depth needed
- a viral dataset simulation module for testing and validating analysis tools
- a module providing several pipelines dedicated to the analysis of viral metagenomes with detection of highly divergent viral sequences.

Methods:

Experimental design module:

Estimation of sequencing depth, often called target coverage, is a common design problem in sequencing experiments. While sequencing depth estimation for single organism experiments is straightforward, coverage estimation for metagenomic sequencing is far more complex, being one of the most difficult parts of metagenomic experimental design, as it involves advanced probability theory. We have written a high-precision mathematic module to easily help with this otherwise daunting step, and implemented it to solve an adaptation of Steven's theorem for coverage estimation.

Dataset simulation module

This module has two functions; first it can read a set of sequencing data, creating a statistical profile, and secondly, simulate datasets from such a sequencing profile. The user is asked to estimates the number of species in the sample, and then the module downloads random viral genomes, generating two output files. One is a Sanger Fastq file with read statistics corresponding to the profile, and the other one is a key file, a list of comma-separated values (csv) describing the dataset contents.

Analysis pipelines module

Several different analysis pipelines are implemented in the analysis module of MetLab. All of them include a quality control step and a taxonomic classification step. Each pipeline also offers optional intermediate steps such as:

- removal of host genomic sequences (for animal sample analysis)
- assembly of the good quality reads into contiguous sequences

The taxonomic classification is performed using a multi-steps workflow dedicated to the detection and classification of viral sequences. This is done by execution of similarity searches at nucleotide and protein level, e.g. KRAKEN, BLAST. The nucleotides sequences that could not be classified with similarity search tools are then translated into protein sequences. Then, a similarity search is performed on this protein sequences. Sequences for which no similar proteins are found in this step are then scanned with specific viral profile hidden Markov models (profile HMMs).

Results: MetLab is written as a two-part application, the computational framework and the graphical user interface (GUI). The framework is composed of three main modules previously described. Each module can be used independently as a standalone command-line application, or as a part of a pipeline in a server environment, while the GUI provides an easy-to-use alternative for those who seek to use the application as-is.

The experimental design module enables the user to estimate the sequencing depth needed for the experiment, in order to be able to even detect species of low abundance. Given estimated species abundance in the metagenomic community and an estimated genome size, the module calculates the probability of achieving a complete coverage assembly in a theoretically optimal assembly. If the module does not reach the desired probability, iterations are performed adding supplementary sequencing runs to the experiment up to a maximum of 10.

The dataset simulation module provides the user with datasets of known taxonomic content, allowing testing and validating assembly and taxonomic classification tools. A program to validate assemblies of simulated data-

set is also available in MetLab, as well as validators for several taxonomic classification methods (e.g. Kraken, Blast+LCA, MEGAN, ProViDE).

The module providing analysis pipelines allows the user to process his/her data using different solutions, allowing flexibility in the choice of steps to be performed. The workflow proposed for taxonomic classification is well adapted to the detection of viral sequences, even those highly divergent from known viral species, as it offers a scan of the dataset with profiles HMMs specifically built using viral proteins.

Conclusion: The *in silico* environment presented here provides scientists with a wide range of uses before, during and after the metagenomic experiment. MetLab supplies tools for design, simulation, validation and analysis, which will be of value for estimating the feasibility of using metagenomics to answer biological questions, and for actually solving those questions.

Capsid proteins of porcine circoviruses interact differentially with the cellular protein gC1qR

Guy Kouokam [1], Claire De Boisséson [1], Cecilia Bernard [1], Lionel Bigault [1], Annette Mankertz [2], Yannick Blanchard [1], André Jestin [1], Beatrice Grasland [1]

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The post weaning multisystemic wasting syndrome is a swine pathology first described in the 1990's in Canada. It is manifested clinically by wasting and the major histopathologic lesion is lymphoid depletion. PMWS is caused by a small non-enveloped DNA virus called porcine circovirus type 2 (PCV2). This virus is different from the non pathogenic porcine circovirus type 1 (PCV1) that was primary isolated from PK15 cells. In both circoviruses, two main ORFs encode three proteins: the Cap, unique component of the capsid and Rep and Rep' proteins, involved in the replication. The PCV2 viruses are clustered in two major genogroups: PCV2a and PCV2b.The percentage of similarity in term of amino acids between PCV2 Cap and PCV1 Cap is about 63%.The molecular mechanisms triggering the pathology are not well understood for the moment.

It has been reported that the PCV1 Cap and PCV2 Cap can interact with the cellular protein gC1qR (also named p32/HABP1) (Finsterbush et al., 2009). This protein is a pleiotropic multifunctional protein mainly found in the mitochondria but also in the nucleus and at the cytoplasmic membrane of cells. Its functions imply the membrane reception of the C1q protein of the complement, a contribution to the apoptosis mechanism and the regulation of the transcription in human cells.

Objective: The objective was to study the interaction between the gC1qR protein and the Cap proteins of circoviruses in order to know if a difference of interaction between the Cap proteins of the porcine circoviruses with gC1qR may explain the difference of pathogenicity between PCV1 and PCV2.

Methods: *Amplification* of gC1qR: The gC1qR cDNA was amplified by RT-PCR from PK15 cell extracts. The nucleotide 205-846 of the putative ORF of the porcine gC1qR was amplified, cloned into the pGAD-T7 plasmid and named $gC1qR_{205\text{-}846\_PK15}$. The plasmid pCMVmyc- gC1qR constructed by Finsterbusch et al. from a cDNA pig spleen library was also used. From this plasmid, the region corresponding to nucleotide 205-846 of the putative ORF of the porcine gC1qR was cloned into the plasmid pGAD-T7 plasmid and named $gC1qR_{205\text{-}846\_spleen}$.

Cloning of PCV Cap proteins: The Cap proteins of a PCV2a, PCV2b and PCV1 strains were cloned into the pGBK-T7 plasmid.

Double Yeast Hybrid assay:

Yeast were cotransformed with a combination of one pGAD-T7 and one pGBK-T7 plasmid and grown on double drop out SD medium (-Leu,-Trp) for four days at 30°C. Yeast patches were made on double drop out SD medium (-Leu,-Trp). After three days of growth the patches were replicated on quadruple drop out medium (-Le,-Trp,-His,-Ade) and double drop out medium (-Leu,-Trp) and the plates were incubated for two days.

Results: Analysis of patches on SD-4 plates showed that the gC1qR205-846_spleen and gC1qR205-846_PK5 interact with the two Cap proteins of PCV2 (PCV2a and PCV2b). Furthermore, gC1qR 205-846_PK15 interacts also with the Cap protein of PCV1 unlike gC1qR205-846_spleen which does not show any interaction with the Cap protein of PCV1.

$gC1qR_{205\text{-}846\_spleen}$ isolated from a spleen cDNA library and $gC1qR_{205\text{-}846\_PK15}$ amplified from PK15 differed in one amino acid .The mutation was observed at the amino acid level: S203P.

Conclusion: The gC1qR protein translated from spleen cDNA interacts with the Cap protein of PCV2 but not with the PCV1 Cap. This result is intriguing since the interaction between gC1qR with the PCV1 Cap had already been described by Finsterbush et al.,2009. Whether this observation is due to difference in gC1qR or in PCV1 Cap will be studied in future works.

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Superinfection exclusion during infection with African swine fever virus

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African swine fever virus (ASFV) is the agent of a highly contagious disease of domestic swine and wild boar threatening worldwide pig husbandry with no effective vaccine available. It a complex virus with a double stranded DNA genome of 170-190 kbp predicted to code for 150 to 167 genes. Mechanisms of virulence still lack elucidation. Superinfection exclusion (SIE) has been observed to occur in several viral infections, from bacteriophages to RNA and DNA viruses from animals and plants, whereby a virus restricts a secondary infection by the same or closely related virus. This prevents the simultaneous infection and replication of different viruses within the same cell, potentially selecting for rapidly replicating virus and maintaining viral fitness by avoiding deleterious recombination.

Objective: SIE hasn't been described for ASFV and hence we assessed whether it also occurs for this viral infection.

Methods: Two homologous ASFV recombinants expressing either GFP (NHV-GFP) or DsRed (NHV- dsRed) under the control of viral p72 late promoter were used in infection of susceptible WSL cells, either simultaneously or at different time intervals of 4h, 8h and 12h. A bovine herpesvirus 1 (BHV-1) recombinant expressing dsRed under immediate early CMV promoter (BHV-1/DsRed) was also used in combination with ASFV NHV-GFP. Cells exhibiting autofluorescence and hence effective viral gene expression and thus probably replication were visualized by confocal microscopy 22h after the simultaneous or second infection.

Results: In simultaneous NHV-GFP and NHV-DsRed infection we observed several cells with both GFP and DsRed autofluorescence. However the majority of infected cells exhibited only one type of auto- fluorescence signal. The number of double infected cells further decreased when infections were separated by a 4h or 8h interval and with 12h interval between infections double autofluorescent cells were no longer observed. This clearly showed SIE occurring during ASFV infection, at least with closely related viruses. Using high MOI infections, the first virus also decreased the number of cells infected with the second one in a time interval increasing dependent manner. When we tested infection with the unrelated DNA virus BHV-1/DsRed, added to cells 12h after NHV-GFP, once again no double autofluorescent cells were detected, only individual GFP or DsRed expressing cells. This was also the case when BHV-1/DsRed infection was followed 12h later by NHV-GFP, indicating that these viruses are mutually exhibiting SIE.

Conclusion: SIE occurs during infection with related ASFV viruses. Exclusion of the second virus increases as the time interval between infections rises until 12h after the first infection when the effect appears to be maximal.

Interestingly ASFV also excluded superinfection by the unrelated herpesvirus BHV-1 and vice versa. Further studies are underway to explore potential mechanisms of this SIE process and whether more distant ASFV strains also exhibit SIE, contributing to our knowledge on ASFV infection.

Epigenetic regulation of the viral RNA telomerase subunit over-expressed in lymphoma induced by Marek's Disease Virus

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Objective: Marek's disease virus serotype-1 (MDV-1) is an oncogenic chicken alphaherpesvirus that induces the rapid onset of a highly malignant T-lymphoma, consistent with the involvement of one or more virus-encoded oncogenes. Marek's disease pathogenesis begins with a primary, semi- productive infection of B lymphocytes, followed by a switch to latent infection, mainly in CD4+ T cells. Latent infection is characterized by the persistence of the viral genome in the absence of viral antigen expression or the production of infectious virus. Reactivation may then occur, resulting in late productive infections. The transformation of latently infected $CD4^+$ lymphocytes is the ultimate consequence of interactions of MDV with the host cell. In susceptible chickens, the transformed cells proliferate to form gross lymphomas 3 to 4 weeks after infection, potentially leading to the death of the animal. This study focuses on the mechanisms involved in this switch between productive and latent phase of MDV life cycle. The objectives are to determine whether epigenetic modifications are involved in this transition and to establish patterns of DNA methylation in the promoters of lytic and latent genes involved in the regulation of MDV life cycle. In the first step, methylation patterns were established for three genes of interest, 38 kDa phosphoprotein (pp38), 14 kDa phosphoprotein (14 KDa) and viral telomerase RNA subunit (vTR) at the key steps of the viral infection. In the second step, the impact of DNA methylation was assessed through promoter reporter assay.

Methods: Bisulfite Genomic Sequencing Assay (BGSA) was used to assess pp38, 14 kDa and vTR promoter methylation patterns at three relevant steps of MDV life cycle. For the productive phase, infected chicken embryo fibroblast cells (CEF) were used. For the latent phase the chicken lymphoblastoid cells (MSB-1) were used. This cell line was transformed and latently infected with MDV-1. 5'azacytidine, an inhibitor of the DNA methyltransferase was used to induce viral reactivation in MSB-1 cell line. PCR design was adapted to the Bisulfite treated DNA followed by cloning of PCR product and sequencing. Biostatistical analyses of 5 clones per condition and of individual CpG locus were performed. To know whether a correlation between the methylation status of the analysed promoters and the abundance of the related transcripts existed or not, transcription activities were assessed by comparing the expression levels through quantitative real time PCR assay. Reporter vectors were constructed to evaluate the activity of vTR, pp38 and 14 kDa promoters. From the genome of MDV-1, the sequence of promoter of interest was cloned into CpG free reporter vector pCPGL. Second step was methylation of plasmid constructs using CpG methyltransferase M.sssI. The impact of methylation on the activity of different promoters was studied. For that purpose, the constructs were transfected on three cell lines, chicken hepatocellular carcinoma cell (LMH), chicken embryo fibroblast cells (CEF) and human HeLa cells. Luciferase activity of non methylated promoter versus methylated ones was measured. Finally, specific response elements in the vTR promoter were studied through E-boxes binding sites of the transcription factor c-Myc mutagenesis. Earlier studies showed that c-Myc is active on vTR promoter and involved in vTR transcription1. Mutation of E-boxes and the methylation on the mutated promoters were investigated. E-box mutations were generated by site directed mutagenesis.

Results: Global methylation patterns of the promoters differed from one gene to another, from one cell type to another during the MDV viral cycle. A correlation was noticed between the level of methylation observed in the studied promoters and the level of the expression of the corresponding genes. The pp38, 14kDa and vTR promoters analyzed by BGSA showed high levels of methylation of CpG dinucleotides in during latency, from 65%

to 90%, respectively. Following induction of the reactivation, the methylation level dropped to around 20%. In the condition of lytic infection, these promoters were not methylated. The transcription of genes associated with the replication (pp38) was significantly induced after DNA demethylation. Using the same demethylation agent on vTR (associated with latency and MDV induced tumorigenesis) its activity was considerably decreased. Impact of DNA methylation was then analysed by luciferase promoter reporter assay. There was significantly low activity of methylated promoters compared to non-methylated ones in all of the cell lines. The hybrid promoter EF1/CMV known to be not sensitive to methylation was used as negative control. E-boxes were mutated by PCR mutagenesis, obtaining E2, E3 and E2E3 mutants. On non- methylated promoter E2 mutation had no repressive effect in all of three cell lines, but E3 and E2E3 mutations showed repression of vTR promoter, without significant difference between them. However, methylation seemed to mask the effects of mutations.

Conclusion: Specific DNA methylation signatures were found in the MDV genome during latency. It was shown that DNA methylation induces transcriptional silencing of lytic gene and demethylation is associated with reactivation of the gene expression. Also DNA methylation positively influences the expression levels of vTR and the activity of the viral induced telomerase. The results showed that demethylation is associated with decreasing telomerase activity. The cellular transcription factor c- Myc plays a role in the expression of the gene vTR during the latency phase of the MDV and these results showed that the E3 box is involved in regulating transcription of the vTR. The double mutation of E2 and E3 sites did not induce complete inhibition of transcription. This suggests the involvement of other transcription factors regulating the activity of vTR promoter. In all three cell lines, the activity of the double mutated methylated vTR promoter was significantly higher than that of methylated wild-type. The same observations were made for mutations in E2 and E3 in LMH and CEF cell lines. In HeLa cells no significant difference between the methylated mutated (E2 or E3) vTR promoter activity and methylated wild-type promoter was found. The effects of mutations were masked by methylation. DNA methylation is one of the epigenetic modifications and it would be interesting to conduct further investigation of the histone marks in the selected promoters and to determine relations with non coding RNA.

1. Shkreli, M., Dambrine, G., Soubieux, D., Kut, E., Rasschaert, D. (2007): Involvement of the oncoprotein c-Myc in viral telomerase RNA gene regulation during Marek's disease virus-induced lymphomagenesis. Journal of Virology, 81(9), 4848–57

Parallel ESVV Session 9: General virology 2

Wednesday 2nd September 2015
09:15-10:30
Room Sully 2

Chair: Thierry Van den Berg & Stéphane Bertagnoli

Molecular analysis of canine parvovirus type 2 in New Zealand

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Objective: Canine parvovirus 2 (CPV-2) is a well-recognized cause of acute haemorrhagic enteritis in dogs worldwide. The antigenic differences between CPV subtypes are a consequence of amino-acid substitutions in five loci in the viral capsid protein VP2. These changes are thought to be associated with the antigenicity, host range

and pathogenicity of the virus. Although clinical implications of infection with various types of CPV are currently poorly understood, CPV-2 is an evolving virus, and monitoring of the on-going changes is important. Thus, the aim of the current study was to identify which CPV-2 subtypes currently circulate among dogs in New Zealand, and to investigate the evolutionary patterns of contemporary field CPV-2 viruses.

Methods: Faecal samples were collected from 79 dogs with suspected CPV-2 infection over the period of 13 months, and tested for the presence of CPV-2 DNA by PCR. The amplicons were assigned to specific CPV-2 subtypes based on the presence of selected amino-acid residues at specified positions. In addition to the contemporary survey samples, 3 vaccine strains of CPV-2 and 9 archival CPV-2 isolates were included in the study. CPV-2 sequences with good quality sequence data and the reference sequence were trimmed to produce a data set containing 72 contiguous sequences of equal length, which were used to investigate the genetic structure of CPV-2 within New Zealand. Additional CPV-2 VP2 sequences (n=95) originating from various countries were obtained from the National Centre for Biotechnology Information (NCBI) database. The selection of 27 full length contiguous sequences obtained in the current study were aligned with those obtained from the NCBI database, and the reference sequence. The resulting dataset of 123 CPV-2 sequences was used to assess the New Zealand CPV-2 sequences in the context of the worldwide radiation of CPV-2.

Results: Of 70 CPV-2 positive samples, 69 were subtyped as CPV-2a and one as CPV-2. A majority of CPV-2 positive samples were collected from not-fully vaccinated puppies $\leq$ 6 months of age. The haplotype network produced from New Zealand CPV-2 sequences showed no structure when assessed based on location, vaccination status or age of the animals sampled. International haplotype network indicated that, unlike CPV-2 from other countries, the population of CPV-2 in New Zealand appeared to be monophyletic.

Conclusion: CPV-2 was most commonly detected in samples from young, unvaccinated puppies, underscoring the importance of puppy vaccination for prevention of CPV-2 gastroenteritis. Our data suggest that the on-going introduction of CPV-2 from other countries is not a common occurrence in New Zealand. This may be explained by the physical separation of the country coupled with strong border security. The relative homogeneity of New Zealand CPV-2 sequences may also be a reflection of the limited choice of available CPV-2 vaccines and lack of a number of wild carnivore species susceptible to infection with CPV-2 or related viruses in New Zealand.

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Canine infectious tracheobronchitis in Swedish dogs – what does a metagenomics approach reveal?

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Objective: Canine infectious tracheobronchitis (CITB), or kennel cough, is an upper respiratory infection affecting dogs. There are several causative agents for CITB with the main contributors considered to be canine parainfluenzavirus (CPiV) and B*ordetella bronchiseptica*. Recent findings indicate that also canine respiratory coronavirus (CRCoV) can cause CITB. In addition, viruses and bacteria other than CPiV, *B. brochiseptica* and CRCoV have been considered as causative agents to CITB, either as a single infection or in co-infections. As the diagnosis of CITB primarily is made without any laboratory diagnostics, there is currently limited knowledge about which viruses and bacteria that actually causes CITB.

Metagenomics is the method by which all genomes within a sample can be analysed simultaneously and without the need of prior knowledge of the genomes. This approach is particularly useful for discovering new or unexpected disease causing agents.

This study aims to: I) identify viral and bacterial agents associated with CITB using metagenomics, II) genetically characterize the most interesting findings and, III) establish a baseline prevalence of viral and bacterial agents in healthy dogs.

Knowledge about disease causing agents for CITB and the dogs´ commensal microbiome is important for improved understanding of the disease and for disease prevention and control.

Methods: Swedish privately owned dogs with characteristic upper respiratory signs of CITB (dry cough) for up to 7 days (n=77) and 17 healthy controls were enrolled in the study from April 2013 to February 2015 (the study is still ongoing). Oral and nasal swabs for virological and bacteriological analyses were collected from all dogs. Real-time PCR/RT-PCR was used to assess presence of canine adenovirus type 2 (CAV-2), canine herpesvirus (CHV), canine influenzavirus (CIV), canine parainfluenzavirus (CPIV), canine respiratory coronavirus (CRCoV) and *B. bronchiseptica.*

For metagenomic studies, a sub-set of the dogs were selected within the following groups; A) dogs with CITB that were positive for at least one of the agents assessed by real-time PCR/RT-PCR (n=4), B) dogs with CITB that were negative for all agents studied by real-time PCR/RT-PCR (n=4), C) healthy controls (n=4).

In brief, oronasal swabs were freeze-thawed, vortexed, and centrifuged at 600g for 10 min. The supernatant was filtrated using a spin filter (0.65um pore size) and treated with TURBO DNase and RNase cocktail (Ambion®, Life Technologies). DNA was extracted using QIAamp DNA Mini Kit (QIAGEN), and RNA was extracted using Trizol/chloroform and RNeasy Mini Kit (QIAGEN). Thereafter, the DNA and RNA were amplified using sequence independent single-primer amplification. The amplified DNA was purified using GeneJET PCR purification kit (Thermo Scientific). The sequencing libraries were made using Nextera XT sample preparation kit (Illumina), normalization was done based on concentration measurements from High Sensitivity DNA Chip (2100 Bioanalyzer, Agilent Technologies), followed by sequencing at the MiSeq platform using the MiSeq Reagent Kit v3 600 cycles (Illumina).

The output datasets from MiSeq were quality controlled with PRINSEQ software to filter out bad sequences. The remaining dog sequences were removed by mapping good quality reads on a dog genome (*Canis lupus familiaris* 3.1 from Ensembl), using Bowtie2. Then the un-mapped reads were assembled into contigs using Ray assembler, and those contigs as well as the unassembled reads were taxonomically assigned by Blast analyses.

Results: This is currently a work in progress. The preliminary results from the metagenomics work have validated the *B. bronchiseptica* positive case included in this study, suggesting that the approach is valid for identifying disease causing agents. Further investigations and validations are on-going.

Conclusion: Metagenomics analysis is a powerful tool for discovery of disease-causing agents and characterization of the commensal microbiome. This approach has great potential to increase our knowledge about CITB, which will be important for disease prevention and control.

Staining and expression kinetics of the feline immunodeficiency virus envelope glycoprotein

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Objective: The feline immunodeficiency virus (FIV) is an enveloped RNA virus with only one glycoprotein embedded on the envelope. The envelope glycoprotein is composed of two parts that are non-covalently linked; the surface protein (gp95) is important for host cell recognition, while the transmembrane protein (gp36) anchors the glycoprotein to the viral envelope and contains a fusion peptide which is key to infection of the host

cell. Apart from recognition and infection of cells, the envelope protein also plays a role in immune-evasive mechanisms of FIV. The protein contains hypervariable loops that are prone to a high mutation rate, which allows the virus to escape from the humoral immune response.

Although a lot of research has already been done on the FIV envelope protein, it is still not known when in the viral replication cycle the protein is expressed. The localisation of the viral envelope protein expression and the availability of different epitopes in the hypervariable loops of this protein can give a lot of information about the immune-evasive strategies. Therefore, the present study reports the expression kinetics of the FIV envelope protein and the staining of different epitopes on this protein.

Methods: The expression kinetics of the FIV envelope protein and the staining of different epitopes were analyzed in three FIV expression systems. Therefore, an adherent feline cell line (CrFK) was either transfected with a molecular clone of FIV-PPR strain (1st) or infected with virus of the same strain (2nd). In addition, since FIV mainly infects monocyte/macrophages and lymphocytes, the analysis was also made in peripheral blood mononuclear cells (PBMC) infected with FIV-PPR (3rd). The viral envelope protein was stained intra- and extracellularly with antibodies targeting epitopes in the different variable loops of the surface part (gp95) of the envelope protein (Custom Monoclonals Int). Expression was stained 3, 6, 9, 12, 18, 24, 36 and 48 hours after transfection or infection.

Results: A large difference was found between expression characteristics of the viral proteins in a transfection environment in CrFK compared to infection in both CrFK and PBMC. The expression in the transfection system gave an over-expression of the viral proteins. Most epitopes could be stained both in the endoplasmatic reticulum and the golgi-apparatus. In infected cells, the majority of the glycoprotein content was retained and expressed in the golgi-apparatus. In the adherent cell line, both after infection and transfection, the protein was expressed on the plasma membrane in a scattered manner. However, it was noticed that epitopes in variable loop 2 that are heavily competed out by sera from naturally FIV infected cats, were not available for staining. This suggests that cats mount an immune-response to epitopes that cannot be recognized on infected cells or virus particles, giving the opportunity to the virus to escape from the immune system. In infected PBMC the plasma membrane expression was very restricted and polarized to certain areas, most likely to lipid rafts containing areas. These areas could also be responsible for initiation of the viral synapse as has been seen for the human immunodeficiency virus. Staining of the FIV envelope protein in PBMC also revealed, for the first time, the visualization of cell-to-cell spread of FIV in PBMC.

Conclusion: The FIV envelope protein is expressed in a tightly controlled manner during infection. The low amounts of viral antigen on the plasma membrane and the masking of highly immunogenic epitopes on the envelope protein, hide infected cells from the immune system. Furthermore, the envelope protein is targeted to sites that are responsible for cell-to-cell contact, increasing the probability of formation of a viral synapse. This viral synapse allows FIV budding and infection of cells in a sealed area, protected from recognition by the immune system. These properties of the FIV envelope protein show that this protein is actively involved in the immune-evasion of the virus.

Evaluation of genetic differences between the avirulent and virulent pathotypes of feline coronaviruses

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Objective: Feline coronaviruses (FCoV) are large, spherical, enveloped, positive-sense RNA viruses belonging to the family *Coronaviridae* of the Order *Nidovirales*. Two pathotypes of FCoV can be distinguished, the low- or avirulent feline enteric coronavirus (FECV) and the virulent feline infectious peritonitis virus (FIPV). Whereas FECV is endemic in many cat populations, FIPV occurs quite rarely. FECV and FIPV are genetically and antigenically highly related. Most experimental and field studies support the hypothesis - known as “internal mutation theory”- stating that FIPV evolves from FECV by mutation(s) in the individual cat. The genes involved in the FCoV virulence shift are

unknown but mutations in the spike (S) and the accessory genes, particularly genes 3c and 7b have been implicated. While the 3c gene is intact in all the FECVs, in about two thirds of the FIPVs the gene is variably mutated leading to a non-functional 3c protein (1). Recently differences in the S gene that distinguish virulent from avirulent pathotypes have been identified (2,3). In our previous study the full genomes from 11 pairs of the two pathotypes were compared which revealed one difference hot spot in the S gene. Sequencing hundreds of additional viruses in this particular region of the S gene led to the identification of two alternative amino acid differences in the putative fusion peptide of the S protein that together distinguish FIPV from FECV in >95% of cases (2). Other differences were subsequently also detected in the furin cleavage site located between the receptor binding (S1) and the fusion domain (S2) of the spike protein by Licitra et al. (3). All FECVs were shown to have a conserved furin cleavage site. In contrast, in most FIPVs $\geq$ 1 substitution in the S1/S2 motif was detected.

The objective of the current study was to gain insight into the relative importance of the various differences between the FCoV pathotypes in the 3c and S genes. In this analysis we also included the second proteolytic cleavage site S2' which occurs in the fusion domain (S2) of the spike protein, just upstream of the fusion peptide. Thus we analysed the sequences of large numbers of FECVs and FIPVs at these different genomic locations and determined the relative frequencies of differences at these sites.

Methods: Blood samples, ascitic fluids and/or tissue lesion samples from cats with FIP were used as a source of potential FIPVs. The samples were derived from cats in which the clinical diagnosis was confirmed by post-mortem examination and in most cases supported by a positive immunochemistry result. Faecal samples from apparently healthy cats were collected as a source of FECV strains. RNA was extracted from these samples using different RNA extraction kits following the manufacturers' protocols (QIAamp Viral RNA Mini Kit for faecal supernatant and ascites; RNeasy Mini Kit for organ tissue homogenate and RNA Blood Mini Kit for white blood cells; QIAGEN, Valencia, Ca, USA). After synthesizing cDNA using random primers (Invitrogen,NY,USA), sequences of the 3c gene and two spike gene fragments covering the S gene sequences of interest were amplified using two sets of specific primers in RT-nPCR. The PCR products were purified by electrophoresis in a 1.5% agarose gel, extracted from the gel using a gel extraction kit (Macherey-Nagel, Germany) and subsequently sent to Macrogen Inc for sequencing. Multiple sequence alignments were constructed by using Clustal X (http://www.clustal.org/clustal2/) with the Lasergene Megaline and MEGA 6 software programs. Phylogenetic analysis was performed by using features of the MEGA6 suite of programs. Phylogenetic trees of these sequences were obtained by using the neighbor-joining method. The bootstrap consensus tree, inferred from 1,000 replicates, was prepared; positions containing gaps and missing data were eliminated from the dataset.

Results: Sequences of 42 FCoVs from faeces of healthy cats were obtained. An intact 3c gene was found in all samples. Also, in all faecal FECVs the conserved motif at the S1/S2 cleavage site (R/K(P5)-R(P4)- S/A(P3)-R(P2)-R(P1)-S/A(P1') as published by Licitra et al. (3) was present. A consensus sequence was determined for the S2' cleavage site and the cleavage core motif (K-R-S-A-V/I) was detected in all FECVs. Analysis of the fusion peptide revealed a M and a S residue at position 1058 and 1060 respectively in all FECVs corroborating our previous findings (Chang et al). Of in total 113 FIPVs, sequence data of all the four sites (3c and the 3 sites on the S protein) could be obtained. In all but one of the FIPVs mutations were found at one or more of the 4 sites. With regard to the 3c gene, 31% had an intact open reading frame comparable to the 3c gene of FECVs. In 69% of the 3c gene sequences various mutations leading to premature termination of translation, truncation of the 3c polypeptide or loss of initiation or stop codon were detected. The two alternative amino acid differences in the putative fusion peptide of the S protein that were shown to distinguish FIPV from FECV (M to L at position 1058 and S to A at position 1060) were found in 92.7% of all the FIPVs. With regard to the S1/S2 cleavage site, in 74.2 % of the FIPVs several differences were found compared with the conserved furin motif of the FECVs from asymptomatic cats. Most variation was found at the critical position P1. The S2' cleavage motif was more conserved with 59.7% of the FIPV sequences having the same motif as found in FECVs. In total 82.3% of the systemic FIPVs contained variations at either or both of the two cleavage sites.

In more than half of the FIP cats, FCoV sequences could be detected in the feces. In the majority (approximately 60%) of these FCoVs an intact 3c gene and the M and S residues at positions 1058 and 1060 correlating with the FECV phenotype were found. Mutations at the two cleavage sites were present although at a lower frequency and with less variability (table 1).

	FCS	S2'	FP	3c
FECV	0	0	0	0
FIPV	74.2	40.3	92.7	55.9
FIPV feces	44.1	17.1	42.9	40.6

Table 1. Percentages of FCoV sequences in which mutations were found at the indicated sites. FECV: FCoV derived from fecal samples of healthy cats; FIPV: FCoV derived from blood and/or tissues from cats with FIP. FIPV feces: FCoV derived from the feces from cats with FIP. FCS: furin cleavage site; S2': S2' cleavage site; FP: fusion peptide; 3c: 3c gene

Conclusion: Several differences in the 3c and spike protein were found that distinguish virulent from avirulent pathotypes. As compared to the FECV pathotype, the mutations in the S protein fusion peptide of FIPVs were found most frequently. While all FECVs had a methionine and a serine at aa position 1058 and 1060, respectively, in 92.7% of FIPVs sequenced in this study either of the two were mutated. Also a relatively high number of mutations were found in the S1/S2 and S2' cleavage motifs of the systemic FIPVs. Since the S protein is responsible for cell attachment and membrane fusion it seems obvious to hypothesize that these mutations play a role in the virulence shift from the FECV to FIPV pathotype. Whether these differences indeed represent the virulence mutations that turn avirulent FECV into lethal FIPV remains to be demonstrated. To prove the role of these mutations in the virulence shift a suitable reverse genetics system and studying the effects of mutations in animals will be required.

Although feces from cats with FIP are not considered infectious (4), sequences correlating with the systemic FIP phenotype could be detected in FCoVs present in their fecal samples. In 10 of 19 FIP cats the typical virulent genotypic sequences found systemically were also observed in the feces. We hypothesize this to be due to passive leakage of systemic FIPV into the gut.

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Molecular epidemiology, prevalence and risk factors of FCV infection in European domestic felines

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Objective: Feline calicivirus (FCV) is a highly contagious pathogen causing upper respiratory tract disease (URTD), a common problem in feline medicine - especially where animals are housed in groups. Acute infection with FCV usually leads to mild signs of URTD including mouth ulcers, sneezing and serous nasal discharge but can also cause lameness, has been associated with chronic gingivostomatitis and, more recently, hypervirulent strains have been identified that cause severe and fatal outbreaks of often fatal, virulent systemic disease characterized by a systemic inflammatory response syndrome, vascular compromise and multi-organ failure. Feline calicivirus is a member of the *Caliciviridae* and is a small non-enveloped, single-stranded, positive sense RNA virus. High levels of genetic and antigenic diversity have been observed for FCV. The plasticity of its genome

makes the virus adaptable to new environments and is important for viral persistence and the emergence of new strains. The objectives of this study are to provide insight into the prevalence, molecular epidemiology and risk factors for infection with FCV, for the first time, at a European level.

Methods: Sixty-three randomly selected (stratified random sampling by regions within countries to ensure geographic coverage) veterinary practices in five European countries (France, Germany, Italy, Netherlands, Sweden and the UK) were asked to collect oropharyngeal (OP) swabs from cats attending each practice. A short questionnaire was completed by the assisting veterinary surgeon with the assistance of cat owners for each sampled cat. Diagnosis of FCV was by isolation in cell culture by standard techniques. RT-PCR was conducted on all isolates followed by consensus sequencing. Neighbour-joining phylogenetic trees were constructed based on viral capsid and polymerase sequences. Multilevel multivariable analysis was used to identify risk factors associated with FCV isolation using data obtained from questionnaires.

Results: Fifty (79.4%) of the recruited practices returned a total of 1521 OP swabs. A total of 150 samples tested positive for FCV (9.9% - 95% CI 8.4, 11.4). Phylogenetic analyses showed high strain diversity evidenced by a radial phylogeny containing 109 strains, with more than one strain isolated in each country. Field strains were restricted to one country with no evidence of widespread international transmission as seen for other caliciviruses. Regarding risk factor analysis, neutered animals and increasing age were associated with a lower likelihood in testing positive for the virus, whereas animals from multicat households and suffering from chronic gingivostomatis where more likely to be shedding FCV.

Conclusion: Taken together, results indicate that FCV remains an ongoing international threat to cats, with high prevalence and strain diversity emphasising the need for ongoing surveillance and vaccination.

Parallel ESVV Session 10: Emerging virus & diseases 2

Wednesday 2nd September 2015
09:15-10:30
Room Sully 3

Chair: Trevor Drew & Antonio Lavazza

A devastating outbreak of orthopoxvirus infection in Macaca Tonkeana

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Objective: Orthopoxviruses are known to infect humans and a broad range of domestic and wild animal species (Essbauer S. et al., 2010; Carletti F. et al., 2009; Cardeti G. et al., 2011). Human cowpox virus (CPXV) infections usually cause self-limiting skin lesions, even if severe and fatal cases have been described in immunocompromised persons. Cowpox infections in nonhuman primates were reported in Europe twice (Martina, B.E.E. et al., 2006; Mätz-Rensing K. et al., 2006). Small rodents are considered the principle viral carriers in which the infection remains mainly asymptomatic.

The present report describes an Orthopoxvirus outbreak occurred in 2015 in a group of Tonkean macaques (*Macaca tonkeana*) housed in a sanctuary for wild and exotic animals located in a wooded area of Central Italy.

Methods: Twelve macaques (*Macaca tonkeana*) of a group of 18 animals, died between 48 hours and 7-8 days from the beginning of symptoms and were submitted for post-mortem examination.

Severe depression, respiratory distress and in most of cases skin lesions were observed. Major organs and skin were routinely processed for histological observation.

Virus detection by transmission electron microscopy was carried out from samples of skin and gut content. Tissue homogenates (mainly from skin, lungs, spleen, liver and brain) were tested by a real time PCR for the differential diagnosis of orthopox- and herpesviruses (Carletti F. et al., 2005) and inoculated on Vero cells. Viral isolates were submitted to molecular investigations to exclude Monkeypoxvirus (MPXV) etiology and for further characterization.

Data relative to animal introduction and movements were recorded to identify a probable source of the infection.

To evaluate the possible diffusion to other sensible species present in the sanctuary, a preliminary serological investigation was performed on 12 cats, 1 roe deer, 1 mouflon, 1 badger and 4 llamas by seroneutralisation test, using a cell adapted CPXV strain isolated in a previously described outbreak (Cardeti G. et al., 2011).

Results: All monkeys were in a good body condition. Animals dead within 48 hours (N=2) showed severe lung congestion and hepatosplenomegaly; erythematous papular and pustular lesions on face, oral and tongue mucosa and at the inguinal region, were evident in subjects dead in 7-8 days. Histologically, cutaneous lesions were characterized by focal epidermal necrosis, acanthosis and acantholysis and early vesiculation with eosinophilic intracytoplasmic inclusion bodies in enlarged degenerated cells.

Affected lymph nodes showed severe necrotising lymphadenitis associated with haemorrhages and histiocytosis.

Transmission electron microscopy detected Orthopoxvirus particles in the skin lesions of all tested animals. Virus was constantly isolated on Vero cells from the third post infection day. Cytopatic effect was characterized by foci of rounded cells, cytoplasmic bridging and syncytia and the isolated Orthopoxvirus was again confirmed by transmission electron microscopy.

The real time PCR validated the first morphological diagnosis, detecting the Orthopoxvirus genome. The preliminary characterization of the strain carried out on the haemagglutinin (HA) and crmB genes keeps out the presence of MPXV.

Data collections exclude animal introduction in the sanctuary in the last years and contacts of monkeys with other species maintained in captivity. However free ranging synanthropic rodents circulate in the area.

Wild animals of the centre and ranging cats resulted negative at the seroneutralisation test.

Conclusion: Orthopoxvirus infections in Tonkean macaques (*Macaca tonkeana*) had not been previously described. That could be also due to the limited presence of this species in captivity out of Sulawesi, the Indonesian island in which those animals are endemic.

Pathological and histological findings observed were similar to those described in New World monkeys in a fatal outbreak occurred in Germany in which a closely related Cowpox virus was isolated (Mätz-Rensing K. et al., 2006).

The disease severity indicates a high susceptibility of this species of macaques to Orthopoxvirus infection.

As preliminary epidemiological investigations and molecular characterisation exclude the introduction of an exotic Orthopoxvirus strain, and as Ectromelia virus infection is limited to mice, Cowpox virus is suspected responsible of the outbreak. Further phylogenetic analysis will contribute to precise the etiology and to compare the isolate to other circulating Orthopoxviruses. Epidemiological studies to define the source of the infection are in progress.

As in the last decade the number of cases in wild and domestic animals is continuously increasing, even affecting humans following the ceased variola vaccination, Cowpox should be considered an emerging pathogen to be valued also in terms of public health.

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New bornavirus discovered in variegated squirrels: Potential link to fatal human infections

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Following the occurrence of lethal encephalitis in three breeders of variegated squirrels (Sciurusvariegatoides) in Germany from 2011 to 2013, investigations on a possible infectious origin were carried out.

By using ametagenomic approach with a comprehensive deep-sequencing strategy, the presence of high RNA-loads of a novel bornaviruscould be detected in samples from a squirrel in one of the holdings. Subsequent intensive molecular biological and immunohistological tests also confirmed the presence in brain samples of the deceased patients. Phylogenetic analyses demonstrated that this novel virus, tentatively named variegated squirrel 1 bornavirus (VSBV-1), forms a separate lineage in relationship to the known bornavirus species. Investigations of further squirrel samples from different holdings in Germany identified additional positive animals, and serological studies revealed the presence of high bornavirus-specific antibody titers in both the PCR-positive squirrels and one of the deceased patients. The results of virological and serological analyses will be presented and discussed, and recommendations for the detection of this newly discovered pathogenin live animals will be provided.

In conclusion, it is very likely that the fatal disease of the three patients was caused by transmission of the novel bornavirus"VSBV-1"directly from infected squirrels. However, the transmission path so far is unknown, but bites and scratches appear to be likely means of acquiring infection.

Wobbly possum disease: the proof of causation

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Objective: Wobbly possum disease (WPD), a neurological disease of the Australian brushtail possum (*Trichosurus vulpecula*), was first described in 1995 in a group of captive possums at a New Zealand research facility. It was subsequently observed in free-living possums, and reproduced under experimental conditions. The early stages of WPD are characterized by behavioral changes (loss of appetite, decreased interest in the environment, temperament changes), followed by progressive ataxia. Histologically, the disease is characterized by inflammation of the brain and of several other organs including liver, kidney and spleen. Recently, we have identified a novel virus in archival tissues from WPD-affected possums using a combination of next generation sequencing and traditional molecular tools. The objective of the current study was to confirm the aetiological involvement of the novel virus in WPD.

Methods: The virus was grown in primary liver macrophages and purified using density gradient ultracentrifugation. Possums caught from the wild (*n*=16) were individually housed. Following acclimatization period of up

to 4 weeks, each possum in group 1 (*n*=4) received 1 mL (~1 x 10^7 $TCID_{50}$) of a purified cell culture isolate, each possum in group 2 (*n*=4) received 1.8 mL (~1 x 10^6 $TCID_{50}$) of a cell lysate from inoculated cultures, and each possum in group 3 (*n*=4) received 1 mL of a previously used standard inoculum that had been prepared from tissues of WPD-affected possums. Control possums (*n*=4) received either a gradient material prepared from uninoculated cultures (*n*=2) or nothing (*n*=2). All inoculations were performed via intraperitoneal injection.

Results: All but one possums in groups 1-3 developed clinical signs of WPD approximately 2 weeks post inoculations and were euthanized. Decreased appetite was observed in 12/12, weight loss in 11/12, and severe neurological signs in 11/12 of infected possums. One possum died prior to the development of neurological signs, and 3/5 joeys died. Control possums did not demonstrate any clinical signs of WPD. High levels of viral RNA were detected in tissues from all possums that received infectious inocula, but not from control possums.

Conclusion: We have reproduced the disease experimentally using a purified viral isolate, thus providing final proof of the aetiological involvement of this novel virus in WPD. Further work to characterize the WPDV is currently underway in our laboratory.

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Detection and characterization of a novel reassortant Mammalian Orthoreovirus of bats in Europe

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Objective: Orthoreoviruses, with mammalian orthoreovirus (MRV) as the type species, are non-enveloped viruses with a segmented dsRNA genome. MRVs have a wide geographic distribution and can infect virtually all mammals, including humans. Three MRV serotypes have been recognized, based on the capacity of anti-MRV sera to neutralize viral infectivity and inihibit hemagglutination.

A renewed interest in Orthoreoviruses has recently grown for several reasons:

- in the last few years, MRVshave been often described as responsible for severe illnesses in humans including hemorrhagic enteritis, acute respiratory infections, encephalitis, and others(1);
- novel MRVs have recently been identifiedinseveral hosts, particularly in bats in Italy and Germany (2, 3);
- a novel Orthoreovirus with high similarity to MRVs found in bats in Europe was detected in Slovenia from a child with acute gastroenteritis requiring hospitalization (1);
- the segmented nature of Orthoreovirus genome poses risks regarding the potential onset of novel reassortant virus with unpredictable biological properties;
- a bat-borne fusogenic Orthoreovirus with zoonotic potential has been detected from healthy and legally imported flying foxes (*Pteropus vampyrus*) from Indonesia to a Europe (4);
- MRVs have been evaluated as oncolytic agents in experimental cancer therapy (5).

Bats, as the most abundant, assorted and geographically disperse vertebrates, are increasingly known as reservoir hosts of viruses that can cross species barriers to infect humans and other animals. In a previous study, we showed that MRVs are quite frequently detected from bats and appear genetically more differentiated in comparison to those Orthoreoviruses found in other animal species (6).However, to date the unique MRVs identified in bats belong to the serotype3 (Europe) and 2 (Asia).

This study reports the first isolation and characterization of a new reassortant MRV type 1 from Lesser Horseshoe Bat *(Rhinolophus hipposideros*) in Italy referred to as BatMRV1-IT2011.

Methods: *Sampling*. Fresh fecal samples were collected for virogical investigations from a knownreproductive

colony of Lesser Horseshoe Bat (Rhinolophus hipposideros) using a clean plastic sheet placed under the roost site for approximately 24h before sampling.

Viral isolation. Cell cultures used in this study were VERO (African green monkey kidney cells) and MARC- 145 (Foetal monkey kidney) cells. Fecal samples were homogenized in minimal essential medium (MEM) (1 g/10 ml), inoculated in confluent monolayers in 24-well plate and observed daily for viral cytopathic effect (CPE). In the absence of CPE, the cryolysate were subcultured twice into fresh monolayers.

Electron microscopy. Faecal samples and the supernatant fluids from cell cultures showing CPE were submitted to negative staining electron microscopy (nsEM) using the Airfuge method. Viral particles were identified based on their morphological characteristics.

Molecular analysis. RT-PCR with specific primers for a conserved region of the L1 viral gene common to the different serotypes of MRV was used (7) for viral identification. The whole genome sequence of BatMRV1-IT2011 was obtained with the Ion Torrent NGS platform as previously described (1). The sequences of the ten gene segments were compared with those of reference strains obtained from GenBank under alignment with the program Clustal W. The phylogenetic trees for each genome segment were generated by Neighbour-joining method using the Kimura 2-parameter model. The evidence of genetic reassortment was obtained by analysis with SimPlot and RDP3 programs on the manually concatenated full-genome sequences.

Virus neutralization (VNT).VNT was carried out using 100 TCID50 of BatMRV1-IT2011 grown on MARC- 145 cells and rabbit and guinea pig immuno sera produced against the MRV prototype strains: Type 1 Lang (T1L), Type 2 Jones (T2J) and Type 3 Abney (T3A).

In order to obtain a preliminary and rapid overview on seroepidemiology of MRVs, we tested 100 sera collected in the local areas from different species (bovine, pig, horse, dog) for the presence of neutralizing antibodies against BatMRV1-IT2011 and T1L, T2J, T3A.

Results: BatMRV1-IT2011 was isolated from fecal samples of *Rhinolophus hipposideros* by cellculture.nsEM performed on supernatants of infected cell culture revealed a non-enveloped icosahedral virus with ~75 nm in diameter morphologically related to reoviruses (Figure 1). Virus identification was firstly confirmed by RT-PCRs specific for MRV L1 gene.

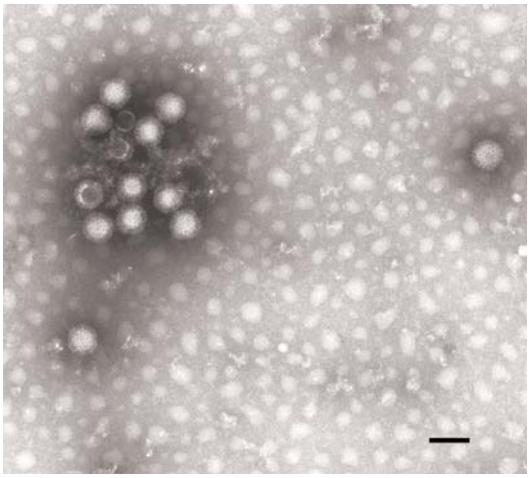

Figure 1. Ns EM of reovirus particles (bar = 100nm)

During the sampling activities no relevant mortality or clinical signs referable to infectious diseases of bats and normal reproductive success (number and mortality of juveniles until fledging) were observed.

The full genome sequence revealed that BatMRV1-IT2011 was a novel serotype 1 MRV, with the S1 segment similar to the bovine MRV T1/bovine/Maryland/Clone23/59 and C/bovine/Indiana/MRV00304/2014, but in other segments, it was more similar to MRVs of different host, origin and serotype. Results of the Blast analysis showing the highest similarity for each genome segment are reported in table 1.

The results showed that BatMRV1-IT2011 is a reassortant strain that contains MRV genes of bat, human, bovine and pig origin. The most MRVs related to BatMRV1-IT2011 have been associated with enteric/respiratory disease and encephalitis in animals and humans.

SimPlot and RDP analyses were performed by comparing BatMRV1-IT2011 to some representatives

MRVs belonging to the serotype 1, 2 and 3 to confirm the general sequence identity data. Virus neutralization confirmed that BatMRV1-IT2011 belongs to serotype 1, being strongly neutralize at high titre from rabbit and guinea pigs immune sera produced against the MRV T1L with no or low neutralisation byanti-T2J, T3Aimmune sera. Additionally, a high neutralizing antibody rate was found in serum samples of different mammals species

(bovine, pigs, horses, dogs) collected in the local areas against MRVs T1L, T2J, T3A and BatMRV1-IT2011. The results suggest that MRVs are widespread between animal populations and multiple infections with different serotypes are common including BatMRV1-IT2011 or other MRV strains with a similar S1 gene.

Table 1: Highest nucleotide similarityfor each gene segment of novel BatMRV1-IT2011.

BatMRV1-IT2011	% similarity	MRV strain	Serotype	Host	Disease	Country	GenBAnk Acession no.
L1	93	MRV-HLJ/2007	3	Pig	Fever,respiratory illness	China	HQ642769.1
	90	Porcine reovirus SHR-A	1	Pig	Unknown	China	JX415466.1
L2	91	MRV-HLJ/2007	3	Pig	Fever,respiratory illness	China	HQ642770.1
	90	T3/bat/Germany/342/08	3	Bat	Hemorrhagic enteritis	Germany	JQ412756.1
	90	SI-MRV01	3	Human	Acute gastroenteritis	Slovenia	KF154725.1
L3	95	MRVTou05	2	Human	Encephalitis	France	GU196308.1
	94	MRV-HLJ/2007	3	Pig	Fever,respiratory illness	China	HQ642769.1
M1	98	T3/bat/Germany/342/08	3	Bat	Hemorrhagic enteritis	Germany	JQ412758.1
	98	SI-MRV01	3	Human	Acute gastroenteritis	Slovenia	KF154727.1
M2	92	MRV-HLJ/2007	3	Pig	Fever,respiratory illness	China	HQ642773.1
	92	4 Ndelle virus	Putative 4	Mouse	NA	Cameroon	AF368034.1
M3	93	MRVTou05	2	Human	Encephalitis	France	GU196314.1
	92	Austria/729	2	Pig	Encephalitis	Austria	JN799425.1
S1	90	T1/bovine/Maryland/Clone23/59	1	Bovine	NA	USA	AY862134.1
	88	C/bovine/Indiana/MRV00304/2014	1	Bovine	Diarrhea	USA	KJ676385.1
S2	95	China/MPC/04	3	Civet	NA	China	GQ468273.1
	94	T3/bat/Germany/342/08	3	Bat	Hemorrhagic enteritis	Germany	JQ412762.1
	94	SI-MRV01	3	Human	Acute gastroenteritis	Slovenia	KF154731.1
	94	MRV-HLJ/2007	3	Pig	Fever,respiratory illness	China	HQ642776.1
S3	91	SC-A	3	Pig	Diarrhea	China	DQ411553.1
	91	Feline/California/Cornell/1968	3	Cat	NA	USA	U35362
S4	95	MRVTou05	2	Human	Encephalitis	France	GU196313.1
	95	MRV-HLJ/2007	2	Pig	Fever,respiratory illness	China	HQ642778.1

Note: L, large segment ; M, medium segment ; S, small segment ; NA, not available.

Conclusions: In our previous study, performed in the context of a virological survey on bat populations in Italy, we characterized 15 MRVs suggesting that MRV type 3 are widespread among bats (2). A similar study conducted in Germany confirmed this observation (3) and, very recently, a serotype 2 MRV has been detected in bats in China (8). To the best of our knowledge, before this study, the only MRVs identified in bats belonged to the serotype3 and 2.Here we reported the isolation and characterization of a new serotype 1 MRV from Lesser Horseshoe Bat (*Rhinolophus hipposideros*) named BatMRV1- IT2011, with evidence for genome segment reassortment with MRVs of different host, origin and serotype.

This finding extends the current knowledge on bat-MRVs and stresses the importance to continue and improve the Orthoreovirus surveillance in bats and other mammals even through the development and standardization of specific diagnostic tools.

Acknowledgments: This research was supported by the Italian Ministry of Health (WFR GR-2011-023505919).

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Genetic characterization of a novel adenovirus detected in captive bottlenose dolphin (Tursiops truncates) suffering from self-limiting gastroenteritis

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Objective: Even though adenoviruses have a wide host range and are common pathogens in vertebrates, it has only rarely been detected and correlated with disease in cetaceans. Recently, a novel adenovirus was detected in captive bottlenose dolphin (Tursiops truncates) suffering from self-limiting gastroenteritis, as described by Rubio-Guerri et al. [1]. The initial analysis of partial pol and hexon gene sequences indicated that this was a hitherto unknown adenovirus with less than 80% sequence identity to previously published sequences. However, full genome analysis would allow a more comprehensive classification and also enable analysis of genetic elements associated with e.g. tropism. The aim of this study was therefore to further genetically characterize this novel adenovirus by using a high-throughput sequencing approach for whole-genome sequencing.

Methods: Fecal samples from affected bottlenose dolphins were collected as previously described (1).

Samples diluted in ice-cold PBS were homogenized using an Omni homogenizer with disposable soft tissue tips and centrifuged at 3400 rpm for 10 minutes. The supernatant was transferred to a spin filtrate column (0.22 µm) and centrifuged for 4 min at 3400 rpm.

The filtrate was treated with 6U of TURBO™ DNase (Ambion), in reaction volumes of 100ul, and incubated for 30 min at 37°C. A total volume of 400ul was treated with QIAGEN Protease (dissolved in AVE buffer) and Ambion RNase Cocktail™ for 5 min at room temperature. DNA was extracted using QIAamp MinElute kit (Qiagen). The DNA concentration was measured using Qubit 2.0 Flurometer dsDNA HS (high sensitivity) kit.

Sequencing libraries were made using Nextera XT Library Preparation Kit (illumina), normalization was done using concentration measurements from Agilent High Sensitivity DNA Kit (2100 Bioanalyzer, Agilent Technologies), followed by sequencing at the MiSeq platform using the MiSeq Reagent Kit v3 600 cycles (illumina).

All sequence reads were assembled using the MIRA assembler with the standard settings for de novo assembly of Illumina data. The longest contigs were used to identify the most similar reference genome using BLASTn searches. To generate a draft consensus sequence, alignment of matching contigs against the reference genome was performed using the CodonCode Aligner software (CodonCode Corporation).

To allow verification by PCR and Sanger sequencing, the web-based Primer3 software was used to designed primers according to the obtained consensus sequence. The same approach was also used for gap-filling and primer walking to obtain a near full-length genome.

The analysis of phylogenetic relationships was conducted in MEGA 5 as previously described [2].

Results: In a previous study, two shorter gene fragments of a novel adenovirus genome were obtained. In this study, high-throughput sequencing allowed the recovery of more than 90 percent of the adenovirus genome and regions with lower quality values were verified and by PCR and Sanger sequencing. The preliminary phylogenomic analysis indicates that the most similar complete genome is the California sea lion adenovirus 1 strain Zc11-030 (KJ563221), which also was recently published [3], followed by the Bovine adenovirus type 2 complete genome (AF252854).

Conclusion: We here describe the near full-length sequence of a recently identified adenovirus most genetically close to the adenoviruses found previously in marine mammals. However, this virus is clearly different from previously published adenoviruses, demonstrating less than 80% sequence identity also when compared at the whole genome level. The virus has been associated with gastroenteritis in dolphins and a more in-depth analysis of the obtain sequence data and individual genetic elements should allow predictions to be made regarding

its tropism. The study also serves to demonstrate the usefulness of high-throughput sequencing to obtain near full-length sequence of genetically divergent viruses.

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Parallel ESVV General Assembly

Wednesday 2nd September 2015
11:00-12:00
Auditorium Pasteur

Parallel EPIZONE Session 1: Epidemiology, surveillance & risk assessment 1

Wednesday 2nd September 2015
15:00-16:30
Auditorium Pasteur

Chair: Beatriz Martínez-López & Thierry Lefrançois

Development of a Pipeline for the High-Throughput Sequencing of FMDV: An Application to a Large Outbreak

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Objective: The importance of a rapid, cost-effective and accurate response to an outbreak is essential to any effective outbreak management strategy. Next generation sequencing offers the opportunity to produce large volumes of high quality sequence data, derived from the parallel sequencing of large numbers of samples. This technology has the capability to generate complex datasets for the analysis of viral molecular epidemiology, which forms an essential component for monitoring the trans-boundary viral spread during any outbreak. We have developed and optimized a sample processing pipeline for the high-throughput genome sequencing of Foot-and-mouth disease virus (FMDV) using a MiSeq benchtop sequencer (illumina). We report here the optimization and adaption of an in-house protocol for the high-throughput sequencing of large numbers of samples.

Methods: Animal tissues infected with a single field isolate (O/UKG/35/2001) were used for the purposes of optimization. Methods were compared and contrasted at the following stages: Tissue homogenization, RNA extraction, reverse transcription, library quantization and library normalization. The final pipeline was described as follows: Infected epithelial tissue was weighed and placed in lysis buffer, prior to homogenization using the Tissuelyser LT (Qiagen). Viral RNA was extracted from tissue homogenates using the Magmax express-96 nucleic acid extraction platforms and the Magmax 96-viral RNA extraction kit (Life Technologies). FMDV genomic double-stranded DNA for constructing sequencing libraries was generated using an in-house protocol (Logan & Freimanis *et al*, 2014) and quantified using Quanti-fluor (Promega) as per manufacturer's instructions. Sequencing libraries were constructed using the Nextera XT DNA sample prep kit (illumina) and sequenced on an illumina MiSeq using 2x300bp reads version 3 MiSeq reagent packs (illumina). Bioinformatic analysis was performed using a standard analysis pipeline as described previously (Logan & Freimanis *et al*, 2014).

Results: The pipeline was initially validated using 24 FMDV field isolates. After validation, the method was then applied to generate consensus level sequences from a larger sample set i.e. 200 FMDV-positive tissue samples collected during the UK-2001 outbreak. Samples were processed in batches of 96 and sequenced, alongside appropriate controls, on three MiSeq runs of 96 samples. Those 24 samples, repeated on a 96-sample format produced identical consensus sequences. Bioinformatic analysis using established protocols determined factors of library complexity (including quality scores, total numbers of reads and percentage genome recovered at consensus level). Average coverage depth and reproducibility between runs were also evaluated. All three sequencing runs successfully generated high-quality genome sequences for between 70-80% of samples, with results correlating with viral load. Consensus sequences of samples repeated on different runs were identical. Additionally, 5 samples were sequenced on both the MiSeq and Sanger platforms, with the Sanger derived sequences considered to be 'gold-standard'. All MiSeq consensus sequences were identical to Sanger derived 'gold-standard' sequences.

Conclusion: This sample processing pipeline represents a step forward in the capability to produce large numbers of consensus-level genome sequences, using novel technologies, such as next- generation sequencing. The protocol demonstrates the potential for use of this technology within a diagnostic laboratory environment. The large datasets produced here have been validated using consensus sequences produced by Sanger sequencing and also provide an opportunity to investigate sub-consensus diversity, where coverage depth permits. Such

datasets provide important information on the molecular epidemiology of FMDV during outbreaks and will be applied to further outbreak tracing studies to identify and determine patterns of viral transmission and mechanisms of evolution.

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Classical Swine fever in wild boar: Surveillance strategies under the microscope

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Objective: Classical Swine Fever (CSF) is a highly contagious viral disease, which affects all suid species. CSF infection in wild boar can play an important role in disease introduction to commercial pig holdings. Due to its high economic impact, efficient but also cost-effective surveillance strategies have to be implemented not only in commercial pig holdings but also in wild boar.

In Germany, the last CSF outbreak occurred in wild boar in 2009. Consequently, vaccination had been implemented until 2012. Since June 2012, Germany is considered to be free from CSF. Following this, active surveillance in wild boar has been carried out according to the directives of the European Union (Council directive 2001/89/EC). Germany's implementation of these regulations plan to take 59 samples per district per year to be capable to demonstrate freedom from disease on district level (conventional method).

Compared to conventional surveillance, risk-based surveillance approaches may achieve similar performance at lower cost or better performance at the same cost. Within the framework of the European project RISKSUR (http://www.fp7-risksur.eu/) we conducted a simulation study to compare the performances of different surveillance approaches for CSF in an unvaccinated wild boar population in an area free from the disease.

The aim of the study was to find out whether surveillance with the objective of demonstrating freedom from disease in wild boar could be designed more effectively using risk-based or alternative methods compared to using conventional methods.

Methods: For the simulation model, R (www.r-project.org) was used for statistical computing and displaying graphs. A virtual wild boar population was generated and an infection initiated within this population. As study area we used the federal state of Rhineland Palatinate. The population size estimates for the considered region, determined using fecal DNA samples, were used to calculate the total number of wild boar within the simulation study. To assure a general pattern of the population structure we chose districts from three areas with different epidemiological situations. The simulated population was structured containing information on age, gender and the type of carcass (shot healthy, shot sick, injured through road traffic accident and found dead). The setup of the infection was done on the basis of data from Mecklenburg-Western Pomerania to estimate the increase of the seroprevalence at the beginning of an infection.

The risk factor analysis used to define the risk-based surveillance approaches was done through literature search, expert opinion and bivariate analysis of infection data. Due to a lack of appropriate information in Rhineland Palatinate, infection data of the federal state of Mecklenburg-Western Pomerania were used as data basis as well.

The simulation was conducted on the basis of real hunting data of Rhineland Palatinate and the surveillance approach, performed following the regulations of the European Union was simulated.

With these simulations, the probability of case detection and the time until the first case detection were determined and used as reference values. These values were then compared to the values resulting from the simulation of different alternative and risk-based surveillance methods. Alternative surveillance methods were increased sampling in the age class at higher risk of infection or in the season with an assumed higher detection probability. Furthermore we simulated the sampling in dependence of the population density in the different districts in two different ways. One approach was to sample only in districts were the population density was above a predefined threshold, whereas the other approach was to determine the sample size in the individual districts in dependence of the population density.

Results: The results of the fecal DNA analyses showed an estimated mean of 2,593 wild boar per district with a maximum of 8,524 and a minimum of 30 wild boar (median: 2,163). The real hunting data collected in specified districts (27 districts) of Rhineland Palatinate between 2003 and 2014 constituted the basis for the age and sex structure of the simulated population. In total we had 105,439 records. In the analyzed data the sex proportion of shot male and female animals was almost equal (male: 52.4%; female: 47.6%). The age distribution showed that the number of shot animals aged less than one year were highest (55.1%) and the number of samples coming from animals over 2 years was lowest (10.8%). Almost all samples (99.6%) were collected from hunted healthy animals, i.e. from active surveillance. Most samples were taken in the months of November (15.92%), December (13.68%) and January (12.57%). For the simulation of hunting, averaged values of the hunting bag data from 2003-2011 were used. In average 1,340 wild boar/district/year were hunted (min: 0; max: 4,573; median: 1,113).

For the setup of the infection into the simulated population, data from Mecklenburg-Western Pomerania were used. They consisted of 17,492 data sets resulting from the years 1993 and 1994, which were collected in six non-vaccination districts. Within this dataset, 2,652 samples had tested seropositive for CSF.

Due to the low number of positive cases identified through serological and virological testing in the data set of Rhineland-Palatinate, surveillance data from non-vaccination districts within Mecklenburg-Western Pomerania were used for risk analysis. The data consisted of 85,105 data sets from 1994-2000. The sex, age and carcass distribution showed a similar pattern as the data from Rhineland-Palatinate. Bivariate analysis showed that age plays a role in the probability of being serologically or virologically positive. These findings were supported by the findings of the literature search as well as by expert opinion. In the literature, it was found that the probability of samples gathered through passive surveillance being positive is assumed to be higher. However, the low number of available passive surveillance data made it impossible to support this assumption through statistical analysis. Additionally, experts suggest that seasonality of the hunting and infection as well as population density may be considered as risk factors for infection. The following initial results refer to simulations in a defined area of Rhineland Palatinate consisting of three neighboring districts. From 1000 simulation repetitions of sampling 59 samples per district over the year, by serological examination the infection was detected 1000 times per district (reference value for detection probability). On average 331 cases were detected in the first month of infection (reference value for time until first case is detected). By serological investigations of 59 animals aged less than 1 year infection was not detected at all in 11 simulation runs. On average 162 cases were detected within the first month. However, investigating 59 animals aged over 2 years serologically, the infection was detected 1000 times and 688 times already in the first month of infection.

Conclusion: The first results of the simulation model indicate that alternative surveillance strategies show a similar probability of detection. However, serological surveillance of animals aged over two years reduced the time until the first case was detected significantly.

Additionally, it might be assumed that through changes in sampling strategies e.g. based on population density thresholds, a reduction of costs is possible. The resulting financial resources could be used to increase sampling following the risk based approaches, therefore increasing the detection probability. They could also be used for campaigns supporting for example passive surveillance. However, the effectiveness of the single surveillance approaches should not only be measured by the outputs of the model, but also through a comprehensive evaluation of the whole surveillance system, including acceptability and practicability of the system. Moreover, the surveillance could be designed taking different risk factors into account and could therefore have the potential to result in a better performance.

If higher numbers of animals found dead were investigated, passive surveillance would be an alternative. However, also in the future it will be difficult to reach higher numbers of samples from passive surveillance.

Finally it has to be mentioned that due to the limitation of getting accurate population abundance data in wild life, designing surveillance schemes and the establishment of effective surveillance strategies will always constitute a certain challenge.

Quantification of African swine fever virus transmission parameters in carriers and the possible role of indirect virus transmission

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Objective: In 2007 African swine fever (ASF) virus (ASFV) was introduced in Georgia, and through Russia it reached the EU (Poland and the Baltic states) in 2014. So far the disease seems to be a typical disease of backyard holdings, with virus spread mainly through direct contact and swill feeding, with only occasional introductions in industrial herds. Wild boar are heavily involved in many areas, and while no one doubts they play a role in the spread of the virus, the extent of their role is unclear and under discussion. The Eastern European ASFV strain seems almost 100% lethal, at least under experimental circumstances. Infections with less virulent strains, however, result in some pigs that survive the infection and remain persistently infected for several months (1), while showing no clinical signs. These carriers could potentially play a major role in the virus being sustained in wild boar population. This will, however, also depend highly on the infectiousness of these carriers, and it is still unclear how that compares to the infectiousness of pigs in the acute phase of the infection.

Besides through direct contact or swill feeding, spread of virus may occur through indirect routes as a result of contamination of the environment or fomites. A high stability of the virus in the environment will facilitate the virus to become and remain endemic in a population. Furthermore, this would, especially for industrial herds, facilitate the spread of the virus to other farms. Indirect transmission routes, which are difficult to identify and control, are for instance a major problem during outbreaks of classical swine fever (CSF). Both the excretion patterns from infected pigs and the infectious dose of ASFV suggest that indirect transmission may be of limited importance for ASFV as compared to CSF virus (CSFV). This hypothesis has, however, never been tested under controlled circumstances.

The objective for the current study was to estimate quantitative parameters for ASFV transmission by carriers and to elucidate the possible role of indirect virus transmission.

Methods: Twenty pigs, housed in four pens, were successfully inoculated with ASFV through the intranasal route with the moderately virulent strain Netherlands ’86. In previous experiments with this strain, roughly half of the inoculated pigs tend to survive and become a carrier. Excess faeces were removed on a daily basis from the pens with a shovel, but no further cleaning and disinfection was carried out.

Contact infections by carriers: Six pigs, divided over three pens, survived the acute phase of the infection and became carriers. Four weeks after the inoculation, these six carriers were moved to new, clean pens, one pig per pen. In each pen, one contact animal was added for a maximum of 13 days, or until the contact animal became infected. After 13 days, all remaining contact pigs were removed and 6 new contact pigs were added 24 hours later for another 13 days (or until they became infected). Contact animals were tested frequently by PCR on oropharyngeal swabs and blood for evidence of an infection. The six carriers were regularly tested for viremia and virus excretion by PCR and virus isolation. Transmission rate parameters (β) were estimated for each period separately and for both periods together.

Infections from the environment: The four pens were repopulated with two sentinel piglets each, 24 hours after the inoculated pigs were removed, for a period of 14 days. The sentinel pigs were frequently tested by PCR on oropharyngeal swabs and blood for evidence of an infection. Environmental samples were collected from the pens on a regular basis and tested for virus by PCR and virus isolation.

Results: *Contact infections by carriers:* In the first group of contact pigs, who were in contact with the carriers for 13 days between day 28 and 41 after the inoculation, none of the contacts became infected with ASFV. In the second group of contact pigs, who were in contact with the carriers for 13 days between day 42 and 55 after

inoculation, two of the six contacts became infected with ASFV. The estimated times of infection were day 42 and day 44 respectively. These results in transmission rate parameters (?'s) of resp. 0 (0-0.038) and 0.036 (0.012-0.099) day^{-1} for each of the 13 day periods, or 0.015 (0.002-0.038) day-1 overall (between brackets: 95% confidence bounds).

Infections from the environment: During a period of 14 days, none of the sentinel pigs in the four contaminated pens became infected.

Conclusion: The overall transmission rate in carriers was estimates to be 0.015 day^{-1}. This is much lower than in the acute phase of the infection, where transmission rates for this particular virus range from 0.45 to 0.92 day^{-1} (2). The two observed contact infections took place around day 42 and 44 after inoculation of the carriers. This coincides with a second peak of virus excretion observed in these carriers, both in this study (data not shown) and a previous study (1). With an estimated infectious period for an average carrier of approximately 40 days (2), and an overall transmission rate of 0.015 day-1 in this period, the reproduction ratio (R) of the virus in the carrier phase would approximately be 0.6 for any carrier in direct contact with one naïve pig on average at any time during the infectious period. Although contact infections by carriers are thus expected to be rare, especially in wild boar that may have limited contact with each other, these might still be of importance as movements of the carriers during the long infectious period enhance the probability that these cause new spatial foci of infections. In combination with the higher local transmission rates in the acute phase, such low-rate longer-distance transmission could be essential to keep the infection chain going. Especially for wild boar it remains to be seen if and to what extent the presence of carriers would facilitate an endemic situation of the virus in the population. The high mobility of these apparently healthy carriers should definitely be taken into consideration, as it allows for virus spread over large distances.

No infections were observed in the sentinel pigs in the contaminated pens. This suggests that indirect virus transmission through the environment may be difficult to achieve for ASFV. This is likely to also apply to indirect virus transmission between industrial herds (e.g. through transport trucks or fomites), suggesting that ASFV spreads more difficult through these indirect routes than for instance CSFV. This is also in line with previous findings that virus excretion from ASFV infected pigs is limited compared to pigs infected with CSFV (especially when chronic infections with CSFV occur), while the infectious dose for ASFV is also much higher than for CSFV (1).

In wild boar populations, the environmental contamination may in addition also result from carcasses from wild boar that died from ASF. Virus amounts in these carcasses are expected to be high. Especially in winter time, with temperatures potentially below freezing for a long time, the carcasses may constitute reservoirs of virus that may contribute to sustained presence of the virus in the population. In summary: Virus transmission from carriers and environmental contamination may be crucial factors in the ability of the virus to become endemic in wild boar, even though this kind of virus transmission is very limited. For industrial pig herds, the findings support the hypothesis that indirect transmission between pig herds is likely to play a minor role and that the disease may be relatively easy to control and eradicate under those circumstances.

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Lessons learnt from a cross-sectional field survey: how to implement a serological monitoring of BVD-free herds in the Belgian BVDV eradication programme?

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Objective: In January 2015, Belgium started a national eradication programme for Bovine Viral Diarrhoea (BVD). Mandatory testing of all newborn calves within seven days after birth followed by the quick elimination of persistently infected (PI) animals are pivotal during the first years of the eradication program. In a later phase, it is foreseen that herds in which all animals are certified non-PI will be given the opportunity to apply for a BVDV-free herd status. Serological surveillance based on BVDV- specific antibody ELISAs is a commonly used tool to ascertain freedom of infection. In a previous study (De Baere et al.) the intrinsic characteristics of all available commercial BVDV antibody ELISAs were determined using a limited panel of samples. Based on these results, six different ELISA kits were selected for further evaluation on a large number of field serum and milk samples. The serological results were analyzed taking into consideration epidemiological parameters at individual and at herd level. These parameters have been collected through a questionnaire implemented in each of the visited farm. The goal of this study was to provide recommendations to the stakeholders of the BVD eradication program regarding the monitoring of BVDV-free herds by means of serology. Factors with relation to age of sampled animals, sample matrix, type of ELISA test, number of animals to sample, design prevalence and vaccination status were evaluated.

Methods: The study was conducted on 51 cattle farms (dairy, beef, mixed) selected in Belgium and based on a representative list of herds that participated in the voluntary BVD control program. Each farm was visited once, by a veterinarian between December 2013 and March 2014. During this visit, blood samples (serum) were taken from all young stock (aged 6 to 18 months) as well as from 20 adult cows (> 24 months) randomly selected, except in mixed herds where 20 dairy cows and 20 beef cows were sampled. In dairy herds, individual milk samples were taken from the same cows which had been sampled for blood, as well as a bulk milk sample.

Herd-level database

An epidemiological survey was conducted in each farm at the moment of sampling. Hereto, a questionnaire was designed and filled out together with the farmer and, when possible, the private veterinarian. The objective of this survey was to characterize each herd and to collect epidemiological information regarding the BVD status, control measures in place, vaccination practices, risk factors for BVD introduction, detection of PI animals in the herd, etc. Answers to all questions were recorded in an Access® database for further statistical analysis. Through this epidemiological survey it was possible to classify the herds into five different categories regarding BVD infection, in order to correlate the serological results with the BVD herd status: (1) BVD free, (2) PI-free, (3) BVD controlled, (4) BVD infected less than one year, (5) BVD currently infected.

Animal-level database

In total, 3159 sera, 557 individual milk samples and 28 bulk milk samples were tested for BVDV- specific antibodies, using different commercial ELISA kits (four ELISAs for serum samples and two ELISAs for milk samples). The obtained results were used to assess the performances of each ELISA kit on serum or milk individual and pooled samples, taking the Virus Neutralization (VN) assay as the reference test (De Baere et al., data not shown).

For serum samples, a positive, negative or discordant final serological status was assigned to each sample, depending on the outcome of all four commercial ELISAs and of the results of VN test which was performed in case of a discordant result between one or more ELISAs . A database for all the individual samples was created in Excel® with other variables such as ID number, age, geographical localization, herd type, etc. This information was extracted and obtained from the national livestock databank (SANITEL).

Using SAS 9.2. ® Software, the two databases (animal- and herd-level) were merged taking the herd ID as matching variable. Descriptive statistics were performed to identify factors that influenced the serological status of samples and herds: variations in the proportion of seropositive animals were observed in function of several of these factors such as age, vaccination, BVD herd status, herd type, etc. Subsequently these factors were included as covariates in a univariate logistic regression using the serological status of the samples as dependent (response) variable. Significant covariates were kept and put in a final multivariable logistic model.

Results: Among several explanatory variables, the herd production type, the participation to auctions, the vaccination status against BVD, the age of the animals as well as the BVD status of the herd clearly influenced the serological results obtained by the ELISAs. After taking into consideration the seroprevalence observed in function of the age classes, vaccination and the BVD herd status, the following recommendations could be provided to the stakeholders with relation to the serological monitoring of BVD-free herds:

a) Due to the life-long persistence of BVD antibodies in animals exposed to BVD virus and to the transient persistence of maternal antibodies in young stock, serological monitoring should preferably be performed in animals at the age of 6 to 18 months.
b) Due to the interference of BVD vaccination in the serological results (20 to 30 % of seroconversion due to vaccination, depending on the ELISA kit used), only unvaccinated animals should be sampled for the monitoring as it is not possible to differentiate between a vaccinated and an infected animal.
c) Due to the currently high prevalence of seropositive cows even in herds which have become BVD- free, bulk milk testing cannot be used at this stage in Belgium to monitor the possible exposure of the herd to BVDV infection.
d) Based on the seroprevalence observed in young unvaccinated animals in function of the BVD status of the herd, it is advised to use a case detection protocol for an annual serological monitoring of BVD-free herds, designed to detect a minimum prevalence of 15%, a higher seroprevalence indicating a possible recent circulation of BVDV in the herd.
e) Taking into consideration sensitivity and specificity of the ELISA tests which was evaluated in parallel in this study, an annual monitoring of BVD-free herds can be performed by testing 15 to 19 (in function of the herd size) young unvaccinated animals. All tested animals should be seronegative to confirm the BVD-free status of the herd.
f) Alternative strategies can be used to monitor BVD-free herds such as continuous testing for BVDV of all new-born calves, annual testing for BVDV of all animals which have not yet been certified non- PI, serological annual testing of larger number of animals to evaluate with an acceptable precision the herd seroprevalence. The choice of the monitoring system to be used should be adapted to each herd and take into account practicability and financial cost in function of the herd size.

Conclusion: This cross-sectional serological survey coupled with an epidemiological survey, delivered very useful data and information to anticipate the serological monitoring of BVD-free herds in the Belgian BVDV eradication program initiated in January 2015. It has been shown that serological monitoring can be used as a tool to maintain a BVD-free herd status provided that it is adapted to the epidemiological field situation and in particular takes into consideration age of the animals, vaccination status and herd seroprevalence observed in function of exposure to BVDV.

One World One Health: The STAR-IDAZ Global Network for Coordination of Animal Disease Research

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Animal diseases can cause serious social, economic and environmental damage and in some cases also threaten human health. It is estimated that 60% of human pathogens are of animal origin while 75% of emerging animal diseases can be transmitted to humans. Disease risks have increased over recent decades, especially as a result of the increased globalisation of trade and animal product movements, and the consequent transfer of associated fast evolving pathogens. An increasing number of the major disease problems or threats faced by the livestock industry are of a global nature.

STAR-IDAZ (Global Strategic Alliances for the Coordination of Research on the Major Infectious Diseases of Animals and Zoonoses) was a 48 month EU-funded project to improve coordination, at international level of research activities on the major infectious diseases of animals (including zoonoses) and hasten the delivery of improved control methods. This was achieved through the establishment of an international forum of R&D programme owners/managers and international organisations for the purpose of sharing information, improving collaboration on research activities and working towards common research agendas and coordinated research funding on the major animal diseases affecting livestock production and/or human health. It involved partners from 19 countries around the world with organisations from more than 30 other countries involved at a regional level through networks established for the Americas, Asia and Australasia and Africa and the Middle East to complement the existing European network.

A number of databases and reports were produced and are available from the project website at www.star-idaz.net. A Publications Database maps animal health related scientific publications with abstracts in the ISI Web of Science from 2006 to 2013 according to disease/pathogen group, scientific discipline, animal species group, research organisation and country. It allows users to identify the major research institutes publishing on a specific topic across and within countries and an analysis of collaborations between research institutes based on their co-publications is included in a report. A Research Organisation Database, provides an overview of research centres, funding organisations, programmes and facilities, in the field of animal health across member countries.

A report on Mechanisms for Networking has been produced as has an inventory of animal health related foresight studies and related risk assessment activities of partner countries. Criteria for Priority Setting, to be used in prioritising research needs depending on the needs of the user, were established through a number of exercises including a literature review, workshop and online surveys. Regional foresight workshops and online activities were conducted with the objective of identifying the scientific, technological and related needs to prevent, control or mitigate animal health and zoonotic challenges for the next 20 years. The outputs of these exercises were developed further and integrated at a Global Foresight Workshop in Moscow, held under the EU-Russia Year of Science, and form the basis of the Global Strategic Research Agenda - Meeting Future Research Needs on Infectious Diseases of Animals and Zoonoses (SRA). Better understanding of the role of wildlife, vector borne diseases including alternative methods to control vectors, antimicrobial resistance, vaccinology, knowledge management systems including big data, partnerships and collaborations, maintenance of capacity and investment in basic research all rated highly in the Global SRA.

STAR-IDAZ now moves forward as a self-sustaining network and partners will have the option to participate in a higher level of engagement where they will commit to aligning their programmes on priority diseases and cross-cutting issues. Those priorities identified by STAR-IDAZ partners include Influenza, Mycobacterial Diseases, Foot and Mouth Disease (FMD), Salmonella, Helminth Parasites, Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Brucellosis, African Swine Fever (ASF), Rabies, Alternatives to Antibiotics, Vaccinology, and the Reduction of Livestock Greenhouse Gas Emissions through improved Animal Health. Working Groups have been or are being established with research gap analyses published on the STAR-IDAZ website as they become available. As well as the network partners working together to fund these gaps, the benefit of such a network is being able to collectively respond to the rapidly evolving disease threats and together create the tools for improved control of the major animal diseases threatening the livestock industry and/or of public health.

Parallel EPIZONE Session 2: Intervention strategies

Wednesday 2nd September 2015
15:00-16:30
Room Sully 1

Chair: Linda Dixon & Marie-Frédérique Le Potier

Classical swine fever virus marker vaccine strain CP7_E2alf: shedding and dissemination studies in boars

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Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a severe multi-systemic disease of pigs, which can lead to tremendous economic losses. While prophylactic vaccination is prohibited in most countries with industrialized pig production, emergency vaccination is foreseen to reduce the socio- economic and animal welfare impact of CSF outbreaks. However, the lack of suitable marker vaccines has so far prevented implementation of emergency vaccination in domestic pigs.

Over the last decade, the pestivirus chimera “CP7_E2alf” was presented as a promising modified-live marker vaccine in several studies, and recently it has been licensed by the European Medicines Agency. However, some safety issues are still not completely elucidated. The presented study was undertaken to gain further background data with regard to distribution and shedding of the vaccine, especially in urine, faeces, and semen of reproductive boar.

In detail, after a single intramuscular vaccination with a tenfold vaccine dose, the dissemination and shedding pattern of the vaccine strain “CP7_E2alf” was assessed in twelve adult boars. Upon vaccination, neither local nor systemic adverse affects were observed in the experimental animals. Four and seven days post vaccination, six animals were subjected to necropsy and triplicate samples were obtained from reproductive and lymphatic organs as well as urine, faeces, blood, and several additional organs and matrices. The sampling days were chosen based on pre- existing data that indicated the highest probability of virus detection. It was confirmed that primary replication is restricted to the lymphatic tissues and especially the tonsil. While viral genome was detectable in several samples from the lymphatic tissues at four and seven days post vaccination, infectious virus was only demonstrated at four days post vaccination in one tonsil sample and one parotid lymphnode. Sporadic detection at a very low level occurred in some replicates of liver, lung, bone marrow and salivary gland samples. In contrast, viral genome was not detected in any sample from reproductive organs and accessory sex glands, in faeces, urine or bile.

The presented data on the dissemination of the vaccine virus “CP7_E2alf” in adult boars are supplementing existing safety and efficacy studies and indicate that the use of the vaccine is also safe in reproductive boars.

First expression in baculovirus of major capsid proteins belonging to two new lagoviruses

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Objectives: Rabbit Hemorrhagic Disease (RHD) and European Brown Hare Syndrome (EBHS) are two similar acute and lethal forms of hepatitis caused by two distinct species-specific caliciviruses belonging to the *Lagovirus* genus that emerged around 1980 in China and in northern Europe, respectively. Although genetically closely related, RHDV and EBHSV are antigenically distinct. A third type of lagovirus has been detected in Italy in 1996 in domestic rabbit, this virus causes a non clinical infection of the intestinal tract without pathogenicity

and was named rabbit calicivirus (RCV). Since then, other non-pathogenic related virus have been detected in Europe (RCV), United States (MRCV) and in 2009 in wild rabbits in Australia (RCV-A1). These RHDV- like viruses can persist both in commercial farms and wild population, inducing cross-reactive antibodies with RHDV that interfere significantly in RHDV specific serological testing. Moreover, since their non-pathogenicity they are present in limited yield in tissues of infected animals, hence their use as antigens or immunogens is very difficult.

Recently some authors have described the emergence of two new types of lagovirus, one, related to RHDV, has been named RHDV2 (1, 2), the other is a non-pathogenic lagovirus isolated for the first time in hare and hereafter referred as HaCV (3). RHDV2 has shown pathogenic and antigenic differences with RHDV that might be related to substitutions in the capsid gene. HaCV is the first non-pathogenic lagovirus described in hare.

In this study, we expressed both the capsid protein (VP60) of RHDV2 and HaCV in a baculovirus expression system. It is well known from many studies that calicivirus major capsid protein can self-assemble in virus like particles (VLPs) when expressed in baculovirus. It has been described that RHDV VP60 assembly correctly in virus like particles (VLPs) after expression in baculovirus, and also in this case VLPs have shown structural and antigenic features of native viral particles, besides they were obtained at high yield (4,5). The main objective of this work was the production of RHDV2 and HaCV VLPs in order to obtain high yield of recombinant assembled protein to evaluate, especially for HaCV, their antigenic profile, to be used as source of antigen in diagnostic assays and, for RHDV2, as potential form of vaccine.

Methods: The entire VP60 coding region from RHDV2 and HaCV was cloned in pFAST-Bac plasmid, recombinant baculoviruses were obtained using Bac-to-Bac expression system (Life Technologies) and used to infect Sf9 (Spodoptera frugiperda 9) insect cell line. The Sf9 were seeded at a concentration of 1210^6 cells in 175 cm^2 tissue culture flasks and infected with the two recombinant baculoviruses (RHDV2 VP60 and HaCV VP60) respectively. After 72h post- infection medium and cells were harvested by centrifugation, both were evaluated for VP60 expression and VLPs presence. The samples were concentrated by ultracentrifugation through a 20% (wt/wt) sucrose cushion, then analyzed by electron microscopy and Western Blotting (WB). For WB was used a cross-reactive VP60 specific MAb produced against an internal conserved epitope common between lagovirus. One panel of RHDV2 specific MAbs were also used to evaluate the structural and antigenic properties of recombinant RHDV2 VP60 using sandwich ELISA assays.

Results: The Western Blotting analysis on the samples before being submitted to ultracentrifugation evidenced the high level expression of both VP60 recombinant proteins, efficiently recognized by the cross-reactive 5G3 MAb at the expected molecular weight of 60Kd. However only HaCV VP60 was clearly detected in high amount in the pellet of ultracentrifugation in which electron microscopy put in evidence the presence of regularly assembled VPLs. Differently, results with RHDV2 were variable in relation to the lot of productions. In any case, also in production where some VP60 was found in the pellet and MAb RHDV2 specific resulted partially reactive, the quantity of the VLPs found in the pellet after ultracentrifugation was just in trace in comparison to the case of HaCV. For RHDV2 most of the VP60 was instead found in the supernatant of ultracentrifugation.

Conclusion: We have efficiently expressed in baculovirus expression system both recombinant VP60 proteins from RHDV2 and HaCV lagovirus, but only HaCV VP60 resulted to self-assembly in VLPs, enabling the antigenic characterization of this new lagovirus (3). Moreover the high yield of VLPs obtained will allow to the development of specific serological tests. The inability of the RHDV2 VP60 to self-assemble adds a further data in favor of the idea that RHDV2 is not variant of RHDV but rather a new emerging virus. In fact, this results is inconsistent with those reported in literature regarding the self-assembly of recombinant RHDV VP60 alone (4,5). Probably, difference in the primary structure between the VP60 of RHDV and RHDV2 cause some instability in the precursor subunits of the latter and so not allowing the correct and stable capsid assembly. In the case of RHDV2 the co-expression of the minor capsid protein (VP2) could be an important factor to obtain stable VLPs.

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In vivo testing of deletion mutants as candidate vaccines for African swine fever in vaccination/challenge models in pigs

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Objective: African swine fever (ASF) is a highly infectious disease affecting both domestic and wild pigs, from which there is not vaccine. It is acute form induces high mortality rates causing enormous economic and health losses in affected countries. The naturally attenuated ASF virus (ASFV) NHV/P68 /P68 has been shown previously to protect immunized pigs against lethal challenge with some virulent strains including ASFV-genotype II Armenia 2007 isolate. However, adverse reactions including fever and joint swelling were observed in some pigs after the post-inoculation with the attenuated strain. In the current study two deleted viruses, the NHV/P68 –ΔA238L and NHV/P68 –ΔA276R were constructed from the NHV/P68 ASFV genome. These genes have previously been shown to be involved in virulence and immune evasion. The ASFV A238L protein inhibits key pathways involved in activating transcription of immunomodulatory genes (Sánchez et al., 2012), whereas the ASFV A276R gene inhibits the induction of IFN-β (Correia et al., 2013). The deleted viruses were used for pig immunization. The main goal was to assess whether the NHV/P68-Δ A238L and NHV/P68 - Δ A276R were able to induce protective immunity in pigs against a lethal challenge and their ability to reduce the adverse clinical signs produced by the attenuated strain.

Methods: The *A238L* and *A276R* genes were deleted from the ASFV NHV/P68, a non haemadsorbing and low virulence ASFV isolate (Leitao et al., 2001). Recombinant viruses NHV/P68-ΔA238L and NHV/P68-ΔA276R were grown in COS-7 cell line or in primary porcine alveolar macrophages (PAM), respectively, in parallel with the parental NHV/P68 viruses. The viruses obtained from COS-7 cells were purified by Percoll gradient to eliminate any cellular debris which could induce an adverse immune reaction and diminish the levels of protection. Fifteen European domestic pigs were used for the animal experiments. The pigs were grouped into four groups. Two groups of five pigs were immunized with either NHV/P68-ΔA238L-COS (group 1) or NHV/P68-ΔA276R (group 2). For comparative analysis two groups of 3 and 2 pigs were respectively immunized with the parental NHV/P68 virus produced in COS-7 (group 3) or in PAM (group 4). All pigs were intramuscularly (IM) inoculated with 10^2 TCDI50 of each virus. Clinical examination and rectal temperature were daily monitored. At 29 days post immunization (dpi) these groups of pigs were IM challenged with 10 HAU50 of the virulent ASFV Arm07 isolate. Two control pigs which had not been immunised were also challenged with the virulent strain. Paired EDTA-blood and sera samples were collected at 0, 7, 14 and 29 dpi and at 3 day intervals post challenge until the end of the experiment (65 dpi). Viremia and virus presence in organs was achieved by real time PCR (Fernández-Pinero et al., 2013) and further virus isolation into porcine peripheral blood macrophages (PBM) (OIE 2012). Detection of ASFV-specific antibodies was performed in serum using a commercial ELISA (INGEZIM, PPA COMPAC K3) and by Indirect Immunoperoxidase Technique (IPT) (Gallardo et al., 2012).

Results: After the initial immunisations, animals immunized with NHV/P68 -ΔA238L-COS (group 1) remained asymptomatic, whereas its parental control (group 3) exhibited slightly raised body temperatures and joint swelling, corresponding to lesions previously described in chronic type ASFV infection (Leitao et al 2001), 14 to 29 dpi. With NHV/P68 –ΔA276R-PAM (group 2) and NHV/P68-PAM (group 4), both groups of pigs showed similar clinical scores. Individually, three pigs from group 2 and one pig from group 4 showed adverse reactions in the form of swollen joints. Low levels of ASFV genomic DNA post-immunisation were detected in all groups in combination with high antibody titers from 14dpi.

Despite to the few number of pigs included in group 3, similar proportion of the pigs immunized with viruses produced in COS cells, survived the lethal challenge with ASFV Arm07. A total of three pigs survived comprising: 2 out of 5 (40%) immunized NHV/P68-ΔA238L-COS and 1 out of 3 (33.3%) in the group receiving the parental virus (NHV/P68 -COS). Interesting, the survivor animal in the parental group developed more intense chronic-type lesions and viremia prior to the challenge. All dead animals showed similar clinical signs to that observed in the two unimmunized pigs which died at day 8 and 9 post-challenge (dpc) following the typical acute ASF course. However, while immunized animals with the deletion mutant died between 8 to 14 dpc, a delay on the onset of the disease was observed in the NHV/P68 -parental-COS immunized pigs dying between 15 to 22 dpc. High viral loads and post-mortem lesions typical of acute ASFV infections were found in organs in both control and immunized animals. By the end of the experiment, at day 65 p.i. survivor pigs were totally recovered, not showing clinical signs of ASF, although viremia was intermittently detected across the experiment in the immunized pigs.

The five immunized animals with the NHV/P68 -ΔA276R-PAM virus died between 7 (2 pigs) to 9-10 (3 pigs) dpc. Interesting, the 2 pigs which died at 7 dpc did not present acute lesions of ASF and were ethically slaughtered due to the severe chronic-type lesions. Nor acute virus (Arm07) was found either in blood or tissues in these animals as it was estimated by the haemadsorbing assay. The pigs dead at 9 and 10 dpc exhibited the typical course of ASF acute disease.

In clear contrast with that observed in the group immunized with the deletion mutant, the two immunized animals with the parental virus survived to the infection without exhibiting significant clinical signs related to acute disease. All of the pigs in group 4 had clinical scores below 4 during the 36 days they were kept post-challenge and only one of the two animals presented significant viremia between 7 to 17 dpc. In agreement with previous observations, the pig which developed more intense chronic-type lesions after the immunization did not present viremia or clinical signs after being challenged.

Conclusion: In this study we investigated whether deletion of genes from the ASFV low virulence isolate NHV/P68 would reduce adverse clinical reactions post-immunisation but maintain induction of high levels of protection post-challenge with virulent isolate Arm07. In addition, to determine if COS cells can be used to generate ASFV recombinant virus, we have carried out the experiments immunize the pigs either with viruses obtained from PAM, or with viruses produced in COS cells and purified in a Percoll gradient.

In vivo immunisation of pigs showed that the pigs inoculated with the deletion virus NHV/P68 - ΔA276R were not protected against challenge with virulent ASFV whereas the two pigs inoculated with the parental strain NHV/P68 were fully protected. Furthermore, there were no clear differences in the adverse reactions observed after the initial immunisation with the recombinant or parental viruses originated from PAM. In contrast, the pigs inoculated with NHV/P68 -ΔA238L produced in COS and purified by Percoll did not develop any clinical signs after the immunization in comparison with pigs inoculated with the parental virus. This data could suggest that the A238L could be involved in the control of the immune response, or that the purification is an important step to reduce adverse clinical signs in the immunized animals. Nevertheless, after challenge with the virulent strain, a similar behaviour was observed in both recombinant and parental COS groups resulted in 60% and 67% of mortality, not conferring full protection.

The data presented in this study confirm previous findings in which immunized pigs with the ASFV NHV/P68 produced in PAM cells were 100% protected after the challenge with heterologous viruses without the intermediated challenged using the homologous viruses (Gallardo et al., 2012a). The development of deletion mutant ΔA238L in PAM could be a good approach in order to decrease the adverse clinical signs induced by the attenuated strain.

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Comparative study of protection in pigs immunised by different routes with attenuated African swine fever virus isolate OUR T88/3 and evaluation of the role of immunomodulatory cytokines.

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Objective: African swine fever (ASF) is one of the most devastating pig diseases, against which there is not vaccine available. Different attempts to develop an efficient and safe vaccine against ASF virus (ASFV) have been made, so far with not very consistent results. Thus, immunizations with inactivated, recombinant and DNA vaccines either failed or gave controversial results, providing just partial protection against homologous virulent ASFV isolates. On the other hand, despite biosafety issues such as attenuated virulence or induction of sub-clinical and chronic infections in pigs, previous studies using attenuated vaccines have demonstrated their potential to protect.

Methods: The present study is an extension of previous works where protection induced by the attenuated OUR T88/3 genotype I isolate was demonstrated after intramuscular immunization using a fixed dose (10^4 HAD/ml). Here, three different doses of OUR T88/3 (10^3, 10^4 and 10^5 HAD/ml) and two different routes of administration (intramuscular-IM and intranasal-IN) were tested in order to evaluate the induction of adverse reactions and grade of protection after challenge with a closely related virulent isolate OUR T88/1 (IM, 10^4 HAD/ml). For this purpose, six groups of six animals each (7-week-old Large White and Pietrain crossbred male piglets) were immunized following the doses and routes describe above, and challenged (21 days after immunization-dpi) along with a control group containing three non-immunized pigs.

Results: Our results have demonstrated a complete protection (100%) in pigs immunized with 10^3 and 10^4 HAD/ml intranasally. Pigs displayed minimal and transient adverse reactions after immunization and after challenge with OUR T88/1 (transient moderate joint swelling, moderate increase of temperature, inappetence and apathy) as well as scarce and mild macroscopic lesions in lungs, compatible with secondary bacterial infections, and joints mainly. However, both in group of pigs immunized intranasally with 10^5 HAD/ml and in all groups immunized intramuscularly, the rate of protection conferred was lower.

All protected pigs from different immunized groups displayed significant high levels of antibodies to Vp72 structural protein that were prolonged throughout the experiment. However, fluctuations along with low levels or absence of antibodies were observed in 50% of immunized pigs that died or were euthanized after challenge. These results suggested the potential role of humoral immune response in protection mechanisms, although the existence of ASFV-neutralizing antibodies has not been successfully demonstrated so far.

Although the mechanisms underlying the induction of protection in pigs to ASFV as well as the role played by cytokines remain unclear, our results clearly suggest the existence of differences in serum cytokine profiles evaluated by ELISA between immunized surviving pigs and pigs that died or were euthanized. So, the kinetics and concentrations of pro- (IL-1β and TNFα) and anti-inflammatory cytokines (IL-10) were similar among groups of immunized surviving pigs, without significant changes after challenge and with minimal individual variations. Nevertheless, an increase of IL-10 serum concentrations was detected before death in 10 out of 11 immunized pigs dead after challenge, including non-immunized control pigs. In addition, statistical analyses displayed a transient significant increase of IFNγ levels at 3 day-post challenge in those groups surviving pigs immunized intranasally (10^3 and 10^4 HAD/ml) where 100% of protection was demonstrated. Interestingly, in these pigs the levels of IFNγ were moderate in comparison with those observed in immunized pigs dead after challenge, and that shown the highest levels of IL-10.

Conclusion: Intranasal inoculation of pigs with low-moderate doses of OUR T88/3 provided a complete protection against homologous virulent ASFV isolates with minimal adverse reactions. Regarding immune protection mechanisms, together with the possible role of humoral immune response, our results suggested that survival of pigs after challenge was associated with a balance between pro-inflammatory (TNFα and IL-1β) and anti-inflammatory (IL-10) cytokines along with moderate levels of IFNγ, whereas in animals that died existed an imbalance linked to an exacerbated increased IL-10.

Deoptimization of codon pair usage of the major capsid protein VP72 of African swine fever virus: effect on protein expression in vitro

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The possibility to artificially re-synthesize large segments of DNA made it possible in recent years to intensely and rapidly modify viral genomes leading to attenuated virus with potential use as vaccines. A biased preference for certain synonymous codon-pairs exists in different species, and this has been exploited successfully for the development of several attenuated viruses through the recoding of ORF sequences with underrepresented codon-pairs, but leading to a identical aminoacid sequence of the encoded proteins (e.g. Coleman et al., 2008, Science 320:1784-1788). This synthetic attenuated virus engineering (SAVE) allows for the development of altered virus with similar epitopes and antigenic potential as the virulent parental virus, which furthermore does not easily revert due to the hundreds of nucleotide alterations in its sequence. The mechanism of attenuation is not fully understood, but has been attributed to the reduced translation efficiency of the altered genes. African swine fever virus (ASFV) is a large dsDNA virus causing a highly contagious disease of domestic swine and wild boar. Different approaches for obtaining an effective vaccine have been unsuccessful so far, but development of attenuated viruses with all the potential epitopes for immune response through this SAVE approach may open new possibilities.

Objective: Our aim has been to study the translatability of VP72, the major viral capsid protein of ASFV, after large scale alteration of its sequence with underrepresented codon-pairs in the viral genome, for further assessing the potential of SAVE in ASFV attenuation.

Methods: Sequences of all ORFs from the genome of a high virulence strain ASFV-L60 were concatenated and analyzed for the frequency with which each possible synonymous codon pair was found (codon pair score – CPS). A clearly biased CPS was detected for several codon-pairs and this information was used to re-design the approximately 2 kbp sequence of the VP72 ORF. New VP72 sequences were synthesized with over 200 nucleotide substitutions, either for underrepresented codon-pairs (deoptimized codon pair usage) or overrepresented ones (optimized codon pair usage) for comparison. For assessing the expression of the different ORF versions, these were cloned in expression plasmids in fusion with the reporter gene Renilla luciferase, at the 3' end of the VP72 ORFs. The fused constructs were placed under the control of the VP72 promoter. To support correct folding of the reporter protein after translation, a linker sequence was added between both ORFs, translating into four tandem units of one serine and four glycines. The native VP72 and a synthesized VP72 with the codon usage of swine were similarly cloned for comparison purposes. Additionally, the Renilla luciferase ORF was also cloned under direct control of the VP72 promoter, in order to compare its expression without fusion. The wild boar cell line WSL was transfected with the different reporter constructs and a plasmid expressing Firefly luciferase under control of MCMV promoter for normalization. After 5h transfection, cells were infected with ASFV and luciferase expression levels were recorded 22h post-infection.

Results: No significant differences were observed in the expression levels of the different fusion constructs, either containing the native, codon-pair deopti/optimized or swine codon usage VP72 modified sequences. Although Renilla luciferase expression directly under control of the VP72 promoter was approximately six times higher than in the fusion constructs, indicating significant interference of the VP72 sequence with the reporter expression, the levels of expression by the fusion constructs were always considerably higher than in mock transfected controls, suggesting that the lack of differences in expression is not due to insensitivity of this system.

Conclusion: Codon pair deoptimization of the VP72 sequence with regard to the viral ORFs codon pair usage bias did not lead to an evident decrease of the translatability of the protein in an in vitro expression system. However, in the viral genome context such modification may still have an impact for the viral replication. Further work is underway in order to substitute the native VP72 in the viral genome with this codon pair deoptimized version, and assessing its effect for viral attenuation.

Efficacy of foot-and-mouth disease vaccines A22 Iraq 64 and A Malaysia 97 against challenge with a recent South East Asian serotype A field strain in cattle and sheep

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Objective: Foot-and-mouth disease (FMD) is a contagious disease affecting economically important livestock species. There are 7 serotypes, and numerous subtypes within these serotypes with variation in distribution in different regions of the world. There is also significant variation in clinical presentation and vaccine responsiveness with different strains and in different species. As FMDV mutates rapidly, with the potential for antigenic variation, and a limited number of vaccine strains are available, it's important to ensure those vaccines will be efficacious against contemporary field strains. By testing two serotype A vaccines (A Malaysia 97 and A22 Iraq 64) against isolates from SEA from the past 4 years using *in vitro* vaccine matching assays, we observed a number of isolates that did not match, or only partially matched the vaccine strains. One of these viruses, A/VIT/15⁄2012, poorly matched both of the vaccine strains. We therefore tested whether vaccination with A Malaysia 97 or A22 Iraq 64 would protect cattle and sheep against challenge with this strain.

Methods: In total, 4 groups of 5 cattle were vaccinated with either A22 Iraq 64 or A Malaysia 97 vaccine either 21 or 7 days prior to challenge by intradermal lingual inoculation. Cattle were considered protected if there was no development of lesions on the feet (evidence of systemic spread). In total, 6 sheep were vaccinated with A22 Iraq 64, 4 days prior to challenge by direct contact with infected sheep. Eighteen non-vaccinated donor sheep (3 per pen) were infected by coronary band inoculation 4 hrs prior to contact with one non-vaccinated sheep and one vaccinated sheep. Sheep were considered protected if there was no development of lesions. Animals were monitored for lesions up to 35 days post infection and blood, nasal and oral swabs were collected and tested for virus by virus isolation and RT-qPCR.

Results: Both vaccines protected 100% of cattle, 21 dpv. The A22 Iraq 64 vaccine protected 80% (4 of 5) of cattle 7 dpv and the A Malaysia 97 vaccine protected 60% (3 of 5) of cattle 7 dpv. All cattle became infected and excreted virus in oral and nasal fluids and cattle in all challenged groups developed antibodies against non-structural protein, however overall viral loads were lower in the groups challenged 21 dpv. All 18 of the donor sheep developed clinical FMD, with severe lesions on the feet, gums and tongue. Five of six non-vaccinated in-contact sheep developed lesions. Only one of six vaccinated sheep had clinical FMD with a lesion on one foot. However all vaccinated as well as non-vaccinated sheep were FMDV positive in nasal and oral swabs and seroconverted to FMDV non- structural protein.

Conclusion: Both of the vaccines could be suitable for preventing clinical disease and most likely will reduce spread in an outbreak with A/VIT/15⁄2012 or related viruses particularly when the immune response is fully developed. While vaccination just 4 or 7 days prior to challenge prevented clinical disease it did not prevent infection as anti-non-structural protein antibodies were detected in all vaccinated cattle and sheep and all were shedding virus in nasal secretions and saliva.

Parallel EPIZONE Session 3: Diagnosis

Thursday 3rd September 2015
10:10-12:10
Auditorium Pasteur

Chair: Anette Bøtner & Antonio Lavazza

Applicability of faeces samples for detecting antibodies against African swinve fever

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Objective: The viral disease African swine fever (ASF) is a highly lethal haemorrhagic disease of domestic and wild pigs with no available vaccine or effective treatment. The introduction of ASF in a free country implicates immediate restriction measures with important associated economic losses and a great risk of spread to neighboring countries. The numerous outbreaks that have occurred recently in such geographically distant regions of East Europe demonstrate the capacity of ASF virus for continuous spread. Recently, wild boar population has been assigned an essential role in the spread of ASFV being responsible of the entrance of the diseases in European countries. Therefore, effective surveillance and monitoring programs on wild boar population are required. Among others methods, faeces samples have been recently reported as a basis for non- invasive sampling for antigen detection by polymerase chain reaction (PCR). Therefore, the objective of this work was to study the potential use of faeces samples to detect antibodies against ASFV as the first step in evaluating its possible use in surveillance and control programs.

Methods: Two groups of pigs were experimentally infected with an attenuated isolate of ASFV (Ken 05). Group 1, consisting of 6 pigs was inoculated with 10^1 50% haemadsorbing doses (HAD50) of the virus, while the group 2, consisting of 4 pigs was inoculated with 10^3 HAD_{50}. To analyze the presence of antibodies against ASFV in faeces samples, we established two indirect enzyme- immune-liked assays (ELISA) based on semi purified vp72 protein and purified vp30 recombinant protein expressed in mammalian cells.

Results: Antibodies against ASFV were detected in faecal samples of nine out of ten pigs in similar conditions than in serum samples using the two ELISA tests that showed a positive correlation using faeces and serum samples. In particular, five pigs showed antibodies in faeces and serum samples at the same day post infection (the earliest detection of antibodies was at 14 dpi in group 1 and at 9 dpi in group 2), while the rest four pigs showed antibodies in faeces only 1.5±1 days after when compared to serum. These results indicate that this kind of sample seems to be a promising alternative for testing serum samples.

Conclusion: These results confirmed the presence of ASFV antibodies in faecal samples from pigs with non-haemorrhagic diarrhoea, which offers a non-invasive alternative sampling particularly useful to screen for the presence of ASFV under field conditions.

Towards serological surveillance in the Belgian BVDV eradication programme: evaluation of commercial ELISA kits for the detection of antibodies against BVDV in serum and milk using a large collection of field samples

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Objective: Since January 2015, Belgium started a national eradication program against Bovine viral diarrhea or BVD. Mandatory testing of all newborn calves within seven days after birth and the quick removal of persistently infected (PI) animals are pivotal in the first years of the eradication program. In a later phase, herds in which all animals are certified "non-PI" may apply for a "BVDV free" herd status. Serological surveillance based on antibody ELISAs is a commonly used tool to ascertain freedom of disease. In a previous study (De Baere et al.) the intrinsic characteristics of all commercial BVDV antibody ELISAs were determined using a limited panel of samples. It was found that all blocking ELISAs in serum and both the blocking and indirect ELISAs in milk showed a lack of diagnostic sensitivity. A good sensitivity was only observed for the total antibody indirect ELISAs in serum. Based on these results, kits in each category were selected for further evaluation on a large number of field serum and milk samples. The goal of this study was to assess whether it is possible to certify and/or monitor "BVD free herds" by means of serology on either serum and/or (bulk) milk samples in the Belgian BVDV control program.

Methods:

Selection and characterization of the herds involved in the study and sample collection

Different herds, representative for the Belgian cattle sector regarding location, type of herds, herd size, BVD status and vaccination status were selected for sampling. 51 farms spread all over the country were visited twice. The average herd size was 168, but the smallest herd contained 13 and the largest herd 635 bovines. During this visit, blood samples (serum) were taken from all young stock (aged 6 to 18 months) as well as from 20 randomly selected cows, except in mixed herds where 20 dairy cows and 20 beef cows were sampled. In dairy herds, individual milk samples were taken from the same cows which had been sampled for blood, as well as a bulk milk sample from the milk tank. During the visit a questionnaire was also filled out together with the farmer, to collect epidemiological information regarding BVD in their herd. The purpose of this questionnaire was to assign a status to each herd regarding BVD and BVD vaccination, in order to correlate the serological results with the BVD herd status. Five different BVD herd statuses were attributed: (1) BVD free (all animals in the herd tested, all newborn calves tested since at least one year, no PI detected), (2) BVD eradicated (all animals tested, all newborn calves since at least one year, PI detected but removed from the herd since at least one year), (3) BVD controlled (part of the herd tested for BVDV, detected PI removed from the herd), (4) BVD infected less than one year (PI detected in the last 12 months and removed from the herd), (5) BVD infected (PI detected and present in the herd).

Sample testing and evaluation of the performances of each ELISA kit

Based on our previous study, 6 ELISA kits were selected. For serum, 1 anti-NS3 and 1 anti-E0 blocking ELISA and 2 total antibody indirect ELISAs were selected. For milk, 1 anti-NS3 blocking and 1 total antibody indirect ELISA was selected. All serum and milk samples were tested and analyzed according to the kit manufacturer's guidelines. When an ELISA result was interpreted as doubtful according to the manufacturer's instructions it was considered as a positive result. Then, a positive, negative or discordant serological status was assigned to each sample, depending on the outcome of all ELISAs. For serum, the virus neutralization test (VNT)(BVDV-1) was used as a gold standard to confirm the sample status when at least one ELISA gave a result different from the others. When the VNT did not confirm the status, or when two ELISAs were positive and two were negative, the sample was classified as "discordant".

Next, all sera from unvaccinated herds were tested in a VNT (BVDV-1) and VNT results were used as a reference. The VNT result of the corresponding serum sample was used as reference for all milk samples. Using 2 by 2 tables we calculated, for each selected ELISA kit, true and false positive and true and false negative results. When an ELISA result was interpreted as doubtful according to the manufacturer's instructions, it was considered a positive result. These results were imported in STATA (StatCorp LP, 2010, 10) to calculate the following parameters for each ELISA kit: sensitivity (Se), specificity (Sp), Youden index (= Se + Sp -1), positive predictive value, negative predictive value, Kappa of Cohen and area under ROC curve.

Results: In total, 3159 sera, 557 individual milk samples and 28 bulk milk samples were tested in 6 BVDV ELISA kits (4 kits for serum and 2 kits for milk) and were assigned a positive, negative or discordant status. For serum samples, the percentage of positive samples was slightly higher for the two indirect ELISAs than for the blocking ELISAs. This in contrast to the results obtained in a previous study using serum panels, where blocking ELISAs seemed to be much less sensitive than indirect ELISAs. For milk, the blocking ELISA performed better than the indirect ELISA, since the percentage of positively identified samples (73.2%) was similar to the percentage of the corresponding serum samples (75.9%), whereas the indirect ELISA seemed to be less sensitive (64.6%) if doubtful results are not considered, although the difference in proportion of positive results between the two kits was not statistically significant. Again, this does not confirm results from a previous study on serum panels, in which there was a better sensitivity for indirect ELISAs on milk samples compared to blocking ELISAs.

To assess the performance of each ELISA kit, only field samples from unvaccinating herds were used, to exclude interference of vaccination. In total, 1308 serum samples and 293 individual milk samples were selected, obtained from 19 herds. Three of the four evaluated ELISA kits on serum scored well for all parameters, whereas the anti-E0 blocking ELISA showed a lack of sensitivity (93%), compensated by an increased specificity (99.1%), possibly due to an inappropriate cut-off value. No lack of sensitivity of blocking ELISAs compared to indirect ELISAs was observed unlike results from a previous study. For milk, we found anew that the blocking ELISA performed slightly better than the indirect ELISA both in terms of sensitivity and specificity, again contradictory to previous findings.

As a last part of the evaluation, pooling of serum and milk samples was investigated in each of the selected ELISAs since bulk milk samples are convenient and cheap samples to obtain, and pooling of sera could reduce the costs of tests for the monitoring. Our study showed that pooling should not be performed with serum samples, since weak positive samples were missed by each of the evaluated kits, even when using small pool sizes (e.g. 5). Similarly, weak positive milk samples were missed by each of the evaluated kits, even using small pool sizes.

Conclusion: A thorough evaluation of selected commercial ELISA tests to detect antibodies against BVD was performed using field samples. To avoid possible interference of vaccination against BVD with the serological results, we restricted the evaluation to samples from non-vaccinating herds. We found that all selected kits performed well on individual serum samples, observing no difference between blocking or indirect ELISAs. Yet, when using milk samples, a decreased sensitivity was observed. Pooling of serum samples and the use of bulk milk samples showed a lack of sensitivity for the detection of positive samples even at small pool sizes. This observation, combined with the lower sensitivity of the ELISAs using milk as a matrix, indicates that it may be impossible to guarantee a "BVDV free" status of a herd based only on bulk milk testing. Of note, the observations in this study differ somehow from a previous evaluation using a limited serum and milk panel. This discrepancy is probably due to interference of vaccination, since only samples from unvaccinated herds were considered in the current study, whereas samples from vaccinated animals were included in the evaluation based on sample panels. Moreover, a subsequent study (paper in preparation) clearly shows the interference of vaccination when crossing the serological results obtained in this study with data from the epidemiological survey performed through a questionnaire in each farm where the samples were obtained. Importantly, the determination of the characteristics of BVD antibody ELISA kits, especially in terms of sensitivity and specificity, was a first step which was necessary to assess the possibility of implementing serological monitoring of BVDV free herds in the future. The results described above were combined with the analysis of the epidemiological survey and allowed CODA-CERVA to define specific and practical recommendations regarding serological surveillance to be implemented in the Belgian BVDV eradication program, which was launched in January 2015. These recommendations have already been presented to the stakeholders involved and, if approved, should become effective in a second phase of the program.

Cartridge-based real-time molecular diagnostic assays for the rapid and simple detection of African swine fever and foot-and-mouth disease virus

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Infectious animal diseases such as African swine fever (ASF) and foot-and-mouth disease (FMD) belong to the major threats impacting upon the productivity of farm animals. To limit the impact of outbreaks and to take rational steps towards a timely detection and eradication of these diseases, rapid and reliable diagnostic systems are of utmost importance. However, most detection systems are limited to the use in sophisticated laboratories and are thus lacking in many rural areas throughout the world, where human and technical resources are limited. Nowadays, advances in technology allow implementing modern and reliable techniques for quick and simple pathogen detection either in basic laboratories or even at pen-side level.

Such a diagnostic system is e.g. the cartridge-based ENIGMA Mini Lab (ML) system, which comprises an automated nucleic acid extraction with a subsequent real-time PCR and printout of the results. The system is easy-to-use and operators do not require special training. Furthermore the assays are based on freeze-dried reagents, so no cooling of the reagents is required and implementation in arid or tropical areas is feasible.

In this study, routine diagnostic (RT) qPCR assays for the detection of ASFV and FMDV were transferred to the cartridge-based automatized system. The assays were evaluated with EDTA-blood dilution series of three different ASFV genotypes and bovine, ovine and porcine samples including vesicles and saliva from three different FMDV serotypes. The results obtained from the ENIGMA ML were compared to results from manual nucleic acid extractions and results from the nucleic acid elutions produced by the ML. For both ASFV and FMDV, the results were in good concordance to the gold standard real-time (RT)-PCR results and the limit of detection was only one log less sensitive. However, even dilutions and samples with very low viral loads (cq-values ranging from 36-39) were detected as positive, and therefore also the detection of acutely infected animals is very likely.

Taken together, our results show, that the developed cartridge-based assays for the detection of ASFV and FMDV are reliable and useful molecular based diagnostic tools for the early and rapid disease detection e.g. in remote areas.

Keywords: ENIGMA ML, rapid diagnostics,real-time PCR, African swine fever virus, foot-and-mouth disease virus

PCR detectability and stability of Aujeszky's disease virus in porcine oral fluid

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Objective: Aujeskzy's disease (AD) is an economically important disease caused by the porcine alphaherpesvirus pseudorabies virus (PRV). Although the disease is eradicated in the domestic swine population in several European countries, the threat of a reintroduction remains since the virus is still circulating within the wild boar population. To maintain an AD negative status domestic pigs are routinely monitored, mostly by serological screening after blood sampling. Since oral fluid has recently been described as an alternative diagnostic sample that is easily collectable at pen-level and allows virological and serological monitoring of several porcine viruses, e.g. PRRSV and swine influenza virus (SIV), this study evaluated the potential usefulness of oral fluid for virological PRV detection.

Methods: To evaluate if oral fluid collected by cotton ropes at pen-level could be useful to detect an emergence of PRV by PCR, an in vivo infection experiment in BSL3 animal facilities was performed. Fourteen pigs were housed in three different pens. Pen A and B each contained 5 pigs of 15 weeks old that were intranasally inoculated with 10^5 $TCID_{50}$ of the virulent NIA3 strain. Pigs in pen A were naïve to PRV at the moment of infection while pigs in pen B had been vaccinated twice with a commercial PRV vaccine (Geskypur) at 5 and 2 weeks before intranasal infection. Pen C contained 4 24-weeks old pigs that were intranasally inoculated with 10^6 $TCID_{50}$

of the low virulent BEL24043 strain. Nasal swabs were collected from all individual pigs during the first 2 weeks post infection, while oral fluid samples were collected at pen-level with cotton ropes via the method described by Pricket *et al*., 2008. The presence of PRV DNA in swabs and oral fluid samples was tested via a real time PCR detecting a fragment of the gene encoding for glycoprotein B. Furthermore, the effect of conservation time and temperature on PRV detectability by PCR in oral fluid was evaluated. NIA3 virus stock was spiked under laboratory conditions in oral fluid collected from PRV negative pigs to a final concentration of either $10^{6.5}$ or $10^{3.5}$ $TCID_{50}$/ml. Spiked samples were then conserved either at 4°C or at room temperature (22 ± 2°C) and aliquots were collected at 0, 0.5, 2, 4, 8, 24, 48, 72 and 168 h post spiking and tested via real time PCR. Virus isolation on PK15 cells was also attempted for all collected aliquots.

Results: Both in pen A and B, first positive swabs were already detected at 1 and 2 dpi, while corresponding saliva samples remained negative. Starting from 3 dpi till 11 dpi, all pigs in both pens excreted the virus and also all corresponding oral fluid samples were found PRV positive. At 14 dpi, the surviving pig in pen A was negative in the swab, while it was still positive in its oral fluid. Also in pen B, PRV excretion decreased at that time point since only 3 out of 5 pigs were still low positive in swabs, but the corresponding oral fluid sample still tested positive. The low virulent character of strain BEL24043 was confirmed since all pigs in pen C excreted the virus only for a short time (till 6dpi) and at low quantities as detected in swabs. Interestingly, only the oral fluid sample collected at 3 dpi from pen C was found positive. These results indicate that oral fluid can be used for the virological detection of a PRV outbreak, but the sensitivity is lower compared to swabbing of individual animals during the early phase of infection and might depend on the virulence of the strain.

Since the results described above indicate the potential of using oral fluid for virological PRV detection, we subsequently evaluated the effect of conservation time and temperature on virus detectability by PCR. Our experiments showed that for both PRV concentrations tested, no significant loss in PCR detectability occurred over a 7 days conservation period in saliva when samples were stored at 4°C. When the virus was conserved at room temperature, a moderate 10 to 20 fold decrease (Ct from 24.3 to 27.9 for $10^{6.5}$ $TCID_{50}$/ml and Ct from 34.1 to 38.2 for $10^{3.5}$ $TCID_{50}$/ml) in detectable PRV DNA was observed over the 7 day period for both concentrations. PRV however remained detectable till the end of the experiment. Interestingly, not only viral DNA remained intact but also virus infectivity remained largely unaltered when conserved in saliva at 4°C since PRV could be isolated on PK15 cells till 7 days post spiking at the highest concentration. In contrast, conservation at room temperature or spiking at the lower concentration strongly reduced the isolation efficiency. These results indicate that PRV genetic material (DNA) is more stable in oral fluid than that of other porcine (RNA) viruses like PRRSV and SIV. Nevertheless, it seems advisable to follow the guidelines formulated for diagnosis of these latter viruses in oral fluid and cool the oral fluid samples to 4°C as soon as possible after collection and send them refrigerated to the lab.

Conclusion: This study indicates the usefulness of oral fluids for virological PRV detection and underlines the stability of PRV DNA in this diagnostic sample. Current monitoring however mostly relies on antibody detection in serum. We are therefore in the process of evaluating the suitability of oral fluid collected at pen-level for anti-PRV antibody detection.

Approaches to DIVA assays for West-Nile virus

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Objective: West Nile virus infection is identified by several diagnostic tools, being the most commonly ones focused on the identification of the agent (RT-PCR), the virus neutralization test or the detection of IgM and IgG against structural proteins of the virus. Over the last decade, several outbreaks caused by West Nile virus have been detected in different parts of Europe, which increased the vaccination of horses in many countries. The existing diagnostic methods based on antibody detection cannot differentiate infected from vaccinated animals. The main goal of this work is to deal with the problem in differentiating WNV vaccinated and infected animals when both events may occur in a horse population at the same time. Based on the difference of antibody respon-

se between infected and vaccinated horses against the major structural protein (E protein) and the non structural protein (NS1 protein), a DIVA (differentiating infected from vaccinated animals) assay has been designed.

Methods: A panel of Monoclonal antibodies (Mabs) against E and NS1 proteins was first obtained and two of them (one specific for E protein and one for NS1 protein) were selected to coat ELISA plates. After that, the inactivated whole culture virus was added and each specific protein was captured by the specific Mab. Finally, the plates were washed to remove the unbound proteins and blocked. Horse samples were analysed in an indirect ELISA DAS format (IDAS ELISA) using a mouse monoclonal antibody anti-horse IgG-HRPO as detector. Different groups of animals were used for this for study: vaccinated and infected animals from controlled experiments bled at different days post infection/vaccination, vaccinated and/or infected field animals (with clinical symptoms compatible with WNV and tested as positive to WNV antibodies using commercial assays) and samples of horses tested as negative using the same commercial assays.

Results: Firstly, to set up the assay the sera from the experimentally infected and vaccinated animals collected from 4 upon to 100 days post-infection/vaccination were analyzed. These experimental samples reveal a positive result in the ELISA based on E protein with the same high OD values. Nevertheless, the antibody response against NS1 in vaccinated animals showed decreased OD values comparing with those obtained with infected animals. The ratio OD with E protein/ OD with NS1 protein was higher than 4 in vaccinated animals whereas this ratio, in infected animals, was lower than this value. Similar results were obtained using the field samples from infected and vaccinated horses. Additionally a group of 90 negative samples showed a very low signal in both assays reporting negative results.

Conclusion: We have observed a different antibody response to the structural E protein and to the non-structural NS1 protein in infected and vaccinated horses. By using an IDAS-ELISA we could differentiate vaccinated from infected horses by the ratio OD obtained with E and NS1 protein as antigen. The design of this assay carried out during this study could help to the development of a DIVA assay. Further experiments are needed to adjust the ELISA conditions with a bigger panel of infected sera to check the utility of the assay in field.

Optimized antigen expression and presentation for diagnostic purposes – a new potential for Equine Herpesvirus 1 as a vector

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Objective: Equine herpesvirus type 1 (EHV-1) has been previously used as a viral vector for vaccination. For the development of diagnostic tools, expression of proteins or protein complexes in a heterologous, but related viral vector, could be of great advantage to reduce serological cross reactivity. Bovine herpesvirus type 1 (BoHV-1) is able to induce respiratory and genital tract disease in cattle. Glycoprotein E (gE)-deleted marker vaccines are important tools for BoHV-1 eradication and control in many European countries. The accompanied serology to verify infection with a BoHV-1 field virus is based on the detection of gE-specific antibodies. gE, together with gI, forms a functional complex that facilitates direct cell-to-cell spread and is an important virulence determinant acting on neuronal spread *in vivo.*

Methods: In order to assess the potential of EHV-1 as a heterologous, but related antigen vector, an EHV-1-recombinant expressing BoHV-1 gE and gI (gE/gI) was constructed. A porcine teschovirus-1 2A peptide (P2A) sequence was inserted between BoHV-1 gE and gI to ensure expression of both proteins at equimolar levels. Using a two-step *"en passant*" mutagenesis, a synthetic *BoHV-1 gE-P2A- gI* cassette was inserted into an infectious bacterial artificial chromosome (BAC) construct of the EHV-1 clone, RacH. An additional EHV-1 gE/ gI-deletion mutant expressing BoHV-1 gE/ gI was also constructed. Expression of both BoHV-1 gE and gE/gI complex in infected cells was analyzed by Western Blot, immunofluorescence microscopy and ELISA. The incorporation of BoHV-1 gE/gI into *de novo* assembled particles was analyzed by EHV-1 gB and BoHV-1 gE/gI antibody staining. Relative accumulation levels of these proteins in particles were calculated after determining the total amount

of the respective protein in each lysate and its corresponding supernatant by using AIDA software (Raytest).

Results: A recombinant EHV-1 gE/gI-deletion mutant (EHV-1ΔgE/gI), an EHV-1ΔgE/gI expressing BoHV-1 gE and gI (EHV-1ΔgE/gI+BoHV-1 gE/gI), and an EHV-1 expressing BoHV-1 gE and gI (EHV-1+BoHV-1 gE/gI) could be recovered and propagated. While wild type virus (RacH-wt), EHV-1 ΔgE/gI+BoHV-1 gE/gI and EHV-1+BoHV-1 gE/gI grew to comparable titers, the titers of infectious EHV-1ΔgE/gI in the supernatant of infected cells showed a 0.5 – 1.0-log reduction over time. Additionally, the plaque size was significantly reduced by 75% only in EHV-1ΔgE/gI-infected cells. Analyzing the release of viral particles through detection of EHV-1 gB by Western Blot showed an impairment of 40% in case of EHV-1ΔgE/gI and EHV-1ΔgE/gI+BoHV-1 gE/gI when compared to RacH-wt and EHV-1+BoHV-1 gE/gI. Furthermore, the incorporation of BoHV-1 gE/gI into *de novo* assembled virions was 4 times lower for EHV-1ΔgE/gI+BoHV-1 gE/gI than for EHV-1+BoHV-1 gE/gI. To assess the potential of EHV-1+BoHV-1gE/gI as an antigen vector for ELISA diagnostics, microtiter ELISA plates were coated with the respective lysates or virus particles purified from the supernatant of infected cells. BoHV-1 gE/gI complex-specific antibodies showed high optical density (OD) ELISA values when the plates were coated with EHV-1ΔgE/gI+BoHV-1 gE/gI- and EHV-1+BoHV-1 gE/gI-infected cell lysates. Whereas only purified EHV-1+BoHV-1 gE/gI virus particles, but not EHV-1ΔgE/gI+BoHV-1 gE/gI, were positive for the presence of BoHV-1 gE/gI.

Conclusion: The insertion of a BoHV-1 gE-p2A-gI cassette into EHV-1 (RacH)-BAC clone resulted in infectious recombinant viruses and high expression levels of BoHV-1 gE and gI in infected cells. BoHV-1 gE/gI has a potential to substitute EHV-1 gE/gI-functions in terms of cell-to-cell spread (plaque formation) but it seems not to be incorporated into the *de novo* assembled virus particles. EHV-1ΔgE/gI+BoHV-1 gE/gI and EHV-1+BoHV-1 gE/gI-infected cell lysates have shown a promising potential as antigen delivery systems for BoHV-1 gE ELISA diagnostics. To our knowledge, this is the first time that EHV-1 was used as an antigen vector for antibody ELISA. The presented EHV-1 expression system has the advantage of being a highly efficient system that guarantees correct processing, folding and complex formation of different BoHV-1 proteins with the complete absences of other BoHV-1 encoded proteins, which are suspected to cause unspecific ELISA reactions in multi- vaccinated cattle. Further analyses will address whether EHV-1 expressing BoHV-1 gE/gI is a suitable antigen source for a BoHV-1 gE-blocking ELISA.

Tracking of African swine fever outbreaks through variation of intergenic I73R/I329L region.

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African swine fever is a highly contagious viral disease of pigs and wild boars. The disease is characterized by fever, hemorrhagic lesions and inflammatory in different organs and can run either an acute lethal, subacute or a chronic course (Penrith M.-L. *et al*., 2009). ASF is caused by a large, double-stranded DNA virus that was only allocated to its own genus, *Asfivirus*, the only member of the family *Asfarviridae* (Dixon *et al.* 2005). Currently there are XXII genotypes of ASF virus, that was defined by phylogenetic analysis of partial sequence of B46L gene, that encodes major capsid protein P72 (Bastos A.D. *et al.,* 2007).Based on the standard ASFV genotyping protocol (C-terminus of the p72 gene, p54 gene and CVR within the B602L gene) all strains from the Caucasus region, the Russian Federation and EU were identical (Gallardo *et al.*, 2014; Malogolovkin *et al*., 2012) and belong to genotype II. Genetic variations among isolates of ASF virus, circulating in the Russian Federation and the European Union first described by (Gallardo et a l, 2014). Also known, that intergenic site I73R/I329L is genetic marker, that allows differentiate closely related ASFV virus isolates. The intergenic region between I73R/I329L was further investigated by Goller et al., 2015 where they have shown the suitability of this region for deeper phylogenetic analyses of ASFV isolates.

Objectives: The aim of this study was to evaluate the relevance of TRS genetic marker as a complementary tool for molecular epidemiology of ASFV. The ultimate goal was to reveal the distribution of ASFV variants based on TRS features.

Methods: In order to determine genetic variation between ASFV isolates from different regions, we used PCR for intergenic region I329L/I73R previously designed by (Gallardo *et al,* 2014) with following alignment with ClustalW algorithm. Spatial and temporal patterns were evaluated using a map generated with ArcMap package implemented in ArcGIS software 10.1 (Esri CIS Limited, USA).

Results: The ASFV isolates from 18 regions across the Russian Federation originating from 2012-2015 were used for nucleotide sequencing. Total 70 ASFV isolates were sequenced, analyzed and mapped according to outbreaks geographical location. The results show the presence of two genetic variants formed by ASF virus isolates based on insertion/deletion in TRS intergenic region. We have notified that the relative number of ASFV isolates with additional insertion of TRS was higher from 2012 onwards than parental (identical to ASFV Georgia 2007/wb). We could not find any significant differences among ASFV TRS variants isolated from domestic pigs and wild boar. This finding can preliminary suggests that there is no specific restriction for TRS changes between domestic and wild boar population.

In terms of geographical location the most of ASFV variant with additional TRS insertion were identified in Central and Southern part of the Russian Federation. Interesting that the same ASFV variant was identified in Ukraine/2012, Belarus/2013 and in all EU ASFV isolates.

The most of “older” ASFV isolates which are genetically identical to Georgia/2007/wb were located in Central and North-Western part of the country. In the central part of the Russian Federation two variants of ASFV are present. This region is a place of active social, transport and economical activity that leads to potential increasing of ASFV introduction from different sources.

However even in this kind of complex region where the main source of ASF virus introduction and transmission remains unrevealed the genetic analysis of intergenic regions I73R/I329L allows to trace ASF outbreaks from primary introduction to secondary outbreaks. We found that even in neighboring regions where ASF outbreaks are detected the intergenic region I73R/I329L in ASFV genome is different. Even more that in case of wild boar of natural or artificial obstacles these two ASFV variants can evolve independently.

Conclusion: These facts suggest about possibility of determination the origin of first introduction of the ASF virus in certain region and might help differentiation of initial and secondary outbreaks of the disease by analysis of intergenic region I73R/I329L. These data can be useful for further ASFV molecular epidemiology studies.

Serology and molecular diagnostics of epizootic hemorrhagic disease virus (EHDV)

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Objective: Epizootic hemorrhagic disease virus (EHDV) is a member of the genus *Orbivirus* in the family *Reoviridae*, and is closely related to Bluetongue virus (BTV). EHDV causes disease in cattle and wild cervids, and has been associated with BTV-like disease in cattle. The virus has important implication for the international livestock trade and has been isolated from cattle throughout the world (North America, Morocco, Japan, Australia, Israel, and Turkey). Differentiation of BTV and EHDV is necessary because diagnosis of infection caused by these viruses is often confused. We have developed two complementary diagnostic tests for EHDV detection: a real-time (rt) RT-PCR and an ELISA. This paper reports (i) the validation of the PCR kit with different EHDV strains (ii) and the development of a blocking ELISA for serodiagnosis.

Methods and Results: The LSIVetMAX EHDV kit is a duplex rtRT-PCR assay using (i) a set of primers and probe labeled with FAM targeting a sequence of the VP6 gene for EHDV detection, and (ii) a set of primers and probe labeled with VIC targeting the Beta-Actin gene (Internal Positive Control). As an External Positive Control (EPC), a plasmid obtained by cloning was used. RNAs isolated from 9 different EHDV genotypes and different BTV strains (from different locations world-wide) were used to evaluate the specificity and the sensitivity. The limit of detection of the EHDV rtRT-PCR was determined by testing a quantified plasmid. All the EHDV strains were detected by the EHDV rtRT-PCR assay. No cross reaction was identified between the EHDV assay and different

specific genotypes of BTV. The detection limit of each PCR is 3 copies of plasmid per PCR.

The LSIVet™ Ruminant EHDV - Serum ELISA uses the VP7 protein to detect EHDV specific antibodies in serum samples. Due to the antigenic similarity of BTV and EHDV, the sensitivity and specificity of the EHDV assay was evaluated and compared to BTV samples. The sensitivity of the kit was shown to be 100% based on a large panel of EHDV positive sera including the 9 existing serotypes. The specificity was found to be >99%. No cross reactivity with BTV samples was observed. The design of the kit was optimized to match with the most stringent regulations, including a certified serum in the positive control and the conjugate diluents. It shows a high robustness and stability and its fast protocol enables to have results in 55 minutes.

Conclusion: In summary, a combination of ELISA and PCR diagnostic test kits for EHDV should allow improved testing scenarios for epidemiologic purposes.

Parallel EPIZONE Session 4: Epidemiology, surveillance & risk assessment 2

Thursday 3rd September 2015
10:10-12:10
Room Sully 2

Chair: Beatriz Martínez-López & Claude Saegerman

MINTRISK, a Method for INTegrated RISK assessment of vector-borne livestock infections

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Increasing occurrence of vector-borne livestock infections (VBADs) in Europe bring about the need for tools to evaluate and compare the risk of exotic VBAD. Although several methods exist for risk assessment, they do not take into account the specific needs of VBADs. Therefore, we developed a tool for risk assessment of VBADs allowing for comparison of the risk for different diseases. The tool can integrate various aspects of risk but still distinguish uncertainty, probability and impact.

An integral calculation method was developed in Excel and Visual Basic to assess the risk of VBADs based on the structure provided by FEVER, a recently developed Framework to assess Emerging VEctor-borne disease Risks for livestock. Knowledge and information about VBADs is summarized using an extensive questionnaire. An underlying quantitative model combines the answers to the questions, while taking account of the indicated uncertainty for each answer using Monte Carlo simulation. Results are presented for the overall risk, but also separately for the different steps in the model. Furthermore, the model indicates which answers and uncertainties contributed most to the risk. The method was developed and tested on several infections. Adjustments were made, resulting in an effective tool for rapid assessment and comparison of disease risks. By separating probabilities, impact and uncertainty in a summarizing graph of the result for each infection, insight in the relevant risk aspects is gained and visualised. The evaluation for the most influential answers and the main sources of uncertainty can guide towards effective control and influential data gaps to be resolved. MINTRISK is a useful tool to prioritise in VBAD management. Results of MINTRISK can be used to support management decisions and prioritise resource allocations for research or disease risk preparedness.

Recent incursions of vector-borne pathogens in Europe such as Schmallenberg virus demonstrate the importance of knowledge of and preparedness for VBADs. MINTRISK has already been used to deal with recent requests from both national and international policy makers. 7 vector-borne diseases were evaluated for the risk they pose for the Netherlands and over 40 infections will be evaluated for the risk they pose for the EU.

Assessment of African swine fever diagnostic tehniques as a response to the epidemic outbreaks in eastern European Union countries: how to improve surveillance and control programmes

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Objective: African swine fever (ASF) is a complex and lethal viral disease affecting swine that has a significant socio-economic impact on both the developed and developing world. The first cases of ASF in wild boar in Lithuania and Poland were reported in early 2014 in areas bordering on Belarus (Gallardo *et al.*, 2014). According to the OIE, during 2014 nearly 260 ASF cases or outbreaks in wild boar and domestic pigs were detected in EU countries (Latvia, Lithuania, Estonia and Poland). This situation has created a need to review the sensitivity and specificity of current diagnostic tests and their ability to diagnose ASF in both domestic and wild Suidae in affected areas. To this end, the EURL has performed in collaboration with the National Reference Laboratories (NRLs) of the four affected EU countries a comparative study of the current ASF diagnostic tests used at EU level throughout the analysis of experimental and field samples collected from both domestic and wild pigs. This study describes the ability of the diagnostic techniques in use to provide reliable information derived from the different tests employed during the epidemic.

Methods: A panel of 314 field samples collected during the 2014 outbreaks occurred in the East EU countries were tested. The samples represented a total of 122 animals, 91 wild boar and 34 domestic pigs. In addition were included 150 paired serum and EDTA-blood and 450 tissue samples collected from three experimental infections with virulent P72 genotype II ASFV isolates.

For ASFV genome detection 295 collected-field samples and 600 experimental samples were tested using the OIE conventional and real time PCRs (OIE 2102) and the UPL real-time PCR (Fernandez- Pinero *et al*., 2013). In addition, 272 samples (92 field and 180 experimental) were tested for ASF antigen detection using the commercially antigen detection ELISA (®INGENASA-INGEZIM PPA DAS K2, INGENASA). Virus isolation was assessed in PCR-positive samples using a haemadsorption assay into porcine blood monocytes (PBM) (OIE 2012).

For ASF antibody detection 150 experimental and 21 field serum samples were tested using four different ELISAs and the confirmatory immunoperoxidase test (IPT). The ELISA assays comprised the OIE indirect ELISA (OIE 2012) and the three current commercial screening ELISA tests from INGENASA (®INGENASA-INGEZIM PPA COMPAC K3), IDVET-ELISA, (ID Screen® ASF Indirect) and SVANOVA (SVANOVIR® ASFV-Ab). The performance in the detection of ASF antibodies in tissue exudate was assessed by the analysis of 90 experimental exudates. 210 negative tissue exudates from 70 non-ASF-infected animals were included in the study. Additionally, the experimental samples, 140 field-tissue exudates, 26 blood and one fluid samples taken during the outbreaks within the EU were tested.

Results and discussion: To detect the ASF virus, three different PCR assays were evaluated in parallel using 785 field and experimental blood, serum, fluid and tissue samples. In the data obtained, 3.3% more samples were shown to be ASFV positive with the UPL-PCR than with the OIE conventional PCR which failed to give positive results for samples with Ct>30, especially those collected during early stages of the disease. This lower sensitivity could be due to the presence of one nucleotide mismatch close to the 3' end in the forward primer, identified in the target sequence of ASFV genotype II isolates that include Georgia 2007, Krasnodar 2012, Lithuania 2014 and Poland 2014.

The results showed almost perfect agreement between the UPL-PCR and the OIE real-time (κ= 0.94 [95% CI, 0.91-0.97]). The OIE-prescribed assay failed to detect the asymptomatic pig experimentally exposed to Lithuanian ASFV (Gallardo *et al.,* 2015) and three field-derived samples from hunted wild boar in Lithuania and Latvia. Although no virus could be recovered from these samples, the presence of antibodies indicates ASFV exposure in these wild boars confirming the specificity of the results. In agreement with previous work, the UPL-PCR has superior diagnostic sensitivity in the detection of carriers, as well as its ability to rapidly detect the dis-

ease even when the typical clinical signs are as yet not evident (Fernández-Pinero *et al.*, 2013).

The sensitivity of the antigen ELISA was poor (77.2%) compared to the UPL-PCR, above all in the case of field-derived samples, even when there was a high virus load. Field samples in poor conditions can decrease the effective sensitivity of the test. The attempt to isolate infectious virus from each UPL- PCR positive animal gave irregular results in experimental and field samples. Although the virus was easily isolated in experimental samples when Ct < 36 ± 3.5, variable results were obtained when field-derived samples were subjected to virus isolation even despite the higher values of viral DNA detected. This could be related to the poor state of samples, which will affect the viability of the virus, above all if we bear in mind the high percentage of material received from hunted or dead wild boar.

The examination of experimental serum samples indicated greater sensitivity for the IPT than for the ELISA assays. The IPT was able to detect ASF antibodies at an earlier stage of serological response than the ELISA. The same result was obtained from the analysis of field-derived samples despite the limited number of tested sera. The sensitivity of the ELISA tests was significantly lower than the IPT as a reference test, and ranged from 22.22% (OIE-ELISA) to 50% seropositive pigs detected (INGENASA-ELISA). IDVET- and SVANOVA- ELISAs were not statistically different from each other and was both superior to the OIE- ELISA. This data suggests that seroprevalence rates may be underestimated when the ELISA assay is employed for surveillance in areas where virulent strains inducing acute infections are circulating, as is the case in certain EU countries.

The search for antibodies from hunted or dead animals is essential for obtaining a complete picture of the epidemiology, and for determining the date of the infection, that is, when infected animals were exposed to the ASFV (regardless of whether antibodies were present or not). The performance of the IPT and the four ELISA tests was evaluated to detect specific ASFV antibodies in tissue exudates. Based on the analysis of experimental samples the IPT revealed to be the most sensitive and specificity when compared with the ELISA tests. Therefore, field samples from wild boar and domestic pigs were tested using IPT. The presence of antibodies was confirmed in 60.37% of the wild boar and in 46.8% of the domestic pigs. Moreover, the IPT antibody titres stressed that wild boar generally had higher levels of antibody titres than domestic pigs. Animals with the highest antibody titres had been previously diagnosed at the limit of the detection with the UPL-PCR test but resulted negative when tested with the OIE-prescribed virological assays. These data suggest that, despite the fact that the ASFV isolates affecting EU countries correspond to a virulent strain that leads to high mortality in affected wild and domestic pigs, some animals can survive for over a month and are able to recover from the infection or even remain sub-clinically infected (Mur et al., 2014; Gallardo *et al*., 2015).

Conclusion: The data presented here shows that the UPL-PCR in combination with the IPT are the most trustworthy methods for the early detection of ASF in affected EU countries. These techniques are accurate and specific for reliable diagnosis of ASF. OIE real-time PCR tests can be also used with great confidence since this technique shows almost perfect agreement with the UPL-PCR. Antibody detection techniques are needed in order to get complete information that will assist control and eradication programs. In this context, the IPT is the best test given its performance with tissue exudate samples, which is particularly relevant for wild boar surveillance and control programs. This study illustrates the capability of ASF diagnostic techniques to provide reliable information generated by different types of tests.

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Prevalence of some potentially zoonotic pathogens in the Dutch equine population

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Objective: the Dutch equine population is estimated around 500,000 horses and most of these horses are used for recreation and sports. The contact between horses and humans is often intensive and therefore the ministry of Economic Affairs was interested to learn more about the prevalence of certain potentially zoonotic pathogens in the equine population, risk factors for these pathogens and their putative attribution to the disease burden in the human population.

Methods: the five pathogens investigated in this study were Salmonella spp., *Cryptosporidium* spp., Giardia *duodenalis*, *Clostridium* difficile, and *Rhodococcus equi*. Faecal samples were collected from four subpopulations: foals with diarrhoea, healthy foals, adult horses with diarrhoea, healthy adult horses. In total 24 (2 per province) equine veterinary practices were recruited, and each practice was asked to deliver 7 faecal samples for each subpopulation. Samples were collected from different sectors: riding schools, boarding stables, livery yards, studs, animal farms. Sampling kits and instructions were provided and all samples were submitted to the Animal Health Service in the Netherlands. The prevalence of *Salmonella* spp., *Clostridium difficile,* and *Rhodococcus equi* was investigated by selective culture methods in combination with typing/confirmation methods such as Maldi-TOF and PCR. The prevalence of *Cryptosporidium* spp. and *Giardia duodenalis* was investigated with pen-side tests based on lateral flow immunochromatography in combination with PCR based confirmation tests. CVI Lelystad carried out the *Clostridium difficile* culture and confirmation in collaboration with the Leiden University Medical Centre (LUMC). The submission forms were combined with short questionnaires.

Results:

	Samples (n)	*Positive (n)*	*Prevalence*	*95% confidence interval*
Salmonella spp.	193	7	3,6%	1,5-7,3%
Cryptosporidium spp. (both tests positive)	193	33	17,9%	13,6-23,3%*
Cryptosporidium spp. (1 of 2 tests positive)	193	66	34,0%	27,6-41,2%*
Giardia duodenalis	193	2	1,0%	0-3,7%
Clostridium difficile	77**	26	32,1%	22,2-43,4%
Rhodococcus equi (VapA positive)	52***	21	40,4%	27,0-54,9%

* Corrected for herd effects
**: Only samples of horses and foals with diarrhoea were investigated
***: Only samples of healthy foals were investigated.

In faecal samples positive for *Salmonella* spp. *Salmonella typhimurium* and *Salmonella enteritidis* were detected. In one sample we detected a monophasic *Salmonella typhimurium* strain that appeared to be resistant for most of the antibiotics investigated. The low prevalence did not allow for risk factor analysis.

For *Cryptosporidium* spp. a high prevalence was found. Even if samples were only considered positive when both lateral flow tests showed a positive result a prevalence of 17.9%. None of these positive samples were confirmed by real-time PCRs for *Cryptosporidium parvum* or *Cryptosporidium hominis*. In one sample *Cryptosporidium cuniculi* was detected. In the risk factor analysis there appeared to be a protective effect of feeding concentrates (odds ratio 0.22; 95% CI: 0.07-0.69) and horses and foals with diarrhea > 7 days were more likely to be test positive than healthy horses (odds ratio 2.4; 95% CI: 1.1-5.3).
The prevalence of *Giardia duodenalis* appeared to be very low, and did not allow for risk factor analysis.

The prevalence of *Clostridium difficile* was 32.1%, the prevalence of toxinogenic *Clostridium difficile* spp. was about 10%. For the latter category ribotype 078 was predominant. This ribotype is also of zoonotic importance with increasing prevalence and pigs have been implicated before as a possible zoonotic risk. Horses and foals that were stabled or were fed concentrates in addition to grazing had a lower risk of testing positive than horses and foals with an "only grass"diet.

The prevalence of *Rhodococcus equi* in faecal samples from healthy foals was 60%. In 40% of all samples the *VapA* gene was detected by real-time PCR (3-5 colonies per plate were tested). For all *VapA* positive *R. equi* isolates MIC values were determined against a panel of relevant antibiotics, and all strains appeared to be sensitive for the commonly used antibiotics rifampicin, erythromycin, clarithromycin and azithromycin. Older foals (6-10 weeks of age) were slightly more likely to test positive than younger foals (odds ratio 1.3; CI: 1.1-2.2).

Conclusion: the apparent prevalence of Giardia duodenalis and *Salmonella* spp. is very low to low in the Dutch equine population. Additional research is not considered necessary, although for *Salmonella* spp. a higher prevalence can be expected when 3 or more sequential samples per horse would have been collected. Although the most relevant *Cryptosporidium* spp. from a zoonotic perspective were not detected in this study additional research is recommended to determine the *Cryptosporidium* spp. in the equine population. The detection of several *Clostridium difficile* strains with ribotype 078 warrants additional research to determine the zoonotic relevance of these findings. MLVA typing and additional samplings are recommended. The high prevalence of *VapA* positive *R. equi* implies that many healthy foals are shedding this micro-organism and that also the environment of many horse yards will be contaminated with this micro-organism. The zoonotic implications seem only relevant for immunodeficient persons, and it is recommended that this category should take proper precautions when handling foals and horses or might even better avoid contact.

Spatio-temporal network analysis of pig movements in Great Britain: implications for disease transmission and control strategies

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Objectives: Animal movements represent an important means for disease transmission among animal holdings over large geographical distances. Therefore, understanding the dynamic patterns of animal movement networks is needed to inform models of disease spread and target control and risk-based surveillance strategies. Here, we conduct a spatio-temporal analysis to explore pig movement networks throughout Great Britain (GB) over a 5-year period (2009-2013) with a view to identify spatial and temporal patterns, characterise the monthly network topology and identify trade communities.

Methods: Descriptive statistics were initially generated including all premise types. A directed and weighted monthly network was then built, considering each premise (only farms, gathering areas and market premises) as a node and each daily pig movement between two premises as an edge.

Results: Over the 5-year study period, the pig movements included 48,976 active premises and 888,613 movements, involving 64,121,604 pigs. Most of the movements originated from farms (96.0%) and gathering areas (3.8%) and were directed to slaughter houses (68.3%), farms (23.7%) and gathering areas (7.8%). The distance covered by 50%, 75% and 95% of the pig movements was 31km, 65km and 175km, respectively. A seasonal pattern was observed, with increased trade movements occurring in autumn and spring. East Anglia, North West, South East and South West England and Yorkshire and Humberside represented the major sources and receivers in term of number of movements and pigs. The monthly network exhibited both scale-free and small-world properties. The 10 largest trade communities including 18% of premises were identified and associated with specific regions, providing a basis for defining zoning areas in the context of control of endemic and epidemic disease spread.

Conclusion: This study demonstrates how the spatio-temporal and functional organisation of pig trade in GB can be investigated to reveal hot spots in time and space for disease spread. This information can be used to parameterise epidemic models and also to directly inform the design of targeted disease surveillance and control strategies.

Risk of transmission of Coxiella burnetii during the caprine in-vitro produced embryo transfer

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Objective: The aim of this study is to analyze the risk of transmission of *Coxiella burnetii*, during the caprine *in-vitro* produced embryo transfer. After the last Nederland epidemic episode of transmission of *Coxiella burnetii* to humans, this disease is a major zoonotic disease in Europe and all the fighting means against this infection must be improved.

The 3 objectives of this study are:

To evaluate the ability of *Coxiella burnetii*, to adhere to the intact Zona Pellucida of in-vitro produced caprine embryos, infected in-vitro, by Coxiella burnetii.
To test the potency of IETS recommended rules for the washing of bovine embryos to eliminate *Coxiella burnetii.*
To see and localize *Coxiella burnetii*, on *in-vitro* produced caprine embryos, after the ten washings recommended by IETS.

Methods: 90 caprine embryos are produced by in-vitro fecundation and distributed in separated eleven groups. 9 groups are contaminated by *Coxiella burnetii*, Cb C1, phase 1, produced by ovoculture, at a concentration of 10^9 bacteria/ml. 2 groups of embryos are used as non contaminated control probes. *Coxiella burnetii* DNA is detected by C-PCR, previously published technique, with a target sequence in the *IS 1111* gene. *Coxiella burnetii* DNA is quantified by Q-PCR, previously published technique, with a target sequence of 76 pb DNA fragment in the *icd* gene. The detection of *Coxiella burnetii* is made by using Immunofluorescence labeling and analyzed by confocal microscopy.

Results: *Coxiella burnetii* DNA is detected in all the 9 contaminated caprine embryos groups, after ten successive washings. No *Coxiella burnetii* DNA is detected in the two control groups of the non contaminated probe caprine embryos. After ten successive washings, the bacterial charge is variable but under 10^4 bacteria/ml. The detection of *Coxiella burnetii*, in all the caprine embryos contaminated, demonstrate the capacity of the bacteria to adhere or to penetrate the Zona Pellucida.

The ten washings recommended by the IETS rules for bovine embryos are not able to eliminate the bacteria in the in-vitro produced caprine embryos. The observation, by confocal microscopy, of the contaminated caprine embryos demonstrates that *Coxiella burnetii* is present in the external part of the Zona Pellucida, without profound penetration.

Conclusion: This study demonstrate that *Coxiella burnetii* adsorb strongly to the Zona Pellucida of *in-vitro* produced caprine embryos after in-vitro contamination and that the ten washings protocol recommended by IETS, to eliminate the pathogenic agents of bovine embryos, is not able to eliminate these bacteria. The presence of these bacteria is observed on the surface of the Zona Pellucida, with different bacterial charges, from one embryo to another. This difference of bacterial charge may be due to differences of the ultrastructure of the Zona Pellucida, that should be further studied by electron microscopy.

First Isolation of Canine Parvovirus in Morocco

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Canine parvovirus (CPV-2) is an acute infectious and highly contagious virus which has evolved since its emergence in the mid-1970s, giving rise to new antigenic variants (CPV-2a, CPV-2b, CPV-2c) with increased pathogenicity and extended host ranges. CPV-2(s) are considered to be the most dreaded enteric pathogen of canine population worldwide, in spite of widespread vaccination of domestic dogs.

Although clinically suspected in Morocco since 1984, first evidence of CPV-2(s) circulation was confirmed in 2011 (Drif, 2011), even in dogs "regularly" vaccinated. Further studies typed autochthonous strains as the original strain CPV-2 and the new antigenic variant CPV-2c (Benlafquih, 2012). The present study was conducted to isolate autochthonous CPVs in cell culture (Madin Darby Canine Kidney MDCK) from suspect lethargic dogs suffering from vomiting, and diarrhea.

Eighty-three clinical specimens (Rectal swabs, gut contents, colon, liver, spleen and myocardium) were collected from 6 "regularly" vaccinated and 23 unvaccinated dogs. As an initial step, the panel was screened with Haemagglutination (HA) test. Virus isolation was carried out on samples that had tested highly HA-positive. After 3 passages, viral replication was confirmed firstly by HA and secondly by Real Time PCR assay which has become the principal rapid, specific and sensitive diagnostic tool for detection of CPV-2(s) DNA.

HA test was able to detect CPV-2(s) antigens in 24/83 analyzed samples collected from 18 symptomatic dogs. According to the results of HA and Real time PCR assays, cultivation of canine parvovirus in MDCK cell lines was successfully established from 11 specimens collected from 10 symptomatic dogs. Additional passages of cell cultures were done in order to obtain a high yield stock of viruses for subsequent assays. To our knowledge, this study reports the first isolation of canine parvovirus in Morocco.

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African Swine Fever in Sardinia: evidence of several persistent clusters of infection during the re-epidemic wave 2012-2014

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Objective: African swine fever (ASF) is a devastating notifiable disease that affects domestic and wild pigs worldwide. It can only be distinguished from the similar disease classical swine fever (CSF) by laboratory testing.ASF disease ranges from very mild to severe, and in its most severe form, up to 100% of affected pigs may die. ASF is caused by African swine fever virus (ASFV), which is transmitted through direct contact with infected pigs, contaminated material in the environment, and infected swill or meat scraps fed to pigs. Most of the international spread of ASFV has been associated with swill feeding near international airports or seaports. In Africa, the soft argasid tick maintains ASFV in the warthog population. The virus is very stable over a wide range of acid and alkaline levels (pH 4-13) and temperatures even below freezing. It can survive in contaminated pigpens for at least one month and can survive for many months in unprocessed frozen meat. Pigs can remain carriers of the virus for long periods, and perhaps for life.

ASF was first reported in Kenya in 1921, and thereafter, ASFV spread throughout the African continent. The virus was subsequently introduced into European countries: first into Portugal (1957) and then Spain. While the disease became endemic in the Iberian Peninsula, many other outbreaks were registered in different continents (in

Brazil, Cuba, and the Dominican Republic in the Americas and France, Belgium, the Netherlands, and northern Italy in Europe). The virus arrived in Sardinia in 1978, most likely by contaminated waste fed to pigs. Sardinia was the only endemic region outside of Africa from the 90s until 2007 when ASF was reported in the Caucasus. Following this introduction, the virus diffused through the Russian Federation as well as other ex-Soviet countries, increasing attention on this infection. Severe measures were imposed in the second half of 2011 to stop the reemergence of outbreaks. In spite of these efforts, the disease spread widely in certain zones of Sardinia, and sporadically even in territories where the disease was not typically observed.

This work aimed to describe where ASFV circulation was occurring from 2012–2014, in order to identify the source of virus persistence.

Methods: The National Informative System (SIMAN) for the notification of infectious diseases was used as a source of data. In this system, data flows from the referring local emergency unit to the European Union or to other international institutions and organization, and is used to transmit disease alerts. This system also registers zootechnical information, such as farm positions and the number of bred pigs. These data were used to verify the position of cases and to carry out a spatial analysis of viral circulation. Our starting hypothesis was that in spite of the increased efforts to stop transmission, the virus continued to circulate in the same areas as it did historically. Thisstudy focused on evaluating the distribution of outbreaks in Sardinia in 2012, 2013, and 2014. We used kernel density estimation to obtain a non-parametric estimate of the probability density function of a random variable. The biweightkernel function was used to create a density (heatmap) raster of an input point vector layer. This heatmap allows easy identification of “hotspots,” or clustering of cases. A radius of10km specified the distance around a point where the influence of the point would be felt. QGIS 2.4.0 ‘Chugiak’ software was used to perform the analysis. The output of the study consisted of several maps that defined the areas of ASFV circulation.

Results: A total of 373 ASF outbreaks were considered this study, with 91 outbreaks registered in 2012, 176 in 2013, and 106 in 2014.For each year, outbreaks were mapped and the territory where virus was circulating was shown. The concentration of outbreaks was represented on a scale of 1 to 5, from low to high, and hotspots were symbolized.

Several relevant clusters of cases could be individuated during the study period: two hotspots were identified in 2012 (Goceano, AlàdeiSardi), two in 2013 (Nulvi, AlàdeiSardi), and three in 2014 (Goceano, Nulvi, Nuoro). A small number of outbreaks were recorded in the historically endemic area in the Nuoro and Ogliastra Provinces, as well as in the northern part of Cagliari Province (Sadali, Seulo).In the Goceano area, disease clusters were identified in 2012 and 2014.A large number of cases were registered also in 2013, but in a scattered distribution. This characteristic spread of the disease was even observed in the historical endemic area, where many outbreaks were registered over a large territory.

Conclusion: This study confirmed that ASF cases over the last three years in the Sardinia Region were distributed over a large area, but not evenly. Several sporadic outbreaks were registered in areas such as Oristano and Cagliari Provinces. In contrast, outbreaks were continuously recorded in at least four areas where the most cases were detected. Results of the spatial analysis seem to suggest that the virus travels easily, as it was detected in almost all of the Sardinian territory, but that the reservoir of infection is located in areas where virus circulation is uninterrupted.

The measures to curtail outbreaks were particularly effective in the area of AlàdeiSardi, where in the 2014 viral transmission was arrested. In this territory no recent outbreaks have been reported in the domestic pig population, whereas in wild pigs, sporadic detection of serological positives shows the last signs of earlier viral circulation.

More worrying is the epidemiological situation of Nulvi and Goceano areas. In these territories, there is evidence that the virus is still circulating and many cases are being detected in a small area. This suggests that the containment measures implemented were not able to interrupt ASFV transmission. Finally, the absence of ASF case clusters in the historical endemic area was probably due to the lack of exhaustive information. It is important to remember that, in this area, a large part of pig farms are illegally conducted as free range. Consequentially, it is likely that many ASF cases remain undetected and unreported. In this epidemiological situation, it may be impossible to accurately estimate the persistence of ASFV and the incidence of ASF disease.

Modelling economic impacts of an epidemic spread of West Nile virus in Belgium

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Objective: West Nile virus (WNV) is a vector-borne pathogen, member of the genus *Flavivirus* (family *Flaviviridae*). Its main vectors are mosquitoes belonging to the *Culicidae* family, genus *Culex*. The infection is maintained in a bird-mosquito enzootic cycle, and birds, especially passeriforms, are the primary reservoir hosts. Horses and men are considered as accidental dead-end hosts, and considered not to transmit the virus to other mosquitoes. The disease generates clinical signs mainly in horses and humans, while most affected birds are not clinically affected in Europe. The majority of horses remain asymptomatic, and approximately 10% of clinical cases develop neurological signs. In humans, after a two-week incubation period, two main clinical pictures can be observed: a flu-like syndrome and a neuro-invasive form. In Europe, the virus is constantly expanding its geographical distribution and has recently emerged in previously free countries. The present study aimed at estimating, in a prospective scenario, the potential economic impacts of a West Nile virus epidemic in free country such as Belgium, both for the equine sector and for the human health sector.

Methods: The modelling of risk areas, based on the habitat suitable for *Culex pipiens*, allowed determining equine and human populations at risk. Characteristics of the disease based on European past experiences allowed estimating morbidities among horses and humans. Regarding the human health aspect, only short-term costs and losses were estimated for patients who developed the neuro-invasive form of the disease, as no vaccine is available yet.

Results: The economic costs associated with the viral disease per horse were monetary estimated. When considering global estimations, the main costs are related to vaccination, followed by the replacement value of dead/euthanized horses. The costs incurred per patient affected by the neuro- invasive form of the disease, as well as the associated production loss were estimated. Global monetary estimations highlight the important part of hospital costs (64% of total costs), compared to insurance claims paid to the beneficiaries after the death of patients (19% of total costs).

Conclusion: The main costs for the equine sector were prevention measures such as vaccination and replacement value for dead/euthanized horses. Hospital charges would be the major financial consequences of the West Nile virus epidemic in humans. In horses, the choice of the vaccination strategy will have important consequences in terms of costs. The modelling economic impacts of an epidemic spread of West Nile virus in European countries appears to be useful in terms of awareness and to making mitigation measures proactively and appropriately.

Parallel EPIZONE Special session 1: Host / Virus Interactions & Viral Immunity 2

Wednesday 2nd September 2015
15:00-16:30
Room Sully 2

Chair: Alejandro Brun & Stephan Zientara

Cowpox virus: virulence studies in different animal species

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Objectives: Cowpox virus (CPXV) reservoir hosts are rodents, primarily voles. But many other species like rats, cats, cattle, several zoo animals and humans, can be naturally infected. The objective of our study was the analysis of CPXV virulence of different strains. Therefore, several host species were experimentally infected using various CPXV strains to estimate pathogenicity.

Materials & Methods: The tested CPXV strains originated from accidental or reservoir hosts. The viruses were characterized *in vitro* in comparison to an established reference strain and whole-genome sequencing was performed. For *in vivo* analysis Wistar rats (animal model species), cattle and new world camelids (accidental hosts) or common voles (reservoir host) were used.

Results: *In vitro* analyses revealed no differences between the CPXV strains. In contrast, morbidity and mortality rates were quiet different. Using the Wistar rat model, only one virus isolate caused very severe disease in Wistar as well as fancy rats resulting in a 100% mortality rate. Other CPXV strains caused no or some mild to moderate clinical signs, but no mortality. Interestingly, common voles infected with the CPXV strain highly virulent for rats exhibited clinical symptoms, characterized by nasal discharge and dyspnoe. In contrast, infection of voles using the CPXV isolate from the reservoir host induced subclinical infection. Experimental infection of cows resulted in mild skin lesions only. Recent descriptions of severely diseased alpacas were examined by experimental infection of both alpacas and lamas, which resulted in 100% morbidity but no mortality.

Conclusion: The variable pathogenic potential of CPXVs was confirmed even in the reservoir host.

Equine monocytic cells as a 'Trojan horse' for equine herpesvirus type 1 (EHV-1) dissemination within the host

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Equine herpesvirus type 1 (EHV-1) is a main cause of respiratory disease, abortion and neurological disorders in horses worldwide. Upon entry in the host, EHV-1 is known to replicate in the epithelial cells of the upper respiratory tract and disseminate through the body via a cell-associated viremia in peripheral blood mononuclear cells (PBMC).

Secondary replication in the endothelial cells (EC) of target organs such as the pregnant uterus and/or central nervous system can cause vasculitis and thrombosis and may lead to severe symptoms such as abortion and/or neurological disorders. Commercially available vaccines do not provide full protection against severe symptoms, as EHV-1 can cause viremia in the presence of neutralizing antibodies. Like other herpesviruses, EHV-1 has developed sophisticated strategies to evade elimination by the host immune system. *In vivo* and *ex vivo* studies of the pathogenesis of EHV-1 in the equine upper respiratory tract have identified CD172a monocytic cells as one

of the main carrier cells of EHV-1. Our research aims at unraveling the mechanism by which (i) EHV-1 hijacks immune cells and uses them as 'Trojan horses' in order to disseminate inside its host; (ii) EHV-1 is transferred from immune cells to EC.We designed an *in vitro* study to compare replication kinetics of EHV-1 in RK-13 cells, a cell line known to be susceptible to EHV-1 infection, and CD172a$^+$ cells. We also investigated how EHV-1 inoculated CD172a$^+$ cells adhere and subsequently transmit EHV-1 infection to equine venous endothelial cells.We found that the replication of EHV-1 was restricted to 4% of CD172a+ cells compared to 100% in RK-13. In susceptible CD172a+ cells, the expression of immediate-early (IEP) and early (EICP22) proteins was delayed in the cell nuclei by 2-3 hpi compared to RK-13, and the formation of replicative compartments by 15 hpi. The intracellular and extracellular virus titers produced by inoculated CD172a+ cells were 3 to 4 logs lower than those of the control RK-13 cells. The percentage of inoculated CD172a$^+$ cells, which produced infectious EHV-1 was less than 0.02%. All these findings were similar for CD172a$^+$ cells isolated both from nasal mucosa as from blood. In addition, we showed that trichostatin A and sodium butyrate, two inhibitors of histone deacetylases (HDACs), relieved the infection block in CD172a$^+$ cells by increasing viral permissiveness and EHV-1 proteins expression at very early time of infection. Besides, we demonstrated that EHV-1 infection of CD172a$^+$ cells resulted in a 3 to 5-fold increase in adhesion to EC. Antibody-blocking experiments indicated that $\alpha 4\beta 1$, $\alpha L\beta 2$ and $\alpha V\beta 3$ integrins mediated adhesion of infected CD172a+ cells to EC. EHV-1 infection induced CD172a+ cell adhesion via an ERK-MAPK dependent signaling pathway at a very early time of infection. In addition, EHV-1 replication was activated in CD172a$^+$ cells upon adhesion to EC via the production of TNF- α of EC origin. In the presence of neutralizing antibodies, infected CD172a$^+$ cells were able to transfer cytoplasmic immediate-early (IEP) and late gB proteins to uninfected adjacent EC. Approximately 0.01% of inoculated CD172a+ cells produced and transmitted infectious virus to neighbouring cells. Together, our results provide evidence that the restriction and delay of EHV-1 replication in CD172a$^+$ cells is part of an immune evasive strategy and moreover, that it involves silencing of EHV-1 gene expression associated with histone deacetylation events which play a major role in chromatin remodeling. EHV-1 infection uses specific signaling pathways to manipulate cellular changes in CD172a$^+$ cells essential for their adhesion to EC.Both cell-to-cell contacts and production of TNF-α by EC activate EHV-1 replication in CD172a$^+$ cells, which subsequently facilitate transfer of viral cytoplasmic material to EC but do not typically lead to a productive infection. These novel findings are bringing new insights in the complex pathogenesis of EHV1-related problems.

Systemic spread of wild boar hepatitis E virus in pigs

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Objective: Hepatitis E virus (HEV) is a small, non-enveloped virus with a positive sense RNA genome. An increasing number of animal species have been recognized as susceptible to HEV. Direct zoonotic transmission to human beings, followed by symptomatic infection has several times been documented, as in the case of uncooked meats from wild boar, deer and pigs infected with HEV. The particular epidemiological role of wild boars in the HEV transmission route has recently been investigated. The aim of this study was to investigate the early consequences of pig infection with a wild boar HEV strain (WbHEV).

Methods: A genotype 3 subtype of HEV positive liver from wild boar hunted in Belgium and a HEV negative swine liver collected from a specific pathogen free (SPF) pig, were used for production of inocula. The livers were homogenized in 1 volume of PBS, centrifuged at 3000 RPM for 20 min at 4°C and filtrated through 0.45 µm microfilters. Before inoculation, infectious particles were quantified by qRT-PCR after RNase treatment with RNaseA, followed by RNase inhibitor (QIAGEN). The five eight-weeks-old piglets were obtained from the French Agency for Food, Environmental and Occupational Health & Safety's SPF pig herd. The herd is known to be HEV-free, thus

lacking maternal antibodies against HEV. All piglets were tested free of HEV infection by qRT-PCR and ELISA at the time of inoculation. The piglets were divided in two groups. Group 1 included three piglets (A, B and C) inoculated with a wild boar HEV strain (WbHEV). Group 2, representing a negative control, was composed of two piglets (D and E) inoculated with the HEV-free liver homogenate. The experiment was conducted over a period of ten days in biosafety level 3 animal facilities. Pigs were intravenously inoculated via the auricular vein at day 0 with 2 mL, containing either 8.4 x 10^6 genome equivalent/mL (ge/mL) (group 1) or 0 ge/mL (group 2). Faeces and sera were sampled at days 0, 4, 7, 8, 9 and 10; necropsies were performed at days 8, 9 and 10 post-infection. The qRT-PCR protocol used has been described previously. RNA extractions were performed with the QIAamp Viral RNA kit mini kit and the RNeasy mini kit (QIAGEN). Nucleotide homology was determined with the BioEdit software (Hall, 1999). Sera from days 0, 4, 8, 9 and 10 were analysed with a double antigen sandwich ELISA (HEV ELISA kit 4.0V, MP Biomedicals. Aspartate transaminase (AST) (reference value: 31-58 IU (45±14)) and Alanine transaminase (ALT) (reference value: 32-84 IU (61±26)) were measured at days 0, 4, 8, 9 and 10. Histopathology was performed on liver, duodenum, jejunum and colon of all pigs.

Results: HEV RNA was detected in serum, bile, liver, spleen, duodenum, jejunum, colon, lung, gastro-hepatic lymph nodes and faeces in all group 1 piglets. The qRT-PCR showed 4.1 x 10^6, 3.7 x 10^7 and 5.8 x 10^6 ge/gr in the livers of pigs A, B and C, respectively. Pigs from both groups remained seronegative until the end of the experiment and hepatic enzymes remained within the normal range. Furthermore, no clinical signs were observed. The sequences (270 bp from ORF2) detected in the livers of the inoculated piglets (A, B and C) showed 100 % nucleotide homology to the inoculum. Histopathological lesions seen in livers from group 1 pigs showed multiple sites of focal infiltration of lymphocytes, plasma cells, eosinophils and macrophages in the portal triads and hepatic sinusoids. No histopathological differences were observed between the pigs of both groups in duodenum, jejunum and colon samples.

As demonstrated above, a G3f HEV strain isolated from a wild boar is able to induce an early productive infection in domestic pigs. In European countries, HEV was detected in several species. An especially high prevalence has often been observed in pigs and wild boars. This epidemiological situation, combined with the capacity of the virus to cross the wild boar - pig barrier, raises the question of natural transmission events occurring between these animals, at least in the context of outdoor breeding. This study investigated the acute phase of infection and especially the distribution of viruses in pigs. No seroconversion was observed during the infection time in the inoculated pigs, implying that at least ten days are necessary for swine to mount an antibody response against WbHEV.

These results allow a fuller understanding of the pathogenesis of WbHEV infection in pigs and especially complement the interesting data presented in a previous publication (Schlosser et al., 2014) in the following points. Firstly, HEV RNA was detected in all inoculated pigs between days 8 and 10 in this study but only in one pig on day 11 in Schlosser et al. This could be explained by the difference in the amount of inoculated virus; an inoculum dose-dependent effect can thus be hypothesized regarding the onset and spread of viraemia. Secondly, this experiment extends the virus distribution in organs of experimentally infected pigs to lungs and gastro-hepatic lymph nodes. As early as day 10, we observed liver lesions similar to those previously observed after 28 days (Schlosser et al., 2014). Interestingly, histopathological hepatic lesions induced neither clinical signs nor an increase in hepatic enzymes, suggesting that asymptomatic WbHEV infections could be observed in swine up to 10 days after infection.

Conclusion: HEV is considered a threat for both food industry and public health. This study supports the epidemiological role of wild boars in HEV transmission. It provides experimental evidence of the early spread of the WbHEV strain in pigs. Further work is required to investigate the course of WbHEV infection in pigs over a longer period.

A cell culture-adapted Classical swine fever virus phenotype does not require the 476Arg Erns mutation

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Classical swine fever virus (CSFV) is a small, enveloped RNA virus of the genus *Pestivirus* within the *Flaviviridae* family, and previous studies gave further insights into cell – virus- interactions of CSFV.

It was e.g. demonstrated that the envelope glycoproteins E^{rns} and E2 play the major role for virus attachment and entry. While E^{rns} mediates the initial contact to the host cells, binds E2 the specific receptor, porcine complement regulatory protein CD46. It is known that in vitro propagation of CSFV leads to cell culture-adapted phenotypes that are characterized by superior infection rates and viral growth in vitro. These virus variants are able to use cellular Heparan sulfate (HS) as a complementary receptor to attach to the host cells. It was described for CSFV strain "Brescia" that a single amino acid change in the envelope protein E^{rns} (aa 476 Ser to Arg) is responsible for the HS binding. DSTP-27 a N,N`bisheteryl derivate of dispirotripiperazine was shown to bind to cellular heparan sulfates, and is thus able to inhibit infection of cell culture- adapted virus variants of CSFV.

In the framework of recent receptor studies, field-type and cell culture-adapted variants of the CSFV strain "Roesrath" were investigated. The cell culture-adapted phenotype was represented by the 50^{th} cell culture passage. The adaptation was proven by a strong influence of HS-binding compound DSTP-27 that was able to block infection to a very high extent. Despite the obvious phenotype, partial and full- length sequencing showed that the observed ability to bind HS after passaging was not accomplished through an expected amino acid exchange in the E^{rns} at amino acid position 476. The original field strain as well as the 50^{th} passage of the virus still presented a Serin at this position. Instead of the expected mutation, the propagated virus variants showed an exchange from Asp to Asn in the E^{rns} (aa355) and from Leu to Ser in the glycoprotein E2 (aa763).

In order to investigate the impact of these changes, the point mutations, leading to both amino acid exchanges, were integrated into the consensus sequence of the original field variant of CSFV "Roesrath" (GenBank entry: GU233734) with the help of a pBelo BAC reverse genetics system. The resulting clones are under investigation with regard to growth kinetics, receptor usage and mutation rate.

Irrespective of the final outcome of our studies regarding the impact of the described mutations in the E^{rns} and E2 of CSFV, investigations of the alternative way of cell culture adaption will shed light on the infection process of CSFV in vitro.

Next-generation sequencing fails to identify viral miRNAs encoded by PCV2 in subclinically infected pigs

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Objective: MicroRNAs (miRNAs) are small non- coding RNAs with post- transcriptional regulation functions. These small RNAs are expressed by a huge variety of organisms, from mammals, to plants and recently discovered, viruses. Within the viruses, it has been demonstrated that some DNA viruses are capable of express miRNAs. PCV2 is a small single stranded DNA virus with 1.76 Kb and is the aethiological agent of post weaning multisystemic wasting syndrome. In this study, small RNA libraries were constructed from two tissues of subclinically PCV2 infected pigs to explore if PCV2 can encode viral miRNAs.

Methods: Firstly, *in silico* prediction was carried out in order to check if the PCV2 genome encodes possible miRNA precursors by using Vmir software. Then, four animals were inoculated with $7x10^{4.8}TCID_{50}$ of PCV2 iso-

late Sp-10-7-54-13 and two animals were inoculated with PBS. At 21 days post-inoculation samples were taken and total RNA extraction was carried out in order to construct small RNA libraries. A total of 12 small RNA libraries were constructed in a two-step ligation procedure with the 3' and 5' adaptors from IDT technologies. Libraries were (HT) sequencing with the GS FLX 454 device (Roche). From the total reads obtained (1,106,437), primer sequences were trimmed and only those insert sequences between 15 and 29 nucleotides and with total number of sequences ≥3 were kept for further analysis. For viral miRNA discovery, sequences were blasted to PCV2 isolate Sp-10-7-54-13 genome (accession number: GU049342) considering 100% of alignment and identity (perfect match). In order to search potential viral miRNAs, a blast against viral genome was done with an increased mismatches number in the extremes due to the miRNA variability (isomiRs). Besides, in some cases, a blast was done allowing internal variations with a <100% of alignment in order to consider the variability of the viral genome. Also, sequences were blasted to the output of Vmir hairpin PCV2 structures.

Results: No hits with a 100% of homology against the viral PCV2 genome sequence were found. One potential candidate with a copy number of 58 was detected by allowing <100% of alignment. As the exact candidate was not found in the viral sequence it was not considered a viral miRNA. The candidate sequence was blasted to the porcine genome obtaining a 100% of homology in the region corresponding to the ssc-miR-29a hairpin precursor.

Conclusion: PCV2 cannot express viral miRNAs in its natural host in a subclinical infection. If the virus is capable of expressing them *in vitro* or other infection time points, it has to be determined.

Studying CSFV specific immune response using dendrimeric peptides

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Classical swine fever virus (CSFV) impairs the immune system of the host. The degree of immune compromise is one of the determining aspects in the outcome of the disease. Previous studies have shown the existence of B and T cell epitopes in the virus, mainly in the E2 structural protein, as well as the NS3 non-structural protein. Dendrimers represent a promising tool for the multimeric presentation of epitopes in candidate vaccines. Multimerisation is a nature-mimicking strategy of antigen presentation that has proven successful in the development of human-made vaccines, particularly by means of dendrimeric (e.g., branching) designs. This strategy facilitates the interactions between different epitopes to potentiate the elicited immune response, and can be useful for basic investigations of the mechanisms governing the induction and control of immunity. The aim of this work was to evaluate the CSFV specific immune response generated by different epitopes previously described within E2 and NS3 CSFV proteins, combining them with a T helper epitopes reported from Foot and mouth disease virus (FMDV) and Peste des petits ruminants virus (PPRV), using the multimerisation strategy.

Five dendrimeric constructs in different conformations were formulated and later inoculated in five groups (four animals each) of six-week old pigs (Landrace x Large White), while another group (control group) was inoculated with NaCl 0.9%. The epitope from E2 glycoprotein (Tarradas et al., 2011) was conserved in all the constructions, but varied in number of copies (4 or 2). Two doses of 2 mg each of the corresponding construct, dissolved in 1 mL of saline solution and mixed with 1 mL of Montanide v206 adjuvant (Seppic), were administered at days 0 and 21 of the trial. An experimental challenge with CSFV was performed with 10^5 $TCID_{50}$ of CSFV (strain Margarita) 15 days after the second immunisation by intramuscular (i.m.) injection in the neck region. Additionally, four pigs (vaccination controls) were immunized with one dose of a commercial live-attenuated vaccine (C-strain) and were challenged with the same inoculum at 16 days post vaccination (dpv). Humoral as well as cellular immune response were evaluated in the 28 pigs at 8 different dates after vaccination and chal-

lenge. The experiments were approved by the Ethics Committee for Animal Experiments of the Autonomous University of Barcelona (UAB), according to existing national and European regulations. Different levels of partial protection from clinical signs were observed in the five dendrimeric immunized groups. Interestingly, the best clinical protection was found in two groups inoculated with the same peptides in different conformations (B4T or B2T). In terms of CSFV specific humoral response analysed against the E2 protein, one of the dendrimeric peptides developed a faster humoral response at 8 days post challenge. The cellular response was evaluated through INF- γ producing cells, with two groups showing INF- γ levels after stimulation with PHA on the day of Challenge (0DPI) and one of these groups increasing this response even further at 8 DPI. The specific humoral and cellular response against every peptide and CSFV will be presented. This results show the capacity of a previously reported epitope for a monoclonal antibody to induce immune response in pigs, making it a new prospect for the generation of DIVA vaccines and diagnostic tools against CSFV, thus making safer the use of vaccines in eradication campaigns.

Parallel EPIZONE Special session 2: Focus On PED & SBV

Thursday 3rd September 2015
10:10-12:10
Room Sully 3

Chair: Bernd Hoffman & Christopher Oura

Study of the nuclear targeting of the NSs protein of Schmallenberg virus

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Objective: In 2011, an emerging arbovirus named Schmallenberg virus (SBV) and belonging to the *Bunyaviridae* family was identified in German cattle herds, and has rapidly spread through Europe. SBV infects ruminants and although the infection is mild in adult animals, females' infection during gestation can lead to stillbirth and foetal abnormalities due to SBV ability to cross the placental barrier. Among bunyaviruses encoded genes, the non-structural protein NSs is a virulence factor involved in transcription and interferon inhibition. Indeed, a SBV NSs-deleted recombinant virus is unable to disrupt transcription and interferon synthesis *in vitro* and *in vivo*, referred to the wild type strain. This effect of NSs requires its targeting to the nucleus through an unknown mechanism. Thus, we wanted to determine the amino acids sequence responsible for nucleo-cytoplasmic shuttling of NSs. In addition, to further characterise NSs functions during SBV infection, we aimed to identify its cellular partners.

Methods: To identify NSs domains involved in nuclear targeting, we generated multiple GFP or GST-fused deletion mutants designed on structural or subcellular in silico predictions. Their respective subcellular localisation was appreciated using immunofluorescence and cellular fractionation assays in human and ovine cell lines. In parallel, we used the yeast two-hybrid method to screen several cDNA libraries, using NSs as bait, to find out its cellular partners.

Results: As expected, immunofluorescence assays allowed us to visualize full length GFP-NSs protein in the nucleus. Interestingly, the amino-terminal part of NSs appeared sufficient to trigger nuclear targeting of the protein while carboxy-terminal constructs seem to be retained in the cytoplasm. In another study, a yeast two-hybrid screen allowed us to uncover several putative cellular partners of NSs, including the dynein light chain type 1 (DYNLT1) and the Major Vault protein (MVP). DYNLT1 is part of the dynein motor complex involved in retrograde transport of proteins and vesicles from the cytoplasm to the nucleus inside the cell. MVP is a multifunctional ribonucleoprotein notably involved in nucleo-cytoplasmic transport of proteins through its associa-

tion to the microtubules. Use of NSs deletion constructs showed that the first 33 amino-acids residues of the protein are sufficient to mediate NSs-DYNLT1 interaction in a pairwise interaction yeast two-hybrid test. In contrast, NSs-MVP interaction was completely abolished with all deletion mutants, suggesting that MVP binds NSs through a highly specific conformational epitope. Importantly, in SBV infected cells, inhibition of DYNLT1 or MVP synthesis by means of siRNA lead to a high decrease of SBV replication as assayed by western blot, quantitative PCR and titration, suggesting that both proteins are important for SBV replication.

Conclusion: In this study, we confirmed that NSs of SBV is addressed to the nucleus and that the amino terminus of the protein contains a nuclear targeting signal. Interestingly, by using the yeast two-hybrid methodology, we also found that NSs interacts with DYNLT1 and MVP, two proteins that use the microtubule network for cellular trafficking. We thus think that NSs interacts with DYNLT1 and/or MVP to be addressed to the nucleus in a retrograde manner, where NSs could act as a transcriptional inhibitor. Further investigations are in progress to confirm this hypothesis including the use of microtubules polymerisation inhibitors. We also aim to design recombinant viruses devoid of DYNLT1 and/or MVP interaction ability to address their respective role in an infectious context both in cell culture and using the IFNAR-/- mouse model.

Schmallenberg virus incursion into Great Britain: identification of mutations and a large deletion in the M segment of British field samples

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Schmallenberg virus (SBV) is a vector-borne orthobunyavirus from the family Bunyaviridae. The virus causes overt clinical disease in new born lambs and calves and mild disease or is asymptomatic in adult ruminants. SBV emerged in north-western Europe during the summer of 2011. In Great Britain (GB) SBV was initially reported on the 23rd of January 2012 through cases of malformed lambs in Norfolk, Suffolk and East Sussex and has since spread widely across GB.

Objective: To pinpoint and summarize the incursion and spread of SBV across GB and the Channel Islands during the first years after introduction (2011 - 2012). Analyze the S and M gene segments to identify sequence variability over time, species and geographical location with the aim to describe how SBV evolved in GB.

Methods: Field samples from all clinically affected farms in England, Wales and the Channel Islands were sent to the Animal and Plant Health Agency (APHA) for diagnosis using RT-qPCR. Results were collated to pinpoint when each county had its first reported case of SBV. Subsequently, 21 positive samples from a variety of counties, dates and both ruminant farm species (18 ovine and 3 bovine) where selected for Sanger sequencing. Overlapping sequence fragments were designed to amplify the ORFs of the S and M genes to enable full coverage of each segment. Resulting data was phylogenetically analyzed using MEGA5. In addition serum samples stored from 2011 were re- examined for the presence of SBV antibody to delineate the earliest possible incursion data.

Results: This study describes the SBV incursion into GB, which resulted in clinical disease initially between January and May 2012 and predominantly affected the south and east of England followed by the Midlands and Channel Islands. After a quiet period in late spring/early summer new cases of SBV infection and further spread across the Midlands, Wales and northern England were first detected by seroconversion in cattle followed by new cases of malformations in autumn.

Sequence analysis revealed the S segment to be more conserved in comparison to the M segment, which had more amino acid changes throughout. Contrary to previous published findings no specific region of high sequence variability was observed within the analyzed M segments. One sample displayed a 193 amino acid deletion in the M segment and a second sample displayed a 132 and a 16 amino acid deletion. Phylogeny of the 21 sequences showed no distinct clustering between species, geographical locations or over time.

Conclusion: It appears that the likely route of introduction was through multiple incursions of midges brought over the English Channel in the wind from mainland Europe. The initial cases seen in the south east and east of England and atmospheric dispersion event models published by the UK Meteorological Office suggest midge incursions occurred during August 2011 from the Netherlands and Belgium. The following reported case seen in Cornwall on the 17th of February 2012 suggests a separate incursion from France during September 2011. These incursions resulted in an outbreak, which largely affected the south and the east of England. SBV seroconversion and new cases of malformations seen post May 2012 confirmed that the virus had managed to overwinter and that midges were still active during January and February 2012. One reason SBV was able to spread so rapidly was due to there being a delay in detection which can be months depending on the length of gestation. The sporadic spread and resulting randomized phylogenetic data is as expected for a vector borne disease. Amino acid changes and deletions appear to be a common factor in the M segment, however it is not yet known how large deletions such as those seen in this study will affect replication within the host.

Reoccurrence of Schmallenberg virus 2014/2015 in Germany

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Schmallenberg virus (SBV) emerged in Germany in 2011, thereafter the virus spread rapidly across Europe and almost disappeared in 2013.

New infections were detected in late summer 2014 in adult cattle in the context of export investigations. Virus genomes with a very high sequence identity to the first SBV-samples from 2011 could be repeatedly detected in the blood of subclinically infected adult cattle in 2014. Full-genome analysis revealed only a small number of amino acid substitutions in comparison to the original SBV. Animals experimentally inoculated with samples from 2014 developed an identical course of viraemia for 4 to 6 days as after inoculation with the original SBV-isolates. Subsequently, the reoccurring virus circulation during the 2014 vector season, primarily in an area less affected in the two previous years, resulted in an increased frequency of the birth of malformed offspring in the first months of the year 2015.

SBV-seroprevalence studies showed a reduction of SBV-antibody levels over the months for individual animals, but most SBV-infected animals showed a robust SBV-antibody titre for a long time period. Nevertheless, a marked drop in the herd seroprevalence could be seen after 2012 due to a high turnover and seronegative offspring. This seroprevalence situation was comparable to the BTV-8 outbreak 2006 to 2008 in northern Europe. However, the substantial reoccurrence of SBV in 2014/2015 after a period of markedly reduced virus activity (at least in livestock animals) could indicate a different and endemic situation. A possible explanation could e.g. be the persistence of SBV within the insect vector.

The data about SBV-reoccurrence as well as the seroprevalence studies will be presented and possible implications e.g. due to an endemic SBV-status will be discussed.

Is flock renewal a risk for novel Schmallenberg virus episode in sheep flocks?

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Objective: No evidence of Schmallenberg virus (SBV) circulation was detected since late 2012 in a Belgian research sheep flock composed by around 400 ewes producing 700 lambs a year for breeding and meat industry. This flock underwent SBV emergence in September 2011 and SBV re-emergence between mid-July and mid-October 2012 (Claine et al., 2013). If long-term immunity exists in adult animals, the number of SBV seronega-

tive sheep was supposed to increase yearly as soon as colostral immunity disappeared due to absence of novel viral episode. So, flock renewal could be a risk for novel SBV episode in sheep flocks. The objectives of this study is to evaluate within-flock SBV seropositivity over years in a sheep herd that encountered two SBV episodes and to monitor evolution of SBV seropositivity within different groups of animals in relation to their year of birth.

Methods: The entire research sheep flock of the University of Namur was used for this study and SBV seropositivity was evaluated each year in early January and in late December in 2012, 2013 and 2014. Due to flock renewal, the total number of sheep kept for breeding changes continuously. The table 1 summarizes the number of adult animals (born before SBV emergence in September 2011) and lambs present at the farm in early January and late December for each year since 2012 until 2014.

Number of animals						
	2012		2013		2014	
	January	December	January	December	January	December
Adult animals	432	344	344	205	205	135
Lambs born in autumn 2011	178	44	44	34	33	28
Lambs born in 2012		170	167	52	52	50
Lambs born in 2013				154	154	100
Lambs born in 2014						161
Total	**610**	**558**	**555**	**445**	**444**	**474**

Table 1. Number of animals (adult and lambs) composing the research sheep flock at the different time points of SBV seropositivity evaluation.

SBV seropositivity was evaluated at the previously mentioned time points in those animals by performing serum neutralization tests (SNT) with serial 2-fold dilutions. Results of SNT were considered positive if the dilution leading to 50% of virus neutralization (ED50) was > 11.22.

Results: The evolution of within-flock and within-group SBV seropositivity is detailed in table 2. It appeared that for adult animals born before September 2011 and that underwent both two SBV episodes, a constant 100% SBV seropositivity was observed since January 2012 until December 2014. SBV seropositivity evaluated one year after birth in lambs born in 2011, 2012 and 2013 equaled 66%, 12% and 4% respectively and differed significantly ($p<0.05$). Among lambs born in autumn 2011 during or just after the first SBV circulation period, the percentage of SBV seropositive animals was approximatively 63% over years since 2013. In late 2014, only 10% and 4% SBV seropositive animals were observed in lambs born in 2012 and 2013 respectively while about 50% of lambs born in 2014 were seropositive at the same time. At a herd scale, it appeared that SBV seropositivity showed slight a decrease between early 2012 and early 2013 (96% vs 90%) while it equaled 50% in late 2014.

SBV seropositivity (%)						
	2012		2013		2014	
	January	December	January	December	January	December
Adult animals	100%	100%	100%	100%	100%	100%
Lambs born in autumn 2011	85%	66%	66%	62%	61%	61%
Lambs born in 2012		76%	76%	12%	12%	10%
Lambs born in 2013				48%	48%	4%
Lambs born in 2014						48%
Total	**96%**	**90%**	**90%**	**69%**	**69%**	**50%**

Table 2. Evolution of % total within-flock and % within-group SBV seropositivity over years from early 2012 until late 2014.

Conclusion: The evolution of SBV seropositivity showed significant differences between animals in relation to the date of birth. Adult animals born before September 2011 showed 100% seropositivity over years resulting in existence of long-term immunity in those animals. Comparatively, the decrease of SBV seropositivity was lower for lambs born in autumn 2011 than for lambs born in 2012, 2013 and 2014 certainly due to the fact that those lambs completely lose colostral protection at the time of SBV re- emergence and developed active immunity. Lambs born in 2012 were for most of them still protected by colostral protection during second viral episode resulting in higher decrease of SBV seropositivity as observed in lambs born in 2013 and 2014 that never underwent viral infection. Due to the absence of SBV novel episode since late 2012, the number of seronegative animals has thus increased yearly. So, flock renewal could promote novel hypothetical novel SBV episode. Moreover, it could be interesting to focus on seropositive animals born after viral episodes that are supposed to never have been SBV infected after birth and are now out of colostral protection.

Evolution of Schmallenberg virus seropositivity over years among sheep naturally infected at different ages

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Objective: Schmallenberg virus (SBV) emerged in early September 2011 in a Belgian research sheep flock and the period of SBV circulation was determined to extend to 26th October 2011.Clinical evidences of SBV transplacental infection were detected during the lambing periods of January and March 2012. They consisted in musculo-skeletal defects (arthrogryposis, scoliosis…) and abnormal development of central nervous system tissues (cerebellar hypoplasia, hydrocephalus…).Between mid-July and mid- October 2012, SBV re-emergence was assessed by Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) and Serum Neutralization Test (SNT) in 50 female lambs of the same flock (Claine et al., 2013).Since late 2012, no serological evidence of novel viral circulation was detected. The objective of this study was to monitor evolution of SBV seropositivity over years among different groups of sheep born before, during or after first virus episode in 2011 and that underwent SBV re-emergence in 2012.

Methods: A maximal number of 742 animals was used for this study. Group A animals (n=432) were adult sheep born before SBV emergence. Group B animals (n=73) were born in October 2011 during the first SBV episode. Animals from groups C (n=105), D (n=132) and E (n=142) were lambs born in November 2011, January 2012 and March 2012 respectively, from ewes that underwent SBV infection at different stages of pregnancy. Ewes that gave birth to group C animals were SBV infected in late pregnancy while lambs born in January (group D) and March 2012 (group E) were from ewes SBV infected during the most sensitive period of pregnancy (between 30 and 50 days) or in early gestation respectively.

Anti-SBV antibody titers were evaluated in those animals four times a year (March, June, September, and December) from 2012 until 2014 by serum neutralization tests (SNT). Animals were considered SBV seropositive if the dilution neutralizing 50% of the challenge virus (ED50) was > 11.22.

Results: All adult sheep (group A) born before SBV emergence (September 2011) seroconverted and remained 100% seropositive until December 2014. In March 2012, the percentages of seropositive animals equaled 65%, 80%, 100% and 100% for groups B, C, D and E respectively. Two months later, in June 2012, just before the estimated period of SBV re-emergence, the percentages of SBV seropositivity decreased for all animals and equaled 27%, 29%, 8% and 71% for groups B, C, D and E respectively. After the second viral episode, an increase of SBV seropositivity was observed in groups B, C and D animals but was only significant for lambs born in October 2011 (group B) ($p<0.05$). In December 2012, the percentage of seropositive group D animals was lower than in June 2012 (20% vs 71%). Since December 2012, the percentages of SBV seropositivity as presented in table 1 remained constant in all groups.

	SBV seropositivity (%)			
	March 2012	June 2012	September 2012	December 2012
Group A (n=432)	100%	100%	100%	100%
Group B (n=73)	65%	27%	80%	87%
Group C (n=105)	80%	29%	49%	55%
Group D (n=132)	100%	8%	23%	27%
Group E (n=142)	100%	71%	25%	20%

Table 1. Evolution of SBV seropositivity among sheep that were naturally SBV infected in 2011 and/or in 2012 from March 2012 until December 2012.

Conclusion: By considering results obtained for sheep born before September 2011 that underwent SBV emergence and second SBV episode, it seems that an at least 3 years long-term immunity exists in those animals. Lambs born in March 2012(group E) were certainly still under colostral protection during SBV re-emergence. Contrastingly, animals from groups B (born in October 2011), C (born in November 2011) and D (born in January 2012) should have lost passive immunity acquired by colostrum ingestion and absorption at the time of SBV re-emergence. However, levels of SBV seropositivity in those groups were surprisingly high in June 2012, especially for groups B and C animals. This could be explained by the fact that SBV transplacental infection of pregnant ewes may have led to development of active immunization in fetuses in addition to passive immunization transmitted by their dams.

Re-emergence of Porcine epidemic diarrhea virus in Germany

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Porcine epidemic diarrhea (PED) is an acute and highly contagious enteric disease of swine that results in severe enteritis, diarrhea, vomiting, and dehydration. Especially in suckling pigs, mortality can be very high. The causative agent, porcine epidemic diarrhea virus (PEDV), is an enveloped positive single-stranded RNA virus that belongs to the family *Coronaviridae*, genus *Alphacoronavirus*.

After its first recognition in the 1970s in Europe, the disease caused considerable economic losses, especially in Asia. In May 2013, a highly virulent PEDV variant emerged in the United States (US), with swine farms experiencing explosive epidemics affecting all age classes of animals, with up to 95% mortality in suckling pigs.

Since May 2014, several cases of PED were reported from the southern and western part of Germany with numbers of cases rising in winter. In most cases, fattening pigs were affected showing high morbidity with almost non-existent mortality. However, some breeding herds also reported high mortality rates with up to 85% losses in suckling piglets. More than 20 representative virus strains were subjected to next-generation sequencing on the Illumina MiSeq platform, and the resulting full-length genomes were used for phylogenetic analyses and molecular epidemiology. Moreover, additional strains were sequenced in the S-gene segment to obtain a broader picture.

Comparative analyses of the whole genomes showed high identities (>99%) among all German PEDV-strains which are highly related with the PEDV-strain OH851 (GenBank accession KJ399978; app. 99.5% identity) that was recently reported from the US, and was linked to mild clinical cases in fattening pigs. Lower identities of approximately 98.7% were found with currently circulating highly virulent US strains and strains from China.

Historical PEDV-strains from Europe (CV777) are even less similar with 97 % identity on a full-genome basis.

So far, no links were found between different disease courses and genetic variations. However, indications exist that secondary infections of viral and/or bacterial genesis may have an important impact on the disease outcome in suckling pigs. Moreover, management of diseased piglets seems crucial.

To date, there is still a serious lack of data and comprehensive background information pointing to the need of further extensive investigations preferably in collaborative approaches joining all forces to overcome the burden of PED.

Description of the first case of porcine epidemic diarrhea in France in December 2014 and the duration of viral shedding in the herd

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Porcine epidemic diarrhea was first described in England in 1971 and then spread throughout Europe till the end of the 1990's. The etiologic agent of PED is an Alphacoronavirus, the PED virus (PEDV). Since April 2013, a severe epizooty of porcine epidemic diarrhea (PED), characterized by watery diarrhea and vomiting, has been striking USA that was previously free from this disease. Suckling piglets are the most affected by PED with up to 90-95% mortality. In France, no PED case has been described since late 1990's and the immune status of the pig population against PEDV is low. In France, as a first measure for the control of potential outbreak, PED has been recently classified in the list of the first category of animal health hazards, making notification to the veterinary services compulsory.

Objective: The objective of the study was to characterize the PEDV isolate in case of PED outbreak in France and to evaluate the duration of viral shedding in feces.

Methods: An outbreak occurred in a farrow-to-finish herd located in the North of France in December 2014. Samples collected were jejunum from 3 affected animals that had died within the day and pools of feces from 5 affected animals. Next-generation sequencing and data analysis were performed on RNA extracted from the jejunum samples in order to obtain the full-length genome sequence. RT-qPCR targeting the N gene was performed on feces sampled every 2 weeks approximatively for 2 months to assess the duration of viral shedding in the herd.

Results: A PED case was confirmed in the North of France in December 2014. The isolated strain named PEDV FR/001/2014 was genetically related with the recent German strains PEDV GER/L00719/2014 (99.9% of identity) isolated in May 2014. This PEDV strain showed an insertion and deletion in the S gene and has been gathered with other US "InDel" strains. The viral shedding in feces lasted up to 29 days for affected suckling piglets and 20 days for fattening pigs.

Conclusion: The French isolate was an "InDel" strain presented mild pathogenic characteristics as other "InDel" PEDV strains. The shedding of the virus in feces seems to be shorter than other US strains.

Acknowledgements: We thank the French Directorate General for Food (DGAl) for the financial support and also the "Direction départementale de la protection des populations du Nord" and the pig farmer for their assistance in sample collection.

Genetic characterization of porcine epidemic diarrhea virus strains isolated from novel outbreaks in the European Union, Belgium.

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Objective: Porcine epidemic diarrhea virus (PEDV) is an enteropathogenic virus, which causes diarrhea and vomiting in pigs. It was first detected in Europe in the 1970s, after which it circulated for two decades in Europe. Since then, only sporadic outbreaks have been observed. Later in the 80s it appeared in Asia, staying endemic in the population causing mild problems until 2010 when severe outbreaks with high mortality occurred. In 2013 genetically related strains started to circulate in the US, causing severe problems in US swineherds. In 2014, novel PEDV strains with several deletions and insertions in the spike gene were detected. These strains, called INDEL strains, are associated with milder disease symptoms. In May 2014 a first case of PEDV in fattening pigs was detected in Germany. Full genome sequence of isolated strains revealed a close genetically relation between these strains and the US INDEL strains. At the end of January 2015 an outbreak of diarrhea occurred on a Belgian farm in fattening pigs. The faeces were tested for the presence of PEDV RNA and were found positive using an in house RT-qPCR. Because this was the first case of PEDV in decades in Belgium the strain was fully characterized by sequencing. Furthermore samples of other suspected PEDV affected farms where collected and tested for the presence of the virus. For the samples which tested positive in RT-qPCR, the complete spike gene was sequenced and compared with known sequences. In that way we wanted to obtain an idea about which PEDV strains were circulating in Belgium.

Methods: For the full genome sequencing of the first novel Belgian isolate (BEL/15V010/2015) next generation sequencing was applied. Viral particles were purified, RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) and whole transcriptome amplification was performed (WTA2 kit, Sigma Aldrich) to generate cDNA. Sequencing library was prepared using the Nextera XT DNA Library Preparation Kit (Illumina). Sequencing was performed on a HiSeq™ 2500 platform (Illumina) for 301 cycles (150 bp paired end reads). Raw reads were trimmed for quality and adapters using Trimmomatic, and reads were mapped using BWA against the German PEDV strain L00721 (Genbank LM645057). Remaining gaps were closed using Sanger sequencing.

For the spike sequences of the other isolates (n=x), cDNA was generated, followed by PCR using 4 sets of primers to cover the entire open reading frame. PCR products were used for Sanger sequencing using forward and reverse primers. All sequences were analysed using 4Peaks software and alignments and phylogenetic trees were made using the MEGA 5.2.2. software.

Results: PEDV strain 15V010, isolated from a Belgian farm with an outbreak of diarrhea in 2015, contained a genome of 28,028 nucleotides, excluding the poly A tail. The genome organization resembled that of other PEDV strains and genes were arranged as follows 5'- ORF1a/1b-S-ORF3-E-M-N-3'. The 5' UTR had a size of 292nt and the length of the ORF1a/1b, spike, ORF3, E, M, N and 3' UTR were 12,308nt, 4,151nt, 674nt, 231nt, 680nt, 1,325nt and 333nt respectively. This novel Belgian isolate was most closely related (>99%) to the German PEDV strain, PEDV/GER/L00721/2014 (LM645057), whereas it was less closely related to the historic Belgian isolate CV777 and the contemporary highly virulent US strains.

Analysis of the different spike open reading frames showed that all novel Belgium isolates belonged to the S INDEL strains, sharing high similarity with US OH851 strain (KJ399978) and German strains. Spike genes of different new Belgian isolates showed, although isolated in distinct geographically locations, a high genetic relatedness (99.6%-99.9% nucleotide similarity), whereas they were only moderately related to the ancient Belgian isolate CV777 (96%).

Conclusion: A full genome sequence and spike gene sequences of novel Belgian PEDV strains were determined and phylogenetic analysis showed highest similarity to recent German PEDV isolates and US INDEL strains, whereof the latter are thought to be associated with milder disease symptoms. All different isolates showed high relatedness to each other and were distinct from the old Belgian isolate CV777. These findings demonstrate that PEDV is still or again circulating in Europe.

Parallel EPIZONE Coordinating Forum Meeting

Wednesday 2nd September 2015
17:00-18:00
Room Joffre 5

Parallel EPIGENESIS Session: Animal Health In The Caribbean

Wednesday 2nd September 2015
15:00-16:30 17:00-1800
Room Sully 3

Chair: Cedric Lazarus & Nathalie Vachiery

Research and surveillance on Animal Health in the Caribbean

Cedric Lazarus

Livestock Development Officer FAO, Sub-Regional Office for the Caribbean - Barbados

Prior to the formation of the Caribbean Animal Health Network (CaribVET) there was no formally agreed process for the sharing or exchange of animal health surveillance information and no mechanism existed for collaboration among the Caribbean countries. The Caribbean Veterinary Network (CaribVET) established in 2005 with the objective of improving the regional sanitary situation and contributing to the harmonization and reinforcement of animal disease surveillance and control activities in the Caribbean. It is a collaborative network of thirty-one Caribbean countries and territories involving official veterinary services, research institutes, laboratories and regional and international organizations. The Network takes into consideration the importance of animal production to livelihoods, food and nutrition security and the socio-economic development of the countries and through its programs seeks to safeguard animal health within the region. Within the context of animal health research and surveillance CaribVET plays a leading role in harmonizing animal health programs, building collaborative partnerships and improving coordination and at the same time the Network leads efforts to mobilize resources and strengthen communication among the stakeholders of the Network. The Network through it various Working Groups develops and provides tools, protocols and standards to support surveillance activities at the national level and serves as a forum for providing information on emerging and reemerging diseases that are a potential threat to the Caribbean. The universities and institutes who are members of the Network undertake research in areas that impact on animal health and livestock production in the Caribbean and provide training for animal health professionals in veterinary laboratory diagnostic procedures, risk analysis, veterinary epidemiology and other related areas. Over the ten years of its existence the network has developed into a credible source of veterinary surveillance information within the Caribbean and is seen by the countries as a partner providing support in the area of animal health. This paper outlines how the network designs and develops veterinary surveillance strategies that support the delivery and development of animal health services across the Caribbean.

Orbiviruses in the Caribbean - should we worry about them?

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The best known and most significant of the Orbiviruses affecting animals in the Caribbean are Bluetongue virus (BTV) and Epizootic Haemorrhagic Disease virus (EHDV). These are segmented, double stranded RNA viruses with multiple serotypes, which are capable of undergoing reassortment of their genetic segments in a similar way to influenza viruses. EHDV predominantly causes disease in deer, although some strains of the virus can cause severe clinical signs in cattle. BTV predominantly causes disease in sheep, however cattle, goats and deer can also be clinically affected under some circumstances.

In the Caribbean and the Americas, BTV has for some time been considered to be a virus that causes limited or no disease, and the virus has been seen as being a block to the free trade of ruminants within these regions. This situation did change a little when a virulent and reproductive strain of BTV-(BTV-8) appeared and spread in Northern and Western Europe from 2006-2009. Since 2007 strains of BTV have also been discovered to be capable of being transmitted through both direct and oral routes, dispelling the previous belief that BTV was only capable of being transmitted through the bite of Culicoides biting midges. It is now clear that some strains of BTV are potentially more 'dangerous' than others, so the Caribbean and the Americas need to be on their guard, through continued surveillance, in order to monitor which of the BTV serotypes and strains are present.

Limited levels of surveillance have been and are being carried out for BTV and EHDV within the Caribbean region. In surveillance studies carried out in the early 80s and the early 90s, BTV was detected in Trinidad, Tobago, Barbados, Puerto Rico, Jamaica, Martinique, Guadeloupe and the Dominican Republic. Serotypes 1, 3, 4, 6, 8, 11, 12, and 17 were detected by serum neutralisation tests (SNT) and serotypes 1, 3, 4, 6, 8, 12 and 17 were detected by virus isolation. In the mid-2000s, BTV 2, 10, 11, 13, 17, 18, 22, 24 were isolated from Martinique and BTV-5 and 7 were isolated from Guadeloupe.

We recently carried out a study to establish if BTV and EHDV are currently circulating in the Republic of Trinidad and Tobago (T&T) and, if present, identify the specific serotypes circulating. Fifty-nine naïve dairy cattle (54 cows; 5 bulls) imported into T&T from the USA were bled monthly for six months from the time of their arrival in T&T. By month 3 all of the imported cattle had seroconverted for BTV and were group-specific RT-PCR positive for BTV RNA. Serotype-specific RT-PCR (for serotypes 1-26) and virus isolation on *Culicoides* (KC) cell lines were carried out on samples with a CT value of less than 30. Of the 18 samples tested by serotype-specific PCR, two were positive for BTV-3, six for BTV-5; three for BTV-12 and six for BTV-17. Four samples were co-infected, one co-positive for BTV-5 and 17 and three co-positive for BTV-12 and 17. In five of the 18 samples the serotype was not identified by serotype-specific PCR. BTV serotypes 1, 2, 5, 12 & 17 were isolated from the samples on *Culicoides* (KC) cells.

Samples from the same group of imported cattle were also tested for the presence of antibodies to EHDV, as well as for EHDV RNA. By month 6, all of the imported cattle had seroconverted for EHDV and were group-specific RT-PCR positive. Serotyping and virus isolation to further identify and characterize the EHDV serotypes / strains that are circulating is currently ongoing.

It is clear from these and previous results that multiple serotypes of BTV (and possibly EHDV) are co-circulating in countries across the Caribbean region. The presence of multiple serotypes of BTV, in combination with limited or no disease, points towards an endemically stable situation. BTV is therefore not likely to cause disease in indigenous ruminant populations within the Caribbean, except in the case when naïve ruminants, especially sheep, are imported into the region.

Similar BTV serotypes are circulating within the Caribbean and the Americas making the risk posed by the introduction of strains through the movement of BTV infected animals within the region low. Even if a new BTV serotype was introduced into a country, it is likely that the indigenous population would have developed some level of cross-immunity to the 'new' strain, due to the fact that the 'local' population is likely to have been infected with multiple BTV serotypes. It is however recommended that all countries within the Caribbean region wishing to trade in ruminants carry out surveillance to identify which serotypes of BTV they have circulating. In this way countries can scientifically assess the risk of introducing new BTV serotypes through trade in animals, and the likelihood that disease could result from the 'new' introduced serotypes should they go on to infect the indigenous 'local' ruminant populations.

Molecular epidemiology of two important viral diseases of pigs in Cuba: classical swine fever and swine influenza

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Since 90's decade, when the economic crisis in Cuba started, the pig industry gained a relevant importance, mainly due to the collapse of the dairy, cattle and broilers as animal protein sources for the Cuban people. However, the high yield expected from the swine had been affected, especially by the sanitary situation of the swine herds in Cuba.

On the one hand, the respiratory diseases have been one of the most common problems affecting the Cuban swine herds. Different viral pathogens have been identified linked to this sanitary problem, such as porcine circovirus type 2 and influenza A virus (IA). On the other hand, since 1993, classical swine fever virus (CSFV) has been the most important viral agent devastating the pig industry in Cuba. Even though CSFV has been under a control program, the epidemiological situation of the disease in the country is highly complicated, with the emergence of several strains with different virulence degree. The current work was focused on providing an overview about the molecular epidemiology analysis of two important viral diseases affecting pigs in Cuba: influenza A virus and classical swine fever virus.

Regarding influenza A virus, in 2010 a study of molecular surveillance to detect pandemic H1N1/2009 influenza virus in Cuban swine herds was conducted. As result, the presence of pandemic influenza A virus was detected for the first time. In addition, phylogenetic analysis and molecular characterization of three viral isolates were performed. Phylogenetic relationships confirmed that all of the eight genes of the three isolates were derived from the pandemic H1N1/2009 virus. The Cuban isolates, formed an independent cluster within the pandemic H1N1/2009 influenza strains. Different molecular markers, previously described in pandemic H1N1/2009 influenza viruses, related with adaptive evolution, viral evasion from the host-immune response, virulence and dissemination were also present in Cuban pandemic H1N1/2009 isolates. More recently, the emergence of reassorted H1N1 swine influenza virus, originated from a reassortment event between the H1N1 pandemic influenza virus (H1N1p/2009) and endemic swine influenza virus in Cuban swine population has been described.

On the other hand, in Cuba, classical swine fever (CSF) has become an endemic disease with several outbreaks each year, despite the implemented vaccination program. Interestingly, a trend towards a milder presentation of the disease has been observed among the animals during the last years. A recent study showed that the positive selection pressure acting on partial E2 gene of CSFV Cuban isolates is a mechanism driving the evolutionary process for CSFV in swine populations under regular vaccination. The results obtained also highlighted a possible association between escape viral variants and the alterations observed in the virulence and pathogenesis of the virus. Therefore, it was proposed that while the vaccination programs have not led to a genotype change, alterations in virulence were suggested to arise.

Situation of the Porcine encephalomyelitis with teschovirus in the Republic of Haiti

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In February 2009, outbreaks of Porcine Encephalomyelitis with Teschovirus (Teschen Disease) were observed for the first time in the Republic of Haiti. There was no plausible explanation regarding the origin of the disease because officially, the disease didn't exist in the American continent. The occurrence of the Teschen disease had complicated the sanitary situation of pig production since the country has been re-contaminated in 1996 by the Classical Swine Fever virus (CSFV) after its eradication in 1984. Subsequently, there were two transboundary diseases that affected pig production in Haiti, causing important losses in the country's rural economy.

The agent of the disease has been isolated from brain samples of sick pigs by the American veterinary laboratories: National Veterinary Services Laboratory (NVSL) -Foreign Animal Disease Diagnostic Laboratory (FADDL) and NVSL -Ames (Iowa) in March 2009 and identified as Porcine Teschovirus (PTV type 1 (PTV-1)), genus

Teschovirus, family *Picornaviridae*. The clinical signs of the Teschen disease were similar to those of the Classical Swine Fever but more severe so that the pig producers didn't take time to consider it as the most important swine disease in the country. The rates of morbidity and mortality were respectively estimated at 60% and 40% in some areas at the beginning of the outbreak. Clinically, it is characterized principally by central nervous system (CNS) disorders (ataxia and paralysis).

To face this situation, the Ministry of Agriculture has requested technical assistance from the Food and Agriculture Organization (FAO) to determine the current epidemiological situation of the disease and help in developing a program for its control or eradication. But there were no commercial Teschen vaccines available in the international market and the international Laboratories were not interested to produce them for Haiti. The Ministry of Agriculture had to wait three years before being granted access to the Teschen vaccines produced by Newport Laboratory (USA), thanks to U.S. Department of Agriculture's Animal and Plant Health Inspection Service (USDA/APHIS). Consequently, PTV-1 was spread from the Artibonite Valley where the first outbreak occurred to the other regions of the country, including areas near the border with the Dominican Republic. Other institutions such as USDA/APHIS and the Inter-American Institute for Cooperation on Agriculture (IICA) have joined FAO to conduct a survey, in cooperation with the Haitian and Dominican Veterinary Services during the year 2010, in order to determine the level of circulation of the Teschovirus, particularly in the departments that were first infected. At this moment, the morbidity and the mortality were evaluated respectively at 30% and 10%.

The purpose of the survey was also i) to determine if there were no other disease agents circulating in the Haitian swine population in addition to CSFV and PTV-1, such as Porcine Circovirus (PCV) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and ii) to find out if the immunosuppressive effect of these eventual agents had facilitated the severe expression of Teschen disease (PTV-1) under the Haitian conditions of pig production.

This survey has been conducted in 35 swine premises located in 10 areas distributed in two geographical departments (Artibonite and Plateau Central) where sick pigs have been observed. Different types of samples have been collected to be tested at the American Laboratories NVSL-FADDL and NVSL-Ames. A total of 111 sick and normal pigs were examined and the major clinical signs observed were central nervous system disorders (ataxia and paralysis). Pigs of different ages were affected. 109 serums and 63 tissues samples were collected: serums from 23 sick and 86 apparently healthy pigs, blood samples from 21 sick pigs and brains, spinal cords and other tissues from 8 pigs humanely euthanized. Few lesions were observed at post mortem.

The Laboratory results indicated that of the 109 serums, 49.5% were positive for Porcine Teschovirus type 1 (PTV-1), 58.7% for Porcine Circovirus type 2 (PCV-2) and 49,5% for CSFV in serology, 7.3% for Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). Swine Influenza Virus (SIV) has been also isolated: H3N2 (11.9%) and H1N1 (22.0%). Among the 54 positive pigs for antibody to PTV-1 , 35 were tested positive for antibody to PCV-2 (64.8%) and 5 for antibody to CSFV (9.25%). But the results were negative for antibodies to Pseudorabies, Encephalomyocarditis (EMC) and Porcine Hemagglutinating Encephalomyelitis (PHE). On the 8 euthanatized pigs, the Laboratory results post mortem have revealed that i) PTV-1 has been detected in 6 of 8 brains and 6 of 8 spinal samples by RT-PCR and ii) PCV-2 in 6 of 8 tonsil samples by PCR. The sequencing of the virus has also been done by the American Laboratories NVSL-FADDL and NVSL-Ames. The molecular analysis has put in evidence a high similitude between the PTV-1 virus present in Haiti and the one existing in Madagascar.

It's important to mention that almost all the sick pigs observed in the field from 2009 to 2013 were generally considered by the veterinary technicians as infected by PTV-1 because the Veterinary Services didn't get access to Teschen vaccines to fight against PTV-1 circulation. Therefore, the peasants refused to cooperate with the CSF Control Program. They couldn't t understand why their pigs continued to die after being vaccinated against CSF. They concluded that the CSF vaccines were responsible of the mortality of their pigs.

From 2012 to 2015, the epidemiological surveillance didn't achieve a lot of progress because of a lack of financing. But 47 blood samples were collected on sick pigs with clinical signs related to both Swine diseases: Classical Swine Fever and Teschen Disease. They were tested by conventional PCR at the National Veterinary Laboratory of Haiti. The results of these tests are: one (1) pig positive for PTV-1, (2.1%) and 25 pigs positive for CSFV (53.2%). The occurrence of Teschen Disease has blocked the development of the CSF Control Program and the prevalence of the latter is now higher than the former. Since 2010, the country has lost all its advances in the process of CSF control.

In areas with high risk level, vaccination campaigns are organized with the Teschen vaccines bought from the Newport Laboratory. Clinically, the severity of the Teschen symptoms has considerably decreased. But, it doesn't mean that there is establishment of a natural immunity as mentioned in the literature. An initiative of active epidemiological surveillance is going to start next September to collect recent data on the evolution of the Teschen disease and Classical Swine Fever, and to develop a program for the eradication of both pig diseases.

Rabies in the Caribbean

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10: Pan American Health Organization - Trinidad and Tobago

Objective: The Pan American Health Organization (PAHO) regional program introduced to Latin America and the Caribbean (LAC) in 1983 was ratified in 2009 with the goal of eliminating dog-transmitted human rabies by 2015. Despite a >90% decrease in cases, the latter has been extended to 2018 due to persistence in certain areas. In discussions about rabies, LAC are often grouped as one. However, data from Latin America generally obscures the Caribbean situation so that it is inadequately represented in the literature. The present study aims to independently analyse the rabies situation in the Caribbean through use of an internal Caribbean Network (Caribbean Animal Health Network), and to examine the changing epidemiology of the disease in comparison to Latin America.

Methods: A questionnaire was developed and administered in February 2014, to the 33 countries or territories of the Caribbean Animal Health Network (CaribVET), through the Adobe Forms Central Web-based platform. Country submissions were collected from April 2014 to June 2015. Responses from30 countries were analysed from June to July 2015 and results were summarized into a regional situation analysis. Complementary information was obtained through a comprehensive literature review using internet searches and the institutional libraries of the PAHO Office in Trinidad and Tobago and the Caribbean Public Health Agency (CARPHA). Selected literature was then summarized and collated with questionnaire responses.

Results: Rabies is a notifiable disease in almost all islands and territories of the Caribbean region, where the disease is present in ten countries and/territories (Trinidad and Tobago, Belize, Grenada, Guyana, Suriname, Cuba, Puerto Rico, Dominican Republic, French Guiana and Haiti). In most instances, the disease is endemic in wildlife with periodic spill over into domestic animals; however urban (canine) rabies still occurs in Hispaniola (Dominican Republic and Haiti) and Cuba. Caribbean sylvatic rabies is maintained by two main reservoir hosts, the vampire bat and the mongoose. The main reservoir host is the mongoose in Grenada, Puerto Rico and Cuba, and the vampire bat in Trinidad and Tobago, Guyana, Suriname, Belize and French Guiana.

In four (40%) rabies endemic Caribbean countries and/territories (French Guiana, Dominican Republic, Haiti, Cuba) human cases have occurred within the last 10 years. The estimated number of human rabies cases per year was highest in Haiti (6-10 cases) and lowest in French Guiana (0-1 cases). Sporadic human cases also occur in Suriname every 8-10 years, with the last case reported in 1998. Rabies cases were reported in the animal population, within the last 10 years, in all endemic countries except Suriname (last case reported in 2002). The average number of cases reported per year ranged from 0-1 (French Guiana) to 90 (Cuba). Cattle were the most significantly affected species, particularly in countries with vampire bat rabies.

Human bite incidents from potential rabies vectors are reportable in 8 (80%) rabies endemic countries, (exclu-

ding Trinidad and Tobago and Suriname) and in 8 (40%) non-endemic countries. In contrast, animal bite incidents are reportable in 7 (70%) endemic versus 6 (30%) non-endemic countries. All endemic countries/territories (except Suriname) have passive or enhanced passive surveillance programs for animal rabies. Active surveillance in the stray domestic carnivore population is carried out in Cuba, Haiti, Dominican Republic and Grenada, whereas active surveillance is conducted in wildlife (bats and mongoose) exclusively in Trinidad and Puerto Rico. None of the non-endemic countries conduct rabies diagnostic testing for humans or animals. Among endemic countries, only Cuba, Dominican Republic and Puerto Rico conduct human rabies diagnostics, and all but Belize, Suriname and Guyana conduct animal rabies diagnostics.

Most rabies-free countries did not implement rabies control programs to attain their disease free status and the risk of rabies introduction was considered by the local veterinary authorities to be low in 60% of these countries, with illegal importation of dogs being the most relevant route. Conversely, national legislation for animal rabies control and prevention exists in all endemic countries apart from Haiti, Belize and Suriname.

Animal vaccination strategies for the prevention and control of rabies in the Caribbean, mainly target bovine and domestic carnivore animal populations, and governments provide vaccines free of charge in most (60%) rabies endemic countries. Annual mass vaccination programmes are carried out for both cattle and domestic carnivores (dogs and cats) in French Guiana, Grenada, Puerto Rico and Belize. The domestic carnivore populations are targeted in Cuba, the Dominican Republic and Haiti with estimated vaccine coverages of >90%, 80% and 40-50% respectively. The bovine population in Trinidad is largely covered by herd immunity with 70% vaccine coverage, compared to the 10% in Guyana that vaccinates only in outbreak situations. Vaccination is legislatively mandatory for the bovine population of Trinidad, and for both the bovine and domestic carnivore populations in French Guiana.

Almost all countries in the Caribbean have rabies import health restrictions. In these countries the most regulated species are dogs and cats, with rabies vaccination being a requirement for entry. Countries without rabies-related import restrictions are Grenada, Dominican Republic, Puerto Rico and Sint Eustatius.

Conclusion: Canine-transmitted rabies occurs only on two Caribbean islands (Hispaniola and Cuba) compared to five countries in Latin America (Brazil, Bolivia, Peru, Honduras and Guatemala) with disease control in Haiti presenting the major challenge to elimination in the Caribbean. As of 2004, the prevalence of rabies transmitted by wildlife in the Americas exceeded that of domestic dogs which is mainly reflective of bat-transmitted cases in North and Latin America. However, although the mongoose is the main rabies reservoir host in 30% of endemic Caribbean countries, it is often overlooked as a vector in the LAC literature due to the ubiquitous presence of the vampire bat in Latin America. Furthermore, although vampire rabies predominates among existing reports in humans and domestic animals, other bat species should not be excluded from routine surveillance activities when warranted, as they have previously been implicated in rabies viral transmission in the region. Therefore, the long term objectives of rabies elimination programs in LAC therefore need to take into account the diverse rabies epidemiology within the Caribbean.

Results of this study underscore the importance of regional animal health networks such as CaribVET in coordinating expertise and resources, facilitating regional training and sharing of information and knowledge. The Caribbean has limited rabies diagnostic capacity compared to Latin America. However, regional collaboration through CaribVET can increase the laboratory capability in countries without independent diagnostic facilities. Also a recent regional training workshop organized by CaribVET resulted in standardization of the rabies diagnostic testing regime throughout the Caribbean which will increase confidence in results. Regional collaboration can also facilitate the development of common protocols and regulations for the control and prevention of rabies in countries with similar epidemiological circumstances.

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West-Nile in the Caribbean

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Since its discovery in Uganda (1937), West Nile (WN) fever historically remained confined in Africa and Middle East with sporadic incursions in Southern Europe. However, it has expanded in the last decades, and is now one of the most widespread arboviruses in the world. Nowadays WN remains present over six continents.

The disease is produced by a virus (WNV, genus *Flavivirus*) and transmitted by mosquitoes among susceptible hosts, usually birds. The virus can also affect dead-end hosts, like humans and equines. The identification of the drivers for WN emergence and spread is difficult. At local scale, WN transmission cycle can occur through different ecosystems with involvement of different species of vectors and hosts. High viral genetic variation and wide range of vectors and hosts makes WN a complex arthropod-borne disease. Recently, up to nine different WNV lineages have been proposed. WNV has been detected in more than 60 mosquito species in 11 genera. However species in the *Culex* genus are considered the main WNV vectors worldwide. Major amplifying hosts are birds, with more than 300 species of birds supporting infection. Mammals are generally considered as dead-end hosts as they are not efficient WNV amplifiers. Nevertheless, multiple mammalian species, amphibian and reptiles are susceptible to WNV infection.

WNV emerged in the New World in New York, 1999. Since then the virus provoked in the USA the major WNV epidemics ever recorded globally. Disease burden was high, causing significant morbidity and mortality in birds, horses and humans. The disease further spread northward (Canada) and southward. The southern spread of WNV into the Caribbean, Central and South America was apparently silent. In contrast to USA and Canada, WNV has caused no or very limited health impact on animal and human populations in the Caribbean. The apparent absence of bird mortality and clinical manifestations among humans or equines makes difficult to track WNV spread in the region. Thus, evidence for WNV circulation is mostly based on serological evidences in a region with other antigenically cross-reacting viruses potentially co-circulating. In the Caribbean, the disease was recorded for the first time in October 2001 in Cayman Islands, on a patient without previous history of travel. First serological investigations were implemented by 2001/2002 across the Caribbean Sea in Mexico, in the Greater Antilles (Dominican Republic, Jamaica and Puerto Rico) and in the Lesser Antilles (Guadeloupe). Such early wave of activities serologically enabled to detect WNV circulation among birds and equines in the Caribbean. Since then, other serological studies supported evidence for consistent WNV circulation in the Caribbean region, Central and South America, including records of sporadic human and equine cases.

The Great Caribbean region is very diverse and heterogeneous. Wide environmental and climatic variation is found along its number of islands but also some continental countries/territories. The Greater and Lesser Antilles are situated at the Carrefour of North and South America, along the “Mississippi and the Atlantic migratory flyways”. The most likely way of WNV introduction in the region is through infected wild birds flying from North to South America. What remains unclear is whether endemic WNV cycles were established or whether detection follows regular introduction by wild birds. Also in some islands (Martinique at least, Guadeloupe’s sister island) several serological investigations have been conducted in horses. However these investigations never succeeded in evidencing WNV circulation suggesting heterogeneous distribution of WNV in the region due to

(still) undetermined factors. Also, the diversity of climate and environments in the Caribbean (Greater Antilles vs Lesser Antilles) suggests different epidemiological cycles.

Unfortunately, information on mosquitoes and hosts is scarce and heterogeneous, and viral isolations have been much more challenging than expected. Therefore the dynamics of WNV in the Caribbean still remains puzzling. Identification and characterization of viral strains circulating, enzootic and bridge vectors and potential amplifying hosts remains a key issue. Similarly, the marked difference in epidemiological patterns and significant differences in morbidity between North and Southern continent remains unexplained. Ongoing activities on WNV in the region aim at reducing knowledge gaps on WN fever in Caribbean ecosystems.

Avian influenza in Belize

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Belize implemented its active surveillance programme for avian influenza (AI) in 1999. This was as a result of the high threat arising from the avian influenza H5N2 outbreaks which started in 1994 in the neighbouring country of Mexico. When Guatemala and El Salvador reported low pathogenic avian influenza H5N2 in 2000 and 2002, respectively, the active surveillance for avian influenza was strengthened. In 2009, through the implementation of the Belize Poultry Improvement Plan (BPIP), avian influenza monitoring of chicken broiler and layer breeder flocks commenced with breeder flocks being tested three times in their life. 2014 was no different than other years in avian influenza surveillance activities including active and passive surveillance and monitoring of breeder flocks.

Blood samples collected from active and passive surveillance for avian influenza in 2014 all tested negative. Blood samples collected under BPIP in 2014 also all tested negative to avian influenza except for samples collected in Spanish Lookout, Cayo District from an 8000 chicken broiler breeder flock 39 weeks of age, in early December, 2014. The sera that tested positive as well as swab samples from AI antibody positive birds were sent to the National Veterinary Services Laboratory in Ames, Iowa, USA, a reference laboratory for the World Organisation for Animal Health (OIE). Thus, by the 22 January 2015 the Belize Agricultural Health Authority (BAHA) had confirmation that there was an exposure to avian influenza H5N2. As there was great uncertainty as to the nature of the exposure particularly as antibody-positive birds were not showing any clinical signs of disease, sentinel birds were placed in known exposed flocks. PCR confirmation was obtained on the 14 February 2015. The virus was sequenced as: North American LPAI H5N2 98.8% similar to A/CK/Mexico/55-12/2012 H5N2

The Belize viruses are highly similar to Low pathogenic avian influenza (LPAI) H5N2 viruses isolated in Mexico. The Mexico LPAI viruses have circulated in poultry in Mexico since 1995 and are well adapted to poultry. Virus characterization results received the 12 March 2015 identified the virus as low pathogenic avian influenza by cleavage site analysis as well as in vivo assay. There have never been any clinical signs associated with the LPAI H5N2 virus in Belize; producers are, in fact, reporting better performance of their poultry but this is probably due to the enhanced management and biosecurity measures implemented.

BAHA responded swiftly to the serological detection of avian influenza by the immediate implementation of quarantine and movement control, enhanced biosecurity at farm and community level and the testing of all long lived poultry in Spanish Lookout, surrounding villages and communities considered high risk. Country wide surveillance has been strengthened with at risk communities having commercial poultry being periodically tested.

The epidemiological surveillance showed that the outbreak was localized in a hot zone in Spanish Lookout and in two nearby villages, Buenavista and Billy White. Once the avian influenza virus was characterized as LPAI H5N2 and virus circulation confirmed, additional control measures were implemented: stamping out of infected flocks and cleaning and disinfection. Vaccination was considered as a control measure but it has not been approved. Movement control at six designated checkpoints considerably reduced the movement of risk poultry and poultry products. Security surveillance in the area has led to confiscations of spent hens smuggled out of the infected

area; BAHA responded swiftly to these confiscations, testing, destroying and disposing of the smuggled birds.

The source of the virus is unknown. Possible entry points considered include illegal importation of avian influenza vaccine, illegal importation of poultry vaccine contaminated with avian influenza virus, wild bird and horizontal transmission through contaminated fomite used in the trade of live spent hens.

Public awareness has been continuous. Initially, the frequency of meetings with community leaders, first responders, producers and the media was quite high. Over time these have decreased considerably; the Chief Veterinary Officer holds biweekly planning meetings with the Belize Poultry Association and Spanish Lookout representatives. BAHA has maintained its OIE notifications.

The avian influenza outbreak affected the most progressive and poultry dense Mennonite community of Belize. Given the economic power of this community control measures were undertaken even though the government has not offered any compensation for stamping out and cleaning and disinfection. As a result of the outbreak, the BAHA laboratory capability has been strengthened and is now able to perform PCR and HI analysis in addition to AGID. The Spanish Lookout community also installed ELISA testing capability within the community to assist in the surveillance work. The Spanish Lookout community has established new standards and guidelines for raising poultry within the community and all farmers are now also very aware of the importance of strengthening their biosecurity measures.

BAHA does a three week cycle ("sweeps") of testing of all long lived birds in Spanish Lookout and satellite villages for avian influenza. These sweeps have detected 26 positive flocks to date with the last infected flock being detected on the 5 June 2015 and disinfected on the 9 June 2015 in Spanish Lookout. A total of 82,145 birds were destroyed through depopulation. Under OIE guidelines a country may regain its free status three months after disinfection of all affected establishments along with the appropriate surveillance in accordance to OIE guidelines. BAHA hopes to regain its AI free status on the 9 September 2015 after completing a total of 7 sweeps, the last sweep being conducted on the 23 August 2015.

Avian diseases in Cuba and the Caribean

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The global poultry sector is characterized by faster growth in consumption and trade than any other major agricultural sector. It is known that poultry meat and eggs contain protein and micronutrients, which could provide an important contribution to the health and nutrition of consumers. In addition to the great biological value of poultry by-products, they haven't religious or cultural restrictions and use provides cheapest products than other livestock sectors at world-wide level. The poultry production has a smaller physical footprint than other livestock systems and may contribute less than other segments of the livestock sector to environmental pollution.

Poultry production at world level is dominate by highly intensive systems but small-scale one survive as long as they are needed for social functions, food security and livelihood support among poor households. This coexistence of system with different levels of biosecurity could propitiate that diseases like avian influenza or Newcastle become endemics and demand science based planning of poultry development, surveillance, improved preventive disease control and biosecurity measures.

The Caribbean region is highlighted by several countries with pronounced levels of poultry production. In fact, over the past decade, the Caribbean poultry industry has seen significant growth and has become the largest agro-industrial enterprise in the process surpassing other traditional agricultural commodities like sugar and rice. Caribbean cumulative growth rates in poultry production are among the highest at the world levels in the last decade. This insular region mainly conformed by small to medium territories, mostly determine either high poultry densities or close proximity of poultry premises that propitiate disease transmission and risk of become endemic. In addition, in such context is difficult overtake in spite of compartmentalization and regionalization

have not been generally accepted. This sanitary treats could be also favored by meteorological event like hurricane that regularly affect the region.

The main diseases with more impact sanitarium in poultry at world level are the avian Influenza (AI) and Newcastle disease (NCD). However, there are other illnesses of viral origin that influence negatively in the productive potential and bear control programs with vaccination, although they don't produce high mortalities levels.

In the Caribbean, the official sanitary information regarding the avian specie is scarce, as well as the poultry industry is not well developed but in Jamaica, Trinidad and Tobago, Dominican Republic and Cuba. There have not been outbreaks of HPAI, while outbreaks of LPAI have been reported in Haiti and Dominican Republic in previous years and in Belize at present. During 2013 and 2014 only five countries reported to OIE avian diseases: Cuba, Dominican Republic, Belize, Haiti, Martinique and Suriname. The most reported diseases are NCD and Infectious Bursal Disease (IBD), both under vaccination programs.

In Cuba there are established vaccination programs in the organized poultry industry. We have been carried out investigations during the last years to know better the behavior of some of these agents like the Infectious Bronchitis (IB) and IBD. In the case of AI, exotic disease for Cuba, the investigations have been aimed to increase the diagnostics capacities at national level and to optimize the active surveillance risk based.

IB. The avian infectious bronchitis (IB) is a highly contagious viral disease of poultry caused by the IB virus. It has been attempted the use of attenuated alive and inactive vaccines for the control of the disease.

The emergence of new genotypes/serotypes and the low crossed protection observed among the known serotypes have complicated the control programs in different geographical regions. In Cuba, the growing epidemiologic importance of the respiratory processes in the chicken farms demands an etiological clarification of them. The infection status by the IB virus, the genetic diversity and the possible implication of the same one in the processes with respiratory clinic in the populations of egg-laying hens in 2008-2012 periods were determined after 20 years control program of this agent.

The studies about the genetic diversity and the phylogenetic relationships evidenced the circulation of two new genotypes in the farms under study, one of them with nephropathogenic potential. An assay of RT-PCR in real time was developed based on SYBR-Green able to detect the virus to differentiate strains of the Massachusetts (vaccinate) genotype of other genotypes in the same reaction. The proposed trial is a useful tool for the quick identification of outbreaks and for programs of surveillance in suspicious cases of IB, mainly in countries with vaccination program.

IBD. Infectious bursal disease (IBD) is a highly contagious and acute viral disease, which has caused high mortality rates in birds and considerable economic losses in different parts of the world for more than two decades and it still represents a considerable threat to poultry industry. The study was designed to rigorously measure the reliability of a phylogenetic marker included into segment B. This marker can facilitate molecular epidemiology studies, incorporating this segment of the viral genome, to better explain the links between emergence, spreading and maintenance of the very virulent IBD virus (vvIBDV) strains worldwide.

Sequences of the hyper-variable region of the VP2 (HVR-VP2) gene from IBDV strains isolated from diverse geographic locations were obtained from the GenBank database; Cuban sequences were obtained. All sequences were analysed by Bayesian phylogeographic analysis, implemented in the Bayesian Evolutionary Analysis Sampling Trees (BEAST), Bayesian Tip-association Significance testing (BaTS) and Spatial Phylogenetic. Reconstruction of Evolutionary Dynamics (SPREAD) software packages. Selection pressure on the HVR-VP2 was also assessed. The phylogeographic association-trait analysis showed that viruses sampled from individual countries tend to cluster together, suggesting a geographic pattern for IBDV strains. Spatial analysis from this study revealed that strains carrying sequences that were linked to increased virulence of IBDV appeared in Iran in 1981 and spread to Western Europe (Belgium) in 1987, Africa (Egypt) around 1990, East Asia (China and Japan) in 1993, the Caribbean Region (Cuba) by 1995 and South America (Brazil) around 2000. Selection pressure analysis showed that several codons in the HVR-VP2 region were under purifying selection. This work is the first study applying the Bayesian phylogeographic reconstruction approach to analyze the emergence and spread of vvIBDV strains worldwide.

AI. Increasing diversity among H5 hemagglutinin (HA) subtype avian influenza (AI) viruses has resulted in the need

of novel sensitive and specific molecular assays. In this study, a SYBR Green-based real-time RT-PCR (RRT-PCR) assay was developed for the detection of H5 subtype AI virus. Sequence analysis of the Mexican lineage H5N2 isolates (subgroup B) revealed several mismatches in the primer/hydrolysis probe set reported in the commonly used RRT-PCR assay for the detection of H5 North American lineage. The assay was designed to circumvent the challenge that these viruses represent for the specific detection of H5 subtype AI viruses. This RRT-PCR assay successfully detected a range of different H5 subtype AI strains from both Eurasian and North American lineages representing different avian H5 HA clades from diverse geographical locations. The sensitivity of the method was determined by using in vitro-transcribed RNA and 10-fold serial dilutions of titrated AI viruses. High sensitivity levels were obtained, with limits of detection of 10^0 50% egg infectious dose (EID_{50})/mL and 4.2 gene copies/ml. The linear ranges of the assay span within 10^6e10^0 EID_{50}/mL and 10^6e10^0 gene copies/ml. The results obtained from this method were directly compared with those of the H5 RRTPCR assay recommended by the OIE. The comparison was performed with 110 tracheal and cloacal swabs from various bird species collected during field and laboratory investigations in Eurasia and Africa in 2006 and 2008 and showed 100% agreement. This assay is recommended as an alternative method, also allowing 'double check' approach detection, to be use mainly in outbreak scenarios with higher risk of poultry infections by Central American/Caribbean H5 AI viruses.

The serological surveys, coupled with passive surveillance activities, are essential to detect sub-clinical infections by LPAI viruses, H5 and H7 subtypes. However the proper planning of an active surveillance system should be based on a careful estimation of its performance. Therefore, the sensitivity of the active surveillance system for AI in the western region of Cuba was assessed by a stochastic model quantifying the probability of revealing at least one animal infected by H5 or H7 subtype. The diagnostic sensitivity of the haemagglutination inhibition assay and different levels of within-flock prevalence (5%, 12% and 30%) were considered. The sensitivity of the surveillance system was then assessed under five different samples size scenarios: testing 20, 30, 40, 50 or 60 animals in each flock. Poultry flock sites in the western region of Cuba with a size ranging from 10,000 to 335,000 birds were included in the study.

The sensibility of the system of surveillance of exotic diseases was determined, as well as other critical indicators of the performance of the surveillance, its systematic evaluation and the establishment of improvement plans. It was also developed a map of quantitative risk of introduction of exotic diseases in Cuba and designed a system of surveillance based on that risk that improves the cost-effectiveness relationship of the activity.

The reemergence of the HPAI in North America, the gaps checked in the levels of biosecurity of the affected farms, their fast dissemination and the increase of trade in the Caribbean region impose the establishment of contingency and emergency plans to stop their dissemination in the event of presence in our geographical area and the increment of the information diffusion and training of human resources. On the other hand, it demands the Caribbean much more sanitary information on the avian species, in particular.

Identification of viruses causing respiratory disease of poultry flocks in Trinidad and Tobago

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Objectives: Despite efforts to curb outbreaks of respiratory infections on poultry farms in Trinidad and Tobago (T&T), they are known to cause significant production losses and mortality. Currently there is little or no information about the naturally occurring viruses circulating on poultry farms in T&T. This study set out to identify which respiratory viruses of poultry, with worldwide and regional significance, were circulating and causing respiratory disease outbreaks in T&T poultry. Avian Influenza Virus (AIV), Newcastle Disease Virus (NDV),

Infectious Bronchitis Virus (IBV), Avian Pneumovirus (APV) and Infectious Laryngotracheitis Virus (ILTV), which are all known respiratory viruses of poultry, were selected for this study. In some poultry farms, vaccination against these viruses are used as a preventative measure, however vaccines may be ineffective against circulating viral strains, and some farms remain unvaccinated and vulnerable. Poultry farmers who vaccinate their flock still experience clinical respiratory signs in their birds. The objective of this study was to improve poultry health through gaining a better understanding of the viruses circulating and causing respiratory disease, so better control and management practices can be implemented in T&T and the wider Caribbean region.

Methods: Serological surveillance was carried out in layer flocks across T&T. Serum samples were collected and tested for antibodies against AIV, IBV, NDV, APV and ILTV in unvaccinated flocks using commercial enzyme linked immunosorbent assay (ELISA) antibody detection kits (IDvet, France; IDEXX, USA). Thirty two farms throughout T&T were identified for sampling based on a proportional sampling distribution of farms at a 98% test sensitivity and a 95% confidence interval. Individual sampling sizes per farm were generated for the various viruses, based on varying predicted prevalence of disease, ranging from 10% to 30% for the different viruses. Clinical surveillance was also carried out through the collection of tracheal swabs, cloacal swabs and serum samples from layer and backyard poultry presenting with clinical signs of respiratory disease. Swab samples from clinically affected birds were tested for the presence of AI, IBV and NDV RNA using real-time reverse transcription polymerase chain reaction (qRT-PCR) commercial detection kits (Primer Design, UK).

Results: Antibodies were detected for NDV, IBV and APV at a rate of 70.0 %, 98.8 % and 89% respectively, while no antibodies were detected against AIV and ILT in the study. Antibody detection across the farms was at a rate of 100 % for NDV and IBV, and at a rate of 96% for APV. Swabs taken from clinically affected birds tested strongly positive by qRT-PCR for IBV RNA, but negative for AIV and NDV RNA. These results indicated that IBV was likely to be the principal causative agent of respiratory disease observed in Trinidad poultry.

Discussion and Conclusion: This study demonstrated a high seroprevalence of three important respiratory viruses (NDV, IBV and APV) in poultry in T&T. Results suggested that IBV is the main causative agent of respiratory disease in layer birds, as well as in backyard poultry in T&T. Vaccination programs are carried out for IBV on some poultry farms in T&T, however there is a high probability that the strains of IBV used in the vaccines may not be protective against the field strains that are circulating and causing disease. The samples found to be qRT-PCR positive for IBV RNA will be further tested by conventional reverse transcription polymerase chain reaction (RT-PCR) using primers to amplify a partial sequence of the S1 gene, a known hypervariable region. Amplicons will then be sequenced and the resulting sequences will be compared alongside other strains of IBV identified worldwide, as well as strains used in the vaccination program in T&T. The S1 sequence comparison of field and vaccine strains will shed light on whether the IBV vaccines currently in use in T&T are likely to be protective against the circulating field strains. Poultry continues to be an important industry within the Caribbean region, so new knowledge in relation to poultry health is of vital importance to this vulnerable industry. The data from this study will be used to improve current disease prevention and control measures used by the poultry industry in T&T and the Caribbean region.

POSTERS SELECTED FOR SHORT ORAL PRESENTATIONS

Parallel ESVV Poster Presentation Session 1

Tuesday 1st September 2015
17:00-18:30
Auditorium Pasteur

Chair: Stephan Zientara & Renata Servan de Almeida

The characterization of avian influenza viruses isolated from wild birds in Vietnam from 2010 to 2014

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Objective: The surveillance of wild birds for avian influenza viruses (AIV) has increased substantially worldwide in recent years due to the spread of the H5N1 HPAI viruses among domestic and wild birds. Wild bird sampling for surveillance of AIV also intensified in South-East Asia including Vietnam, and H5N1 HPAI viruses have been detected in migrating wild birds since 2005. In the present study, due to concerns that wild birds could possibly spread H5N1 viruses, surveillance was conducted to monitor the types of avian influenza viruses circulating among the wild birds migrating to or inhabiting in Vietnam from 20010 to 2014.

Methods: A total of 853 swabs (throat and cloacal) and 147 fecal samples has been obtained from wild birds in Hanoi, Quang Ninh, Nha Trang and Long An provinces, and applied for virus isolation. Each specimen was inoculated into the allantoic cavity of 10-day-old embryonated eggs and incubated at 35 °C for 2 to 3days. The allantoic fluids were tested for hemagglutinating activity and all the hemagglutinating agents were identified in hemagglutination-inhibition and neuraminidase-inhibition tests using specific antisera. Viral RNA was extracted from allantoic fluid and was reverse-transcribed. PCR amplification was performed by using fragment-specific primers. The PCR products were purified with the QIAquick Gel Extraction Kit and sequenced by using the CEQ DTCS-Quick Start Kit on a CEQ 8000 DNA sequencer. Sequence data were compiled with the GENETYX-MAC program, and the nucleotide sequences were compared and the phylogenetic tree generations were conducted using MEGA.

Results: Total 18 influenza A viruses including an H5N6 virus had been isolated (1 H3N2, 3 H3N8, 12 H4N6, 1 H5N6, and 1 H11N9). The H5N6 virus was isolated in 2014 from a Spot-billed Duck that is resident in the southern part of its range from Pakistan and India to southern Japan. Phylogenetic analyses revealed that HA gene of the H5 virus was classified into clade 2.3.4.6. This isolate had a close phylogenetic relationship to H5N6 viruses isolated in South China in 2014. These findings suggest that these H5N6 viruses are circulating and are being maintained in the East, South East and South Asia regions including Vietnam and China. This H5N6 virus showed high pathogenicity to chickens while moderate pathogenicity to domestic ducks.

Conclusion: Taken together, these results also support that wild ducks such as Spot-billed Duck are playing a significant role in the spread and maintenance of avian influenza in the Asia regions including Vietnam.

The effect of Streptococcus suis co-infection on the infection of well-differentiated porcine respiratory epithelial cells by swine influenza viruses

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Objective: Respiratory diseases in swine are responsible for high economic losses in pig industry worldwide. The upper respiratory tract is a reservoir for a heterogeneous community of pathogenic microorganisms and commensals. Pneumonia often occurs due to a combination of various factors including viral and bacterial pathogens as well as environmental factors. A major factor responsible for severe virus infections may be viral-bacterial co-infections. Influenza A viruses are a major cause of acute respiratory disease in pigs which may play an important role in the interspecies transmission of influenza viruses. Swine influenza virus (SIV) strains that are endemic in swine populations worldwide are assigned to the subtypes H1N1, H3N2, or H1N2. Primary target cells for Influenza viruses are the epithelial cells in the respiratory tract. *Streptococcus suis* (*S. suis*) is one of the most important bacterial pathogens affecting the porcine airways causing invasive diseases associated with meningitis, arthritis, septicaemia, and bronchopneumonia. It is an emerging zoonotic agent and it is assumed that *S. suis* adheres to the mucosal epithelium of the upper respiratory tract. Differentiated airway epithelial cells contain special cell types such as ciliated cells or mucus-producing cells that can't be maintained as immortalized cell cultures. We have recently reported a culture system for differentiated respiratory epithelial cells to analyze the infection of porcine influenza viruses in their natural target cells. Therefore, the objectives of this study are to analyze the effect of *Streptococcus suis* (*S. suis*) co-infection on the infection of well-differentiated porcine respiratory epithelial cells by swine influenza virus (SIV).The co-infection studies are expected to provide experimental data how *S. suis* affects infection by influenza viruses with high or low virulence. In addition, the comparison of mono- and co-infection reveals to what extent the bacterial infection enhances the severity of infection by porcine influenza virus.

Methods: Precision-cut lung slices (PCLS) were prepared from fresh lungs of 3 months old healthy pigs and co-infected by porcine influenza viruses of the subtypes H1N1, H3N2 at 10^5 $TCID_{50}$/slice followed byco-infection with *S. suis* wt strain 10 or the non-encapsulated mutant strain 10cpsΔEF at 10^7 CFU/slice, respectively . The effect of co-infection was evaluated by determining: the cytotoxic effect, the amount of infectious virus released into the supernatant, the ciliostatic effect, the bacterial adhesion to and invasion of the respiratory epithelium.

Results: We compared co-infection of PCLS by strains of two subtypes currently prevalent in the swine populations (H3N2, H1N1) followed by subsequent infection with *S. suis* wt strain 10 or the non-encapsulated mutant strain 10cpsΔEF, respectively. The results show that (i) Primary infection by SIV facilitates adherence and colonization of encapsulated *S. suis* (wt.10). (ii) Airway epithelium damage induced by infection of SIV promotes colonization and invasion of both encapsulated and non-encapsulated *S. suis* and this effect is associated with SIV virulence. (iii)Encapsulated *S. suis* affects infection by SIV and reduces the amount of infectious virus detectable in the supernatant.

Conclusion: We observed that adherence and invasion of *S. suis* on PCLS was efficiently promoted by SIV preinfection. The PCLS infection model shows much promise for investigating microbial and host factors to determine the complex mechanisms and dynamics involved in bacterial-viral co-infections.

Keywords: respiratory epithelium, precision-cut lung slices, porcine influenza viruses, *Streptococcus suis*

Investigation of a possible link between vaccination and the 2010 Sheep pox epizootic in Morocco

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Objective: Sheep pox is endemic in most parts of Northern Africa and has the potential to cause severe economic problems. Live attenuated vaccines are used in Morocco, and in many other countries, in order to control the disease. Sheep pox virus (SPPV) re-appeared in 2010 causing a nodular clinical form previously not observed in Morocco. The severe clinical signs observed during the course of this outbreak and initial reports citing similarity in nucleotide sequence between the Moroccan vaccine strain and field isolates warranted a more in depth analysis of this epizootic. It was the purpose of this study to (1) look more closely into the epizootic of 2010 in Morocco (2) investigate the possible link between the Moroccan vaccine used in that period and the virus isolated from the field by exploring sequence similarity in different regions of the genome and by developing PCR methods to differentiate between vaccine and wild-type virus.

Methods: Samples collected from 19 flocks located within four provinces the eastern region of Morocco during the 2010 Outbreak were analysed using real-time PCR panel and an in-house monolayer Elisa. Isolates from different geographic regions were phylogenetically analysed and compared to each other and to the vaccine used in the region. DIVA PCRs were developed to analyze a possible link between the isolates and vaccine.

Results: Aside from scab material, blood was the sample type which most frequently gave a positive result (98% positive) followed by buccal and ocular swabs, 93% and 91% positive, respectively. However, most variability was seen in blood samples when using the Haegeman PCR panel, ranging from 64%, 69% to 85% positivity. Seroconversion for Capx was detected in 80.5% of the animals and in each flock.

Sequence analysis of two genomic regions showed that all isolates obtained from four the provinces of Eastern Morocco were identical and were clearly different from the Moroccan vaccine strain. Using two newly developed DIVA PCRs no trace of wild type SPPV was found in the vaccine and no trace of the vaccine was found in the sampled animals.

Conclusion: Supporting the published findings ocular swabs were found to be a useful sample type to test with a detection rate of the Haegeman PCR-panel of 91%. For rectal and nasal swabs the detection rates were noticeably probably due to a greater sensitivity to the timing of sampling relative to the course of infection. Buccal swabs (detection rate of 93%) were found to be an interesting alternative with the added advantage of being easier to take than ocular swabbing. The PCR-panel detection rate in blood was found to be 98%. However, this sample type may be less suited as the individual PCR detection rates of the PCR-panel were more variable.

Based on the sequences data from the different isolates, it can be stated that a single SPPV strain was responsible for the 2010 epizootic. In addition, no evidence was found linking the vaccine (vaccine strain or presence of wild type virus) directly to the epizootic. However, further analysis is needed to clarify the epidemiological picture in relation to recombination, re-introduction or re-emergence. The two newly developed PCRs, able to differentiate between the RM-65 vaccine strain and wild type SPPV, can be a useful tool in future epidemiological investigations during vaccination programs.

Prevalence of antibodies to selected viral pathogens in wild boars (Sus scrofa) in Serbia

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Objective: There has been a worldwide increase in the number and geographical spread of wild boar populations in recent decades leading to an increase in both the circulation of disease agents and greater contact with domestic animals and humans. Knowledge of diseases circulating among wild boars can be important not only for production and health of domestic pigs, other livestock and wild animals but also for public health. The risk of transmission of pathogens from free-ranging wild boars (Sus scrofa scrofa) to outdoor domestic pigs (S. scro-

fa domesticus) is of increasing concern in many European countries. In Serbia, the wild boar is one of the most important big game species. However, information on the prevalence and distribution of potentially important infectious disease agents among wild boar populations in Serbia is currently very limited, or doesn't exist. The aim of the current study was to investigate the presence of selected viral pathogens in wild boars populations in Serbia, and to assess possible role of wild boars in the epidemiology as reservoirs of these viruses for domestic pigs and other domestic and wild animals and for human population in Serbia.

Methods: Blood samples from 381 wild boars from 53 hunting grounds and 13 out of 25 counties in Serbia, that are 3.66% of predicted number of wild boars (10 409) on the observed territory, and 1.91% of predicted number of wild boars (19 908) from all 12 districts and 142 hunting grounds of Serbia, were collected during the hunting season from October 2011 until March 2012. Blood samples were taken by hunters or by veterinarians from the heart after the wild boars had been shot. Collected blood sera were tested by commercial enzyme-linked immunosorbent assays (ELISAs) for the presence of antibodies against Aujeszky's disease virus (ADV), H1N1 and H3N2 swine influenza viruses (SIV), and hemagglutination inhibition (HI) test was used for detection of antibodies against porcine parvovirus (PPV).

Results: Out of 381 analyzed blood sera samples, antibodies against ADV, SIV H1N1, SIV H3N2 and PPV were detected in 27.03% (103), 4.73% (18), 5.51% (21), and 61.68% (125) samples, respectively. The prevalence of seropositive wild boars to ADV (102/32.38%); SIV H1N1 (18/5.71%); SIV H3N2 (20/6.35%); and PPV (209/66.35%) from 34 hunting grounds on the northern part of the country (315 samples from Vojvodina province) was higher than those found among wild boars (66) from 19 hunting grounds from south part of the country (ADV (1/1.52%); SIV H1N1 (0/all negative); SIV H3N2 (1/1.52%); and PPV (26/39.39%)). Anti-PPV antibodies were detected in wild boars originated from all counties from where the samples were collected. Seropositive wild boars to ADV were detected in all (6 out of 6) tested counties on the northern part and in just one out of 7 counties on the southern part of Serbia. Seropositive wild boars to SIV H1N1 were found just on the northern part of the country in 4 and 3 out of 6 examined counties, and seropositive wild boars to SIV H3N2 were found in 3 out of 6 and in 1 out of 7 examined counties on the northern and southern part of Serbia, respectively.

Conclusion: Our results indicate that wild boar populations throughout the Republic of Serbia are exposed to PPV. Also, our results show that ADV is highly prevalent, especially among wild boars from northern part of Serbia, the area of higher density and intensive pig production. In addition, our results indicate presence of both H1N1 and H3N2 swine influenza virus infections that are more prevalent at the northern part of the country. This is the first comprehensive serologic study on selected viral diseases in wild boars in Republic of Serbia. Our results provide information on the current disease exposure to selected viruses and health status of wild boars in Serbia. The obtained results point on the possibility that wild boars in Serbia may play a significant role in the epidemiology of studied viral diseases and act as a potential reservoir and source of infection for domestic, especially free range pigs, and other animals as well as humans. Further and more comprehensive research is needed including testing of wild boar samples from the whole country and from a few hunting seasons on antibody and virus presence to obtain more conclusive results on presence and role of examined viruses in wild boars on the epidemiology of disease in Serbia.

Keywords: ADV, SIV (H1N1/H3N2), PPV, wild boar, seroprevalence, Serbia

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Is Ornithodoros erraticus able to transmit the Georgia2007/1 African Swine Fever virus isolate to domestic pigs?

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Objective: African swine fever, one of the most devastating diseases affecting wild and domestic swine, is due to a large DNA virus, only member of the *Asfarviridae* family. After ASF introduction in Georgia in 2007, the disease became endemic in the Caucasian region of the Russian Federation and spread towards the Western regions of Europe entering in EU Members States at the beginning of 2014. As no vaccine or antiviral are available to fight against this infection, the only tools to control it are preventive measures based on early detection and actual knowledge of the epidemiological risks. In African sub-Saharan countries, ASF persistence is described to be related to different and complex epidemiological scenarii involving domestic and wild suids and soft tick vectors of the genus *Ornithodoros*. In EU, one species of *Ornithodoros*, *O. erraticus*, is known to be able to maintain and/or transmit some ASFV isolates classified in the genotype I. Recently, the Pirbright Institute also demonstrated that *O. erraticus* was able to amplify the Georgia2007/1 ASFV (genotype II), at least during 3 months. The objective of the current study was to evaluate the in- vitro and in-vivo transmissibility of the Georgia 2007/1 ASFV by infected *O. erraticus* ticks.

Methods: The Georgia 2007/1 ASFV strain belonging to the genotype II, kindly provided by L. Dixon (OIE reference lab), was grown on porcine pulmonary alveolar macrophages to the titre of $10^6$to 10^7 HAD_{50} , then diluted 100-fold into pig blood for tick infection or 1000-fold in medium for intradermal inoculation to pigs.

Ticks were captured in Portugal by F. Boinas and mass reared at CIRAD for one year and a half to obtain a stable and mature population. During this period, several techniques of artificial feeding were tested to optimize the method. In December 2014, 60 adults or last nymphal stages -group A- coming both from field and 1st generation laboratory were artificially engorged on pig blood supplemented with Georgia 2007/1 at a final titre of $10^{4.5}$ HAD_{50}/mL blood. Two other groups of ASFV-free ticks –group B with 60 individuals and group C with 30 individuals- were reared to be used for a second infection directly on infected pigs (group B) and as control group (group C), respectively. Moreover to confirm the possibility to infect ticks through artificial blood meal, another group of 10 ticks was also engorged and tested for virus multiplication three months later. Fifteen other females were also infected and secondarily engorged on ASFV-free pig blood to test in-vitro transmission through virus isolation on second blood meal. Considering that it is difficult to obtain ASFV titres with in-vitro cultivation as high as in infected pigs developing ASFV clinical signs, it seems important to compare ASFV transmissibility between ticks artificially infected in laboratory and ticks directly infected on ASFV-infected pigs and conclude on possible dose effect. In March 2015, 18 Large-White pigs obtained from a high sanitary level field herd were distributed to 4 groups at Anses-Ploufragan high security facilities. Two negative control groups of 3 pigs were either intra-dermally inoculated with MEM or bitten by group C of 30 healthy ticks. One group of 6 pigs was intra-dermally inoculated with 10^3 HAD_{50} ASFV while the last group of 6 pigs was bitten by group A of 60 ticks previously infected through artificial blood meal and dispatched in 10 ticks/pig. Pots of 10 ticks were placed on one ear held there with adhesive tape, then removed after 3 hours. After removal, ticks were numbered in two batches: engorged and unengorged ticks. Finally, as soon as the 6 pigs intra-dermally inoculated with ASFV showed fever and high viremiae, group B of 60 ASFV-free ticks were proposed to engorge on their opposite ear. These ticks would be proposed to secondarily engorge on membrane feeding or healthy pigs three months later.

Post tick feeding or intradermal inoculation, clinical examination and rectal temperatures were recorded daily, until the animals were euthanized or for a minimum period of 18 days. Except on D1 pi, serum and EDTA blood samples were daily collected from all the pigs during the first week pi, then twice a week during the 2 following weeks, and at the day of euthanasia for virological and serological assays. Organ samples were collected during necropsy. The animal experiment protocol was approved by the French national ethics committee ComEth Anses/ENVA/UPEC (10/03/15-9).

Results: Ten ticks from the original batch of ticks that were artificially fed on infectious blood were tested by virus titration. Out of them, 8 were positive with a titre ranging from 10^2 to $10^{4.2}$ HAD_{50}/tick and 2 ticks clearly amplified the virus regarding the estimated amount of virus originally ingested (minimum of 1 log superior). After feeding on pigs, the mean level of engorged ticks was of 62%, whatever the group of pigs.

The experiment, currently running, confirmed the high virulence of the Georgia strain as all the 6 intra-dermally inoculated pigs displayed typical symptoms of ASF including lost of appetite and hyperthermia from D3 pi. The 6 pigs were euthanized from D5 to D7. The group of the 6 pigs bitten by the infected ticks was still healthy at 18 days post feeding, as well as the two negative control groups. However, among the 15 female ticks secondarily

engorged on ASFV-free pig blood, no heamadsorption effect was observed after two passages on alveolar macrophage culture using blood-meal leftovers. Further investigations are needed to confirm the presence of ASF Virus . The final experimental infection of pigs through tick bite using ticks previously engorged on viremic pigs should allow concluding on the ability of *O. erraticus* to transmit Georgia2007/1 and a possible dose effect on this transmissibility. The results will be presented and discuss during the symposium.

Conclusion: The objective of this study was to experimentally assess the ability of the European *O. erraticus* tick to transmit the Georgia 2007/1 ASFV currently circulating in North-Eastern EU. First results showed that ticks artificially infected in laboratory did not induce ASF clinical signs in pigs by biting. However, virus titration in ticks seems to show that the virus is present in the arthropod. Further in-vitro and in-vivo investigations are running to explore the hypothesis of a dose effect. The expected results should clarify the potential epidemiological role of *O. erraticus* ticks in transmission and re-emergence of ASFV in the field, in case of the spread of current active foci from North-Eastern EU.

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A multidisciplinary approach to combat wildlife diseases: Vaccination with hematophagous arthropods as "living syringes"

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In a different approach for combating insect-borne zoonoses, we propose that hematophagous arthropods could be used to carry no longer a pathogen but a vaccine. Using vectors as "living syringes" provides a unique way of reaching in accessible wild host populations. This in order to protect endangered wild species directly, but it could also reduce risk of zoonoses (to humans, cattle…) while protecting the wildlife species that acts as a disease reservoir.

Our research focuses on the protection of the European Wild Rabbit *Oryctolagus cuniculus* (L.) against 2 fatal viral diseases: myxomatosis and RHD (Rabbit Hemorrhagic Disease), using a specific rabbit flea species. There are two major research areas in our project: entomology and virology.

Entomology studies have involved (i) selection of a suitable vector, (ii) laboratory studies to develop an efficient mass rearing, (iii) verification that it has no side-impact on the ecosystems where it is introduced and (iv) definition of a field release strategy that promotes host-parasite contact while minimizing insect loss.

Virology involves development of an efficient vaccine against the 2 diseases that can be transmitted by the insect-vector. Among the various natural strains of myxomatosis, the most suitable according to us is a virus of very low virulence (grade IV), which causes the formation of antibodies in rabbits without mortality, yet protects them against further acute disease. This attenuated strain also maintains a good natural disease resistance among rabbit populations. Regarding RHD, as virus culture is impossible, it was necessary to use a viral recombination RHD/myxomatosis as a potential vaccine against both diseases.
Finally, at the interface between entomology and virology, we are working on a method for introducing sufficient vaccine onto the mouth-parts of mass-reared fleas to assure its transmission to rabbits.

Key words: wildlife vaccination, vectorisation, insect vector, flea, siphonaptera, *Xenopsyllacunicularis*, european wild rabbit, *Oryctolagus cuniculus*, myxomatosis, RHD, recombinant vaccine, mass-rearing, release strategy, biological control

BLV experimental infection in buffalo species (Bubalus bubalis): preliminary data

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Objective: Bovine Enzootic Leucosis (EBL) is the most common neoplastic disease of cattle. The causative agent is Bovine Leukemia Virus (BLV), belonging to the family *Retroviridae* that includes other important members such as human T cell leukemia virus type I and II and simian T cell leukemia virus (Hajj et al.,2012;Rodríguez et al.,2011).These viruses are able to induce tumoral diseases specially associated with the lymphoid system. In most cases, BLV infection remains clinically silent, although, in the past, infection was mainly suspected in cases of persistent lymphocytosis. Because of the low sensitivity of direct laboratory tests, currently the diagnosis of EBL is based on serological methods, such as the immunodiffusion test in agar gel (AGID) or the enzyme- linked immunosorbent assay (ELISA). The transmission (horizontal, vertical, or iatrogenic) of BLV in cattle occurs by transfer of infected lymphocytes from one animal to another. Experimentally, BLV can infect other species, such as sheep, goats, rabbits, and some cell lines from large animal species including humans. The possibility that humans may be susceptible to BLV has been considered, but never demonstrated. Water buffalo are described as a natural host of BLV, but the literature reporting cases of infection is very limited (Molnar et al., 2000). Moreover, since 1995, there has been an active campaign in Italy to eradicate EBL in cattle and buffalo populations, but EBL positive buffaloes have not been found. Agricultural water buffalo (*Bubalus bubalis*) breeding is very common in certain Italian Regions (Campania, Lazio), linked to the production of mozzarella and other gastronomic specialities. Recently, water buffalo have disseminated to other Regions that were previously dedicated to cattle breeding. In this context, experimental infections were performed to investigate the dynamic of BLV infection in buffaloes, and to obtain biological samples to use to standardize procedures of diagnostic methods.

Methods: One male and 8female adult buffaloes were engaged in the animal experiment and inoculated with heparinized whole blood collected from a virologically and serologically BLV positive cow (*Bos taurus)*. The inoculation was performed intramuscularly in the buffaloes and in 3 sheep used as virus controls. All the animals were tended in an adequate structure with a separate pen for sheep. Blood samples were collected at regular intervals to obtain materials for use in direct and indirect diagnostic investigations. The blood samples were stored, registered in a specific database and regularly submitted to serological and virological tests. The serological tests consisted of the AGID test and several commercial ELISAs (IIDEXX, IDVet, SYNBIOTICS), whereas the virological testing was performed using a real-time PCR assay as described in the OIE Terrestrial Manual. The 8 female buffaloes were selected based on different periods of pregnancy to investigate the effects of infection on the fetuses or calves. After farrowing, the newborn calves were tested for the presence of BLV infection. In addition, milk samples were collected regularly to investigate the presence of BLV or BLV specific antibodies. The experimental procedures stated that VLV positive animals should be culled at the end of the experiments or, if possible and convenient, after the discovery of infection. Blood and organs for laboratory investigations were collected from dead animals, following either natural death or slaughter.

Results: The inoculation of infected blood was considered an effective infection method because after 10days post infection (p.i.), all the sheep employed as virus controls were found BLV positive by serological tests. This unequivocal evidence of infection was not obtained in buffaloes. At ten months p.i., only 5 animals out of 10 were found as positive by serological tests. Even among the buffalo testing BLV positive, results were obtained at different times. In fact, the first female tested serologically positive at 28 days p.i., the second female tested positive at 55 days p.i, and the male was found positive after 97 days p.i. Finally, the last two animals were detected positive after 8 and 11months p.i. Results by different ELISA commercial kits were discordant; however, better reproducibility was observed with the most sensitive ELISA kits (IDEXX and IDVet) and with the AGID test. More specifically, the AGID test was performed on 3 out the 5 positive animals and, in 2 animals, detection was about1 or 2 days later than by ELISA testing. More enigmatic were the results of the SYNBIOTICS ELISA: results of serum sample testing fluctuate and are inconsistent, but, above all, were characterized by a low level of sensitivity. Regarding the real-time PCR tests on blood samples, no positive results were recorded using the usual cycle threshold value for bovine samples. In 2 serologically positive animals, however, a positive signal was detected after the 42nd cycle. Practically, this means that all blood samples should be considered as negative by PCR. Negative results were obtained from PCR testing of organs from the male buffalo slaughtered after 9

months p.i. Moreover, two calves born from serologically positive mothers were found negatives in serological and virological investigations. After 8 months p.i., when just 3 animals had been identified as positive, heparinized blood samples were collected from all buffaloes engaged in the study. These samples were inoculated into different sheep. One sheep was inoculated with a pool derived from negative buffaloes' blood samples, whereas the other 2 sheep were inoculated with the blood collected from positive buffaloes. Two months later, all 3 sheep remained serologically negative for EBL. It is relevant to observe that, in the same time, 2 buffaloes became serologically positive (10 months p.i.).

Conclusion: This experiment is still not concluded, but preliminary results are very interesting because they seem not to confirm the findings described in the literature. First, the susceptibility of buffaloes to BLV infection appears to be lower than reported by certain authors: after 10 months p.i., 50% of study animals showed no evidence of infection and remained clinically silent. Moreover, the positive animals had serological confirmation of infection, but no virological or clinical evidence was clearly observed. The results of the serological investigation should be evaluated as equivocal. The reproducibility of results obtained by the IDEXX and IDVet ELISA kits, as well as the AGID test, permit us to consider these tests as effective and reliable for indirect EBL diagnosis in buffaloes. In contrast, the SYNBIOTICS ELISA kit was the least sensitive and reproducible method, and so was inadequate for determining BLV infection. More information can be expected when results of serological testing of milk samples are available. The results obtained in by virological testing were inconclusive. However, the lack of virus detection was not unexpected, considering the well-known poor sensitivity of PCR in cattle. It is relevant, though, that the inoculation of sheep with potentially infected blood collected from the inoculated buffaloes produced no effects. Taking into account that experimental infections in sheep are evident by serological tests, our results seem to indicate that BLV infection can induce the production of antibodies in buffaloes, but either the infection is not persistent or the virus cannot be transmitted. Since 2 buffaloes became serologically positive after the inoculation of the sheep, the second hypothesis should be considered as the most likely. This means that BLV is able to maintain itself, hidden, in buffaloes for a long period, but the infection will eventually be detectable by serological methods. More investigations should be implemented before considering this study conclusive. Certainly, we have to test for antibody in milk samples, but more importantly, it is necessary to investigate the presence of the virus during this "hiding period," when no clinical and serological evidence can be detected. Our preliminary results confirm the susceptibility of buffaloes to BLV infection, but uncertainty remains about their capacity for natural transmission.

Coronavirus diversity and ecology in South African bat populations

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Objective: Emerging infectious diseases (EID) pose a major threat to public and environmental health and may have considerable economic and societal consequences. Searching for relevant infectious agents may serve as an early warning system against EID. However, in order to be useful it needs to be comprehensive, requiring a multi-disciplinary approach that combines virological and ecological expertise to understand the pathogen-host interface. Coronaviruses (CoV) pose significant problems for both veterinary and human medicine and have several characteristics that make them prime candidates for emergence. Their significance has been highlighted most spectacularly through the SARS outbreak in 2002/2003 and the ongoing MERS outbreak. Despite ample evidence supporting the hypothesis that bats (Chiroptera) are the reservoir host from which mammalian CoV emerged, very little is known about the mechanisms of their maintenance and amplification in bats, nor about ways in which bat CoV may be transmitted across species barriers. Our study aims to describe the hitherto unknown diversity of CoV in South African bats and to describe factors determining bat-CoV ecology that might lead to novel CoV emergence from this host.

Methods: Samples include faecal pellets and/or faecal and oral swabs. Sample collection takes place at different

levels: Broadly across the country whenever one in a network of collaborating bat researchers conducts fieldwork; focussing on specific species of bats; and longitudinally, re-sampling individual bat colonies at regular intervals. General surveillance is conducted across South Africa to broadly identify CoV across a wide range of bat species and environments. Secondly, an in-depth study of CoV in Neoromicia capensis bats at different localities across different biomes is underway for the assessment and description of CoV diversity, prevalence and relationship with biogeographical factors. Sample collection is taking place along rainfall, habitat and altitudinal gradients to test the effects of broad scale/regional biogeographical processes on CoV diversity. Thirdly, certain bat colonies are monitored over time to elucidate factors, such as colony size and breeding season, which may influence CoV shedding, prevalence and diversity. A pan-CoV PCR assay targeting the RNA- dependent RNA polymerase gene is used to screen samples for the presence of CoV. Positive samples undergo further PCR amplification to obtain longer gene fragments for better phylogenetic analysis and their viral loads are determined by means of a quantitative real-time PCR. Individual bats' parameters and their reproductive status are being correlated with the abundance and diversity of CoV found, and the possible influence of habitat on virus carriage assessed.

Results: The majority of sequences detected to date are from alpha-CoV, with approximately 20% of N. capensis bat samples screened positive. Co-infection with more than one alpha-CoV sequence has been detected in a N. capensis bat faecal sample indicating the presence of different alpha-CoV within the same bat colony and individual bat. Subsequent clonal sequencing of the fragment confirmed the presence of two dominant sequence types within the colony and yielded greater CoV diversity than expected on the individual bat level. Of particular interest has been the detection of beta-CoV sequences in P. hesperidus bats. Gene fragments amplified and analysed phylogenetically show them to be very closely related to the beta-CoV sequence isolated previously from a N. capensis bat in South Africa, belonging to the species MERS-CoV and thus also of great interest for human health.

Conclusion: Preliminary results indicate a great diversity of CoV in South African bats. With sampling ongoing, analysis of viral diversity and the effect of biogeographical factors should yield interesting results. Furthermore it is hoped that a better understanding of pathogen-host ecology will be gained and that a number of interesting interdisciplinary aspects will emerge.

Ecology of small mammal-borne viruses with zoonotic potential in South Africa

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Objective: Zoonotic outbreaks are caused by a combination of host, environmental and man-made factors such as habitat encroachment, agricultural intensification and climate changes which lead to increased contact of wildlife reservoir hosts with domestic animals and human beings. Of the known ~1400 human pathogens, 58% are classified as zoonotic and 12.5% are classified as (re-)emerging, the majority of which are RNA viruses. Bats and rodents have been implicated as the reservoir hosts of zoonotic viruses such as SARS-CoV, Sin Nombre, Lassa and Nipah.

The relative importance of various wildlife hosts in the emergence of zoonoses remains unclear. Therefore, identifying reservoir hosts is essential for predicting and possibly pre-empting or controlling the emergence of zoonotic diseases. It is therefore important that wildlife populations be sampled and monitored for likely zoonotic agents; the subsequent characterization of these novel agents is essential for insight into host-pathogen ecology and the prediction of the emergence of potentially human-pathogenic viruses.

The aim of this study was to investigate RNA viruses associated with recent zoonotic events in South African bat, rodent and shrew species. Our objectives were to identify and characterise novel viruses in local small mammal species and to study virus-host relationship in bats by conducting longitudinal colony surveillance.

Methods: Faecal and tissue samples from numerous individual South African rodents, shrews and bats belonging to 21, 4 and 20 different species respectively were screened for various RNA viruses associated with recent zoonotic events using broadly-reactive, family- or genus- specific PCR assays.

Sequencing and phylogenetic analyses was conducted to determine the relationship of any viral sequences detected to known viruses of each respective family. Using the sequences obtained, quantitative real-time PCR assays were established and optimised to monitor virus shedding in bat colonies under study. These colonies were sampled every four weeks by collecting 10 faecal pools (10 pellets per pool) from below the roosts.

Results: To date, novel viral sequences belonging to the following virus groups have been identified in various rodent and bat species: arenavirus, coronavirus and paramyxovirus. Novel arenavirus sequences have been identified in three South African rodent species (*Micaelamys namaquensis, Myotomys irroratus* and *Rhabdomys pumilio*), some of which represent a previously undescribed lineage in the Old World arenavirus clade. Of note is also the identification and genome characterisation of MERSr CoV in a South African vespertilionid bat species. This work is ongoing and we are currently conducting longitudinal surveillance of wild bat populations at colony level. We hope to monitor virus shedding and virus (and strain) diversity over time, and answer questions about: shedding patterns (episodic vs transient), how seasonality and habitat affect virus loads and diversity.

Conclusion: In this ongoing study we have identified a great diversity of novel viruses in both rodent and bat species in South Africa. By conducting bat colony studies, we hope to gain a better understanding of bats in their role as virus reservoirs and gain insight into the factors influencing host-pathogen ecology that might lead to the emergence of bat-borne viruses.

Orbivirus screening on dried blood spots from captive oryx in United Arab Emirates stresses the importance of pre-import measures

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Objective: Following reintroduction and conservation programs of the Arabian oryx (Oryx leucoryx) and the scimitar horned oryx (SHO, Oryx dammah) in the United Arab Emirates (UAE), import of animals from wild game ranches in the United States of America (USA) is not uncommon. Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) are orbiviruses that are the causative agents of bluetongue disease (BT) and epizootic hemorrhagic disease (EHD), respectively. BTV and EHDV are endemic in the UAE and the USA. Sheep and some wild ruminant species are usually severely affected by BT whereas EHD mostly affects wild animals and sometimes cattle.

The objective of this study was to estimate the prevalence of these orbiviruses in Arabian and SHO from captive herds in the UAE using serology and molecular virology. Dry blood spot sampling for orbivirus screening is also discussed.

Methods: A total of 175 SHO and 16 Arabian oryx were sampled. The latters were imported from Texas (USA) two weeks before sampling. All sampled animals belonged to captive herds spread over the Al Wathba area. For biosecurity reasons and to simplify blood storage, elutes from dried blood spot were used for serological and virological tests. Drops of about 80 µl of blood were dispensed on Whatman protein saver cards, and then allo-

wed to dry in the dark at room temperature for 48 hours. Blood spots were punched out in paper discs with a 6 mm diameter punch and diluted in 250 µl PBS and Tween 20 0.05%. Eluted samples were incubated overnight at room temperature and then used immediately or stored at -80°C. To assess the most suitable ELISA kit to detect anti-BTV antibodies from the oryx discs, similar discs were prepared using blood issued from BTV seropositive and viremic as well as seronegative and non-viremic cattle. Elutes from discs with dried-blood from cattle were tested by BTV competitive ELISA (cELISA), sandwich ELISA (sELISA) and indirect ELISA (iELISA) and compared to cELISA performed directly on the serum of the same animals. iELISA on cattle paper discs gave the best correspondence with cELISA on cattle serum and was therefore used to test the oryx paper discs. Subsequently oryx paper discs were tested to detect antibodies against EHDV by cELISA. All the paper discs elutes from Arabian oryx and ELISA positive elutes from SHO were also tested by pan-BTV RTqPCR targeting a fragment of BTV segment 5 and detecting all BTV serotypes. Serotype specific end-point RT-PCR targeting a fragment of segment 2 of BTV2, BTV8, BTV10, BTV11, BTV13 and BTV17 were performed on pan-BTV positive samples.

Results: Three out of 175 SHO and eight out of 16 Arabian oryx were found BTV seropositive by iELISA. None of the animals could be found seropositive against EHDV. BTV genome was detected in 1/3 seropositive SHO and in 5/16 of the Arabian oryx, amongst those 2/5 were seronegative. Overall Cq values were high (33-39). End point PCR failed to detect positive samples for any of the tested serotypes.

Conclusion: BTV seroprevalence and RNA detection in SHO was very limited. By contrast BTV could be demonstrated in 5/16 imported Arabian oryx by molecular virology and in 8/16 by serology. The sampling was realized two weeks after the animals arrived in UAE and some oryx were viremic and seronegative, possibly suggesting a recent infection.

Among the local SHO a low BTV seroprevalence was observed (3/175) and no animals were found positive to EHDV. This result was quite surprising because previous studies showed a higher BTV seroprevalence in domestic and wild ruminants of the Arabian Peninsula with wide local variations.

In addition, dried blood spot testing has been demonstrated being a convenient and reliable method of sampling when storage conditions are hazardous. BTV serotypes could not be determined by end- point RT-PCR. At least 15 different BTV serotypes were reported in the USA and at least 10 in the Middle East, thus the oryx could be infected by a serotype not tested so far. Since RTqPCR positive values were high, the sensitivity of end-point RT-PCR might be insufficient to detect BTV out of eluted blood spots. Additional testing will be performed to identify the virus on the serotype level and therefore provide new insights to clarify the origin of the infection of the oryx. These results stress the need for pre-import risk assessment, precaution and implementation of biosecurity measures when considering translocation of wild ruminant species susceptible to BTV and EHDV.

Near-universal contamination of commercial equine serum pools with hepaci- and pegiviruses

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Objective: Novel HCV-related viruses belonging to the genera Hepacivirus and Pegivirus have been recently discovered in different animal species. Until now, non-primate hepaciviruses (NPHV) in horses are the closest relatives of HCV discovered. In addition, two different species of equine pegiviruses have been described.

Methods: The presence of NPHV and pegivirus RNA in horse sera was determined to shed a light on the relevance for biosecurity of serum-based products used in veterinary medicine and research. Individual horse sera (n=119), serum batches for production of veterinary products (n=35), serum products (n=9) and commercial sera (n=6) were screened for viral RNA using a NPHV realtime PCR and a newly developed multiplex realtime PCR for simultaneous detection and typing of the equine pegiviruses “C35” and “Theiler disease associated virus” (TDAV).

Results: NPHV RNA was detectable in 8% of the sera, all originating from the same holding. Independently of the holding, pegivirus RNA was identified in approx. 13 % of the horse sera. Although also multiple serum batches for manufacturing of biological products contained viral genomes, biologicals based on horse sera were exclusively tested negative. In contrast, commercially available equine sera used for cell culture propagation were all positive for NPHV or pegivirus RNA. Molecular analyses revealed a mixture of multiple viral RNAs in a single commercial serum. For the first time TDAV genomes were detected in sera originating from Europe and South America.

Conclusion: The presence of hepaci- and pegiviruses in commercial equine sera can have serious consequences. A putative infection of susceptible hosts by products containing equine sera must be thoroughly examined.

Introduction of the three-tiered diagnostic system in Equine Infectious Anaemia surveillance in Croatia

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Objective: Equine infectious anaemia (EIA) is an infectious disease of equids caused by the Equine Infectious Anaemia Virus (EIAV), a lentivirus with an almost worldwide distribution. EIA is transmitted by biting insect vectors, mainly horseflies and stable flies, as well as iatrogenically. Because the EIAV infected animals stay the source of infection for the rest of their lives, in ~~the~~ most of the countries the infected animals must be compulsorily slaughtered or euthanized, making EIA one of the most important equine diseases economically.

The clinical picture of EIA is variable and most equids enter the inapparent carrier stage in the year following infection, making the clinical diagnosis difficult to achieve. The characteristics of the EIAV make the virological diagnosis of the infection problematic, as is the use of diagnostic molecular methods due to the genetic heterogeneity of the virus and the variation of the virus titre during the infection. Because of this, the diagnosis and surveillance programmes for the disease are usually based on serological testing.

For decades, the agar gel immunodiffusion (AGID) or Coggins test was considered the only reliable serological indicator of EIAV infection. To improve the efficiency of the diagnosis based on serological testing, a three-tiered diagnostic system has been proposed. This system involves the initial testing

by the enzyme-linked immunoassay (ELISA) with the confirmation of the positive samples by the agar gel immunodiffusion test (AGID) and, in the case of the ELISA positive/AGIDT negative results, the final determination by the immunoblot (IB). To improve the efficacy of the National EIA surveillance programme, this system is adopted in Italy.

Since 1974, AGID is the officially recognised method of EIA diagnosis in Croatia. Since then, the surveillance of EIA has been implemented in horse population at various levels, but since 2005 one of the most rigorous surveillance systems has taken place. This system is based on the annual testing of all equids above the age of six months and all animals entering transport. The control to this extent gave excellent results, lowering the EIA seroprevalence from over 3% in 2002 to the level below 0,1% in the last few years.

The objective of this study was to determine the effectiveness of the three-tiered system in the diagnosis of EIA and to determine its advantages, as well as limitations, regarding the current epizootiological situation in Croatia.

Methods: During this research, 14608 sera of equids from all regions of Croatia were tested. These sera were sampled from October 1st 2013 till December 2014 and were collected as a part of the National EIA surveillance programme. All sera samples were first tested using the in-house AGID test. Randomly selected negative and all AGID positive sera samples were tested with the commercial ELISA kit. Altogether 234 samples were ELISA tested. The positive and suspicious samples were retested using the same kit and the positive results in the second testing were confirmed using the in-house AGID test. The AGID positive samples were identified as positive and those sera with discordant ELISA/AGID results were tested using IB for the final conclusion.

The in-house AGID test antigen was made using the gradient purified EIAV treated with ether. The ELISA kit used

was the double antigen indirect ELISA from a well-known manufacturer and readily available on the Croatian market. The ELISA testing was performed in accordance with the manufacturer's recommendations. The lysed gradient purified EIAV was used for IB after denaturation and reduction. Animals were defined as serologically positive for the EIAV when their sera reacted either in AGID and/or with two or more of the major EIAV structural proteins (p26 and gp45 or gp90) in IB.

Results: Based on the results of AGID testing, 9 out of 14608 sera samples were considered positive giving EIA seroprevalence of 0,06%. Out of 5234 ELISA tested sera samples, 108 gave positive and 44 suspicious results. In the second round of ELISA testing of the positive and suspicious samples, only 16 samples gave positive results and 2 suspicious. All sera samples that gave positive or suspicious result in the second ELISA testing were tested using AGID method. Only 9 of them gave positive results by AGID. The remaining 9 samples were tested using IB and all gave negative results.

Comparing the methods, the sensitivity of ELISA was 100%, regardless if samples were tested once or twice, but specificity was 97.26 ± 0.56% if samples were tested once or 99.83 ± 0.15% if tested twice. The results of AGID and IB were 100% identical.

Conclusion: The results of this pilot research proved that EIA in Croatia occurs with a low seroprevalence. This emphasizes the need of optimizing the serological diagnosis of the disease according to the epizootiological situation in order to confirm serologically positive animals, which are not numerous. This is an essential prerequisite for eradicating the disease. In order to improve the serological diagnosis, its effectiveness was tested by using the three-tiered approach in the epizootiological situation in Croatia.

Because of the inadequate specificity and repeatability of the used ELISA kit, some questions aroused. The ELISA kit as the one used in this research would not give the advantage of speeding the process of making diagnosis and making it more accurate. Even more, using the ELISA kit with characteristics like this would make the diagnosis more expensive. Unlike the ELISA method, the IB method proved to be a necessary and functional additional diagnostic technique which ensures a more reliable, objective analysis.

This research showed that the three-tiered approach, recommended by the World Organization for Animal Health, is not entirely appropriate to be used for EIA diagnosis without adjustments and validation. It seems that one of the crucial steps for obtaining the best results of the three-tiered system is to choose the adequate ELISA format.

Based on these limited results, the modified GDP method has to remain the basis of EIA serological diagnosis, but introducing the IB method as a routine method for suspicious results is an objective possibility for improving the diagnosis, which can possibly lead to the eradication of the disease. To make final conclusions more sera samples should be tested using different ELISA kits.

Luminex technology for group and serotype specific Bluetongue antibodies detection

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Objective: The aim of this study was the development and optimization of an immunoassay based on Luminex platform for multiplex antibody detection.

Methods: The technique was developed for Bluetongue virus (BTV) antibodies (Ab) detection in sera from vaccinated or infected animals. The BTV VP7 and BTV serotype 8 VP2 recombinant proteins were expressed and used as antigens for group (BTV) or type (BTV-8) Ab detection. Following infection of SF9 insect cells (for VP2) or Drosophila S2 cells (for VP7), these viral proteins were purified. Their antigenic properties have been verified by ELISA before Luminex beads were coupled with these recombinant antigens.

Five µl of sera from naïve, vaccinated or/and infected ruminants were added into wells (containing the 2 coupled beads) of a filter-bottom microplate. The specific binding of the Ab anti-virus proteins were revealed by

VP7 and VP2 antigens previously biotinylated and streptavidin-conjugated fluorescent protein. By monitoring the spectral properties of the beads and the amount of associated streptavidin-conjugated fluorescence, the presence or absence of specific antibodies were determined.

Results: Results demonstrated that the Luminex technology is suitable for the virus antibodies detection in multiplex assays. Luminex assays performed with VP7 and BTV-8 VP2 antigens allow the Ab detection of vaccinated or infected BTV (all serotypes) and the specific detection of serotype 8 Ab, without cross reaction with other BTV serotypes. Indeed, using biotinylated antigens (instead biotinylated anti-species Ab),this assay permits the specific detection of BTV Ab and BTV-8 serotyping in all susceptible species (domestic and wild ruminants).

Conclusion: The specificity and sensitivity of this technology is similar to conventional serological tools as ELISA or seroneutralization assays.

Large-Scale Nucleotide Sequence Alignment for Universal Screening PCR Assay Design

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Objective: Development of a polymerase chain reaction (PCR) or quantitative PCR (qPCR) assay for universal detection of highly variable viral genomes is always a difficult task. An essential feature of such a screening assay is to detect as many genetic variants of a given virus taxon as possible. Therefore, a crucial step in a diagnostic assay design is the primer and probe selection on the evolutionarily most conserved regions of the targeted virus genome. However, this important step is frequently underestimated in the literature.

We set up a simple workflow how to align, process, and evaluate a huge set of homologous nucleotide sequences in order to reveal the sequences variability. Attention was paid to the quantification and clear graphical visualization of the nucleotide variation at each position of the alignment. All of these steps were performed by using an ordinary desktop computer without the need for extensive mathematical or computational skills. The workflow was demonstrated on aligning more than ten thousand influenza virus (AIV) MP sequences.

Methods: The workflow is comprised by five basic steps: 1) sequence download, 2) multiple sequence alignment, 3) alignment validation, 4) calculation of the sequence variability and 5) graphical visualization of the results. Since the rate of sequence accumulation does not go hand in hand with the development of bioinformatic analysis tools, these particular steps are not integrated in a single comprehensive software application. Therefore, we utilised particular functions of different freely accessible intuitive software tools and Web-based applications.

Results: the amount of variability through each column in the alignment was quantified as the entropy. The information entropy is a measure of uncertainty observed at each particular sequence position. Accordingly, the most variable positions have the highest entropy values (the lowest information content and therefore the highest uncertainty). Conversely, the conserved positions are those with the lowest entropy values (the highest information content) converging or equal to zero. The output of the entropy calculation was visualized in the form of a column diagram, entropy plot, where the entropy values are plotted against the respective positions of the alignment.

Conclusion: We present a quick and easy to perform procedure for nucleic acid sequence variability identification. The methodology was set up as simple as possible to provide a guideline even for an inexperienced user. This procedure enables to process tens of thousands of sequences within a matter of few hours in dependence of the internet connection speed and server occupancy. The entropy plot, as a final result, provides a clear graphical representation of the distribution of the stable and variable domains across the sequence of interest and offers the ability for more advanced approaches.

Parallel ESVV Poster Presentation Session 2

Tuesday 1st September 2015
17:00-18:30
Room Sully 2

Chair: Anette Bøtner & Geneviève Libeau

Emerging pestiviruses and impact on animal production

Nicola Decaro, Leonardo Occhiogrosso, Viviana Mari, Maria Stella Lucente, Gabriella Elia, Eleonora Lorusso, Michele Losurdo, Vittorio Larocca, Canio Buonavoglia

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Objective: Based on the current nomenclature of the International Committee of Taxonomy of Viruses (http://www.virustaxonomyonline.com), the genus *Pestivirus* consists of four recognized species, bovine viral diarrhea virus (BVDV) 1, BVDV-2, border disease virus (BDV) and classical swine fever virus (CSFV). HoBi-like pestivirus represents an emerging group of pestiviruses infecting cattle, which have been detected in various parts of the world. Here, we report an outbreak of HoBi-like pestivirus infection in southern Italy that caused severe economic losses in terms of animal production.

Methods: The outbreak occurred in southern Italy between August and September 2014 causing an abortion storm in pregnant cows and acute gastroenteritis in calves. A total of 14 pregnant cows aborted, while about 30 1-6-month-old calves displayed watery diarrhoea. Virological, bacteriological and parasitological investigations were carried out on three aborted foetuses and 10 faecal specimens from affected calves. HoBi-like pestivirus RNA was detected in foetal tissues and faeces by nested-PCR and real-time RT-PCR. In September 2014, an extensive eradication program for HoBi-like pestivirus was started, which involved the entire cattle herd. In order to bleed all animals of the herd, samples collection was performed under our supervision. Virological investigations were carried out on a total of 780 animals that were sampled twice at 30 days of distance by collecting EDTA-blood samples. All blood samples were tested for HoBi-like pestivirus by nested-PCR and real-time RT-PCR. Animals were considered persistently infected (PI) when they were found to be viremic at both sampling times; in contrast, acutely infected (AI) animals were viremic only at the first sampling, while after 30 days they were expected to become virus negative. Pestivirus testing was also extended to all calves that were born from September 2014 to March 2015.

Results: A HoBi-like pestivirus strain was detected by nested-PCR and real-time PCR in aborted foetuses and faecal samples of diarrhoeic calves. Through the eradication program, the initial screening was able to detect 3 AI and 15 PI animals, which were immediately slaughtered. By pestivirus testing of calves born in the subsequent months, additional 7 AI and 16 PI calves were identified and subsequently slaughtered. Soon after the eradication program started, the farm productions increased markedly in terms of milk production, reproductive performance, growth rates, thriftiness, lower occurrence of other diseases, and decreased mortality among young stock.

Conclusion: HoBi-like pestivirus introduction into the herd has the same outcome as the other, more widespread, pestiviruses BVDV-1 and BVDV-2, whose impact on animal production is well recognised. Therefore, specific prophylactic measures should be adopted whether HoBi-like pestiviruses are found to circulate with high frequency in the European cattle herds.

Emergence of a virulent BVDV type 2C strain in the Netherlands

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Objective: Description of the emergence of BVDV type 2 infections in veal calves in the Netherlands.

Methods: Routinely a veterinary monitoring system is in place in the Netherlands, of which important parts are a veterinary helpdesk, a large post mortem facility and a diagnostic laboratory. For sequencing and genotyping samples have been sent to CODA-CERVA in Belgium and FLI in Germany. Trends in disease prevalence and pathologic findings are monitored regularly.

Results: An unusual number of cases of severe diarrhea and pneumonia with high mortality and morbidity were reported in veal calves. Within a few weeks several herds with the same symptoms were reported by veterinary practitioners. All affected herds had recently imported veal calves from specific regions in Germany. Between March and July 2013 in total 14 veal calf herds were affected. Symptoms commenced 1-2 weeks after the arrival of the calves, starting with mild signs of respiratory distress and temperatures exceeding 40.5 °C. Medication was unsuccessful. Animals became depressed and recumbent, with severe and sometimes hemorrhagic diarrhea. In some cases hemorrhagic diathesis was observed. Ninety percent of the affected calves died within 2 or 3 days. Necropsy revealed severe (pleuro-) pneumonia, petechiae and multiple haemorrhages.

BVDV virus was detected by BVDV antigen test/leukocytes (Idexx) in organ material, confirmed by in- house BVDV realtime PCR. In all herds presence of a BVDV-2 strain was detected, first by sequencing (FLI), later on only genotyping PCR was performed (CODA-CERVA). Sequencing of the 5'UTR region of BVDV strains from 8 herds (FLI) revealed that these Dutch BVDV-2C strains were the same as the BVDV2C strains in Germany.

Mortality ranged from 6-56%. More than 2400 veal calves were killed or destroyed. Measures were taken to prevent spreading of this virulent virus in the Netherlands. Neighboring farmers were informed, collection of dead calves from infected farms by the rendering company trucks was done at the end of the route. From April onwards, imports of calves from the known high risk regions in Germany were regulated: these calves were transported directly to selected Dutch farms and stayed there. At these farms strict hygiene measurements were taken and calves were quarantined and monitored. BVDV 2C has not spread beyond these veal calf herds in the Netherlands.

Conclusion: The source of this virulent BVDV type 2C outbreak in Germany remained unclear. We concluded that monitoring of BVDV infections remains important. Measures that were taken in the Netherlands were effective.

Commercial vaccines may not protect against local strains of bovine viral diarrhea virus

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Objective: Bovine viral diarrhea virus (BVDV) including BVDV-1 and BVDV-2 species causes diverse clinical forms and significant economical loses. Not only the virus species but also the rapidly increasing subgenotypes are striking for prevention. Vaccination is a widely used option beside the essential steps for prevention like elimination of persistently infected animals. Cross- reactivity between BVDV subgenotypes and vaccine strains is important to carry out protective immunization. The aim of this study was to determine the serological similarities among BVDV subgenotypes and to find out neutralization ability of immune response developed by three commercial vaccines against variable BVDV subgenotypes.

Methods: Hyperimmunised sera were acquired by immunizations of sheep using 6 Turkish BVDV isolates from subgenotypes BVDV-1a,b,d,f,h and -l. A cross-neutralization assay was employed to determine serological relationships (R) among tested BVDV subgenotypes. On the other side, for vaccine efficiency testing, 20 calves that

were negative for BVDV antigens and antibodies were divided into 4 groups for three commercial killed BVDV vaccines and one control group. Vaccine containment were as follows: Vaccine-1: BVDV-1a/monovalent; Vaccine-2: BVDV -1a and BVDV- 2/polyvalent; Vaccine- 3: BVDV-1a/ polyvalent. Two consecutive vaccinations by 30 days interval were applied according to instructions. Blood samples were taken from all the animals at day 0, 15, 30, 45, 60 and 75. Serum samples were tested by neutralization-immunoperoxidase monolayer assay against 11 BVDV isolates belongs to one BVDV-2 (BVDV-2b) and 7 BVDV-1 subgenotypes (BVDV-1a,b,d,f,h,l,r).

Results: According to (R) values BVDV-1f demonstrated the weakest antigenic similarity to BVDV-1a and -1b isolates which are widely preferred for vaccine production. Highest similarity was between BVDV-1a and -1b, even BVDV-1h and BVDV-1l showed elevated values against majority of isolates. About vaccine efficiency, convenient responses have detected against BVDV-1a isolate with all vaccines as expected. Conforming to the (R) values, moderately high titers have existed against BVDV-1h and -1l isolates. However BVDV-1a and -1b subgenotypes showed high antigenic similarity, low antibody titers was detected against BVDV-1b by immunization with two of three vaccines. Although there were great differences for BVDV-1f in overall, high level of neutralizing antibodies were detected with tested vaccines. In contrary, low antibody titers have detected against BVDV-1d and -1r with two vaccines which may contribute to inadequacy of protection. Quite low antibody responses were acquired against Turkish BVDV-2 isolate (TR15) with all the vaccines even one vaccine contains BVDV-2 strain.

Conclusion: Results of this study support that vaccination with internationally most common BVDV strains may not protect against field strains belongs to variable subgenotypes.

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Experimental infection of bluetongue virus serotype 4 MOR2009/09 strain in IFNAR (-/-) mice

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Objective: Bluetongue (BT) is a non-contagious, insect-transmitted disease of wild and domestic ruminants caused by the bluetongue virus (BTV), characterized by vascular injury that results in tissue necrosis, hemorrhage, and edema. BTV replicates in lymphoid tissues and infected mononuclear leukocytes secrete proinflammatory and vasoactive mediators that can contribute to BT pathogenesis. In our laboratory, we have reported the interferon α/β receptor knockout (IFNAR-/-) mouse as an animal model for BTV infection. In this animal model, we can closely follow infection and disease progression. Taking advantage of IFNAR (-/-) mice as a suitable animal model, we have studied the histopathology and dynamic of monocytes/macrophages, and B and T lymphocytes in different target tissues (spleen, thymus, and lung) during BTV-4 infection. In addition, the presence of iNOS and caspase-3, an indicator of apoptosis, were also analyzed in these tissues by immunohistochemistry.

Methods: IFNAR (-/-) mice were infected subcutaneously with 10^3 pfu/mouse of BTV-4 MOR2009/09 strain. Whole blood was collected in EDTA from all animals at regular intervals after inoculation. At varying times post-infection, mice were sacrificed and several organs (spleen, lung, and thymus), were collected and (i) homogenized in TRI Reagent Solution (Ambion) using a Tissue Lyser homogenizer for RNA purification and RT-qPCR cytokine analysis or (ii) fixed in 10% buffered formalin (pH 7.2) for histopathological studies and immunostaining with antibodies specific of BTV, CD3, CD79, MAC387, iNOS, and Caspase-3.

Results: The spleen and thymus of BTV-4 infected mice showed severe lymphoid depletion on H&E stained sections. This finding was confirmed by IHC, showing slightly decreased immunopositivity against CD3 in thymus, and scarce immunoreactivity against CD3 and CD79 in the remains of the white pulp in spleen, jointly with an increase in MAC387 immunostaining. BTV-4 infection also induced the expression of caspase-3, in spleen, where apoptotic debris were observed by H&E. A dramatic increment of iNOS immunoreactivity associated to necrotic areas of white pulp was observed, being less noticeable in thymus and lung. To further investigate the pathogenesis of BTV, the induction of pro-inflammatory cytokines in tissues where BTV replicates was evaluated by

measuring transcript levels by RT-qPCR. BTV-4 infection led to enhance transcription of IFN-γ, TNF-α, IL-6, IL-12-p40, and IL-1β mRNA in thymus, spleen and lung and the increase of cytokine induction correlated with the level of virus replication in these tissues.

Conclusions: IFNAR(-/-) mice are susceptible to the infection of BTV-4 MOR2009/09. After infection, BTV infected mice show clinical signs characterized by ocular discharges, apathy and the disease progression led to animal death. Infectious virus is recovered from the spleen, lung, thymus, and blood. Disease progression and pathogenesis (induction of inflammatory modulators, apoptosis, and pro-inflammatory cytokines) closely mimic hallmarks of bluetongue disease in ruminants. IFNAR(-/-) mice are a good choice to facilitate a faster advance in the field of orbiviruses.

Bluetongue and epizootic haemorrhagic disease viruses in Reunion Island

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Objective: Bluetongue (BT) and epizootic haemorrhagic disease (EHD) are arthropod-borne diseases of wild and domestic ruminants caused respectively by viruses belonging to the species Bluetongue virus (BTV) and *Epizootic haemorrhagic disease virus* (EHDV) within the genus *Orbivirus* of the *Reoviridae*. The viruses are transmitted between ruminants by biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae). BTV went undetected in Reunion Island between its first documented emergence in 1979 and two other serious outbreaks with both BTV-3/ EHDV-6 in 2003, and both BTV-2/EHDV-6 in 2009. In these outbreaks, infected animals developed symptoms including hyperthermia, anorexia, congestion, prostration and nasal discharge.

In order to get an overview of the circulation of BT/EHD in Reunion island, an assessment of the prevalence in ruminants native to Reunion Island by a cross-sectional study was undertaken in2011on 67 farms, including a total of 276 cattle, 142 sheep and 71 goats with a total of 489 ruminant samples. Data concerning farm characteristics, type of production, and number of animals were collected through farmer questionnaires for an evaluation of the associated risk factors. In addition, investigation of clinical cases based on the observation of clinical signs was also performed in order to get BTV/EHDV isolates with the aim to track the origins of the circulating strains.

Methods:

Risk factors analysis

Data concerning farm characteristics, type of production, number of animals, closeness to another farm and sugar cane fields, presence of organic and other waste on the farm, exposure to wind, distance to a permanent water point, type of animal housing, presence of ticks on animals, use of treatment against ectoparasites and insects, animal's contacts with other animals or humans, grazing practice, spreading of manure on pastures, presence of *Tenrece caudatus*, rodent control, number of abortions in the herd in the last 12 months, purchasing behaviour, quarantine of newly purchased animals, other biosecurity factors like hygienic precautions taken by the staff or other people entering the farm (truck driver, vets and other visitors) were taken from a questionnaire which was filled in during an interview with the farmers. This questionnaire was pre-tested on five farms in a preliminary study. The final questionnaire comprised 40 questions of which 75% were closed-ended.

Serological assays

Specific anti-BTV antibodies were tested in serum samples with a group-specific competitive ELISA based on the VP7 protein using a commercial kit (LSIVet™ Ruminant BT Advanced II- Serum, Life technologies, France). Specific anti-EHDV antibodies were tested using a blocking commercial kit (LSIVet™ Ruminant EHDV-Serum

ELISA kit, Life technologies, France). A Sunrise ELISA reader was used for reading at 450 nm (Tecan, France). Optical density values were converted to percentage inhibition (PI). According to the cut-off value of the test, test samples with PI values ≥ 40% for BT and ≥ 60% for EHD were considered as positive.

BTV/EHDV genome detection

For the BTV group specific real-time RT-PCR, 6 µl of denatured double-stranded RNA prepared with the EZ1 robot and EZ1® Virus Mini Kit v2.0 (Qiagen, France) were reverse transcribed (RT) and amplified using the one-step QuantiTect Probe RT-PCRkit (Qiagen, France) based on segment 1 developed by Toussaint et al. 2007. For the EHDV group specific real-time RT-PCR, 5 µl of denatured double-stranded RNA were reverse transcribed (RT) and amplified using the commercial TaqVet™ EHDV (Life technologies, France).The subgroup-specific EHDV RT-PCR based on segment 2 was performed according to Sailleau et al., 2012.Embryonated chicken eggs (ECE) were each inoculated as previously described in Sailleau et al., 2012

Sequence analysis, alignment and phylogenetic analysis

To identify the genetic relatedness of the detected virus, phylogenetic analyses were performed with published *EHDV* sequences. Sixteen full-length VP2 gene sequences were cleaned by hand from the results of several BLAST nucleotide searches as well as direct references from available up-to-date literature and then aligned using the ClustalW translation alignment tool in MEGA (Ver. 5.05). Phylogenetic analysis was performed using the neighbour-joining method using distance measures generated by the p-distance algorithm running 1, 000 iterations with Geneious® Pro.

Statistics

A Fisher exact test was used to compare differences in prevalence between diseases and species. All statistical procedures were performed using R.3.0.1. A value of $P < 0.05$ was considered significant. The prevalence rates were estimated as the overall mean and 95% confidence interval (CI).

Results:

The observed EHD prevalence rate in cattle was 63.77% (95% CI [57.99–69.55]), 5.63% (95% CI [0.03–10.99]) in goats, and 3.70% (95% CI [0.05–6.88]) in sheep, suggesting that EHD occurs more often in cattle than in goats and sheep. These findings were supported by a significant statistical difference in the EHD prevalence rate between species (Fisher exact test, P <<2.2e-16).

The observed BT prevalence rate in cattle was 79.62% (95% CI [74.77– 84.47]), 50.70% in goats (95% CI [39.08–62.33]) and 21.48% in sheep (95% CI [14.55–28.40]) with a significant difference in BT prevalence between species (Fisher exact test, P = 4.367e-10).

Additionally, three suspected outbreaks occurred during the 2011 study period, one BTV/EHDV negative, one BTV specific and one combined BTV/EHDV outbreak. In total, 14 EHDV positive cases and 1 BTV/EHDV co - infection case were identified. Two further suspected outbreaks were confirmed to involve EHDV and BTV/EHDV. Isolations of EHDV were successful resulting in the identification of the Reunion -specific EHDV-1 serotype. Phylogenetic analyses of segment 2 showed that the Reunion isolate 6010 _2011 belongs to the group C (hypothesised in Anthony et al. 2009 together with EHDV-1 strains from Australia, 1995, Nigeria, 1967, French Guyana, 2011 and New Jersey, USA, 2011). In January 2014, once more suspected outbreaks occurred on cattle with observed clinical signs such as hyperthermia, congestion and nasal discharge. Virus isolations were successful and led us to identify a new EHDV serotype for Reunion island, the EHDV-7 serotype**.**

Conclusion: Our results confirm that the prevalence of both BT and EHD is high and that both are likely currently circulating. A high risk of BTV and EHDV infections was associated with the introduction of ruminants from neighbouring farms without quarantine, the presence of organic and other waste on the farm, and treatment against ectoparasites and insects. New circulating EHDV serotype 1 and serotype 7 of unknown origin were isolated in 2011 and 2014 respectively. The mechanisms involved in the introduction, maintenance, and perpetuation of both BTV and EHDV orbiviruses in Reunion Island need to be further investigated. How and when the EHDV serotypes were introduced onto the island are unknown, the most likely being the introduction of infected animals from eastern and southern Africa, Madagascar or Australia over a period of many years. The introduction of Malagasy breeds, which could be considered as orbivirus susceptible breeds many decades ago, is one possible hypothesis. Since 1976, importation of domestic ruminants from these countries has stopped. Until 2008, imports were only from mainland France. The maintenance of both viruses in the livestock population could also be due to the presence of reservoirs such as deer as was the case in many places including southern California between 1990 and 2007 (Roug et al., 2012). Pathogens can easily be shared between wildlife and

domestic ruminants which has implications for both the animal production industry and wildlife health. Whether animal reservoirs such as Rusa deer *Cervus timorensis rusa* imported from Mauritius Island and now present in Reunion Island play a role in EHDV epidemiology need to be investigated. The same species of Rusa deer was introduced on the island of Mauritius in 1639 and serological evidence of both EHDV and BTV circulation is documented. Since 1992, in accordance with European Union regulations, importation of live deer from Mauritius to Reunion Island is forbidden. The intermittent detection of certain serotypes and the occasional appearance of new serotypes suggest that, in the past, regular but separate introductions of BTV/EHDV may have also taken place from Madagascar, and from Southeast Asia including Mauritius via windborne *Culicoides*. Although it exists, the observed herd immunity in Reunion Island is not high enough to prevent the maintenance of an enzootic cycle, which could also be related to the abundance and activity of *Culicoides* throughout the year. The findings reported here provide additional hypotheses regarding the ecological characteristics of bluetongue and epizootic haemorrhagic disease and other vector -borne livestock diseases. Sentinel surveillance programmes are a useful way of documenting regionalization zones for diseases, which can be of great importance when securing livestock international markets.

First detection of porcine epidemic diarrhea virus in Slovenia, 2015

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Objective: Porcine epidemic diarrhea (PED) is a viral disease that affects swine of all ages, often leading to high piglet mortality rates. Limited data about the prevalence are known for European countries and no data were available for Slovenia.

Methods: In this study a total 63 samples were collected from 10 herds, where diarrhea was main clinical symptom for suspicion, but without increased mortality in piglets. Feces samples were collected between December 2014 and February 2015. After RNA extraction, PEDV nucleic acids was detected by commercial real-time RT-PCR (virotype® PEDV/TGEV).

Results: First PEDV positive results were identified from six samples collected on January 6 2015 on a pig farm. PEDV was detected by real-time RT-PCR method also from feces samples collected one and two weeks after first positive results on the same farm. The obtained cycle threshold values for positive samples were between 16.3 and 23.9, confirming high viral load in a feces on infected farm. The second PEDV positive farm was confirmed in February 2015, located in the same geographic region as the first pig farm. Three representative positive samples were amplified by conventional RT-PCR and sequenced for phylogenetic analysis. Based on a phylogenetic comparison of 390 nucleotides of the RNA polymerase gene, the detected PEDV strain showed 99.7% nucleotide identity to the closest sequence GER/L00719/2014 detected in Germany in 2014 and 99.2 to 99.7% to strains detected between 2013 and 2014 in USA and China. Preliminary results of commercial ELISA for 92 randomly selected samples, collected between November 2014 and February 2015 from 14 different pig farms, showed that antibodies against PEDV were detected in 51% of tested pigs and in 78% of tested farms.

Conclusion: The origin of this new virus on territory of Slovenia is still unclear, but may be a result of one or more imports of live PEDV positive pigs and then virus was spread rapidly through many swine farms.

Identification and genetic characterization of Aichivirus (porcine kobuvirus) in pig farms in Slovakia

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Objective: Porcine kobuvirus, recently renamed as Aichivirus C, is relatively new discovered virus belonging to the family *Picornaviridae*. This single-stranded RNA virus was for the first time identified in pigs in Hungary (Reuter et al., 2008). Later, virus was detected in pigs originating from China, Thailand, Japan, South Korea, Czech Republic, Italy, Brazil and USA. Aichivirus C was found in both diarrheic and healthy pigs and viral prevalence varies from 3.9 up to 100 % (Khamrin et al., 2014). The aim of this study was to search for this virus in pigs of different age in farms in Slovakia and to perform initial genetic characterization of detected viral isolates.

Methods: Total RNA was isolated using TRIzol (Ambion) from rectal swab and stool samples collected from diarrheic and healthy pigs of different age in Slovakia in the period 2013 to 2014. The cDNA synthesis was carried out using random primers and RevertAid Premium reverse transcriptase (Thermo Scientific). Viral RNA was detected using single PCR employing primers flanking 495 bp long fragment from 3D genomic region coding viral RNA-dependent RNA polymerase - RdRp (Yu et al., 2011). Sequencing of PCR products was performed by commercial company (Microsynth, Austria).

The percentage of nucleotide sequence similarity was calculated by program MegAlign (Lasergene). The phylogenetic tree was constructed by the neighbor-joining method (1000 replicates) of MEGA6.

Results: Of 158 clinical samples tested, 111 (70.3%) were positive for Aichivirus C RNA. High prevalence of virus infection observed is close to 87.3 % positive pigs detected for Aichivirus C in Czech Republic (Dufkova et al., 2013) and to 65% detected in Hungary (Reuter et al., 2009). No significant differences in prevalence rates were observed between animals with diarrhoea and healthy pigs. The group of suckling piglets was slightly more frequently positive for the virus than weaning and finishing pigs but there was no statistical significance.

The nucleotide sequences of eight 404 bp long PCR amplicons obtained from Slovak isolates were 89.4 - 100% similar to each other. When they were compared with 157 sequences deposited in GenBank, the 86.9 – 94.8 % similarity was observed. The phylogenetic tree indicated that viral isolates from Slovakia were clustered in four phylogenetic branches. Interestingly, the isolates were rather clustered with Asian isolates than with isolates originating from neighbouring countries as Hungary and Czech Republic. The differences in clustering of Slovak isolates were due to several unique nucleotide mutations in viral RdRp gene which was used for the phylogenetic study.

Conclusion: This is the first report on the identification of Aichivirus C (porcine kobuvirus) in domestic pigs in Slovakia. The analysis confirmed high prevalence of virus in animals tested. Virus was genetically closely related to other isolates detected in pigs around the world.

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Rotavirus A and C infections in Belgian Diarrheic suckling pigs

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Objective: Group A and C rotaviruses have been identified as important causes of diarrhea in suckling piglets, together with *Escherichia coli*, *Clostridium perfringens* and *Isospora suis*. Though, the importance of rotavirus infections on Belgian pig farms has not been investigated, which hampers the development of good strategies to diagnose and prevent this economical important disease. In this study, it was aimed to investigate the pre-

sence of rotavirus A (RVA) and C (RVC) infections among Belgian diarrheic suckling pigs, in order to optimize current diagnostic strategies used in veterinary practice. Furthermore, the VP7 and VP4 genes of circulating strains were characterized, as this may benefit future vaccine formulation.
Methods: The presence of RVA, *Escherichia coli*, *Clostridium perfringens* and *Isospora suis* in diarrheic fecal samples (n=45) of suckling pigs less than 2 weeks old from 36 farms were investigated at a private diagnostic laboratory. However, veterinarians specified for which pathogens diagnostic tests should be performed. Here, RVA was diagnosed using a fast antigen detection strip, whereas bacteria were isolated on specific agars. Coccidia were purified using flottation, and visualised under a microscope. At the Laboratory of Virology, all samples were analyzed for RVA and RVC using RT-qPCR, and the genes encoding outer capsid proteins VP7 and VP4 were characterized by partial sequencing and phylogenetic analyses.

Results: Many of the common agents involved in the pathogenesis of diarrhea in suckling piglets were not routinely investigated in veterinary practice. However, in 61% of 36 farms tested, high viral loads of RVA (6.96 to 11.95 $\log_{10}$ copies/g feces) and/or RVC (5.40 to 11.63 $\log_{10}$ copies/g feces) could be detected, whereas rotavirus infections could only be diagnosed on 25% of the farms using a fast RVA antigen strip. Seventeen of these RVA strains were characterized, resulting in the detection of 4 different G-genotypes (G3, G4, G5 and G9) and 4 different P-genotypes (P[6], P[7], P[13] and P[23]) in 8 different G/P combinations. VP7 genotypes G5 and G4, and VP4 genotype P[7] were encountered most frequently (29.4% each). All RVC strains belonged to genotype G6 (VP7), except for one strain possessing the G1 genotype. Moreover, VP4 genes of Belgian RVC strains were genetically highly heterogeneous. *Escherichia coli* was also frequently isolated in the present study, but unfortunately the characterization of virulence factors was not requested routinely, making it difficult to interpret diagnostic results. Furthermore, most *Clostridium perfringens* strains were isolated from rotavirus negative samples. *I. suis* was only detected in 2 out of 45 samples, and probably underdiagnosed due to a lack of requests for routine testing in veterinary practice.

Conclusion: As a conclusion, routine testing for RVA and RVC using RT-qPCR in diarrheic feces of suckling pigs is advised, but also diagnostic investigations of other pathogens should be carried out more frequently in order to come to sound conclusions, and to install durable and efficient prophylactic measures on affected pig farms.

Novel astroviruses in the gastrointestinal complex of Suidae - Characterisation of the pathobiome by metagenomics

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Objective: Many observations indicate that infectious agents are closely interacting not only with the host immune system but also with other microbes, in polymicrobial infections (Murray et al. 2014). Understanding these interactions within the "pathobiome", defined as the collected pathogenic agents within the microbial community, is of high importance when making decisions regarding the control and/or the treatment of infectious diseases (Vayssier-Taussat et al. 2014). With the introduction of metagenomics, *i.e.* the methods of unbiased sequencing of all genomics sequences within a sample, it is now possible to identify for example outbreaks caused by previously unknown infectious agents, as well as making in depth analysis of the microbial communities. Thereby metagenomics is becoming a crucial tool for monitoring various infectious diseases, including emerging maladies (Belák et al. 2013; Karlsson et al. 2013). The increasing knowledge about pathogen-host and pathogen-pathogen interaction suggests that we should consider an organism in a holistic way, including its microbiome. Given the close interaction between microorganisms, the wider perspective of the pathobiome, the putatively pathogenic fraction of the

microbiome, must be considered as well as the possible scenario of imbalance in the microbial population.

Intestinal samples were acquired from an outbreak of gastroenteritis in production pigs in Hungary in 2011. By use of viral metagenomics the virome of the intestines were analysed. A number of samples showed signs of polymicrobial infections with two also presenting putatively new variants of astroviruses. Considering the novelty of these findings and the supposed aetiolgical role of the novel astroviruses, both intestinal samples were subjected to deep sequencing to retrieve near full sequences of the infectious agents and to describe the polymicrobial infection to gain insight into the intestinal pathobiome of pigs diseased with gastroenteritis.

Methods: Initially samples were screened by metagenomics according to (Granberg et al. 2013), in brief; Homogenates were centrifuged and supernatant were collected, subjected to nuclease treatment and consecutively DNA and RNA were extracted. Samples were then randomly amplified by Sequence Independent Single Primer Amplification (SISPA) and sequenced on the Roche 454, using the GS FLX Titanium Rapid Library. Data were analysed by *de novo* assembly using MIRA and BLASTn and BLASTx searches. Resulting alignments were *in silico* mined for viral sequence hits and resulting hits were confirmed by PCR and by consecutive sequencing.

Deep sequencing of samples was performed as follows; samples were homogenized and treated as before. A separate sample set was amplified by Multiple Displacement Amplification (MDA), QiaGen Repli-G. SISPA samples were subjected to Ion Torrent sequencing and Repli-G amplified samples were subjected to MiSeq sequencing. Both sample sets were cleaned by mapping them towards the pig genome and consecutively mapped against the best hit genome using MIRA 4 Hybrid assembly, incorporating sequenced PCR products, Ion Torrent Reads and MiSeq reads (Chevreux, Wetter, and Suhai 1999).

Results were visualised using UGENE and multiple alignments to nearest relatives were performed using about 3kb of sequence with good coverage using MUSCLE (Edgar 2004; Okonechnikov et al. 2012). The phylogeny was performed, using PHYLIP Neighbour Joining and visualised as an unrooted tree.

Additionally, *de novo* assembly of the cleaned data were performed and consecutively analysed the same way.

All datasets were subjected to a KRAKEN analysis, using a customised in-house database, for describing the microbiome and discerning the putative pathobiome (Wood and Salzberg 2014). Resulting viral distribution were visualised using KRONA (Ondov, Bergman, and Phillippy 2011).

Results: Ion Torrent Sequencing produced 400,000 reads of good quality for the first run and 600,000 reads for the second. Of these 70% mapped towards the Pig reference genome. MiSeq genereated 1,9m reads with good quality in the first run and 1,7m reads in the second run. Of these around 70% mapped towards the Pig reference genome. The remaining 30%, for both sequencing runs, were subjected to further analysis. The MIRA mapping towards reference genome produced a good coverage over 4kb of the two putative viral genomes.

The detected new astrovirus strains show a relatively low similarity to previously described full genomes of astroviruses, including the Astrovirus wild boar/WBAstV-1/2011/HUN that was present in wildlife in Hungary at the time. Total identity is at the highest 85% with coverage of 80%.

Further investigating the microbiome, both Sapovirus and Rotavirus genomic sequences were detected in the samples, indicating polymicrobial, multifactorial infections in the observed cases of gastroenteritis in swine.

Conclusion: With the increased ability and availability of metagenomics for small and medium scaled labs as well as in resource strained environments investigations into polymicrobial infections as well as the pathobiome will render new insights into host/pathogen — pathogen/microbiome interactions. In this study several omics tools were used for identification, genomic characterisation and metagenomics analysis and characterisation of clinical samples.

Astroviruses have shown an increase in incidence over the last few years with notable changes in how we perceive the virus as the virus passed from a rather common gastrointestetis pathogen into a possible neurological disease agent (Blomström et al. 2010; Li et al. 2013). Given the high variance of astroviruses added knowledge is beneficial for the research community. Herewith, two new variants were described in gastrointestinal disease cases, combined with describing a possible co- infection by other known viruses causing gastroenteritis. This raises the question of a broader screening (since polymicrobial infections might be lost in statistics otherwise) as well as questions of possible synergetic effects of the co-infections of the detected agents in the complex infection biology of gastroenteritis of swine.

Since various forms of gastrointestinal diseases of the swine populations regularly occur at a global scale and cause

tremendous economical and socio-ethical losses worldwide, further research should be aimed at localising new putatively polymicrobial cases and to study the infection biology of porcine gastroenteritis in more detail. The research should include detailed host/pathobiome interaction studies, focusing not only on viruses, but also on further infectious agents, such as bacteria and parasites, in order to further characterise and better understand the complex scenario of infectious gastroenteritis of Suidae.

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New astroviruses detected in wild birds

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Objective: Wild birds are recognized as an important reservoir of viruses. Avian astroviruses (AstV) are classified within the genus *Avastrovirus* and are known to cause infection in domestic poultry. Infection ranges from no clinical signs to enteritis, growth depression, nephritis, hepatitis and mortality. Interspecies AstV transmissions are already described between pigeons and domestic poultry. ORF 1b represents the most conserved region in the AstV genome, making it suitable for detection and phylogenetic analysis. The objective of this study was the genetic characterization of AstV circulating in wild birds and discover the divergence between strains from wild birds and published strains from domestic poultry.

Methods: To characterize AstV in 29 different wild bird species, 239 fecal swabs were tested. Samples were collected during the migration period, in September 2013. AstV nucleic acid was detected by RT-PCR targeting ORF 1b gene, which encodes the RNA-dependent RNA polymerase. From AstV positive samples nucleotide sequences (288 nt) were determined and compared to each other and to published AstV strains.

Results: Out of 29 different wild bird species six of them were detected AstV positive by RT-PCR: Sedge warbler (*Acrocephalus schoenobaenus*), Wood warbler (*Phylloscopus sibilatrix*), Common starling (*Sturnus vulgaris*), Eurasian kingfisher (*Alcedo atthis*), Sand martin (*Riparia riparia*) and Barn swallow (*Hirundo rustica*). Eighteen different nucleotide sequences clustering into 7 different lineages with identity between 53.8 and 98.6 % were detected. When comparing the sequences of detected AstV with published in GenBank, six lineages have no close relatives (< 65 % nucleotide identity) but one avian AstV identified in Common starling has 100 % nucleotide identity with strain detected in domestic polutry (FJ434664 duckling AstV from China).

Conclusion: Our findings indicate high diversity of AstV detected in 6 different wild bird species. To elucidate relationships between wild bird AstV and domestic poultry AstV further surveillance is required.

Update on rabies situation in Serbia

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Objectives: Among all known zoonoses discovered so far, rabies is one of the oldest and still neglected fatal disease. According to WHO (2013), rabies takes every year more than 60.000 human lives and most of the victims are children under 15 years. The majority of human deaths occurred in developing countries of Asia and Africa. India has the highest recorded number of human victims, because of the huge population of stray dogs and inadequate vaccination programme. On the opposite site, many European countries are rabies-free and for these countries the main treat represents importation of rabid animals. In other countries, where Serbia belongs, rabies is maintained in the population of red foxes, which are the main reservoir of rabies virus in wildlife (WHO, 2013; Fooks et al., 2014).

The causative agent of rabies is RNA virus that belongs to the genus Lyssavirus, family Rhabdovirdae, order Mononegavirales. The reservoirs of rabies in Europe are different carnivores, while in North and Latin America bats are the main source of the disease. Rabies is transmitted by the bite of infected animal. Rabies is a neurotropic disease with clinical signs of encephalomyelitis and fatal outcome (Freuling et al., 2013).

After the World War II, rabies existed in former Yugoslavia in both urban and sylvatic form. Extensive veterinary measures and massive vaccination of dogs decreased the number of infected dogs and human exposure. Implementation of these measures resulted in progressive disappearance of urban rabies. The last report of human rabies case was recorded in 1980 (Petrovic, 1987; Aylan et al., 2011).

It is supposed that rabies entered Serbia in 1977 from the north, probably from Hungary, during the large enzootic emergency that spread in red foxes from Russia to Europe in 1940´s. Many researches and field trials have reported that the only effective way for rabies elimination in wild carnivores is oral rabies vaccination (ORV). The first country that implemented ORV of foxes in Europe was Switzerland in 1978, followed by many other countries (Pastoret and Brochier, 1998).

The objective of our study is to present rabies epizootiological situation in Serbia from 2006 to 2014. In 2010 was introduced the programme of ORV of foxes and other wild carnivores in Serbia, and since then, the incidence of rabies has been significantly decreased

Methods: In 2010, Veterinary Directorate of Serbia has started multiannual project of oral rabies vaccination of foxes and other wild carnivores (e.g. jackals), supported and co-funded by EU (financed by Instrument for Pre-Accession Assistance). From November 2010 till the end of 2014, nine campaigns of vaccine distributions were completed with the standard program of vaccine delivery twice a year-in autumn and spring. In the first two seasons was used vaccine *Lysvulpen* (Bioveta, Czech Republic), which consists of the attenuated rabies strain SAD Bern, and starting from the seventh campaign, in October 2013, vaccination was conducted with *Fuchsoral* vaccine, containing strain SAD B19 (IDT Biologica, Germany). The measurement of the efficiency of ORV of foxes and other carnivores was based on: a) *post mortem* laboratory examination of brain tissue of target animals (foxes, jackals and other carnivores) by fluorescent antibody test (FAT), b) detection of antibodies against rabies virus in blood serum samples by ELISA and c) detection of tetracycline biomarker in the mandibles for the evaluation of vaccine bait uptake.

Results: The assessment of rabies situation between 2006 and 2014 is presented in Tables 1 and 2. During the passive surveillance, from 2006 to 2014, 3816 brain samples were tested by FAT and 922 (24.16%) reacted positive (Table 1). Within monitoring of ORV effectiveness, rabies was detected in 17 (0.34%) out of 4943 brain sam-

ples analyzed by FAT (Table 2). The number of rabid animals was reduced dramatically, which corresponds to the beginning of ORV of foxes in Serbia. From 2012, the total number of 26 FAT positive brain samples were examined by RT-PCR and all tested cases belonged to RABV field strain.

Table 1. Surveillance of rabies in Serbia between 2006 and 2014

549	192	34.97%
528	160	30.30%
740	233	31.49%
590	181	30.68%
462	104	22.51%
409	43	10.51%
271	9	3.32%
167	0	0.00%
100	0	0.00%
3816	**922**	**24.16%**

Table 2. Monitoring of ORV of foxes in Serbia between 2012 and 2014

1370	10	0.72%
2069	6	0.29%
1504	1	0.07%
4943	**17**	**0.34%**

Analyzed by animal species, the highest prevalence of rabies was recorded in the population of foxes and just sporadically in other wildlife or in domestic animals (Figure 1). Foxes made 84.66% (795/939) of all positive samples. In the population of domestic animals, the disease was more frequently recorded in cats then in dogs (7.13 % vs. 4.26%).

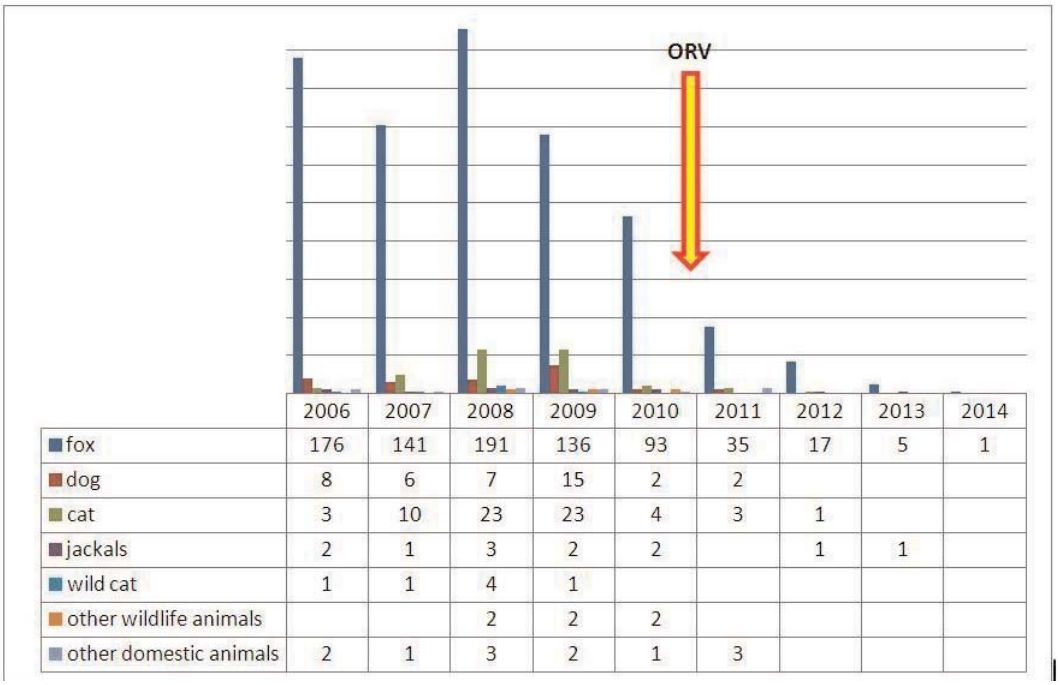

Figure 1: Prevalence of rabies in different animal species between 2006 and 2014

From September 2011 to May 2014, the total number of 4943 brain tissue samples, 4241 blood serum and 4971 mandibles were analyzed. Confirmed rabies-positive brains decreased from 10 in 2011/2012 to 6 in 2012/2013 and eventually to 1 positive case in 2013/2014. The seroconversion rate increased from 10.48% in 2011/2012 to 20.11% in 2012/2013 an 42.23% in 2013/2014. Along with the seroconversion, the number of detected tetracycline positive mandibles demonstrated an increasing tendency in the same period, being: 49.67% in 2011/2012, 62.60% in 2012/2013 and 90.33% in the monitoring programme carried out in 2013/2014.

Conclusion: The presence of rabies in neighbouring countries has enormous influence on the rabies epidemiological situation in Serbia. Lately, the highest number of rabid animals was reported in 2008, with 233 confirmed rabies cases. At the same time, many countries in Balkan region announced elevated number of rabies incidences: 1061 in Croatia, 1089 in Romania, 83 in Bosnia and Herzegovina, 54 in Bulgaria and 43 in Montenegro, and also 3353 cases were reported in Russian Federation and 2164 in Ukraine (WHO Rabies Bulletin Europe, 2008).

Several countries in Balkan region started the programme of oral vaccination: Bulgaria in 2009, Kosovo in 2010, Romania in 2011, Croatia in 2011, Macedonia in 2011, Montenegro in 2011, and Bosnia and Herzegovina in 2011. In Serbia, ORV of foxes and other wild carnivores was launched in 2010. All these countries reported significant reduction of rabies incidence after the implementation of the programme. Oral vaccination of foxes is effective method for elimination of rabies in wildlife population After nine campaigns of ORV in Serbia, the number of rabies-positive animals has declined significantly from 93 in 2010 to only one case in 2014 (Lupulovic et al, 2015).

Implementation of ORV of foxes also had a positive effect on the other animal species. Among the most susceptible wildlife populations are jackals and wild cats. The last case of rabid wild cat was recorded in 2009 and jackal in 2013. Regarding domestic animals, rabies occurred mainly in cats and dogs, as a consequence of sylvatic rabies. The number of rabid dogs and cats is also significantly reduced. In 2009 were registered 15 rabid dogs and 23 cats. The last rabies-positive dog was confirmed in 2011 and rabid cat in 2012.

Acknowledgments: This work is supported by Veterinary Directorate, Ministry of Agriculture and Environmental Protection of the Republic of Serbia, EU IPA projects and by project TR31084, funded by the Ministry of Education and Science of the Republic of Serbia.

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Characterisation in France of non-pathogenic lagoviruses closely related to the Australian Rabbit calicivirus RCV-A1: confirmation of the European origin of RCV-A1

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Objective: *Rabbit hemorrhagic disease* virus (RHDV) is the aetiological agent of a highly pathogenic infectious disease of domestic and wild European rabbits *Oryctolagus cuniculus*. First described in China in 1984, RHD has become enzootic in Europe, Australia and New Zealand, while periodic cases have been reported in domestic rabbits in other parts of the world. RHDV is a non-enveloped single- stranded positive-sense polyadenylated RNA virus (*Caliciviridae, Lagovirus)*. Its evolution shows relatively low genetic variability in the gene coding for the capsid protein VP60 but several genetic groups have been described, among which the antigenic variant RHDVa. In 2010, a new pathogenic lagovirus (RHDV2) affecting European rabbits but distant from RHDV was identified in France and quickly spread throughout Western Europe. Several studies have serologically evidenced non– pathogenic RHDV-like strains circulating in Europe and Australasia. Since the 1990's, several non-pathogenic or a lightly pathogenic (Michigan rabbit calicivirus, MRCV) lagoviruses have been characterised in different countries in domestic and/or wild rabbits. These viruses are genetically related to but distant from each other and from RHDV or RHDV2. In addition to the first non- pathogenic virus identified in Italy (RCV), two viruses for which the non-pathogenicity has been experimental confirmed have been characterised: the French 06-11 strain discovered in domestic rabbits and closely related to the English strain Ashington, and the Australian RCV-A1. The latter constitutes a new genotype and evolutionary analyses suggested that it has an European origin and has been introduced in Australia with introduced rabbits in the mid-19th century. However, RCV-A1 related viruses have not been collected and characterised in Europe yet. We have carried out a study aiming at characterizing non-pathogenic lagoviruses circulating in French wild rabbit populations. We successfully characterised 06-11 related viruses, as well as RCV-A1 related viruses supporting the hypothesis of the European origin of RCV-A1. We provided new molecular data that will contribute to a better understanding of the genetic evolution of lagoviruses.

Methods: Samples of duodenum (315) were collected on wild rabbits killed during the hunting seasons 2007-2008 and 2008-2009. They were collected in 23 locations within different regions of France including three islands. The presence of lagovirus RNA was tested by RT-PCR. Thirty mg of duodenum were homogenised and RNAs were extracted in a preliminary step with TRIzol® Reagent (Invitrogen) before the use of the "RNeasy Mini kit" (QIAGEN). Viral RNAs were reverse transcribed using oligo-dT (Invitrogen) as a primer and SuperScript™ II Reverse Transcriptase (Invitrogen). For the screening of positive samples, we used the pair of PCR primers U38/L337 derived from the one

(Rab1b-Rab2) defined in Strive et al. (2009) and the AmpliTaq Gold® DNA polymerase (Applera Applied Biosystems). The products (318 pb) were analyzed by electrophoresis and purified using UltraClean™ 15 DNA Purification Kit (Mo Bio Laboratories). For some of the samples, two successive amplifications were necessary to obtain sufficient quantities of DNA to allow their sequencing. DNA sequences were determined from both strands of the DNA template using the PCR primers and Big

Dye Terminator v3.1 (Life Technologies) as recommended by the manufacture, then analysed with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The entire capsid protein VP60 gene sequences of several positive samples were obtained following overlapping PCRs performed using Expand High Fidelity enzyme (Roche-Applied-Science). For some of these samples, we used nested- PCRs. The amplified templates were purified and sequenced as mentioned above using PCR and sequencing primers. Genotyping of the partial VP60 sequences obtained during the screening was performed by investigating the most homologous published nucleotide sequences using the BLASTn method available in NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic relationships were inferred using the VP60 sequences obtained and all the rabbit lagovirus VP60 sequences available in databases. Phylogenetic analyses were conducted using MEGA software version 5. Phenetic (Neighbor-joining and Minimum Evolution methods) and Maximum Likelihood analyses were implemented with the pairwise deletion option and based on the Kimura 2-parameter model including transition and translation substitutions. For the cladistic method, Maximum Parsimony was used and the pairwise deletion option was selected. Reliability of the trees was assessed by bootstrap with 1000 replicates. Pairwise nucleotide distance comparisons based on the p-distance model were conducted using MEGA5.

Results: Sequences corresponding to a lagovirus were identified in 19 samples from different locations distributed throughout France. Sequence analysis revealed that three distinct types of virus were characterised. Five viruses were closely related to 06-11 strain previously characterised in French domestic rabbits. Among them, two viruses (JA10 and JA34) were collected in the same location one year apart and were very close to each other (97.8% nucleotide identity). The other viruses (BP1, BO25, and CHA20) were collected in three other dis-

tant locations. These five sequences shared between 94.2% and 98.1% nucleotide identity. Sequencing also revealed 12 viruses more closely related to RCV-A1 than to RHDV, RCV, MRCV, 06-11, or RHDV2 (86%, 80%, 80.6%, 79.6%, 80.5%, 80.8% identity, respectively). They were collected in seven distant locations including one island. The nucleotide identity between these 12 viruses was comprised between 79.6% and 96.2%. Moreover, the VP60 sequences share a same amino acid insertion with RCV-A1 in position 436. They significantly clustered into several distinct genetic subgroups (clades), one of them represented by PLR56 and BT110 being closer to RCV-A1 strains than the other clades. Interestingly, both 06-11 and RCV-A1 related viruses have been detected in two different locations, showing a co-circulation of these viruses in wild rabbit populations. The two remaining characterised viruses (PLR 46 and PLR54) were closely related to RHDV (95.4% nucleotide identity in average) and RHDVa (98.6% nucleotide identity in average). Irrespective of the method used, phylogenetic analyses implemented with the entire VP60 gene sequences including four 06-11 related viruses and five RCV-A1 related viruses for which we obtained the complete VP60 sequences, confirmed these genetic relationships. The nucleotide identities between the sequences of each genetic group (intra-group) and between the sequences of the other lagovirus genetic groups (inter-group) were similar to the values obtained with the PCR screening. Thus, 06-11 related viruses only share an average of 80.1% identity at nucleotide level with RCV-A1 related viruses. The analyses also confirmed the distribution of the five RCV-A1-like sequences into 3 clades supported by significant bootstrap values, the third one (PLR56) being included with all the RCV-A1 strains. Overall, the rabbit lagoviruses were significantly clustered into four monophyletic groups, (i) RHDV and RHDVa viruses, (ii) MRCV, Ashington, 06-11, and 06-11 related viruses, (iii) RHDV2 and (iv) RCV-A1 and RCV-A1 related viruses.

Conclusion: We report the identification of two non-pathogenic lagovirus lineages that co-circulate in French wild rabbit populations with pathogenic lagoviruses. Our data show that the two types of viruses circulate throughout France. In addition, these two non-pathogenic lineages co-circulate at the population scale. The first linage is related to 06-11 strain previously described in France in domestic rabbits showing that it also circulates in wild populations. Otherwise, achievement of four new VP60 sequences shows that these viruses clustered with the Ashington strain collected in the UK, confirming the close genetic relationship between these viruses. This lineage forms a unique genetic group with RHDV-RHDVa, RCV and MRCV, which reveals a common ancestor between RHDV and these non-pathogenic lagoviruses. The second lineage is closely related to RCV-A1 and forms a highly significant monophyletic group with the Australian strains. We propose the name “Rabbit calicivirus Europe”, RCV-E, to distinguish these lagoviruses from the Australian RCV-A1. Our phylogenetic results confirm that RCV-E and RCV-A1 shared a common ancestor and confirm the European origin of RCV-A1. Otherwise, presence of PLR56 grouped within RCV-A1 may suggest that several introductions of RCV-A1 ancestors have occurred in Australia. Compared to 06-11 related viruses, RCV-E viruses show a higher genetic diversity suggesting that they have evolved in European rabbits for a longer time span than 06-11 related viruses did. Studies will be performed to estimate the time to the most recent common ancestors for the different genotypes of lagoviruses and notably for the RCV-E and RCV-A1. The molecular data acquired in this work contribute to understand the evolutionary history of lagoviruses.

Serological and entomological studies on Schmallenberg virus in Poland, 2013-2014

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Objective: Schmallenberg virus (SBV), is a new *Orhobuniaviridae* genus member, which emerged in Europe in 2011 causing fever, milk drop, and congenital abnormalities in ruminants. SBV transmission to Polish cattle was confirmed in the summer of 2012 when two bulls imported to Poland were found SBV seropositive and/or SBV RNA positive during quarantine. The virus spread rapidly in the neighboring herds infecting almost 40% of cattle after two weeks and 91% after ten months without causing any clinical disease. The virus was also confirmed in *Culicoides* midges collected in a trap 5 km away from one of the outbreaks. SBV spread across the country in the second half of 2012 infecting 3.4% of domestic and wild ruminants. In 2013 after overwintering, the virus continued to circulate infecting up to 90% of animals in a Lower Silesia Province in the first half of the year [1].

The aim of the study was to assess SBV seroprevalence in cattle, sheep and goats at province and country level in comparison to SBV monitoring in the midge vector in second and third year of epidemic in Poland.

Methods: A total of 10,038 serum samples from domestic ruminants (5,351 cattle, 4,410 sheep and 277 goats) collected in autumn 2013 and 2014 and originated respectively from 13 out of 16 Polish provinces were serologically tested using multi-species ID Screen Schmallenberg Virus Competition Test. The samples came from BTV monitoring program in Poland and their numbers were calculated using province population statistics estimating 20% seropositivity with 95% probability.

In order to study SBV distribution in the midges, a total of 32907 and 11994 females representing the most prevalent species in the country and four parity status were collected using 24 evenly distributed in the country Onderstepoort UV light traps in 2013 and 2014. The insects were tested in in pools containing 24 individuals on average. First the midges were homogenized in RLT Buffer using Lysing Matrix D Tubes with ceramic beads in Rybolizer. Total RNA was purified using RNeasy Mini Kit (Qiagen) in the automatic station Qiacube (Qiagen). Two pairs of primers designed to detect SBV S segment and a fragment of 18S midge gene as internal control were used in the in-house optimized real-time RT-PCR using AgPath-ID One-Step RT-PCR Reagents (Ambion, Applied Biosystem) kit in StepOne Real-Time PCR system (Life Technologies).

Results: The overall seroprevalences were 32.6% and 46.7% in 2013 and 2014, respectively. The highest seroprevalence was found in cattle (46.7% in 2013 and 58.5% in 2014), while it was lower in smaller ruminants (17.6% and 20.9% in 2013 and 31.6% and 39.8% in 2014 in sheep and goats, respectively). Overall seroprevalence in 2013 was the highest in northern provinces (Pomerania and Warmia-Masuria Provinces 63% and 53.7%,respectively), however in 2014 southern provinces: Lower Silesia and Lesser Poland Provinces had the greatest seropositivity (81.3% and 72.9%, respectively) but previously mentioned had also constantly high number of SBV antibody protected animals.

Among ten species of *Culicoides* genus identified in the samples collected around Poland, *C. obsoletus/scoticus* complex and *C. punctatus* predominated. In 2013, 8 out of 1404 midge pools (0.57%) collected in7 traps tested SBV positive with mean Ct value of 38.2 suggesting individual prevalence at a level of 0.0002%. The SBV positive midges originated from different parts of Poland. In 2014, only 2 pools out of 360 (0.56%) from 2 two traps with mean Ct value 36.8 were detected. Interestingly, in some traps SBV positive midges were found on consecutive years, which suggests that the virus 'overwintered' in the ruminants or in the vector. The latter one might be confirmed since one of SBV positive pools collected in May 2013 consisted of nulliparous (midges that have not taken a blood-meal or oviposited) *C. punctatus* females, while the rest were parous females (that have taken a blood-meal and oviposited at least once) of *C. obsoletus* and *C. punctatus* species.

Conclusion: The seropositivity level increased tenfold from 3.4% in 2012 as shown in previous study [1], to 32.6% in 2013 and subsequently was growing in 2014 to 46.7%. This result indicates that after rapid and vast spread in 2012 Schmallenberg virus successfully overwintered and as in other countries continued to infect immunologically naïve population of domestic ruminants. The presence of Schmallenberg virus decreased in 2013 and 2014 in *Culicoides* vector compared to 2012 [2], both with regard to the number of SBV positive pools, higher Ct values suggesting lower quantities of virus and the number of locations were positive samples were detected. Detection of *nulliparous* SBV-positive *Culicoides* female suggest transovarial route of transmission SBV virus and confirms previous studies [2]. The increase in percentage of seropositive animals between 2013 and 2014 was not significant and together with the very low occurrence of SBV in midges we can speculate that the dynamics of SBV epidemic has slowed down which perhaps will lead to the clearance of the virus from the country in the close future.

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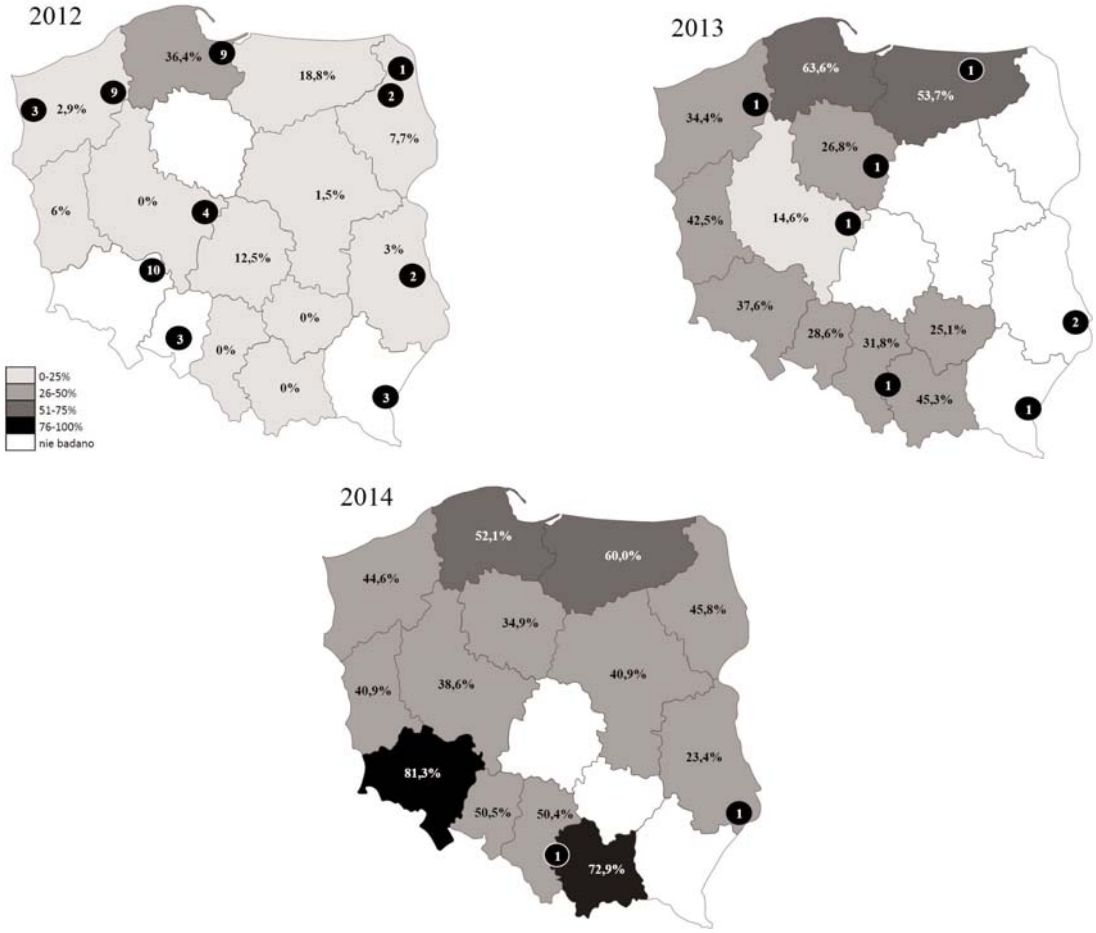

Fig. Maps of Schmallenberg virus infections in Poland in 2012-2014. Black circles indicate locations and the number of identified in RT-PCR SBV positive *Culicoides* pools. In shades of gray, and the percentage number indicates the proportion of seropositive samples from ruminants in each province. In 2012, the percentage of seropositivity includes farmed and free living ruminants [1], while in 2013 and 2014, only livestock.

Detection of neuropathogenic variant of equine herpesvirus 1 associated with abortions in mares in Poland

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Objective: A marked increase in the incidence of Equine Herpesvirus Myeloencephalopathy (EHM) cases caused by infection with neuropathogenic strains of equine herpesvirus type 1 (EHV-1) was reported in the last decade in many western countries. The purpose of the study was to estimate the prevalence of the neuropathogenic (G2254) and non-neuropathogenic (A2254) variants of EHV-1 among isolates associated with abortions in Polish stud farms.

Methods: Tissue samples (lung, liver, spleen, heart, kidney and placenta) from 64 aborted fetuses which were delivered to the Department of Virology of the National Veterinary Research Institute in Pulawy between 1999 and 2012, were tested. To distinguish neuropathogenic and non-neuropathogenic variants of EHV-1, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based on ORF30 specific amplification and *Sal*I restriction has been used. All positive samples were confirmed by sequencing of the target region of ORF30.

Results: The results of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing were consistent and showed that 2 (3.1%) out of 64 abortion cases were induced by neuropathogenic genotype (G2254). All remaining 18 (28.1%) EHV-1 positive abortion cases were caused by non-neuropathogenic genotype (A2254).

Conclusion: Most of the abortions in mares in Poland from 1999-2012 were associated with non- neuropathogenic strains of EHV-1. However, the presented data indicate that neuropathogenic variant of the virus is present in Polish stud farms. Such a presence may suggest that the emerging of EHM in Poland is probable.

Parallel ESVV Poster Presentation Session 3

Tuesday 1st September 2015
17:00-18:30
Room Sully 3

Chair: Carmina Gallardo & Serafin Gutierrez

Infectivity of CBPV major RNAs

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Objective: Chronic Bee Paralysis Virus (CBPV) causes an infectious and contagious disease of adult honey bees. This pathology is characterized by clusters of trembling, flightless, crawling bees and by individual black, hairless bees standing at the hive entrance (Bailey et al., 1983; Ball and Bailey 1997). CBPV was fist isolated in 1963 (Bailey et al., 1963). The viral particle is anisometric and measures 30- 60 nm in length and 20 nm in width. Initially, the genome of the virus was described as segmented in five single-stranded RNAs: 2 majors RNAs (RNA 1 and 2) and 3 minors RNAs 3a 3b and 3c. The complete sequence of the RNA 1 (3674 nucleotides long) and RNA 2 (2305 nucleotides) was determined (Olivier et al. 2008). However the three minor RNAs (1100 nucleotides each) previously described were neither visualized on gel nor detected during the sequencing of CBPV genome. Recently, it has been reported that the inoculation of purified CBPV RNA (naked genome) generates the chronic paralysis of bees (Chevin et al 2012). The complete genome sequence of major RNAs did not allow its classification among the existing virus families (Olivier et al 2008). Consequently, the determination of the essential genetic element for the virus replication is an important concern. In this study, we present the results of experimental *in vivo* inoculations of the two major CBPV RNAs.

Methods: CBPV complete particles were purified from bee heads by a 10-40% (w/v) sucrose gradient ultracentrifugation as described by Olivier et al. (2008). Viral RNAs were extracted using high Pure Viral RNA kit (Roche Diagnostic). CBPV RNAs were separated on 1% agarose denaturing-gel after electrophoresis using MOPS/formaldehyde buffer. The RNA fragments were isolated from the gel and eluted using QIAquick gel extraction kit (Quiagen). The RNAs were quantified by UV spectrophotometry. Purifed CBPV major RNAs were inoculated to CBPV-free emerging bees at amount of 10^9 RNAs copies per injection (Chevin et al 2012). After inoculation, the bees were incubated at 32 °C for 6 days and the clinical signs were observed and recorded daily. Asymptomatic, symptomatic, and dead bees were recovered and stored separately at -80°C. In order to show CBPV replication, we used a specific RT-PCR detecting the negative strand form of CBPV RNA (Cell et al., 2008). The CBPV RNA 1 copies number was estimated by real time RT-PCR carried out on an Applied Biosystems 7500 Real-Time PCR system (Blanchard et al 2007, 2012). RNA 2 copies number was quantified by a new real time RT-PCR specific of CBPV RNA 2 (ORF3 sequence).

Results: Our results showed that inoculation of the CBPV major RNAs induce the chronic paralysis. Five conditions were tested: 1) Bees not inoculated as a negative control, 2) Bees inoculated with CBPV particles as a positive control, 3) Bees inoculated with naked CBPV RNAs (corresponding to the whole CBPV RNAs extracted using

the high Pure Viral RNA kit) as positive control, and 4) Bees inoculated with purified CBPV major RNAs (RNA 1 and 2 extracted from agarose gel and inoculated together). The clinical signs were observed in all inoculated groups except in negative control group within five days post inoculation. Using quantitative RT-PCR, we showed that RNA 1 and RNA 2 copies number increase significantly during the infection. Immediately after inoculation, the CBPV RNA copies number per bee was estimated at 10^3 for conditon 2, 10^5-10^6 for condition 3 and 4). Five days post inoculation the load of RNA copies per bee increase to acheive 10^{11}-10^{12} for conditions 2), 3), and 4). Moreover, we detected the negative strand form of CBPV RNA by specific RT-PCR in these three conditions, showing the replication of the virus.

Conclusion: These results showed that the 2 CBPV major RNAs isolated in agarose gel are infectious and initiate the replication of the virus. Thus, the three minor RNAs initially described by Overton et al. do not seem necessary for the virus replication. Therefore, these results provide essential and useful tools to establish the first reverse genetic systems of CBPV in order to manipulate its genome and study the function of its proteins.

Rare recombination events and occurence of superinfection exclusion during synchronous and asynchronous infection with homologous murine norovirus strains

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Objective: Human noroviruses (HuNoVs) are recognised as one of the major global causes of non-bacterial gastroenteritis with significant morbidity and mortality in developing countries and a high economic impact in developed countries. Spread primarily via the faecal-oral route, HuNoV infection is typically an acute self-limiting gastrointestinal illness. However, chronic HuNoV infection of immunocompromised persons has been identified as a persistent cause of disease and viral populations in such patients have been postulated as possible reservoirs for novel NoV variants.
The *Norovirus* genus belongs to the *Caliciviridae* family of small, non-enveloped, positive sense, single-stranded RNA viruses. This genus is subdivided into at least six genogroups, which infect humans and various animal species. Until the recent report of low-level infection of cultured human B cells, no viable cell culture system existed for the study of HuNoVs. The robustness of this new cell culture system still poses a major hurdle, so that the murine norovirus (MuNoV), replicating efficiently in murine dendritic or macrophage cells, remains the model of choice for in vitro study of noroviruses.

The molecular mechanisms driving viral evolution and specifically that of NoVs, are accumulation of point mutations and recombination, which enables the emergence of new combinations of genetic materials to generate potentially dramatic genomic changes in a recombinant NoV, which clusters within two distinct groups of NoV strains when two different genomic regions are phylogenetically analysed.

The mechanism for NoV recombination is proposed to follow the copy-choice mechanism, involving a template shift during simultaneous replication of two strains infecting the same cell. Numerous NoV recombination events have been highlighted by in silico methods and the phenomenon has recently been shown in vitro with two homologous MuNoV strains.

The object of this study was to qualitatively and quantitatively assess virus progenies generated by the use of different parameters of co- and superinfection of RAW264.7 cells with two homologous MuNoV strains (CW1 and Wu20) and thus help to specify important parameters for the occurrence of recombination events. As prerequisite for recombination events, co-and superinfection are of special interest in viral diseases, such as NoV, for which a persistent stage can be developed.
Methods:

Viruses and Cells
Murine NoVs isolates CW1 and Wu20 were plaque purified and propagated in RAW 264.7 cells (ATCC TIB-

71).Virus stocks were produced by infection of RAW 264.7 cells at an MOI of 0.05 and clarified by centrifugation. Passages 8 and 7 for CW1 and Wu20, respectively, were used for the experiments.
Co-infection and superinfection experiments Monolayers of RAW 264.7 cells were infected with Wu20 at a MOI of 1 on ice. After 1 h, the Wu20 inoculums were removed and stored. The cells were washed twice with PBS and infected with CW1 at various MOI (0.1 ; 1 ; 10) and at various delays of co- or superinfection (0 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h). For co-infections, CW1 and Wu20 were simultaneously inoculated on the cells for 1 h on ice. Twenty-four hours after CW1 co- or superinfections, cells and supernatants were collected.

Molecular analysis

RNA was extracted both from supernatants from the experiment and from propagations of individual plaques, reverse transcribed into cDNA and analysed via two parallel real time PCR reactions allowing discrimination between CW1 and Wu20 at both genomic extremities (regions 1 and 5, located at the ORF1 and ORF3 terminal respectively), as previously described by Mathijs et al., 2010.

For analysis of the supernatants, quantifications were also performed via real time PCR. Accordingly, amplicons corresponding to region 1 were amplified for both CW1 and Wu20, then cloned and in vitro transcribed to provide a standard curve for RNA copies. Following this, values for genomic copies were deduced and results were normalised with GAPDH gene transcripts.

Isolation and screening of progeny viruses

A plaque assay for virus isolation from the co- or superinfection experiments was set up by modifying the protocol described by Hyde et al. (2009). Thus, after 24 h of incubation, 36 plaques were randomly picked for each condition and propagated by inoculation onto RAW 264.7 cells.

After this amplification step, monitoring of recombination events was performed by PCR and Real- Time PCR on extracted, reverse transcribed viral RNA, using two sets of primers to amplify regions 1 and 5. The use of two pairs of TaqMan probes allowed discrimination of the strains WU20 and CW1 in two different regions and identification of recombinant strains.

Results:

Molecular analysis

The Real-Time PCR performed on supernatants collected at 24 h post infection showed a greater number of copies of MuNoV Wu20 cDNA in almost all conditions, except t 0 h, 0.5 h, 1 h, 2 h at the MOI 10, where an increase in the number of copies of the CW1 strain was noted.

In particular, the latter showed a peak at 1 h at the MOI 10 (89%) followed by a rapid reduction in later times (t 8 h: 20%).

Interestingly, for both viruses expected ratios were never attained during the study with the notable exception of the MOI ratio 0.1/1 and the condition t1 MOI 10/1.

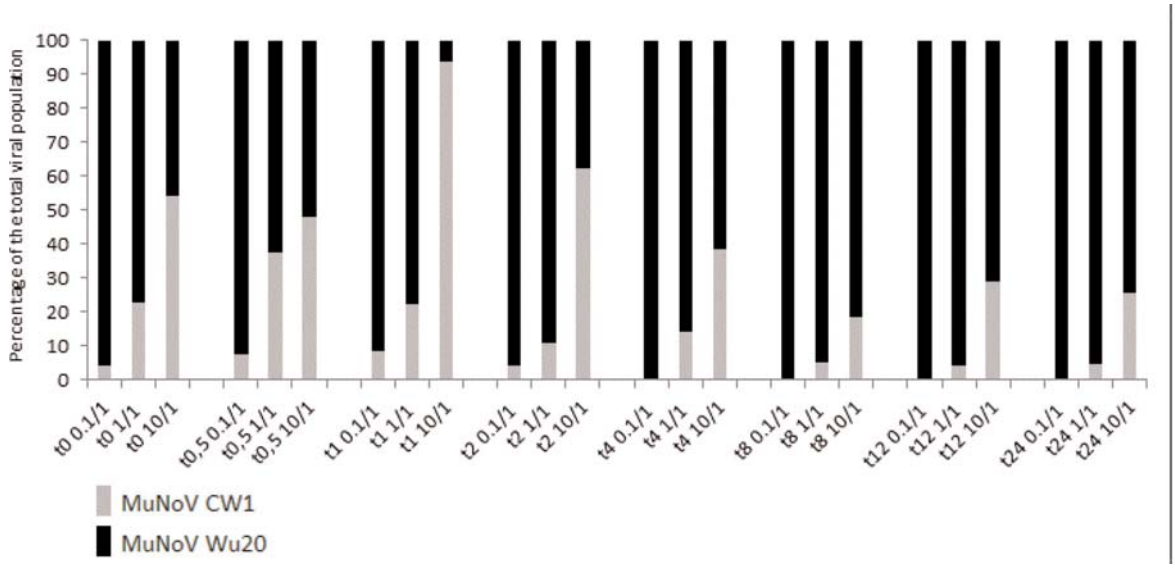

Fig. 1: Genomic quantification establishing the relative percentage of the total viral population 24 h post infection for genomic copies of the two MuNoV strains

Isolation and screening of progeny viruses

Molecular analysis conducted on plaques selected in the condition of coinfection at MOI 1 and 10 highlighted a predominance of the strain MuNoV CW1 (90%) from t 0h to t 2h, followed by a sharp reduction from t 4h leading to complete absence at t 24h.

The Wu20 strain showed a progressive increase from 4h (10%) to 24h (100%).

Overall, the occurrence of recombination events was very rare. Only three putative recombination events were detected at t1 h MOI 1/1 and t 4 h MOI 1/1 (marked by an asterisk in Fig. 2)

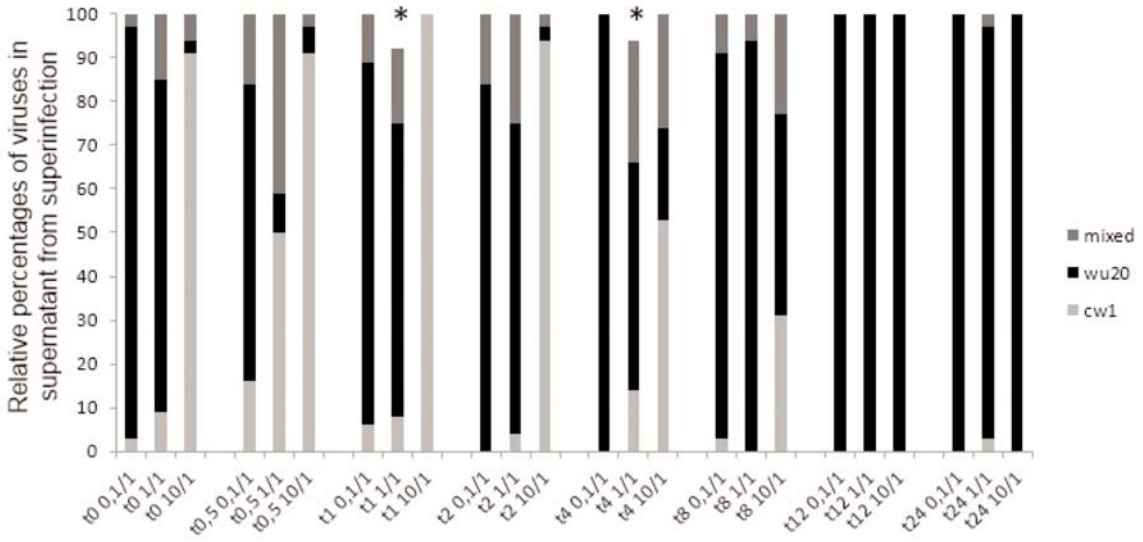

Fig. 2: *Superinfection parameters: time of superinfection and relative proportion of viruses (CW1/Wu20)*

Conclusion: The profiles of viral ratios over time are highly interesting. Particularly the infection with CW1 at the MOI 10, with a relative percentage of genomic quantifications of about 50% at the two first time points is intriguing. While the percentage is changed completely at t1 MOI 1 to give the expected ratio of 90/10, it then gradually decreases over the next time points to less than 10% at 24 h post infection.
The presence of numerous recombinant viruses as a possible explanation for the t1 MOI 1 peak seems likely, as very few putative recombinants were detected during the screening process.

The single-step growth kinetics established by Mathijs et al in 2010, showing great similarities for both strains, indicate that the replicative cycle dynamics of the viruses are probably also not responsible. The decrease, especially marked after 8 h, is suggestive of a superinfection exclusion mechanism, where productive infection with Wu20 induces a resistance of the cells to infection with the homologous CW1. Alternatively, in view of the above-mentioned growth curve at high MOI, the decrease might also be due to the end of the first replication cycle having been reached, with no more viable cells left for infection. Considering the 50% viral ratio estimated by genomic copies for the early time-points, the identification of CW1 as the predominant strain (90%) after plaque purification for the same time appears to be somewhat of a discrepancy and merits further investigation

Although circulating recombinant NoV strains seem to be common, in vitro recombination is a rare event, at least in the protocol described above, and does not seem to be easily influenced by parameter changes such as time of infection and MOI. Parameters where putative recombinations were identified include t1 MOI 1 and t4 MOI 1. The possible recombinants are yet to be confirmed by sequencing reactions.

Further study is necessary to understand mechanisms favouring the predominance of replication of recombinant virus strain in vivo and the challenges of such a replication in vitro. The occurrence of recombination was theoretically limited to one cycle of replication by the protocol (MOI 1 of Wu20).

More than one replication cycle might be necessary to enhance the process of recombination by increasing the number of replicating events that could favour recombination. Thus, initial infection at a lower MOI might be an interesting future consideration. Other mechanisms than a time-dependent coinfection might also be worth exploring.

Acknowledgements: We thank Professor Herbert Virgin and Dr Larissa Thackray (Washington University, St Louis, MO, USA) for providing the MNV isolates and RAW 264.7 cells.

Feline Immunodeficiency Virus (FIV) in free ranging Leopards (Panthera pardus), from the Kruger National Park, South Africa

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Introduction and Objective:
Feline Immunodeficiency Virus is a recently discovered Lentivirus (*Retroviridae*) which closely resembles HIV. The virus is perceived to be host-specific, and is usually transmitted from one individual to another through direct contact. Very few studies have focused specifically on FIV in leopards (FIV_{Ppa}), as exemplified by the availability of only five FIV_{Ppa} *pol*-RT sequences in Genbank. This study aims to screen leopards (*Panthera pardus*) from the Kruger National Park, South Africa, for FIV in order to determine the presence FIV_{Ppa} and assess its molecular epidemiology.

Methods: A total of 26 leopard blood samples (11 males and 8 females) from KNP were screened for FIV using a PCR protocol designed to target a 577bp fragment in the FIV *pol*-RT gene region. Multiple sequence alignment was undertaken using ClustalX Version 2.1 and manually adjusted. Phylogenetic and molecular evolutionary analyses were inferred using Maximum Likelihood methods, implemented in MEGA Version 6.

Results: Overall prevalence of FIV_{Ppa} in leopards is estimated at 73%, with no difference in prevalence between male and female leopards. FIV prevalence appears to increases with age, with a higher prevalence detected in adult (>4 years) (84%) versus sub-adult leopards (<4 years) (43%), consistent with previous studies focused on lions and pumas. Phylogenetic analysis of the FIV *pol*-RT region shows two distinct groupings within FIV_{Ppa}. These two groups may represent a common Kruger National Park Group (n=20), and a more recently introduced lineage from another unknown geographic origin (n=4).

Conclusion: FIV appears to be prevalent in leopards. Evidence to support multiple lineages within FIV_{Ppa} suggests the possibility of geographic variation within FIV_{Ppa} and the possibility of distinct subtypes similar to species such as African lions and puma. The larger FIV_{Ppa} dataset also provides new insights into the epidemiology of this less well-studied FIV strain. With such high prevalence rates, further studies focusing on immunological and clinical consequences of FIV in leopards is required, especially since leopards are increasingly exposed to bovine tuberculosis co-infections.

Intra-host phylodynamic analysis of canine distemper virus

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Canine distemper virus (CDV) (*Paramyxoviridae, Morbillivirus)* is the causative agent of Distemper, a serious threat for domestic and wild carnivores worldwide. Given the characteristics of RNA viruses (high mutation rates, short generational times and huge population sizes) is an intrinsic feature of this group its remarkable inter and intra-host genetic heterogeneity. Phylodynamic analyses were carried out based on sequences obtained by ultra-deep sequencing from four tissues (cerebellum, lung, spinal cord and bladder) of a necropsied dog with clinical Distemper, with the aim of investigating the population dynamics during the infection course. Our study showed the presence of intra-host subpopulations evidencing viral tissue-compartmentalization. The migration patterns analyses revealed the lung with the highest probability for being the ancestral tissue from which the variants disseminated to the remaining compartments. At the cerebellum, the infection showed a persistent pattern without posterior migratory events, which may indicate that this CDV strain is neurovirulent. This integrative analysis is the first report for the *Paramyxoviridae* family, revealing the potential relationship between compartmentalization, migration and disease progression, seeking for understanding the viral pathogenesis.

Molecular phylogeography of canine distemper virus

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Canine distemper virus (CDV) (*Paramyxoviridae, Morbillivirus)* is a worldwide spread virus causing a fatal systemic disease in a broad range of carnivore hosts. Although it is assumed that most CDV lineages follow a geographical pattern, the geographic origin and evolutionary history of the virus still remain uncertain. The aim of this study was to provide an extensive description of spatio/temporal population dynamics of CDV. An approach based on Bayesian inferences was implemented using 208 full-length hemagglutinin gene nucleotide sequences isolated in 16 countries during 37 years (1975 - 2011). The most recent common ancestor was consistently dated around 125 years ago (1886, HPD 95%, 1858 -1913) in the United States, and has diverged into two major ancestral lineages. One lineage diversified into the current America 1 lineage which recently spread to Asia; the other ancestral lineage has diversified and spread worldwide originating the remaining eight lineages characterized to date. The evolutionary history of CDV was characterized by its geographic origin, several migratory events and local differentiation without population expansion. CDV exhibits high spread capacity accompanied by a significant genetic flow between domestic and wildlife hosts; being domestic hosts the main viral reservoirs worldwide.

Genome evolution of canine parvovirus in South America

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Canine parvovirus (CPV) causes one of the most significant infectious diseases of dogs that has to be controlled by early vaccination. Its genome has a linear single-stranded DNA genome (5.2 kb) that codes for nonstructural (NS) and capsid (VP) proteins. In the present study, we analyze CPVs from South America to characterize the strains and to provide new insights into local and global viral diversity and evolution. Samples were collected from Uruguay, Argentina, Brazil, Paraguay and Ecuador in the last decade. The complete genome was obtained in a single PCR reaction and directly sequenced. Phylodynamics analysis was performed to characterize the circulating strains and estimate the time of the most recent common ancestor. We identified four CPV phylogenetic groups or circulating in South America. These groups have different evolutionary history and residence time and are unevenly distributed in South America. Two clear recombinant sequences were detected in Ecuador and Uruguay. Some relevant amino acid chances occur in positive selective sites in the VP and NS proteins. Our findings reveal that several factors contribute to the current distribution of canine parvovirus in South America. There is co-circulation and spreading of genotypes from different geographic origin, local differentiation by the acquisition of particular amino acid changes in positive selected sites, deletions in non-coding sequences (VP1 intron) and recombination. CPV variability reveals a particular dynamics in the continent that provide new insight into the evolution of this relevant virus.

Molecular characterization of Rift Valley fever virus isolates from Mozambique and phylogenetic comparison with selected other isolates

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Background: Rift Valley fever virus (RVFV) is an arthropod borne pathogen causing Rift Valley fever, a zoonotic disease that affects ruminant animals and humans. The disease has the potential to cause severe economic losses and outbreaks have occurred in sub-Saharan Africa, Egypt, Saudi Arabia and Yemen. In the past decade, eastern and southern Africa have also experienced several outbreaks. More recent outbreaks include 2007/10 in Sudan, Kenya and Tanzania, 2007/8 in the Comoros and major outbreaks in 2008/9, 2010 and 2011 in South Africa. However, factors and mechanisms influencing the spread of the virus and the re-emergence of the virus after long inter-epidemic periods are not fully understood. Both animal and human infections have been associated with high rainfall patterns which lead to increased mosquito populations, the principal vectors of the virus. The virus has a tripartite segmented RNA genome, comprising large (L), medium (M) and small (S) segments. The M- segment codes for glycoprotein Gn, glycoprotein Gc and two non-structural proteins. The Gn and Gc proteins form the glycoprotein shell around the capsid. Phylogenetic analysis to infer evolutionary relationships could include parts of all three segments or the entire genome but sequence data from the M-segment is most commonly used. Rift valley fever viruses are grouped into 11 lineages with many viruses from southern and eastern Africa grouping in Lineage C.
Objectives: The first aim of this study was to gain insight into the epidemiological dynamics of RVFV in southern Africa through an evaluation of blood and tissue samples from suspected RVFV outbreaks in Mozambique in 2013/14. The presence of RVFV RNA was determined using reverse transcribed (RT)- quantitative PCR (qPCR) and sequence data generated. A further aim was to compare sequence data from these isolates with selected other isolates from neighbouring countries and other regions where outbreaks have occurred.

Methods: A total of 54 blood, serum and organ samples were collected from two small suspected RVF outbreaks in the Goba and Chibuto areas, Maputo Province of Mozambique. Samples were analysed using RT-qPCR targeting the conserved region of the L-segment of the virus. In view of phylogenetic analysis a conventional PCR assay was used targeting a 490 nt portion of the Gn encoding gene on the M-segment. Phylogenetic analysis was conducted using sequence data from the 490 nt portion as well as the entire M-segment obtained from isolates from Mozambique, unpublished isolates from outbreaks in 2008 in South Africa, Namibia (2004) and Madagascar (2008), obtained from collaborators, as well as sequence data of selected isolates available in GenBank. Whole genome assembly using next generation sequencing and reference mapping of one isolate from Mozambique obtained from foetal liver material was also done.

Results: The RT-qPCR assay detected RVFV RNA in 34 of 54 of the various sample types analysed, confirming the presence of RVFV in the outbreaks in Mozambique. Only PCR amplicons obtained from tissue (foetal material) samples met the minimum concentration requirement for sequencing. The Mozambique isolates along with the unpublished South African isolates grouped together with the Lineage C group of viruses. Phylogenetic analysis showed epidemiological links between the outbreaks in Mozambique and previous outbreaks in Sudan, and isolates from Mozambique were more closely related to isolates from Sudan than to unpublished and published isolates from neighbouring South Africa and Tanzania. The unpublished isolates from South Africa and Namibia grouped together with those from the Kenya I sub-lineage. The Kenya I sub-lineage consists of RVF viruses that caused outbreaks in 2006/7 affecting Kenya, Tanzania, Somalia and in 2008 affecting Madagascar, Mayotte and South Africa. These groupings were not influenced by the sequence length of the M-segment used. The bootstrap support values at the node for the Lineage C group of viruses were 89% for the partial and 100% for the complete sequence analysis.

Conclusion: This study reported genetic evidence of RVFV circulation in 2013/2014 in the Maputo Province of Mozambique with closer links to isolates from the 2007 and 2010 RVF outbreaks in Sudan than to the 2008 outbreaks in neighbouring South Africa. This relationship of the Mozambique isolates to isolates from East Africa might imply introduction of the virus into Mozambique from those countries where viruses within Lineage C are established. There are also reports suggesting that the 2008-2011 RVF outbreaks in South Africa were also as a result of introduction from East Africa. However, the factors underlying this distant spread are largely unknown.

With this new genetic evidence of RVFV in Mozambique, all countries on the eastern coastline of Africa, the Indian Ocean islands of Madagascar and Mayotte as well as Yemen and Saudi Arabia, have reported viruses from Lineage C. This broad geographic distribution pattern of the virus requires further characterisation in order to identify factors important in the epidemiology of the virus.

Experimental infection of domestic pigeons (Columba livia L.) with lineage 2 West Nile virus

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Objective: Wild birds are the natural hosts of West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus*). In Europe, WNV strains belonging to lineage 1 are endemic in the Mediterranean region. Additionally, a lineage 2 strain of WNV emerged in Hungary in 2004. It became resident in central Europe and in the east-Mediterranean region, and caused outbreaks of West Nile encephalitis in humans and animals. The natural transmission cycle of WNV is based on mosquito vectors. However, fatal WNV infections due to the lineage 2 strain were frequently observed in birds of prey in Hungary and in Austria, therefore an alternative route of infection via consumption of infected prey carcasses were hypothesized. Because WNV-affected captive raptors were often fed with pigeons, this species was suspected as a potential source of infection. To estimate the host competence of domestic pigeons to WNV lineage 2 we have performed experimental infections.

Methods: Commercially reared homing pigeons (rock dove, *Columba livia* L.) were quarantined in mosquito - free environment for two weeks. Seronegative birds were placed in BSL-3 animal house facilities and were experimentally infected with a field isolate of WNV lineage 2 (strain 578/10, horse, Hungary, 2010). In a first experiment pigeons were infected with 1 to 10,000 $TCID_{50}$ of WNV and were observed for 21 days. Oral and faecal samples were collected individually every day, blood samples were collected weekly. Animals were put down on day 21, carcasses were necropsied and organ samples were collected. In a second experiment pigeons were infected with 1,000 $TCID_{50}$ of WNV. On every second oral and faecal swabs were collected, two birds were sacrificed, necropsied and organ samples were collected. Swab and organ samples were tested for the presence of WNV RNA with quantitative real-time RT-PCR method. Virus back-isolations from selected, PCR-positive samples were performed in Vero cell cultures and by suckling mouse brain inoculation. Anti-flavivirus antibodies were detected by competitive, WNV ELISA method.

Results: Within both experiments all pigeons remained clinically healthy. In the first experiment, birds infected with 1,000–10,000 $TCID_{50}$ of WNV became seropositive on day 7; those infected with 100 $TCID_{50}$ became seropositive on day 14; while the ones infected with 1–10 $TCID_{50}$ remained seronegative. Birds infected with higher doses of WNV have been shedding the virus by oral discharges and / or faeces between days three and eleven after infection. Virus RNA was detected in the spleen of infected birds even 21 days after infection. In the second experiment 6 out of 12 pigeons inoculated with 1,000 $TCID_{50}$ of WNV have been shedding the virus by pharyngeal discharge and / or faeces between day 3 and 12. Viraemia was detected in 4 birds (DPI 4-12). Viral RNA was detected in the spleen (n=7, day post infection [DPI] 2-12), liver (n=5, DPI 2-8), kidney (n=4, DPI 2-12), intestine (n=4, DPI 4-12), and lung (n=2, DPI6-8) samples. The virus was detected only in one brain sample (DPI 12). Anti-WNV antibodies were detected in three birds (DPI 10-12). Three contact control birds were kept together with the infected ones for 12 days. Within this period they remained WNV-free and seronegative.

Conclusion: The results of the study indicate that pigeons are susceptible host of the lineage 2 WNV strain which is circulating in central Europe. The birds carried the virus for at least three weeks and have been shedding it

through saliva and faeces. However, the viraemic phase was relatively short and no direct transmission to contact control pigeons was recorded. Nevertheless, pigeons might be sources of non-vectorial transmission of WNV to predatory birds.

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Long range RNA-RNA interactions within the genome of classical swine fever virus; influence on viral RNA replication

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Objective: Almost 30 years ago, an alternative mechanism of translation initiation on picornaviral RNA was characterised in which the ribosome could bind internally within the 5' untranslated region (UTR) via translation initiation factors. This RNA element, denoted the internal ribosome entry site (IRES), is able to direct cap-independent initiation of protein synthesis. Besides IRES function, the 5'UTR may also contain cis-acting RNA elements that are important not only for viral translation and replication but also important in regulating the switch between these processes. In hepatitis C virus (HCV), a domain termed 5BSL3.2, in the coding sequence for NS5B (the RNA polymerase), is known to interact with IRES subdomain IIId and this interaction is believed to be pivotal for the translation/replication switch.

A similar interaction within the RNA genome of classical swine fever virus (CSFV), which contains an IRES element similar to that of HCV, is highly likely. A sequence within the coding region for NS5B of CSFV has been identified, from sequence analysis, to have the capability of interaction with the IRES.

Methods: To investigate whether this putative interaction in the CSFV RNA is important in viral replication, a triplet motif of three cytosines (CCC) within NS5B was modified to affect the possible interaction with the IRES domain $IIId_1$ that contains a GGG motif. Specifically, silent mutations in the CCC motif (CAC, CGC and CUC) were generated by site-directed mutagenesis of a CSFV replicon containing Gaussia luciferase. Thus, any variation in replication efficiency should not be due to changes in polymerase activity but rather due to interactions with other parts of the viral genome. The effect on replication was measured using the luciferase reporter system. RNA transcripts generated in vitro were introduced into porcine PK15 cells by electroporation and the replication efficiency was assessed by measurement of luciferase activity.

Results: Initial results indicate that all three NS5B mutants have reduced viral replication efficiency. In particular, the mutant with a cytosine to uracil change at the last base of the codon (resulting in no amino acid change within the protein), exhibited a substantial decrease in replication.

Conclusion: These results indicate that the three consecutive cytosines within the NS5B coding region are required for efficient replication. This effect is not associated with any change in the amino acid sequence of the RNA polymerase but hence is probably due to interactions within the RNA genome. An extensive network of RNA-RNA interactions has been characterised for HCV but knowledge on RNA-RNA interactions within CSFV is still scarce. Further understanding on these putative long range interactions will provide valuable insight into mechanisms underlying viral RNA replication.

Expression and serological reactivity of Hemorrhagic enteritis virus hexon protein

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Objectives: The aim of this work was to express recombinant hexon protein of Hemorrhagic enteritis virus, to establish the diagnostic value of this protein for serological detection of antibodies in turkey serum samples and

to assess seroprevalence of the infection in the Czech Republic.

Methods: The N´ terminal part of the hexon protein was expressed in bacterial expression system and used as an antigen in ELISA test for the detection of hemorrhagic enteritis virus specific antibodies in turkey sera. Validation of the test was performed by comparison with commercially available ELISA test. Serological reactivity was assessed on a panel of 126 turkey sera by newly developed ELISA test. Serum samples were taken from turkey farms with the history of Hemorrhagic enteritis virus infection, from farms with animals free of infection and from turkey farms following vaccination.

Results: Successful expression of polyhistidine-taged hexon protein was performed in *E. coli* Top 10 cells. Two recombinant proteins with molecular weight of 38.6 kDa (HEV1 fragment) and 42.5 kDa (HEV2 fragment) were detected on nitrocellulose membrane with anti polyhistidine antibody which corresponded to calculated molecular weights (including N´ terminal polyhistidine). The reactivity of turkey sera with hexon protein fragments was demonstrated by immunoblotting. Both purified proteins displayed positive reaction with control positive HEV turkey sera. However detection of antibodies on a panel of 126 turkey serum samples has shown that HEV2 protein reacts with significantly lower number of sera compared to HEV1 protein and Synbiotic ELISA. For this reason only HEV1 fragment was used for development and testing of recombinant ELISA test.

All collected turkey 126 serum samples were examined using both ELISA tests; both tests were in accordance in all tested samples. The specificity and sensitivity of HEV1 ELISA test was thus determined to be 100% (sensitivity) and 100% (specificity) when compared to Synbiotic ELISA.
Conclusion: ELISA based on recombinant hexon protein thus proved useful and cheaper for detection of antibodies in turkey flocks infected with Hemorrhagic enteritis virus.

Genetic diversity and pathological findings of Small Ruminant Lentiviruses Virus in sheep and goats in the European bordering region (Marmara) of Turkey

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Objective: Small ruminant lentivirus (SRLV), also known as Maedi/Visna virus and caprine arthritis encephalitis virus, is an RNA virus belonging to genus of lentivirus in the family of *Retroviridae* and effects animal health by causing chronic inflammatory and degenerative lesions in the brain, lung, joints and mammary gland in sheep and goats. Previous studies have shown that SRLV is present in sheep and goat population in Turkey and many serological studies were performed to determine the frequency of SRLV. However, genotypic variations in regions are not well documented. Hence, this study was aimed to investigate frequency of disease in animals with neurological and respiratory symptoms in the European bordering region (Marmara) for the first time and to determine similarity and diversity of circulating strains with other strains detected in the world as well as with those found in Turkey.

Methods: In total, 29 lung and 18 brain tissue samples from 2-24 months old dead sheep (25) and goat (4) originated from the Marmara region were included in the study. Samples from 4 sheep that died with the neurological symptoms and from 25 sheep and goat that died with the respiratory symptoms were taken for pathology and Virology. For histopathology, tissue samples were fixed in 10% formalin solution and further processed and stained with hematoxylin and eosin. The slides were examined under a light microscope.

Viral DNA was extracted from 20 mg tissue samples using a DNA isolation kit (QIAamp DNA Mini Kit- Qiagen) as described by the manufacturer. The amount of DNA in the extracted material was measured using a NanoDrop spectrophotometer (NanoDrop 1000c, Thermo Scientific). Approximately 100-200 ng/µl DNA was used for gag-gene and LTR-gene sequence analyses.

Primers for the sequence analyses LTR region and gag gene region were from published literature and the PCR

reaction was performed as described by others (Extramania et al. 2002; Grego et al.,2007).

Samples found positive by PCR were subjected to sequencing and phylogenetic analyses. Products obtained by PCR using the LTR and gag gene primers were sequenced by a commercial company (REFGEN, Turkey). Multiple alignments of LTR and gag gene sequences were made by using the Clustal W program (Geneious 6.1). Phylogenetic analyses were carried out by using the criterion of neighbor-joining trees based on genetic distance model Tamura-Nei principle.

Results: In total, 4 samples (lungs of 3 sheep and lung of 1 goat) were found to be positive by PCR. Tree sheep samples were found to be positive by LTR gene primers sets. Two sheep and one goat were found to be positive by gag gene primers sets. A phylogenetic tree generated by using the sequences of LTR and gag gene showed that SRLV detected in 2 sheep lungs were clustered in subtype A while SRLV found in one sheep and one goat lung were clustered in subtype B1.

A large quantity of foamy fluid was present in the trachea and in the cut sections of the lungs. Intestinal hyperaemia was observed and the intestinal content was quite watery. There were areas of emphysema and hepatization in the lungs. On histopathology, infiltration of mononuclear cells localized around bronchioles and vesicular emphysema on alveoli and smooth muscle hyperplasia within the alveoli were observed.

Conclusion: The primary results on the detection and presence of SRLV in lung and brain samples in Marmara region shows that the circulating subtype in sheep and goat is found to be B1 and A in the gag region phylogenetic classification. Generally genotype B was reported in goats but in this study one sheep partial gag sequence was identified as subtype B1. The collection of samples, detection and sequencing studies are still going on.

Immunization of Day-Old Chickens with Recombinant Viruses Expressing Chicken Parvovirus VP2 Protein

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Objective: To determine the efficacy of immunization with baculovirus or Newcastle disease virus recombinants expressing chicken parvovirus (ChPV) VP2 protein against challenge inoculation with ChPV ABU-P1 strain in day old chickens.

Methods: A baculovirus (BV) recombinant and a Newcastle disease virus (NDV) recombinant were constructed using the standard BV expression vector and a lentogenic NDV vaccine strain, PHY- LMV-42 inserting the VP2 protein of the chicken parvovirus (ChPV) ABU-P1 strain. Expression of the VP2 was confirmed by immunofluorescence staining in virus infected cell cultures with rabbit antipeptide VP2 antiserum. Day-old broiler chickens were inoculated orally with the BV-VP2 or NDV-VP2 and challenged orally three days later with the pathogenic ChPV ABU-P1 strain. Cloacal swab samples and small intestines were taken at 4, 7, and 14 days post challenge and parvovirus shedding was determined using a chicken parvovirus-specific quantitative real-time PCR (qRT-PCR) assay.

Results: The recombinant virus NDV-VP2 exhibited similar growth characteristics in vitro and pathogenicity in chickens when compared to the parental NDV. Both the BV-VP2 and NDV-VP2 recombinants demonstrated a strong expression of the ChPV-VP2 protein in virus infected Sf9 or VERO cells, respectively. The qRT-PCR data showed that ChPV shedding was significantly reduced in birds that were immunized with BV-VP2 or NDV-VP2 when virus content of cloacal swabs or small intestines were compared to those collected from PBS inoculated and challenged birds.

Conclusion: These results suggest that immunization of day-old broilers with recombinant viruses expressing ChPV VP2 protein may induce a host response that results in decreased parvovirus replication in the intestinal tract and could potentially lead to less severe ChPV-induced enteric disease.

Replication characteristics of respiratory and nephrophathogenic infectious bronchitis virus (IBV) strains M41 and B1648 in respiratory mucosa and monocytes

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Objective: To evaluate replication characteristics of respiratory and nephrophathogenic infectious bronchitis virus (IBV) strains M41 and B1648 in respiratory mucosa and monocytes, and to asses the cell viability of infected mucosal explants.

IBV is a coronavirus that affects both meat and egg laying poultry. Vaccination failure is very common against IBV, due to the existence of many genotypes/serotypes. IBV has a main tropism for the epithelial cells of the respiratory tract and oviduct of chickens. In the beginning of the 1950s, the respiratory Massachusetts (Mass) type of IBV was identified in the United States (prototype: M41). Later, Mass-type strains have been isolated all over the world and variants emerged. Some IBV-strains were described as nephropathogenic since the respiratory infection was followed by a severe renal infection, which leads to clinical signs such as excessive water consumption and wet droppings and increased mortality. Post-mortem examination of birds that died after nephropathogenic IBV (NIBV) infection reveals dehydrated carcasses and swollen and pale kidneys with urates in the tubules. B1648 is a well-studied NIBV, which has affected poultry industry in Belgium, Northern France and The Netherlands. Despite a good knowledge of the pathogenesis of respiratory IBV, the pathogenesis of NIBV is still enigmatic. The main questions are: how is NIBV reaching the kidney.

Methods: The in vitro chicken tracheal mucosa explants were set up for 96h at an air-liquid interface. The chicken tracheal explants were used to study the replication characteristics of both respiratory IBV (M41) and NIBV (B1648) and to examine if there are differences of both types of IBV in replicating and invading in the mucosae. In addition, replication kinetics of respiratory IBV and NIBV were evaluated in KUL01+ cells (blood monocytes, macrophages and interdigitating cells) in order to see if these cells may be a vehicle for IBV and if there are differences between the respiratory and nephropathogenic strains M41 and B1648. Finally, the effect of IBV on cell viability of infected tracheal explants was evaluated using a TUNEL assay.

Results: Replication characteristics of the respiratory IBV strain M41 and the NIBV strain B1648 were compared on tracheal mucosa explants. The presence of IBV M41 and NIBV B1648-infected cells were observed both in epithelium and lamina propria of respiratory mucosa. Analysis revealed that the M41 and B1648 infection pattern was similar in the epithelium and lamina propria of the respiratory mucosa. Double immunofluorescence stainings were performed for identification of IBV susceptible cells. IBV infected KUL01+ cells were observed both in epithelium and lamina propria of the respiratory mucosa for both M41 and B1648. These results show that IBV penetrates the deeper layers of the respiratory tract, using migrating KUL01+ cells as carrier cells. Further, replication kinetics of both strains was compared in monocytic cells (KUL01+ cells). In KUL01+ cells the infection pattern was different for M41 and B1648. B1648 strain caused a fully productive and sustainable replication in KUL01+ cells, which was not the case with M41 strain.

The effect of IBV on cell viability of infected tracheal explants was evaluated using a TUNEL assay, which has indicated a large number of IBV infected tracheal epithelial cells were apoptotic. In addition, a higher percentage of B1648 infected tracheal epithelial cells were TUNEL positive compared to M41. These results implicated that IBV induces apoptosis in infected epithelial cells of tracheal mucosa and that B1648 is more cytotoxic than M41.

Conclusion: No major difference in the replication characteristics was observed between respiratory (M41) and nephropathogenic (B1648) strains in respiratory mucosa. IBV penetrates deeper layer of respiratory tract by using $KUL01^{+}$ cells as carrier cells. The active replication of the nephropathogenic infectious bronchitis virus (NIBV) B1648 *in vitro* in $KUL01^{+}$ cells (monocytic cells), in contrast with the respiratory infectious bronchitis virus M41, may be very relevant for the *in vivo* pathogenesis. However, this hypothesis should be confirmed *in vivo.* A better understanding of the mechanism of the kidney tropism of poultry coronaviruses may also shed new light on the kidney tropism of MERS-CoV (Middle east respiratory syndrome-coronavirus) in humans.

Dissecting respiratory viral co-infections in poultry using a nanofluidic PCR screening assay

Guillaume Croville [1], Alexis Senet [1], Charlotte Foret [1], Mariette Ducatez [1], Faouzi Kichou [2], Mohammed Mouahid [3], Jean-Luc Guérin [1]

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Objective: Respiratory diseases have a major pathological impact in poultry, mainly in the last period of production (broilers and meat turkeys). They are also associated with egg-drop syndromes in layers and breeders. In broiler turkeys, respiratory conditions are not only directly impacting growth and viability, but lead to secondary leg bacterial arthritis, resulting in increased condemnation rates and increased use of antibiotics. Avian metapneumoviruses, avian paramyxovirus type 1, avian infectious bronchitis, *Mycoplasma spp. Ornithobacterium rhinotracheale* (ORT) and/or *E.coli* are considered as key co-infectious agents of respiratory syndromes. Low pathogenic avian influenza viruses and *Aspergillus sp*., *Pasteurella multocida* or *Chlamydia psittaci* may also be involved in respiratory outbreaks in poultry. We designed an innovative quantitative PCR method, based on a microfluidic technology, in order to achieve a comprehensive screening of respiratory avian viruses and bacteria.

Methods: More than 20 respiratory pathogens (viruses, bacteria and aspergillus) were targeted and a Sybr Green PCR assay was optimized for each one on a Light Cycler 480 platform. These primers were subsequently used in a high throughout PCR microfluidic platform, namely Biomark® (Fluidigm) resulting in multiplex-like quantitative PCR assays.

A large set of turkey and chicken flocks facing respiratory syndromes (10 to 20 birds/flock) were swabbed in 2014 (continued during the 1st semester of 2015) in Western France and in the Region of Rabat-Temara in Morocco. Nucleic acids were extracted and submitted to this screening assay.

Results: The set of primers was first validated on reference strains, as well as on sequences cloned into plasmids. The assay was then applied to tracheal swabs taken on turkeys facing acute respiratory syndromes: avian metapneumovirus were extensively detected, as well as avian paramyxovirus type 1 (aPMV-1) of vaccinal genotype. No avian influenza virus could be detected. Surprisingly, *avian infectious laryngotracheitis virus* (LTI) could be detected in birds from 3 flocks. This unusual infection in turkeys has been reported one in Brazil and should be further investigated, to evaluate its pathological relevance. Beside viral infections, bacterial co-infections were extensively detected, as expected.

Conclusion: This assay resulted in very comprehensive pictures of respiratory co-infections in French turkey poults, suggesting widespread co-infections with *E. coli*, *mycoplasma*, ORT, along with viral infections. This screening approach may be used on a larger extend for a comprehensive description of respiratory co-infection profiles in chickens, turkeys or ducks. The set of primers included in this screening assay is continuously improved, in order to address the emergence of novel agents or variants.

Parallel EPIZONE Poster Presentation Session 1

Wednesday 2nd September 2015
17:00-18:00
Auditorium Pasteur

Chair: Claude Saegerman & Geneviève Libeau

The detection of West Nile virus in veterinary surveillance: how to overcome the flavivirus similarity and control the absence of inhibition in RNA extracted

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Objective: West Nile virus (WNV) virus is a single stranded positive sense RNA of about 11,000bp. WNV can be divided into lineages 1 and 2 on the bases of envelope protein analysis. Lineage 1 is found in North America, Southern Europe, Africa, Asia, and Australia, while lineage 2 remained in sub-Saharan Africa and Madagascar until 2004, when it was detected in a goshawk in Hungary. Nowadays WNVLin2 seems to be present in the eastern part of Europe, from Greece to Northeastern Italy across former Yugoslavian countries. In particular, in Italy, the circulation of WNV is overlapped by the circulation of Usutu virus (USUV), another flavivirus which arrived in Europe (in Italy) at least in 1996 and was responsible for mortality in blackbirds occurred in Austria in 2001. USUV is not considered to be a significant human pathogen nevertheless two USUV-positive cases of meningoencephalits were reported in immunecompromised patients in Italy. Moreover, the entomological surveillance has to consider the circulation of Mosquitoes-Only Flavivirus (MOF) demonstrated in many European countries, although these viruses are distantly related with other flaviviruses. Cross reactivity of serological test among flavivirus is well known in literature and the seroneutralization is frequently requested to confirm the serological findings. While less reported is the cross reactivity of molecular tests such as Real Time PCR applied in large vector surveillance system. The first aim of this poster is to propose a WNV/USUV Diagnostic Decision Tree to avoid misidentification of positive reactions during entomological survey.

Nowadays is mandatory for a modern molecular diagnostic laboratory to control the absence of inhibition in RNA extracted from diagnostic samples. There is considerable debate about the most suitable and effective inhibition control. An artificial target, such as a length of DNA contained in a plasmid can be used, but usually because the target for the assay and for the control is the same, competition for the primer and dNTPs may reduce the analytic performance of the assay. An alternative strategy for an inhibition control is to amplify a housekeeping or structural gene such as β-actin. The veterinary surveillance of West Nile circulation is normally conducted considering samples from mosquitoes, bird and lastly horses. Housekeeping gene, such as actin can work well across different species, for this reasons we propose the use of a protocol originally developed to test absence of inhibition in bovine and ovine samples, also in West Nile and other flavivirus veterinary surveillance.

Methods: Fifty-five reference RNA extracted from proficiency tests samples received by the laboratory between 2009 and 2013 were analyzed using difference real time PCR available in literature (Lanciotti et al. 2000; Shi et al. 2001 ; Jiménez-Clavero et al. 2006 ; Tang et al.2006 ; Linke et al. 2007 ; Eiden et al. 2010 ; Del Amo et al. 2013). The result of different methods were compared and ordered in ranks for relative sensitivity. The specificity of different methods in respect to WNV lineage 1 and 2, USUV and JEV were evaluated.

In silico analysis of Sequence of actin gene were obtained from Genebank for mosquitoes (Culex pipiens) and hawk (Falco peregrinus). The theoretical amplification of endogenous control for beta actin originally proposed in Toussaint et al. 2007 for bovine and ovine samples, were checked.

Finally the endogenous control were applied on different species of birds (magpie, crow, jay, blackbird, kestrel, hawk dove, robin) and mosquitoes collected during WNV surveillance in 2014.

Results: The relative analytical sensitivity rank obtained by the seven real time PCR tested was reported in brackets: Lanciotti et al. 2000 (7); Shi et al. 2001 (6); Jiménez-Clavero et al. 2006 (5) ; Tang et al.2006 (1); Linke et al. 2007 (3); Eiden et al. 2010 (2); Del Amo et al. 2013 (3).

In the analytical specificity test Tang et al.2006, the most sensible method, show the worst specificity detecting WNVL1, WNVL2, USUV and also JEV. Lanciotti et al. 2000 and Shi et al. 2001 detect WNVL1 only, Eiden et al. 2010 can detect USUV depending on the viral concentration. Jiménez-Clavero et al. 2006, Linke et al. 2007 and Del Amo et al. 2013 detect only WNL1 and 2.

It is reported (from experts of EU reference laboratories, confidential communication) that it is possible to differentiate PCR positive signals for JEV and USUV on the basis of the analysis of the slope of the amplification curve, but many factors can contribute to the slope of a PCR and the copy number of the target is one of these.

The sequence analysis of actin genes reveal good homology for hawk and some little miss-match in 5' region of the forward primer in *Cx. pipines,* but an amplification of the actin by the real time PCR originally proposed in Toussaint et al. 2007 for bovine and ovine samples, is theoretically possible also in veterinary samples used in WNV surveillance.

The results obtained on different species of birds (magpie, crow, jay, blackbird, kestrel, hawk dove, robin) and mosquitoes (on a total of 59 samples of birds and 60 samples of *Culex* spp. and *Aedes* spp. mosquitoes) confirm that actin can be amplified also in these species.

Conclusion: Considering all the available methods in term of analytical sensitivity, ability to detect at least WNV lineage 1 and lineage 2 and the required specificity, is almost impossible with a single real time PCR to satisfy all the necessities of a mosquitoes and birds survey for the early detection of WNV circulation in a Regions. Moreover if USUV is endemic in a Country a specific PCR for USUV (such as Cavrini et al. 2010) has to be considered. To take in account all of these considerations the following decision tree was applied in our laboratories for the detection of WNV and USUV in mosquitoes and birds pools:

If Tang et al. 2006 is negative -> mosquitoes pool is negative.

If Tang et al. 2006 and Eiden et al. 2010 are POSITIVE -> WNV positive sample, and proceed with PAN-flavivirus sequences (Scaramozzino et al. 2000)

If Tang et al. 2006 and Cavrini et al. 2010 are POSITIVE (Eiden et al. 2010 is negative) -> mosquitoes pool is positive for USUV, proceed with PAN-flavivirus sequences (Scaramozzino et al. 2000).

It can happen that in the same the pool both viruses are present, in this case the samples will result positives in all the Real Time PCR used.

In case of a quick differentiation between WNV lineage 1 and lineage 2, Del Amo et al. 2013 can be used, but the lack in sensitivity of this method could be an important limit.

Following the human cases detected in 2008 in the Emilia-Romagna region a surveillance network was designed and operated in the summer period. A total of more than 25'000 mosquitoes and birds samples were analyzed using the Diagnostic Decision Tree presented. The veterinary surveillance demonstrated positive evidence in terms of sensitivity (capacity to detect the virus circulation even when at the enzootic level), early detection (capacity to detect the virus circulation well before the appearance of human cases) and area specificity (capacity to indicate the spatial distribution of the risk for WNND human cases).

Regarding the possibility to use the actin endogenous control to test absence of inhibition in samples of birds and mosquitoes the results are promising. Despite of 5' miss-match observed in mosquitoes, amplifications were observed in field samples confirming the possibility to test absence of inhibition in both species applying the same protocol.

Mosquito species involved in West Nile and Usutu viruses transmission in Italy between 2008 and 2014

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Objective: West Nile (WNV) and Usutu (USUV) viruses are mosquito-borne pathogens, belonging to the Japanese encephalitis virus complex (genus *Flavivirus*). Natural life cycle of these viruses involves mosquito-bird-mosquito cycles, in which mosquitoes act as vectors and birds as amplifying reservoir hosts. Humans and horses are incidental dead-end hosts, which become infected through mosquitoes. In Italy, the first outbreak of WNV was reported in Tuscany region in 1998, whereas the first epidemic caused by USUV occurred in 1996 in wild birds, yet again in Tuscany region. About 10 years later, USUV and WNV reappeared in the North-Eastern regions of Italy causing death and clinical signs in birds, and horses and humans, respectively. In the following years, these viruses spread in many other Italian regions, involving different mosquito species.

A National Surveillance Plan for WNV is active in Italy, since 2002, including sentinel animals (horses and chicken) and insect collections. Furthermore extensive Regional surveillance plan for monitoring WNV are in place, in Emilia Romagna (since 2008), in Veneto (since 2009) and Friuli Venezia Giulia (since 2011) regions.

The aim of this work is to review the mosquito species found infected by WNV and USUV in Italy, between 2008 and 2014.

Methods: The mosquito collections were performed from 2008 to 2014, using different methods: CO2-CDC light traps; BG-Sentinel traps; CDC gravid traps and aspiration trapping. The collected mosquitoes were identified to species level and divided in pools according to collection site, collection date, trapping method and species. The pools were tested for WNV and USUV by real time RT-PCR. Freshly engorged and unfed females were pooled and tested separately.

Results: WNV was detected, for the first time in Italian mosquitoes, in *Culex pipiens s.l.* and *Ochlerotatus caspius*, collected in Emilia Romagna region in 2008.

Since then, pools of *Culex pipiens s.l.* were repeatedly found infected in Northern Italy (Emilia Romagna, Friuli Venezia Giulia, Liguria, Lombardy, Piedmont and Veneto regions) and in Sardinia and Sicily islands, including overall more than twenty Italian provinces. In addition, pools of *Culex modestus* were found positive in 2011 and 2014, in Sardinia and Emilia Romagna regions, respectively.

Mosquito pools, infected by USUV, were found in eleven Italian regions: Emilia Romagna, Friuli Venezia Giulia, Liguria, Lombardy, Marche, Molise, Piedmont, Sardinia, Tuscany, Umbria and Veneto. In particular, USUV was first detected in *Culex pipiens s.l.* and in *Aedes albopictus* pools in 2009, in Emilia Romagna region. In addition, *Aedes albopictus* was found infected also in 2010, 2011, 2012, in the same region. Finally, USUV was detected in other mosquito species, namely *Anopheles maculipennis s.l., Ochlerotatus caspius*, *Ochlerotatus detritus* and *Culiseta annulata*.

Conclusion: To date, three Italian mosquito species, were found naturally infected by West Nile virus: *Culex pipiens s.l.*, *Culex modestus* and *Ochlerotatus caspius*. *Culex pipiens s.l.* and *Ochlerotatus caspius* resulted positive also for USUTU virus, as well as other four species, namely *Aedes albopictus*, *Anopheles maculipennis s.l.*, *Ochlerotatus detritus* and *Culiseta annulata*. As in other parts of Europe, also in Italy *Culex pipiens s.l.* results definitely the most important vector of WNV and USUV: it is widespread and abundant all over the Country, and it was repeatedly found infected in the field. This species presents two biological forms: a rural and ornithophilic form, namely *Culex pipiens pipiens,* and *Culex pipiens molestus*, urban and mainly anthropophilic.

Another well-known vector of West Nile Disease in Europe is *Culex modestus*, which was found positive also in Italy, even if only two times. However this species should be taken into account, for its anthropophilic behavior and its high vector competence.

Ochlerotatus caspius is a common and abundant species in Italy. Although its competence for WNV resulted low under laboratory conditions, its abundance could improve its vectorial capacity.

Finally, the detection of USUV also in specimens of *Aedes albopictus*, *Anopheles maculipennis s.l.*, *Culiseta annulata* and *Ochlerotatus detritus*, suggests a wide distribution of this virus among the mosquito fauna supporting the virus transmission cycle in nature.

Vector Competence of European Culex pipiens (Diptera: Culicidae) mosquitoes for Rift Valley fever Virus

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Objective: Rift Valley fever (RVF) is an arthropod-borne zoonotic disease responsible for widespread outbreaks in both humans and ruminants. RVF virus (RVFV) (*Phlebovirus* genus, *Bunyaviridae* family) was identified in Kenya in 1930 and re-emerges periodically in Africa. RVFV is largely distributed in Africa, and in 2000, RVFV spread in Arabian Peninsula. RVFV is transmitted by mosquitoes of the *Culex pipiens* complex (Diptera: *Culicidae*). The spread of virus and the presence of possible competent vectors in the countries of Mediterranean Basin cannot discard the possibility of introduction of RVFV in Europe. The main objective of the current study is to explore the vector competence of the European *Cx. pipiens* species, specifically two different populations of *Cx. pipiens* from Catalonia (Northeastern of Spain) for a virulent RVFV strain.

Methods: Two different biotype of *Cx. pipiens* were used. A population of *Cx. pipiens forms molestus* (*Cx. pipiens molestus*) and a population of *Cx. pipiens form pipiens form molestus* (*Cx. pipiens* hybrid). Seven days old female mosquitoes, no bloodfeeded, were used for each population in the oral experimental infections. Mosquitoes were fed using a Hemotek feeding system with titer of virus used was 5.75 $\log_{10}$ $TCID_{50}$/ml. All the mosquitoes were maintained under environmental condition which simulated summer season, the season with most mosquito density and activity at our latitude. Fourteen days after imbibing infective blood mosquitoes were dissected into body and paws to evaluate the viral infection and dissemination. The challenge was performed in Biosecurity level 3 facilities of *Centre de Recerca en Sanitat Animal* (CReSA - IRTA).

Results: The rate of feeding was different between the populations tested. *Cx. pipiens* hybrid showed greater feeding behavior than *Cx. pipiens molestus* (21.25% vs 5.33%). On the other hand, the percentage of mortality at 14 dpi was very similar in the two populations tested (4.61% *Cx. pipiens* hybrid vs 8.33% *Cx. pipiens molestus*). Regarding the infection at day 14 dpi, the 14.28% of *Cx. pipiens molestus* bodies and the 8.95% of *Cx. pipiens* hybrid bodies resulted positives to RVFV by RT-qPCR.
No dissemination was observed in any mosquito tested.

Conclusion: Our results indicated that both *Cx. pipiens* populations tested was able to replicate virus. However, the infection probably remains limited to the midgut of the infected mosquitoes with the conditions tested at 14 dpi. These preliminary results show that more studies are needed to evaluate the vector competence of European mosquitoes, such testing different time points during the extrinsic incubation period and using a higher viral load of the infected blood. All these studies will provide information about the potential of European *Cx. pipiens* species as vector for RVFV and novel strategies to mitigate mosquito-borne arbovirus transmission in Europe.

What is metadata and why is such a good idea?

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Objective: In the management of data about surveillance, disease epidemics, vaccination campaigns, among other, there is a need to compose data across different resources in order to create the necessary basis for decisions. The prerequisites for this is to have the opportunity to discover whether a resource exists, who has it, where it is located, and what are the conditions for accessing it. The 'who, what, where, when, why and how' information are the

elements contained in the 'metadata'. Metadata is a detailed description of the content and quality of a data resource. In particular, each metadata record includes sufficient details about a resource so as to allow a user to make an accurate judgment on its content, quality, currency and conditions of access and re-use.

However, metadata can also serve a variety of further purposes such as to document the available information in a structured way, to bridge the gap between data owners and users, to educate users about the characteristics of the data, to state "what the data are not", and to avoid collecting or purchasing the same dataset more than once.

The Geographic Information System (GIS) office at the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) defined a pilot study for the metadata implementation on a selected set of data that are shared with other veterinary institutes, or have the potential to be shared and are characterized by a geographical component. One of the purposes of the study is to promote awareness within the IZSVe and among the related veterinary community about the opportunities derived by the integration of the metadata within the data management process. Specifically, the pilot study aimed to (i) improve the IZSVe internal awareness on metadata opportunities, (ii) assess methods of collecting and disseminating metadata, and (iii) collect and publish metadata of the IZSVe relevant data that are shared with other veterinary institutes, or have the potential to be shared. Here are presented the preliminary results of the pilot study regarding the analysis of the challenges on implementing metadata for veterinary information characterized by a spatial component.

Methods: The first phase was the evaluation of existing metadata models, in order to assess how they might be tailored towards a metadata process to be used in a veterinary context. This approach was pursued for two reasons. Firstly, the examined standards were defined for geographical information and no veterinary experience on their adoption was found. Secondly, the definition of a "new metadata schema" tailored for the veterinary community was excluded in order to ensure the compatibility with the internationally accepted metadata standards (such as the ISO TC/211 – 19115 or the INSPIRE metadata regulation). Four specific Use Case analyses, organized within a focus group session, were defined in order to assess the fit for veterinary purposes of the chosen metadata standards. For authoring the metadata of the defined Use Cases, the GeoNetwork opensource metadata editor was used (http://geonetwork-opensource.org/).

To make it possible for users to locate the metadata, a catalogue discovery service was implemented. The catalogue service (defined according to the OpenGIS® Catalogue Services Specification) is the solution to publish the metadata in a standard way that enables the discovery of metadata by using simple web client interfaces. A specific solution, based on GeoNetwork opensource application, Laravel framework (http://laravel.com/) on PHP 5.4 (http://php.net/), JavaScripts using jQuery library (https://jquery.com/) and a natural template engine (used to create the template of the web catalogue service (WCS) interface), was developed.

Results: The International Standards Organisation (ISO) Technical Committee for Geographic Information (ISO TC/211) is responsible for creating a suite of standards associated with geographic information, including several standards relating explicitly to metadata. Other metadata standards are in usage around the world, such as Dublin Core [http://www.dublincore.org/] and US Federal Geographic Data Committee's metadata standard (FGDC) *http://www.fgdc.gov/metadata/geospatial-metadata- standards]. The selected metadata schema was the standard ISO 19115 - Metadata. The ISO 19115 standard has been chosen because there are some open source tools that can be exploited to easily editing the metadata records and to set up the catalogue service to publish the metadata records. In this context, the implementation of metadata is, by and large, not a major technical problem: what is needed is to edit the metadata information in a metadata editor and place the metadata record in a web catalogue service (the technological and organizational aspects to set up a web catalogue service are not here discussed). This seems to be simple and, in relative terms, it is. There are, however, two main areas that need to be addressed: the role of semantics in completing the metadata entries, and the WCS Graphical User Interface (GUI).

Semantics. The adopted standard has been designed for the realm of geographic information. During the metadata editing for the identified metadata of the four Use Cases phase, it was observed that the metadata schema proposed by ISO would not be broad enough for the purposes of describing veterinary spatial data. In particular it was recorded that some metadata elements were difficult to interpret by the veterinary operators and some "required" veterinary information were difficult to associate to the ISO 19115 elements. To deal with the limitations expressed by the metadata editors, one achievable solution is the possibility to develop a metadata profile for the veterinary domain. ISO 19115 provides a built-in mechanism to modify the standard to suit the

needs of a particular community. With the ISO 19115 extension rules, the veterinary community should extend the ISO standard metadata schema as appropriate by introducing additional requirements and elements. Developing a "veterinary metadata profile" can give us the opportunity to achieve further standardization within the community to describe the content of veterinary information. Clearly, those extensions will not be interoperable outside the veterinary community.

WCS - GUI. Once the metadata of the four Use Cases were authored and uploaded in the GeoNetwork database (a specific web page to test the WCS – GUI was set up by the IZSVe at the following URL: http://metadati.izsvenezie.it/geonetwork/srv/eng/main.home), consideration should be given to the catalogue service dissemination interface. Dissemination interfaces were evaluated on two fronts by means of a focus group session. The primary means of evaluation involved the graphical aspects of the web pages used to present the metadata records. Secondly, the focus group evaluated the user awareness on the metadata for the focus group participant. Considering a point of view of a not GIS expert, the results of the focus group about the design of the "best" GUI were of particular interest as they showed the preferences for a simple GUI, similar to the Google search style. The GIS typical criteria for information discovery such as geographical extent and time validity seemed not to be relevant or used by the participant to the focus group. As far as the metadata awareness, the findings indicated that the focus group participants were not aware of the presence of a "system" like the catalogue services, because they usually discovered the information about potential data sources thanks to personal enquiry and personal contacts.

Conclusion: Using metadata is no longer a matter of convenience or want, it has become a necessity for disseminating data and related documentations. During the pilot study activities it was recognized that providing access to metadata information is an important data management activity that requires agreements on standards, semantics, and tools. Best practices developed by other groups (like the GIS community) can be used by the veterinary community as examples in implementing the metadata framework.

This work has been supported by the Italian Health Ministry Ricerca finanziata dal Ministero del Lavoro, della Salute e delle Politiche Sociali Dipartimento per la Sanità Pubblica Veterinaria, la Nutrizione e la Sicurezza degli Alimenti (IZS VE 04/11 RC – MetaVet).

ANIHWA project "TRANSCRIPTOVAC": Host response gene signatures associated with FMDV infection, vaccination and persistence

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Foot-and-mouth disease virus (FMDV) is the etiologic agent of one of the most devastating diseases that can affect cloven-hoofed livestock. Currently available vaccines are efficacious as emergency vaccines since they induce an early antibody- mediated protection against FMDV-infection but the duration of immunity is often reduced. This significantly enhances the costs and reduces the efficacy of vaccination programs in endemic areas, which are the source of virus regularly re-introduced into Europe. Moreover, following acute infection of cattle with FMDV, some animals become persistently infected regardless of their immune (vaccinated) status. The mechanisms that underlie the induction of humoral and cellular immunity (including the quality of the immune response at the individual level) and viral persistence are still unknown.

The aim of this project is 1) to identify innate immune gene signatures that are associated with long term anti-

body responses in sheep to define molecular targets for the development of new adjuvants and vaccines. 2) to identify factors within pathogen and host gene signatures associated with FMDV infection and persistence that can be targeted to prevent persistent infection with FMDV. 3) to develop novel viral vectored vaccines that could enhance the adaptive and local immune response against FMDV.

This collaborative project brings together five public research groups from four EU countries, which are leaders in the field of large animal immunology, veterinary virology and FMDV research, and one private international company leader in vaccine development and production. It will contribute to the development of improved vaccines to promote animal health, welfare and food security globally. The knowledge generated in this project will have broad applicability for the design of new and improved control measures.

An unexpectedly major BTV4 epidemic in Greece, 2014

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Objectives: Bluetongue (BT) is an infectious, non-contagious, vector-borne viral disease that affects wild and domestic ruminants. On 29 May 2014, an outbreak of BT was confirmed in the Prefecture of Lakonia (Region of Peloponnese, southern part of mainland Greece). This is more than 10 years since the last outbreak in any part of mainland Greece and the first ever outbreak in the wider region of Peloponnese. One and a half month later, there were BT outbreaks in the northern part of Greece (Thrace region) and the disease gradually covered almost the entire Greek territory.

Total number of outbreaks recorded in the WAHID system until now is 2,895. The morbidity and mortality rates in sheep population varied in several parts of Greece. In goats very low morbidity and mortality were reported while in cattle very few cases recorded with clinical signs. The most common symptoms were: pyrexia, hyperaemia, congestion and erosions of the skin and mucosa (especially oral mucosa), salivation and nasal discharges.

Methods: All samples (blood as well as spleen, lymph nodes or heart) were submitted to the Virology Laboratory in Athens Veterinary Center, which is the NRL for BT in Greece. The laboratory diagnosis is based on serological, molecular (Real-time and Conventional PCR) and virological (isolation of the virus in cell cultures) techniques.

Results: More than 24.000 samples have been serologically tested for BT and approximately 2000 samples have been tested for the presence of BTV virus by PCR. Molecular typing revealed the serotype 4 (BTV-4). Blood samples from Peloponnese were also sent to Pirbright Institute (EURL for BT) for a full length sequencing and phylogenetic analysis. The data demonstrated that although the 2014 Greek virus belongs to the major Western topotype of BTV-4, it is not very closely related to previous European strains but derives from isolates circulating in West Mediterranean and North Africa. More samples were sent to Pirbright Institute from other affected parts of Greece (sequencing data still pending) in order to further clarify the epidemiology of this unusual outbreak.

Furthermore, the current strain of BTV was isolated in BHK-21 (baby hamster kidney) cells, where a characteristic cytopathic effect was observed and the identification of the virus was further confirmed by molecular techniques.

The entomological surveillance was intensified with weekly catches by means of mobile traps in restricted zones of the country. The culicoides vector predominately found is C. obsoletus.

Conclusions: The control measures were set according to the relevant legislation provisions and awareness is being raised including vector control measures at the holdings. In order to investigate the level of immunity developed against BT following natural infection, a serological survey is in progress. Up to now, Greece adopted a non-vaccination policy against the disease but taking into consideration the current BT situation in the country, vaccination is an option.

Baculovirus mediated generation of rabbit haemorrhagic disease virus variant 2 VLPs in SF9 insect cells and RK13 rabbit cells from codon modified VP60b open reading frames

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Objective: RHD (rabbit haemorrhagic disease) is a rabbit disease caused by RHDV, a member of the *Caliciviridae* family, genus *Lagovirus*. RHD emerged in China in 1984 in Wuxi City, Jiangsu Province and subsequently spread to other parts of Asia, Europe, North and South Americas and Africa in Australia and New Zealand where RHDV was released to control the wild rabbit plaque. Transmission of the disease occurs by direct contact and fomites. Mortality rate in non-vaccinated rabbits is approximately 90 %. The vaccines available against classic RHD showed a good coverage for these field strains and vaccinated rabbits are protected. However, in 2010 RHD outbreaks with high mortalities occurred in vaccinated rabbits in North-Western France caused by an emerging new variant of this virus tentatively named RHDV2 or RHDVb. The common vaccines against classic RHDV are less efficacious in protecting rabbits from disease caused by the new variant which leads to an increase in mortality rates and significant economic losses. For development of accordingly adjusted immunogenic and diagnostic tools, we intended to compare VP60 expression and VLP generation in insect cells (SF9) and rabbit kidney (RK13) cells using recombinant baculoviruses.

Methods: For expression in SF9 cells, an artificial ORF encoding VP60b with the codon usage of Autographa californica multicapsid nucleopolyhedrovirus was designed whereas for expression in RK13 cells, the codon usage of bovine herpesvirus 1 was selected to significantly increase the G+C content of the ORF, a strategy which has been proven suitable in the past. Each of the ORFs were then integrated in transfer plasmids containing the baculoviral p10 promoter for expression control in insect cells and the hybrid CAGGS promoter for transcription regulation in mammalian cells. The envisaged recombinant viruses were generated using the Bac-to-Bac System (Invitrogen).

Results: Surprisingly, comparison of the VP60 expression levels and the formation of VLPs in SF9 cells revealed that the codon usage of the VP60b ORF used had no obvious effect. Comparable results with regard to expression levels were also achieved after transduction of RK13 cells with the respective baculovirus recombinants. Work on quantitation of recombinant protein synthesis and VLP production is ongoing and respective data will be presented.

Conclusion: RHDV2 VP60 is efficiently expressed by recombinant baculovirus infection of SF9 insect cells or transduction of RK13 cells. Modification of the codon usage and thus the G+C content from 55.5 % (authentic sequence) to 52.8 (baculovirus) or 64.7 (BHV-1) had no detectable effect on the expression levels.

The prevalence of ten pathogens detected by real-time PCR method in lung tissue samples collected from dead cattle after respiratory disease

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Objective: Bovine respiratory disease complex can have great impact on economic losses in young stock and breeding herds. The etiology is related to viral and bacterial infections, but the disease is also multifactorial, it cause damage, reduce immunity and often resulting in mortality of affected animals. New molecular methods, based on real-time PCR, provide detection of different pathogens from various types of samples.

Methods: During the 2013/2014 autumn and winter period lung tissues samples were collected from 84 dead cattle in which main pathological findings were symptoms of pneumonia. Individual lung samples were homogenized and nucleic acids were extracted. Samples were tested by three commercial and one in-house real-time PCR methods, detecting nucleic acids of total 10 different respiratory pathogens: Mycoplasma bovis (*M. bovis*), Histophilus somni (*H. somni*), Pasteurella multocida (*P. multocida*), Mannheimia haemolytica (*M. haemolytica*), Bovine coronavirus (BCoV), Bovine respiratory syncytial virus (BRSV), Bovine para-influenza 3 (BPI-3), Bovine viral diarrhea virus (BVDV), Bovine herpes virus type 1 (BHV-1) and Bovine adenovirus (BAdV).

Results: P. multocida was detected in 77.38% of samples, H. somni in 34.57%, M. haemolytica in 21.42%, while M. bovis was positive in 16.66% of the affected lungs. The highest prevalence between viral pathogens was observed for BRSV 29.76%, BAdV and BVDV with 15.47%, following BCoV with 11.90%, BPI-3 with 8.33%, the less frequently detected viral pathogen was BHV-1 with 2.8% of positive samples. At least one of six examined viruses was detected in 64% of samples often together with bacteria and mycoplasma. Simultaneous infection with two or more pathogens was present in 77% of samples, but at least one pathogen was identified in 94% of samples.

Conclusion: The new implemented diagnostic tools are very useful for fast and reliable identification of etiological agent in cattle with pneumonia. This strategy can be important part of successful control, veterinarian therapy and preventive actions in herds.

Malignant catarrhal fever in the Czech Republic: four outbreaks and seroprevalence

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Objective: There are several lymphotropic herpesviruses (MCFVs) that are causative agents of malignant catarrhal fever (MCF). In the Czech Republic, the most probable source of MCF is sheep with its ovine herpesvirus 2 (OvHV-2) and less often wildebeest as the source of *alcelaphine herpesvirus 1* (AlHV-1) in Czech zoos. MCFVs belong to the family *Herpesviridae*, subfamily *Gammaherpesvirinae* and to the genus *Macavirus*. This report describes sheep-associated MCF (SA-MCF) in domestic cattle and wildebeest-associated MCF (WA-MCF) outbreaks in Akole-Watusi cattle. Another objective was to determine the prevalence of antibodies against MCFV in small ruminants and captive exotic ruminants.

Methods: We tested samples (6 tissue, 48 blood and 7 swab samples) from animals suspected of MCF by molecular methods. Total nucleic acids of samples were extracted by automatic extractors. The nucleic acid extracts had been stored at -80°C until they were tested. Two real-time PCR reactions were used for sample testing. Ovine herpesvirus 2 method was based on amplification of ORF75 gene. For alcelaphine herepesvirus 1, we used real-time PCR amplification of a part of ORF3 encoding an AlHV-1 tegument protein. A total of 492 serum samples, collected from several species of both domestic and captive exotic artyrodactyls in the Czech Republic, was examined for antibodies against MCFV by a competitive enzyme-linked immunosorbent assay (cELISA) (VMRD, Pullman, USA). Serum samples came from 9 zoos and 20 sheep/goat farms.

Results: The first MCF outbreak appeared in Ankole-Watusi cattle at a Czech zoo in November 2008. The herd of Watusis was in direct contact with white-bearded wildebeest (*Connochaetes taurinus albojubatus*) on a pasture during the grazing season. In a few weeks after the transfer to winter pens, the first clinical signs (diarrhoea, hyperaemia, conjunctivitis, serous-purulent discharge, photophobia, apathy) were observed in five Watusis (aged 6 month to 4 years). All sick animals died following 2 to 10 days of the illness. Necropsies and histopathological findings revealed typical lesions for MCF. The specific AlHV-1 PCR reacted positive for all organs of five Watusis. Other viral and bacterial infections were ruled out. We confirmed three SA-MCF outbreaks by molecular methods from the whole Czech Republic between February 2014 and February 2015. All infected animals showed the clinical signs typical for MCF – cephalic and ocular form. We found three SA-MCFV strains from various districts of the Czech Republic. The source of all strains was sheep farming on the common pasture with cattle. Antibodies against MCFV were detected in 150 of 404 (37%) zoo animals and in 53 of 88 (60%) small ruminants, respectively. There were animals positive with MCF in every zoo or farm where samples had been taken.

Conclusion: Mixed animal expositions of exotic ruminants bring the risk of MCF infection of susceptible animals. We discovered AlHV-1 virus in Watusi, however, there is a possibility of infection with OvHV-2, CpHV-2, AlHV-2 or others MCFVs. The high seroprevalence of carrier animal species supports that assumption. The rising number of mixed herds has a negative impact on cattle.

Survey of pestiviruses in ovine abortions by different detection methods

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Introduction: Bovine viral diarrhoea (BVD) is the most significant endemic viral disease in the United Kingdom from both welfare and an economic perspective. BVD is caused by Bovine viral diarrhoea virus (BVDV) which belongs to the pestivirus genus, family Flaviviridae. Other pestiviruses are Classical swine fever virus (CSFV) which infects pigs and Border disease virus (BDV) which infects sheep. Pestiviruses are not strictly host-species specific and can infect not only domestic but also wild animals.

The BVD eradication program for Northern Ireland (NI) started with a voluntary phase in January 2013 and it is envisaged that a compulsory phase will start during 2015. The program is based on tissue tag testing of calves. As part on the program it would be important to determine the extent of infections in sheep with pestiviruses. Contact with infected sheep or other ruminants may pose a risk to cattle herds in low prevalence areas, as they can act as a viral reservoir and their presence could delay the control of the disease and the objectives of the eradication program.

Objective: To establish whether pestiviruses are present on aborted ovine foetuses in Northern Ireland and to evaluate the suitability of a range of diagnostic methods.

Methods: Two distinct surveys over two lambing seasons were carried out. In the first one, which took place between December 2012 and May 2013, samples were collected from 165 aborted ovine foetuses submitted for post-mortem examination to the Agri-Food and Biosciences Institute. Samples comprised foetal fluid (typically heart blood), spleen, lung, liver, kidney, lymph nodes, adrenal gland and abomasum. The foetal fluid was tested for BVDV antigen using a commercial antigen capture ELISA (HerdCheck BVDV Antigen/Serum Plus Test Kit, IDEXX) and by RT-PCR using a commercially available RT-PCR kit (VetMax Gold BVDV, Life Technologies). Positive samples were tested by virus isolation in lamb kidney cells. Spleen, lung and liver were processed for BDV immunofluorescent antibody testing (IFAT) as they were collected. Otherwise, tissue samples were pooled and stored at -80°C (individual samples and pools) before testing by RT-PCR.

In the second lambing season, foetal fluids from 277 aborted foetuses submitted between January and May 2014 were tested by RT-PCR (as above) and 275 by antigen ELISA (as above). Foetal fluids from 269 ovine abortions were tested both by RT-PCR and by antigen ELISA. The level of agreement (expressed as Kappa value) including 95% confidence intervals between the RT-PCR and the antigen ELISA was evaluated using WinEpiscope 2.0 (http://www.clive.ed.ac.uk).

Samples from all the cases were also collected for histological and bacteriological examination as part of the routine processing to establish the cause of abortion.

Results: 2013 lambing season: Of the 165 cases, 153 were tested by antigen ELISA, 161 by IFAT and 161 tissues and 119 foetal fluids by RT-PCR. Three cases were positive by BVD antigen ELISA in foetal fluids (2%). One of these was also positive by RT-PCR on foetal fluids but negative on tissues. Another of the antigen ELISA positive cases were positive by RT-PCR in lung and placenta but negative on foetal fluids. None of the cases were found positive by IFAT. None of the samples tested by virus isolation were positive.

2014 lambing season: Of the 277 cases tested by RT-PCR, 8 were positive (2.9%), 4 were also positive by ACE and 4 were negative. 18 out of the 275 cases tested by antigen ELISA were positive (6.5%), 4 were positive by RT-PCR and the rest negative. The Kappa obtained when assessing the agreement between the RT-PCR and the antigen ELISA was of 0.278 (95%CI: 0.168-0.387).

Conclusion: The number of ovine abortions with a positive pestivirus result during the 2014 lambing season was similar to levels found in bovine abortions tested within the same laboratories (4-5%). The BDV IFAT polyclonal antiserum used was not able to detect any positive samples, showing low sensitivity. The antigen-detecting ELISA and the RT-PCR were found to be superior to IFAT and virus isolation for detection of pestiviruses in ovine foetuses. Most of the positive results on both surveys were obtained with the antigen ELISA.

The results of this study highlight both the need to use optimal diagnostic methods and the potential role of sheep in the epidemiology of BVDV in Northern Ireland.

Suvaxyn® CSF Marker, the first live marker vaccine against Classical Swine Fever Disease authorized at European level

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9: SCU - China
10: SVA - Sweden
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14: IZSA_M - Italy
15: Spectos - Germany
16: VAR - Belgium
17: FLI - Germany

Classical swine fever (CSF) is a highly contagious viral disease affecting pigs, which can lead to a tremendous socioeconomic impact. Within the European Union (EU) and in other areas with significant pig production, the disease is controlled by strict, mandatory control measures without prophylactic vaccination. However, emergency vaccination is usually foreseen and can be implemented in the case of a contingency. From a trade point of view, only marker vaccines are feasible that allow differentiation of infected from vaccinated animals. First generation marker vaccines, i.e. different E2 subunit vaccines, were hardly ever employed due to the fact that these vaccines lacked efficacy upon single application and did not provide early onset of immunity. Within two collaborative research projects funded by the 6th and 7th EU Framework Programs (Grant No. SSP1-501599 of FP6, Grant No. 227003 CP-FP of FP7), a potent new live marker vaccine was developed, validated and finally licensed as Suvaxyn® CSF Marker through the European Medicines Agency in February 2015.

To choose the most promising vaccine candidate, comparative trials were conducted within the CSFV_goDIVA project. Based on experimental studies and additional data, the chimeric pestivirus "CP7_E2alf" was chosen as final candidate. This chimera has a Bovine viral diarrhea virus "CP7" backbone and carries the glycoprotein E2 of CSF virus (CSFV) strain "Alfort/187". The marker concept is based on the detection of antibodies directed against CSFV E^{rns}.

The safety and efficacy studies required by the European Pharmacopoeia and the OIE Manual of Diagnostic Tests and Vaccines were divided among the project partners.

All safety studies showed innocuousness and complete safety for target and relevant non-target species, and no evidence was found for vaccine virus transmission to contact animals. Moreover, the virus did not cross the placental barrier in pregnant animals and did not affect the reproductive performance. In addition, the vaccine virus in itself is not able to induce persistently infected offspring. Efficacy was proven against highly virulent CSFV strain "Koslov" (genotype 1) and relevant CSFV strains of genotype 2. Varying degrees of protection were seen in young animals with maternally derived antibodies and with regard to vertical protection after harsh and early challenge. Onset of immunity was proven after 14 days (in supplemental studies, protective effects were seen after 2 days) and duration of immunity was at least six months.

Apart from the studies carried out for licensing as a live marker vaccine for domestic pigs that can be intramuscularly injected, the vaccine strain was also tested as oral marker vaccine. Also these studies were most promising and a bait marker vaccine for wild boar is feasible.

Taken together, Suvaxyn® CSF Marker (Zoetis) is the first live CSF marker vaccine that received EU product license by the centralized authorization procedure and for the first time, an emergency vaccination scenario is feasible that would allow deviations from the trade restrictions for vaccinated animals.

Parallel EPIZONE Poster Presentation Session 2

Wednesday 2nd September 2015
17:00-18:00
Room Sully 1

Chair: Jean-François Valarcher & Karl Stahl

Molecular typing of Bluetongue Viruses using nCounter Analysis System platform

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Objective: Bluetongue virus (BTV) is a segmented double-stranded RNA virus belonging to the genus *Orbivirus* of the family *Reoviridae* and it is transmitted mainly by *Culicoides* spp midges. Twenty-six serotypes of BTV are described and a further serotype, the 27th, was recently reported in Corsica. Genome constellation of BTV is composed by 10 segments and among them, Seg-2 encodes for the VP2, the outermost protein of the virion which determines serotype specificity eliciting the production of specific protective antibodies. As for flu viruses, multiple BTV serotypes can co-circulate in the same region, as seen in Italy with BTV-1 and BTV-4 during 2014. Identification of BTV serotype uses multiple typing assays, that tend to be executed based on the known epidemiological situation within a given country. Samples containing multiple serotypes (particularly those containing novel introductions) may therefore be missed. The complete identification of even a single BTV strain can therefore be expensive and time consuming.

The aim of this work is to develop a diagnostic test, based on the nCounter® Analysis System platform (Nanostring technologies), that would all at once, with minimum hands on time and operator steps, simultaneously identify all BTV serotypes, co-infection of BTV viruses in biological samples, including internal organs and blood specimens and in tissue culture adapted BTV strains.

Methods: The nCounter® Analysis System use the principle of molecular hybridization of sequence-specific color code (that work as a molecular barcode) and capture probes. This technology is actually, mainly used for gene expression experiments.

Results: Probes were designed according to all Seg-2 sequences available on line. Experiments were therefore performed with the 26 reference BTV strains, then with field biological samples single infected with BTV-1, BTV-2, BTV-4, BTV-9, and BTV-16 and at the end with some mixed-infected samples also. All samples were previously characterized by a serotype-specific real time RT-PCR. The reference tissue culture adapted BTV strains were correctly identified as well as those infecting field samples, single or mixed infected.

Conclusion: Specificity of the assay needs to be further investigated against a larger panel of BTV collected worldwide and further experiments are currently ongoing to increase the sensitivity of the test and to define a sensitivity threshold with the aim of move the test into daily diagnostic.

Detection of a divergent Alpha Coronavirus in bats in Piedmont (Italy)

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Objective: Bats have been recognized as the reservoir hosts of numerous pathogenic viruses, including Coronaviruses (CoV) which are widely associated with respiratory and gastrointestinal diseases both in humans and animals. In the genus Coronavirus, family *Coronaviridae*, subfamily *Coronavirinae* are comprised enveloped positive sense single stranded RNA viruses. Based on their genetic characteristics, CoV are divided in four main groups known as alpha, beta, gamma, and delta coronaviruses and usually infect only one, or a limited number of closely related animal species. However, in the last decades at least two zoonotic epidemics caused by novel CoVs jumping the species barrier have emerged very likely from wildlife. Severe Acute Respiratory Syndrome (SARS) CoV, an emerging betaCoV responsible for the outbreak of severe acute respiratory syndrome in 2002–2003 worldwide, presumably passed to humans from bats through civet cats, as intermediate host. Moreover, the Middle East Respiratory Syndrome (MERS), a 40% lethal epidemic affecting the Arabian Peninsula since spring 2012, turned out to be caused by a novel betaCoV known as MERS CoV, able to infect also camels and bats.

The aim of the study is to define and characterize the viral strains circulating among the bat population roosting in Piedmont, a Northwestern Italian region. For this porpose, a biomolecular screening for the *Paramyxovirinae* subfamily and the Coronavirus genus was first done. Here, we report the identification in *Myotis nattereri* of a divergent alpha coronavirus strain.

Methods: A minimally invasive samples collection was performed by expert chiropterologists on the regional territory of Liguria and Piedmont starting from July 2013 and still ongoing. Specie, age class, sex, reproductive status, forearm length and weight were determined and registered for each animal. Bats were trapped with mist net and harp trap with the consent of the Italian Ministry of Environment. All captured animals were released in the same place of capture after samples were taken. Bats were trapped with nets and buccal, rectal and urinal samples were collected by swabbing. Swabs were maintained in viral transport UTM medium and stored at -20°C till analyses. Dead animals found in good post-mortem conditions, were collected. Before any further analyses, carcasses were examined by a complete necroscopy and for the presence of the rabies virus antigen by direct immunofluorescent (IF) staining into a BSL3 Laboratory. After the exclusion of the rabid infection, tissues of each animal were pooled together, homogenized in PBS with Tissue Lyser (Qiagen). An aliquot of 200 µl of supernatant was used for the nucleic acid purification with the Qiagen EZ1 Advanced XL Instrument, following the manufacturer's instructions. To avoid any biosafety risk 200 µl of each swab sample were directly inactivated in the Lysis buffer provided by the kit under the BSL3 hood. Extracted RNA was eluted in 60 µl of RNase-free water and stored at -20°C. A pair of consensus primers targeted to the conserved region of coronavirus RNA polymerase (RNA-dependent RNA polymerase [RdRp]) sequences ([1]) was used to screen the RNA samples in a One-step RT-PCR assay (Qiagen). Amplified DNA products were analyzed by agarose gel electrophoresis and bands of the expected size were excised for the DNA sequencing. Amplicon DNA purified band were submitted for direct sequencing (BMR Genomics, Padua, Italy). The obtained chromatograms were manually checked and corrected (Genious R7 ver. 7.1.7). The sequences were aligned (Clustal W) and the alignment was used to evaluate the best evolutionary model (Modeltest ver 3.7) and to draw a bayesian phylogenetic tree (MrBayes ver. 3.1.2).

Results: So far, 27 animals were found dead mostly in Liguria region and 111 animals were trapped in ten different sites of Piedmont regions during 8 capture sessions in 2013 and 11 sessions in 2014. The 138 bats belong to 16 different species and a total of 91 buccal, 31 rectal, and 30 urinal swabs have been collected for analyses. At present 26 carcasses, 37 buccal, 15 rectal, and 15 urinal samples have been analysed by RT-PCR assays. Positivities for Coronavirus have been found in 5 bats collected in three different sites in Piedmont and belonging to *Myotis nattereri* (n=2), *Rhinolophus ferrumequinum* (n=1) and *Pipistrellus kuhlii* (n=2) species. To date sequences from rectal and urinal swabs of two *M. nattereri* have been obtained and a phylogenetic characterization have been performed based on the RdRp gene fragment sequence (382 bp). The highest nucleotide simi-

larity (94.55%) was obtained comparing our new sequences to a *M. nattereri* Hungarian strain (Genbank accession number KJ652333). Interestingly, Italian bats CoV described to date, in Northern Italy, belonged to different lineages, associated to different bat species.

Conclusion: Within the Chiroptera order, some species such as *Myotis nattereri* contain several cryptic lineages/species, especially in the Mediterranean region. Moreover *M. nattereri* bat complex comprises four species whose distributions in the Western Palaearctic correspond to four main glacial refugia (Iberia, Italy, Balkans and Morocco). These species are the result of long-term isolation (remarkable in a flying mammal) over several glacial cycles. Genetic investigations revealed that *M. nettereri Sp A* in present in Italy ([2]). On the other hand, since 2010, the circulation of CoV in Italian bat population have been notified in only few published studies and sequences of AlphaCoV and lineage C betaCoV obtained from rectal swabs are available only for Italian *Myotis blithii* and *Eptesicus serotinus* ([3,4]). In this study we detected and characterized a new divergent lineage of AlphaCoV in *M.nattereri*. Our results confirmed the strict association of bats CoV genetic lineages to their host species and showed how the distribution of different bats CoV lineages is wider than described so far. Sample collection and molecular investigations are still on going, in order to better understand the viral circulation in bats communities and to better investigate the role of the bat species heterogeneity in pathogens distribution and diffusion.

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Experimental infection of domestic pigs with African swine fever virus Lithuania 2014 Genotype II field isolate

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Objective: African swine fever (ASF) is a devastating and complex disease of swine, caused by a DNA arbovirus. Since 2007 the disease is present in East Europe. In earlier 2014, ASF cases in wild boar were reported in Lithuania and Poland, in bordering regions with Belarus (Gallardo et al., 2014). To date (March 2015), nearly 501 ASF cases or outbreaks in wild boar and domestic pigs have been detected in the EU countries Latvia, Lithuania, Estonia and Poland. This situation, combined with an uncertain situation in Belarus, represents a permanent risk for ASF spreading into new regions of the EU. Knowledge about disease dynamics for early detection of ASF is essential in the control of the disease. Therefore, an animal trial has been conducted at the EU Reference Laboratory (EURL) for ASF to obtain a precise description of the clinical, virological and pathological features induced in domestic pigs infected with currently circulating ASFV isolates in the EU.

Methods: The *in vivo* experiment was conducted under biosafety level 3 conditions at the animal facilities of INIA-CISA, in accordance with the EC Directive 86/609/EEC and with local laws and regulations. Eight 3- months old European hybrid pigs were inoculated by the intramuscular route with the Lithuanian LT14/1490 ASF virus (10 HAD_{50}/ml) which was isolated from a wild boar case which occurred in January 2014 (Gallardo et al., 2014).

At the start of the experiment, ten pigs were housed together with the inoculated pigs as in-contact controls. Severity of the disease was expressed by a clinical scoring, obtained by adding the score of eight clinical signs, recorded daily. Paired EDTA-blood and sera samples were collected from pigs twice a week starting at day 3 post-inoculation until death of the animals. Negative control samples were collected at day 0, the day of inoculation. Twenty different types of tissues and organs were obtained from each necropsied animal including bone

marrow and intra-articular tissues of joints. DNA was extracted from organs homogenates and blood samples using the High Pure PCR Template Preparation kit (Roche) and amplified by real-time PCR (Fernández-Pinero *et* al., 2013). The titre of virus was estimated by inoculation of tissues and blood samples into porcine peripheral blood macrophages (PBM) (OIE 2012). Detection of ASFV- specific antibodies was performed in serum using a commercial ELISA (INGEZIM, PPA COMPAC K3) and by Indirect Immunoperoxidase Technique (IPT) validated by the EURL (Gallardo et al., 2012).

Results: An acute, fatal disease was developed in seven out of the eight inoculated animals, which died or were euthanized due to the severity of symptoms between 7-9 days post inoculation (dpi). One inoculated pig showed, however, a delayed course of the disease, resembling the same as that seen in in-contact animals, which died or were slaughtered from 14 to 22 days post exposure (dpe). One in-contact pig remained asymptomatic throughout the experiment and was slaughtered at day 61. Both inoculated and contact animals developed broadly similar clinical patterns, and died or were slaughtered 3-4 days after the appearance of clinical signs, mainly a febrile syndrome (fever, mild anorexia, lethargy, weakness and recumbence) (17⁄18). Post mortem examination of animals dead within 22 days revealed enlarged, edematous and haemorrhagic lymph nodes. Some other lesions included hydropericardium with yellowish fluid, hyperaemic splenomegaly, hepatic congestion and petechial haemorrhages in the renal pelvis and cortex, small and large intestine, gall bladder and urinary bladder. The necropsy of the asymptomatic animal slaughtered at 61 dpe revealed hyperaemic splenomegaly, petechiae in the lungs and moderately enlarged and haemorrhagic lymph nodes.

In seven out of the eight inoculated pigs, ASF genome virus was first detected in blood by PCR at 3.75± 1.4 dpi. In agreement with the clinical signs, one inoculated pig did not show viremia until 14 dpi following the same pattern that the naturally infected pigs in which the ASF genome virus was first detected at 13.7 ± 2.8 dpe. Maximum titres, ranging between $10^{6.4}$-$10^{8.7}HAD_{50}$ /ml, were recorded at 6 dpi or 14 dpe in inoculated and in-contact animals respectively. ASFV genome was also detected in all tested organs and tissues. The asymptomatic animal showed intermittent and weak peaks of viremia at 17, 34 and 38 dpe and virus genome was detected in the limit of the detection in 9 out of the 20 collected tissues including the bone marrow, articular tissues, spleen, lung and the splenic, renal and submandibular lymph nodes. No virus could be isolated in samples from this animal after three passages in PBM cells. ASFV-specific antibodies were detected by ELISA in two in-contact animals (11%) at day 18 post exposure. Using the IPT, six animals (33%, one inoculated/ five in- contacts) yielded positive results between 17 to 21 dpi/dpe. No antibody response was observed in the survivor pig.

Conclusions: In accordance with previous observations (Gabriel et al., 2011; Blome et al., 2012, 2013; Guinat et al., 2014). The type and pattern of clinical signs induced by the Lithuanian isolate in domestic pigs were consistent with the acute form of ASF caused by virulent viruses, which resulted in 94.5% mortality. Upon the primary infection, clinical signs associated to a febrile syndrome were developed after an incubation period of 4-5 days with the appearance of dead or moribund animals from 7 to 9 dpi. An average delay of 12-14 days was observed in the in-contact pigs resulting in a severe disease or fatalities mainly from 18 to 22 dpe. The disease was easily detected by PCR in blood samples prior to the appearance of the clinical signs and from the tested organs at necropsy. ASF antibodies were detected in 33% of the animals, all of them at 17-18 dpi/dpe. One animal survived the infection showing weak and intermittent peaks of viremia, and DNA could be detected in tissues, though virus isolation could not be achieved. On this regard, similar features of infection in absence of detectable clinical signs have been described in naturally infected suids in Africa (Okoth et al., 2012; Penrith, M.L et al., 2004). The potential virus transmission that may result would need further investigation.

Our findings also suggest that recognition of related ASF clinical symptoms in an industrial holding should be suspected when sudden deaths of few animals occur, representing the first evidence of the disease. ASF should therefore be included in the differential diagnosis of such events particularly taking into account the epidemiological situation. The subsequent wave of infection of pigs in the pen-vicinity would require about 12-14 days for first clinical symptoms (usually fever) in the newly infected pigs. A prompt diagnosis would be very valuable to prevent further spreading to other farms. This dynamics of infection has been recently corroborated in a large industrial holding in Lithuania (EC, DG-SANCO 2014).

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The complex serological monitoring of an ongoing Small Ruminant Lentiviruses eradication campaign in Italy

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Objective: Caprine arthritis encephalitis (CAE) and Maedi-Visna (MV) are chronic infections of goat and sheep caused by retroviruses belonging to the genus Lentivirus. Once considered to be species specific, different strains of CAE and MV viruses were later shown to efficiently cross the species barrier and are now referred to as small ruminant lentiviruses (SRLV). Additionally, dual infections were reported, resulting in recombination between variants. Both infections are usually sub-clinic and clinical disease is generally manifested following long incubation periods. SRLV are responsible for important economic losses as no treatments and vaccines are available. Different eradication programs have been developed in Europe to limit their spread. Five distinct genotypes (A to E) have been detected and characterized up to date. Genotype A is associated with the classic MV pathology in sheep and genotype B with the CAE complex in goats. Genotypes A and B are globally distributed; by contrast, the other genotypes (C, D, E) seem restricted to particular geographic areas. In Italy 3 genotypes have been detected: A, B and E (only in goats). Diagnosis of SRLV is based on clinical-epidemiological data and laboratory diagnosis which mainly consists in detection of antibodies through AGID and ELISA tests, both recommended by OIE. Additionally, molecular biology techniques are also used to detect and characterize circulating genotypes. No gold standard laboratory techniques exist to diagnose SRLV infections. Several control programs have been implemented in the past in Europe including Italy. These programs aimed at defining the prevalence of infection and at eradicating the virus. Eradication programs rely upon periodic serological testing, slaughter of positive animals and restocking from certified SRLV-free flocks. Serological testing is routinely performed using ELISAs. Up to date a complete eradication did not succeed, mainly due to unsatisfactory performance of the commercial ELISAs, strictly related to the heterogeneity of the SRLV strains. New ELISA reactions have been recently developed, which show a broader reactivity and, at least to some extent, enable a serological differentiation. Older commercial ELISAs used a whole-virus and single-strain antigen, whereas newer ones are based on a mixture of different recombinant proteins and/or synthetic peptides, often belonging to different SRLV genotypes. In Italy a highly densely populated goat and sheep area is present in Bolzano province. In this province 23,806 goats and 42,187 sheep are present and occasionally reared in mixed flocks. In 2007, the Autonomous province of Bolzano started a compulsory CAEV eradication program based on annual serological surveillance using commercial available ELISAs for detection of *anti*-CAEV antibodies independently of the infecting genotype. The serological surveillance started in November until the beginning of April before the mountain pasture period. All goats older than 6 months were tested for SRLV and positive animals were compulsorily slaughtered. The initial seroprevalence for goats was 13.5% and decreased to 0.5% in 2014. In 2013 a different diagnostic schedule for the serological surveillance was implemented, targeting not only CAEV but all SRLV infections, based on the use of newly available ELISA tests able to differentiate the antibody response to the circulating genotypes. This new approach included testing sheep (older than 6 months) kept in mixed flocks with at least one positive goat. In the present study 4 different ELISA tests were compared on a set of 198 serum samples collected during the sampling period between 2013 and 2014. These samples were selected from farms positive in 2013 but negative during the past 2 eradication programs and from farms were single animals were detected positive. Two ELISAs were used as screening tests. Additionally two ELISAs aiming at identifying the circulating genotypes were compared on the same set of serum samples.

Methods: A total of 198 serum samples from goat and sheep were used in the study. One hundred and twenty one serum samples were collected from goats and the remaining 77 from sheep. Each serum was tested with the following ELISA tests: Idexx, Eradikit which give a positive or negative result for SRLV infection, the “Eradikit

genotyping" which can differentiate the A, B and E genotypes and mixed infections (AB) and the peptide ELISA "SU5" which can differentiate A, (A1, A3, A4), B, (B1, B2) or identify mixed infections (AB). Sometimes both genotyping ELISAs could not determine the infecting genotype, generating indeterminate results. The SU5 was conducted on the same set of samples at IZSVe and at the Institute of Virology and Immunology, University of Bern. The manufacturer instructions were followed for commercial ELISAs. Briefly the peptide SU5 ELISA was conducted dissolving synthetic peptides in carbonate-bicarbonate buffer and allowed to adsorb to ELISA plates overnight at 4°C, Plates were washed three times with PBS and Tween 20. Residual adsorption sites were saturated at room temperature for 1h by incubation with PBS-Tween containing 5% fat-free milk powder. Plates were washed once with PBS-Tween before adding of goat or sheep serum diluted in PBS-Tween supplemented with fat-free milk powder (5%). After 2h of incubation at room temperature, the plates were washed three times with PBS-Tween. One hundred microliters per well of peroxidase-conjugated protein G diluted in PBS-Tween was added, and the mixture was incubated for 2h at room temperature. After two washing steps with PBS-Tween and two washing steps with PBS, bound conjugate was visualized with ABTS solution. Optical density (OD) was measured at a 405-nm wave-length after 30 minutes.

Results: The analysis of 198 serum samples with the Idexx ELISA detected 113 positive, 81 negative and 4 indeterminate sera. The Eradikit detected 185 and 13 negative samples (Table 1). Twelve of the Idexx positive samples were negative in the Eradikit test. The 81 negative samples by Idexx resulted all positive in the Eradikit. Three of the 4 doubtful Idexx samples were positive in the Eradikit and one was negative. Of the 81 negative samples to the Idexx ELISA, 33 were indeterminate, 10 were positive to A genotype, 14 to E, and 24 positive to B. The "Eradikit genotyping" kit produced the following results: 73 samples were classified as genotype B infections, 33 as genotype A and 19 as genotype E, 3 resulted positive for both A and B genotypes while 70 could not be classified. Of these 70 samples more than 50% (40) were collected from sheep. Of the 73 samples indeterminate and positive to A and B in the "Eradikit genotyping", 33 were negative in the Idexx ELISA and 38 positive, whereas 65 were positive to the Eradikit and 8 negative. Considering the results obtained with ELISA tests able to discriminate between the infecting genotypes, the "Eradikit genotyping" identified 33 samples positive to A of which 13 showed concordant results with the SU5, 5 resulted positive to B with the SU5, 11 were indeterminate and 4 resulted positive for A and B (Tables 2-3). Of the 73 serum samples positive to B using the "Eradikit genotyping", 41 were concordant with the SU5, 6 resulted positive to A in the SU5, 10 not determinable and 8 positive to A and B. Nineteen serum samples resulted positive to genotype E using the "Eradikit genotyping", of these 10 were not determinable (all from goats) in the SU5 and 9 resulted positive to A, B or An and B. Of these 19 positive E samples, 7 were collected from sheep. Of these 7 samples 2 resulted indeterminate, 4 A3 and 1 B in SU5 ELISA. The 77 serum samples collected from sheep all resulted positive in the Eradikit and 28 negative in the Idexx test. Of the 70 serum samples indeterminate in the "Eradikit genotyping", some were characterized by the SU5 as positive to A (26), to B (4) and to both A and B (15).

			ERADIKIT
IDEXX	POSITIVE	113	185
	DOUBTFUL	4	0
	NEGATIVE	81	13
TOT		198	198

Table 2. Comparison between Eradikit screening and genotyping data

ERADIKIT SCREENING		**ERADIKIT GENOTYPING**				
		A	**B**	**E**	**AB**	**NN**
POS	185	30	72	18	2	63
NEG	13	3	1	1	1	7
TOT	198	33	73	19	3	70

Table 3. Comparison between Eradikit screening and SU5 data

	SU-5								
Eradikit	**A**	**A1**	**A3**	**A4**	**B**	**B1**	**B2**	**AB**	**NN**
A 33	5	5	2	1	3	1	1	4	11
B 73	3	0	2	1	5	35	1	12	10❖
E 19	0	0	5	1	2	0	0	1	10

❖ *3 samples were not tested in SU5 as run out.*

Conclusion: Preliminary data presented herein show that different ELISA tests applied to the same serum panel generate discordant results in a significant proportion of samples. The genetic variability of the SRLV genotypes circulating in the field may partly explain these discrepancies, pointing to the importance of using a combination of screening and genotyping ELISAs to monitor SRLV infections. Comparing the two screening ELISA tests it appears that each test detects A or B genotypes preferentially. The presence of genotype E seropositive animals and in particular of sheep, considered resistant to this particular genotype, should be further investigated by PCR. The SU5 ELISA test is not able to discriminate the E genotype and this can explain the indeterminate results of E positive samples in this test. The discordant results of some samples identified as genotype E by the "Eradikit genotyping" and A or B by SU5 ELISA should be further investigated. Serological diagnosis of sheep appears to be more complex as the majority of these serum samples produced inconclusive results. The eradication SRLV program in Bolzano province is still ongoing and will permit to continue this comparative work between serological tests, in a well-defined and controlled epidemiological situation. At this point, it will be indispensable to start with the molecular epidemiological characterization of the circulating SRLV strains in this area. Based on sequencing data, we may create serological tools tailored to the relevant field strains, therefore improving the reliability of the serological monitoring. Using these novel tools and a combination of retrospective and prospective serological and virological data it will be possible to precisely pinpoint the strengths and shortcoming of different commercial ELISA in monitoring supra-regional eradication programs.

Diagnosis of respiratory outbreak in swine caused by a H1N1 pandemic virus

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Objective: Swine influenza viruses (SIVs) belong to the family *Orthomyxoviridae*, genus *Influenzavirus* A. These viruses are characterized by a segmented genome of 8 single-stranded RNA molecules and are classified into subtypes on the basis of two surface glycoproteins: the haemagglutinin (HA) and Neuraminidase (N).

Influenza A virus is a common pathogen in animal species including pigs. This animal species plays a crucial role in influenza ecology and epidemiology, primarily because of their dual susceptibility to human and avian viruses. This can lead to co-infection and genetic reassortment of viruses of swine, human or avian origin. Today, influenza is a common infection of pigs worldwide, sometimes causing severe respiratory disease in non-immune animals. Infection is maintained in endemic cycles without clear seasonality. Currently, avian –like H1N1 (avH1N1), human-like H1N2 (huH1N2) and human-like H3N2 (huH3N2) are the predominant subtypes of SIVs worldwide, Italy included, but other virus subtypes have also been isolated occasionally from pigs in some parts of the world, e.g. H1N7, H4N6, H9N2, H3N3 and H5N1.

Soon after the emergence and spread of the pandemic H1N1 (H1N1pdm) in 2009 in humans worldwide, it was noticed the introduction of this virus in pig holdings in several countries. Transmission experiment showed the high susceptibility of pigs to H1N1pdm raising concerns regarding the generation of reassortant viruses between the H1N1pdm and the endemic SIVs. Not wistanding the susceptibility of swine to H1N1pdm, this virus is more often detected as a reassortant viruses with several genes acquired from endemic SIVs or vice versa, suggesting its scarce ability to remain established in the swine population.

In October 2014 a respiratory outbreak was detected in a finishing farm of 1,200 animals in North East Italy.

Vaccinations for Aujeszky disease, Porcine Circovirus type 2 (PCV2) and Mycoplama were in place in the farm.

Serum samples and nasal swabs were collected soon after the appearance of respiratory signs and used to make differential diagnosis for the main viruses involved in respiratory diseases: PRRS, influenza and PCV2.

The present case report describes the diagnostic approach used for the detection of a whole H1N1 pdm virus in a swine farm with acute respiratory signs.

Methods: Animals showed fever (from 39.8°C to 41.5°C), respiratory dyspnea, loss of appetite, decrease reactivity. Affected animals were 8 months old (150kg) and no mortality was registered. Serum samples and nasal swabs were collected from diseased animals. Two consecutive sampling were performed 10 days apart. The first sampling consisted of 5 serum samples and 5 nasal swabs from diseased animals. During the second sampling, a higher numbers of serum samples (10) and nasal swabs (10) were collected.

Serum samples were tested by a type A influenza competitive ELISA (Idexx) and haemagglutination inhibition test according to OIE guidelines.

Briefly, sera were pre-treated at 37°C overnight with Receptor destroying enzyme (RDE, Sigma Aldrich, US) and heat-inactivated at 56°C for 30 min before use. Sera were tested individually against avH1N1, huH1N2, huH3N2 and H1N1pdm. Four haemagglutinin units of each virus and a 0.5% chicken red blood cell suspension were used in the test. The initial starting dilution of all sera was 1:10. Nasal swabs were placed in vials containing 1ml D-MEM medium. Viral RNA was extracted from 200µl of individual or pools of samples using the High Pure RNA Isolation kit (Roche, Germany) according to the manufacturer's instructions. All RNAs were examined for SIV using a Real-Time RT- PCR protocol targeting the matrix gene (M) and an internal control (InType-IC RNA –Qiagen) was co- extracted and co-amplified with each sample to avoid false negative results due to PCR inhibition. Subsequently, all gene M-positives were further characterized as individual samples by Real-Time RT- PCR targeting both M gene and for specific H1N1pdm haemagglutinin (HA) fragment.

PCR amplification for sequencing was performed by using specific primers (primer sequences available on request). The partial coding sequences were generated using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystem, Foster City, CA - USA). The products of the sequencing reactions were cleaned-up using PERFORMA DTR Ultra 96-Well kit (Edge BioSystems, Gaithersburg, MD - USA) and sequenced in a 16-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA - USA). Sequence data were assembled and edited with SeqScape software v2.5 (Applied Biosystem). Maximum likelihood (ML) trees were estimated using the best-fit general time- reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites, and a heuristic SPR branch-swapping search available in PhyML version 3.0.

Results: All but one serum samples collected at the beginning of the respiratory outbreaks resulted negative by type A competitive ELISA. Based on ELISA results HI test was not conducted on this set of samples. Nasal swabs collected at the same time resulted all positive to type A influenza virus by RT-PCR and positive to the specific RT-PCR targeting the HA of the pandemic strain. Serum samples collected 10 days after resulted all positive by type A competitive ELISA and HI test. Animals showed high levels of antibodies to the H1N1pdm strain by HI test. The GMT values were 351 for the H1N1pdm strain, 101 for the avH1N1, 11 for the huH1N2 and 0 for the huH3N2 antigens.

Nasal swabs collected 10 days after the onset of respiratory signs resulted negative to type A influenza virus by Real Time RT-PCR.

Sequence analysis conducted on positive nasal swabs revealed a high similarity (>98%) of all virus genes with strains belonging to the H1N1 "PANDEMIC LINEAGE". The highest identity (>99%) was observed for the HA, NS, NP and MP genes.

Conclusion: To make a prompt diagnosis of influenza infections is essential to collect samples soon after the onset of clinical signs and submit them to the nearest laboratory. In case of influenza infections as they can be sub-clinical, it is useful to collect samples longitudinally as demonstrated for this case report. For the investigated outbreak the availability of both serum samples and nasal swabs allowed a conclusive diagnosis.

The use of influenza type A antibody ELISA is generally neglected as influenza is widespread in pig populations and the general consensus is that the majority of swine are positive to this test. However knowledge on previous health and vaccination status of the herd makes the ELISA test applicable and eventually useful to interpret negative results to HI test and/or plan a second sampling of serum samples and nasal swabs.

The detection of a whole pandemic H1N1 was quite unexpected as it is generally detected as a reassortant virus. The H1N1pdm virus circulated in the affected farm for a short period of time indicating that a sustained infection chain was not established. This may suggest that the H1N1pdm is not able to persist and become endemic in swine, however preserving its capacity to reassort with other swine influenza viruses as reported in literature.

The combination of serological and virological laboratory tests has revealed helpful in understanding the evolution of the infection in the investigated respiratory outbreak. This approach can aid in obtaining a complete laboratory diagnosis and eventually to detect virus circulation. Additionally, the comprehension of infection dynamics at herd level may assist decisions on control strategies such as vaccination.

Influenza is a zoonotic disease and swine play an important role in the generation of new reassortant strains with possible pandemic potential. For this reason, the detection and complete virus characterization have important scientific implications in the global control strategies of influenza.

H9N2 Avian Influenza virus serological study among poultry workers in Iran

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Objective: Since the mid-1990s, H9N2 subtype influenza A viruses have caused infections in the poultry population around the globe, including several countries in Asia, Europe, North Africa and America. H9N2 influenza viruses are currently circulating in the Middle and Far East, as well as in European poultry, and the infection seems to be endemic in most developing countries.

A significant proportion of recent H9N2 isolates contains the L-226 mutation in their hemagglutinin (HA) receptor-binding site and shows preferential binding to analogs of receptors with α2,6-linked sialic acid (SA), which are traits typical of human viruses. Thus, these avian viruses might possess one of the key elements for infection in humans. The H9N2 virus was isolated for the first time from humans in Hong Kong in 1999 and until now different human cases of AI H9N2 have been reported in different countries. The wide circulation of H9N2 viruses throughout Europe and Asia, along with their ability to cause direct infection in mammals and humans, raises public health concerns about their potential to become candidates for the next influenza pandemic (Li KS et al.2003) in addition to the H5N1 virus. Since 1998 H9N2 AI has been reported in Iran (Nili H. 2003) and at present is endemic in the poultry industry; despite the control measures, which include mass vaccination of poultry, this virus has spread rapidly and is currently circulating in the country (Vasfi Marandi M.2013). This study was carried out to reveal the potential exposure to H9N2 AI viruses among Iranian poultry workers.

Methods: A total of 200 subjects took part in this study, including 100 poultry workers and 100 healthy residents. Serum samples were collected and tested for the presence of antibodies against two different H9N2 avian influenza viruses by haemagglutination inhibition (HI) and microneutralization (MN) assays, the analyses were repeated at least three times. Two Iranian H9N2 strains were isolated in 1998 and 2008, which showed different phylogenetic and important molecular differences at aa 226 (Q/L) (H3 numbering), (A/chicken/Iran/12VIR/9630/1998)/Q, (A/chicken/Iran/10VIR/854-5/2008)/L. The viruses were replicated in SPF chicken eggs and used in the serological study as antigens. In addition, all samples were tested for the presence of seasonal H3N2, H1N1 2009 pandemic human influenza viruses to verify the presence of cross positivity with the previously listed influenza viruses.

Results: Serological results showed that 16% of the exposed poultry workers were positive for the A/chicken/Iran/10VIR/854-5/2008 virus in MN test and 12.33% in HI test using the titer $\geq$ 40 as positive cut-off value. Only 2% of the exposed staff was positive for the A/chicken/Iran/12VIR/9630/1998 virus. Seroprevalence of control subjects for both H9N2 strains was very low in both tests (1-2%).

Conclusion: The results of this study have demonstrated that exposure to avian H9N2 viruses had occurred

among poultry workers in the Shiraz region – Iran, with a higher percentage than that recorded in the control population of the same geographic area. Continuous surveillance programmes should be implemented to monitor the presence of avian influenza infections in humans and to evaluate their potential threat to public health and poultry workers.

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H5N8 highly pathogenic avian influenza in Italy: control measures applied to live decoy birds

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Objective: The use of live decoy birds for hunting purposes is widespread in Mediterranean countries, particularly in France, Italy, and Spain. This activity is regulated by Directive 2009/147/EC on the conservation of wild birds. Typically, live decoy birds include larks, blackbirds, thrushes, fieldfares, lapwings, woodpigeons, and mallards amongst others. These birds are used as live baits to lure conspecific birds in proximity to the hunter or to attract them into traps. During the bird hunting season, which usually takes place between mid-September and late-January in Italy, decoy birds of the orders Anseriformes and Charadriiformes may come into contact with wild waterfowl, thereby acting as a potential bridge for transmission of avian influenza (AI) viruses from wild to domestic birds and humans. The risk posed by decoy birds in spreading the 2005 H5N1 AI virus resulted in the enactment of the Commission Decision 2005/734/EC, which banned the use of live decoy birds. Subsequently, with Decision 2006/574/EC, the competent authorities of each Member State were allowed to decide on the use of live decoys under derogation in their own countries, provided that specific biosecurity measures were undertaken. These included: the registration of decoy bird holders, identification of decoy birds by ringing system, strict separation between decoys and poultry, cleansing and disinfection of vehicles and equipments used to transport and manage decoys, restriction of bird movements to prevent contact with open water, implementation of specific surveillance systems, recording and reporting of any clinical and laboratory data.

North-East Italy has a high density of industrial poultry farms (Densely Populated Poultry Area, DPPA) located close to wetlands and marshlands harboring abundant migratory and/or non-migratory wild waterfowl populations. This is where hunting activities employing live decoys, mainly of the order Anseriformes, are common practice.

On December 2014, a Highly Pathogenic Avian Influenza (HPAI) H5N8 outbreak was detected in a meat turkey farm located in the province of Rovigo, North-East Italy, and strict control measures were implemented as per the Decision 2005/94/EC. Control measures were also implemented nationwide as regard to animal movements. In DPPAs, fairs, exhibitions, and markets involving live birds were banned. An additional measure put in place was the ban on the use of Anseriformes and Charadriiformes decoy birds according to Decision 2006/574/EC. Moreover, a monitoring plan on decoy birds already placed in the hunting areas was carry out in the Veneto region. The aim of the surveillance plan was to assess the risk of AI virus introduction into the decoy bird population and its possible spread to poultry, with a consequent risk for the industrial sector, especially in areas close to large hunting grounds. Hereby the methods and results of this monitoring plan are described and discussed, with particular reference to the role that live decoy birds may play in AI virus circulation.

Methods: Initially, the monitoring program focused on a limited area in proximity to the Venice lagoon. Local health units implemented the monitoring program on a sample of regularly registered decoy bird holders. A two-stage sampling scheme was implemented. The first sampling units were the decoy bird holders, whose sample size was determined based on an expected prevalence of 3% and a 95% confidence level. The second sampling units were the decoy birds themselves: at least 10 birds were sampled in each premise with >10 birds therein, or all available birds in premises housing ≤10 birds. Serum samples and/or cloacal swabs were collected from the sampled birds. A competitive Enzyme-linked Immunosorbent Assay (ELISA) and the Haemagglutination Inhibition test (HI) were performed on the serum samples whereas cloacal swabs were screened by real-time Reverse- Transcription Polymerase Chain Reaction (RRT-PCR).

Results: On February 2015, a Low Pathogenicity Avian Influenza (LPAI) virus of the H5N2 subtype was identified in a pool of cloacal swabs from 10 mallards used as decoy birds in the province of Venice. The samples were taken from a premise where a total of 39 decoy ducks belonging to different hunters were kept. According to Decision 2005/94/EC, all these birds were culled and a 1-km restriction zone was declared. Before depopulation, blood samples were taken from all of the birds in the premise to detect antibodies against AI virus type A. One of the mallards tested serologically positive by ELISA and another one for H5 subtype by HI. Virological tests by PCR gave negative results in all of the birds. Between 18 and 24 February, surveillance activities consisted of virological testing of 118 decoy birds belonging to 19 hunters in the province of Venice. Most of these birds were mallards or mallard hybrids; other species tested were wigeons, northern pintails, gadwalls and common teals. All the birds tested were negative for influenza A viruses by RT-PCR.

Conclusion: Live decoy birds are used by hunters to attract wild birds within their shooting range. As they are usually tethered to limit their movements and to prevent them from escaping or swimming, the possibility of direct contact between decoy birds and wild waterfowls and/or water appears to be limited. Nevertheless, decoy birds represent a potential source of introduction of AI viruses from the wild reservoir to poultry, especially if appropriate biosecurity measures are not implemented and/or poultry is kept in contact with decoy birds.

AI surveillance on live decoy birds requires serological and/or virological testing right after the end of each hunting season. As no information is normally gathered during the non-hunting season, prompt virus identification in decoy birds is more difficult during that period of the year. The monitoring program on live decoys implemented in the Veneto region allowed for the early detection of a LPAI virus, although it was not possible to discern whether infection occurred during hunting or whether the virus entered the flock independently. The monitoring plan also allowed for the collection of epidemiological information on the management of live decoy bird holdings, which is useful for future risk assessments. Additional surveillance measures may be implemented to target live decoy birds during the training period, allowing to gain deeper insight on the management practices out of the hunting season.

Modelling the transmission tree of the highly pathogenic avian influenza H5N1 epidemic in Israel, 2015

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Objective: On the 14th January 2015, unexpectedly high mortality was observed in a turkey farm in Israel. Two days later, the presence of highly pathogenic avian influenza H5N1 virus was confirmed. As a response, protection and surveillance zones were established in which culling or monitoring of poultry either in commercial or backyard farms was performed. Within the next four weeks, the virus was isolated in seven other farms, mainly turkey farms, located in the same geographical area. The objective of this study was to unravel the transmission tree using transmission tree modeling and to estimate relevant transmission parameters.

Methods: The method used in this study is adapted from Ypma et al. (2012). Except for the index case that has been infected by an unknown source, all subsequent cases were assumed to have been infected by farms in

Israel in which H5N1 was isolated since January 2015. To reconstruct the transmission tree, it was assumed that the likelihood that farm A infected farm B increased if A was still infectious when B became infected, if A and B were located close to each other and if there was no other infectious farms that could have infected B. It was assumed that all cases were reported. The likelihood was formulated using one parameter describing the decrease of farm infectiousness after the beginning of culling and two parameters describing the decrease of farm infectiousness as a function of the distance to a susceptible farm. The likelihood of a transmission tree could be calculated for any set of model parameters by multiplying together the likelihood of the most likely transmission events for each infected farms (apart from the index case). The effective reproduction number could also be estimated for each farm by summing the probabilities of all transmission events coming from that farm. The likelihood was explored using a Metropolis-Hastings algorithm.

Results: Results indicate that farm infectiousness dropped by 55% every day after the start of the culling (Figure 1). In addition, the average infection pressure exerted by an infectious farm to a susceptible farm at 1 km was 2 times, 3.3 times and 5 times higher than that exerted at 10, 20 and 30 km, respectively. Results also showed that farms 2, 3, 4, 5 and 8 had more than 75% chance to have been infected by farms 1, 2, 3, 4 and 7, respectively. However, it was not possible to determine with at least 50% certainty whether farm 6 was infected by farm 4 or 5 and farm 7 by farm 4, 5 or 6. The most likely transmission events are presented in Figure 2. The effective reproduction numbers were highly variable from 0.41 (95% credible interval: 0.35-0.47) for farm 6 to 1.52 (95%CI: 1.33-1.65) for farm 4. Phylogenetic data, informing about genetic distances between isolated strains and therefore about transmission events, would be a useful additional data source and will be incorporated in a future version of the model to refine the estimates.

Conclusion: This study showed that transmission tree modelling can provide important insights regarding transmission events without being overly computing intensive. We believe that the technique should become a component of outbreak investigations.

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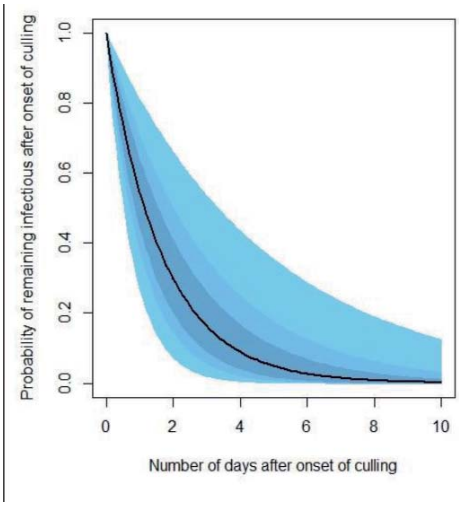

Figure 1: Decays of the probability that a farm is infectious as a function of the time elapsed since the start of the culling. The light, medium and dark blue areas correspond to the 95%, 75% and 50% credible areas for the corresponding distributions.

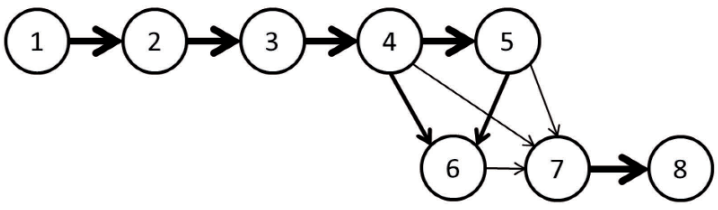

Figure 2: Most likely transmission events with posterior probability greater than 0.3. The thickness of the arrows is proportional to the probability of the transmission events

Reassortant Swine Influenza A detected in a Detection of a divergent Alpha Coronavirus in bats in Piedmont (Italy)

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Swine influenza viruses (SIV) are enzootic in pigs worldwide and infection with the virus causes substantial economic loss for farmers due to secondary infections and reduced weight gain in affected pigs. Further, the presence of Influenza A virus (IAV) in swine also poses a potential health risk to humans due to development of new reassortant viruses with zoonotic potential as recently seen with the 2009 H1N1 pandemic virus (H1N1pdm09)[1]. Due to their natural recombination ability, an enhanced surveillance and the development of more effective countermeasures is necessary to ensure an enhanced surveillance and to address IAV pandemic potential[2].

Objective: The ongoing project aims to investigate the circulation of SIV in pig farms of the Cuneo province, an area at risk for epidemiological conditions. The study will evaluate the circulation of endemic strains and the introduction of emerging/recombinant ones through an active and passive surveillance. The level of occupational exposure in positive farms will be tested on sera of the personnel at risk, in collaboration with the human health sector.

Methods: We designed a preliminary explorative study in order to evaluate the IAVs presence in swine herds located in an high-density area (Cuneo province). We considered 2 farm types: sow breeder and growing finishing farms. To detect the disease, based on a design prevalence of 10%, a random sample of 30 farms by type (populations: N=589 growing finishing farms; N=148 sow breeder farms) has been drawn. As an active surveillance, from 60 farms selected, a minimum sampling of 23 pigs per herd has been carried out, performing at the same time swabs and sera collection from each animal. Sampling was restricted to Cuneo province because it's an area with high density of pigs and poultry farms characterized by various environmental risk factors. The risk factors taken into account were: mixed type farms (pigs and poultry), farms with more than 200 pigs and/or 60 birds, multi-age farms, proximity to wetlands/aggregation sites of sedentary and migratory species reservoir. Epidemiological data were gathered through an on-farm interview with the farmer. Variables were grouped by topic: (a) general data: identification, location, herd size, presence of other domestic animal species; (b) production and health parameters: recent introduction of animals, percentage of mortality or morbidity registered during the last year, vaccination program and records of enteric and respiratory disease outbreaks during the last year.

Total RNA was extracted from pooled swabs with the Qiagen EZ1 Advanced XL Instrument and analyzed by One-step RT-PCR to detect the presence of the M gene of IAV (screening phase)[3]. Positive pool samples were individually re-analyze and then processed for viral isolation on continuous cell lines (MDCK and CACO2) and on S.P.F chicken eggs. Subtype identification was reached through two one-step multiplex RT-PCR methods able to rapidly distinguish between the main SIV subtypes circulating in Europe i.e. hu-like H1N2, av-like H1N1, human derived H3N2 and their potential reassortants4. Phylogenetic trees obtained for HA and NA genes of A/sw/It/240307/2014 were inferred with the maximum likelihood (ML) method implemented in IQ- TREE package 0.9.6. The robustness of the ML trees was statistically evaluated by bootstrap analysis with 1000 bootstrap samples.

Regarding the passive SIV surveillance, we analyzed 27 lung samples from 12 respiratory outbreaks in pigs, using the same workflow and methods described above.

Sera were tested and confirmed to be IAV seropositive or negative by ELISA test that recognizes the presence of antibodies against the internal nucleocapsid protein of IAV. Moreover sera from 15 sows farms resulted vaccinated against swine influenza virus and for that reason were excluded from the seroprevalence evaluation.

Results: At present 43 out of the 60 selected farms have been sampled and a total of 989 nasal swabs and sera have been analyzed. Two farms resulted positive in Real time RT-PCR during the screening phase, with eleven nasal swabs collected from the same pig farm and one from a second farm showing the presence of IAV M gene. Five out of the eleven swabs inoculated have been successfully isolated on cell lines and identified by multiplex RT-PCR as H1N2 SIV subtype.

Sequencing revealed a high nucleotide identity with a recombinant strain currently circulating in Denmark. Phylogenetic analysis showed that the virus, A/sw/It/240307/2014 originates from the reassortment of an avian subtype (H1N1) for the HA gene and a swine subtype (H3N2) for the NA gene. Seven lung samples collected from affected pigs in a third farm resulted positive in the screening phase. Six out of the seven samples have been successfully isolated on cell lines and identified by multiplex RT-PCR as H1N2 IAV but the sequencing analysis is still in progress.

Antibodies against SIV were found in 24 out of 28 farms (86%; CI95%: 98.85–73.15%). Overall within- farm seroprevalence obtained for our sample ranged from 4% to 100% (median: 56.5%; lower quartile (Q1): 25%, upper quartile (Q3): 87%). The animal prevalence of seroconversion was 56% (CI95%: 59.89–52.11%).

The analysis of seroprevalence was quite different between breeding and fattening farms. All breeding farms showed seroconversion (100%; CI95%: 100–100%) but from the animal point of view the antibodies against influenza virus were found in 265/391 sows (67.77%; CI95%: 72.62–63.68%). The observed seroprevalence in fattening farms and related animal resulted lower than in breeding pigs, being 77.78% (CI95%: 100–50.94%) and 41,06% (CI95%: 47.70–34.30%), respectively.

Conclusion: The H1avN2 subtype has been circulating in Denmark since 2003 and originated probably by reassortment between “avian-like” H1N1 and H3N2 Danish IAV strains. Also in Sweden and Italy is reported the isolation of IAVs referring to the Danish5. In Italy the isolation of the A/sw/It/240307/2014 reassortant virus was obtained from piglets introduced from Denmark to Piedmont. This was the first finding in our region, but the observation highlights the need of an active monitoring system for SIV circulation in pigs, but also for early detection of any IAV introduction and for monitoring viral reassortment events in our pig farms.

The seroprevalence obtained in our study are in accordance with other European countries such as Spain (89.9%), Germany (85.2%) or Belgium (94%)[6-7].

The fact that breeding have higher seroprevalences than fattening pigs is in accordance with other studies[8-9] and complies with the idea that the chances for infection with an ubiquitous agent increases with time7.

The antigenic properties of the viruses will be determined on the same sera using hemagglutination- inhibition (HI) tests performed according to OIE procedures using a panel of hyperimmune swine antisera raised against representative strains of H1N2, H1N1 and H3N2 SIVs most circulating in Italian pigs.

By serological survey in human sera the study aims to evaluate also the level of occupational human exposure in the positive farms. The integrated veterinary and human surveillance is crucial to early detect and evaluate the risk of recombination between these zoonotic viruses.

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Generation of engineered BTVs with chimeric structural proteins using reverse genetics - study of functional domain

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Objective: The development of a reverse genetics system for Bluetongue virus (BTV) has broadened the spectrum of possibilities for studies on BTV. We can now engineer bluetongue virus particles to contain genomes of our selection. Mutations and re-assortments between different serotypes can be introduced into the genome of BTV to gain further understanding of the viral protein functions and its replication cycle. This study is focused on BTV serotype 26 (BTV-26), which unlike the majority of all the other serotypes, is unable to grow in either KC cells (*Culicoides sonorensis* cell line) or adult *Culicoides sonorensis* midges. Furthermore, a previous study showed that four BTV-26 structural proteins are involved in the phenotype described above. Here we describe the use of the reverse genetics system as a tool to initially characterize the functional domains of those BTV serotype 26 (BTV-26) structural proteins by generating recombinant BTV particles with chimeric proteins and assessing them in various mammalian and insect cell lines.

Methods:
1. Generation of plasmid clones: Genome segments from BTV-1 RSArrrr/01 and BTV-26 KUW2010/02 were previously cloned as described in Pullinger et al. (in preparation). Chimeric BTV segments were generated by Spice Overlap Extension (SOE).

2. Generation of recombinant BTV-1 containing chimeric structural proteins using reverse genetics: Viruses were generated using a modified reverse genetics system. Briefly, viruses were rescued by transfecting expression plasmids coding for VP1, VP3, VP4, NS1, NS2 and VP6 from BTV-1, into 90% confluent BSR cells in a first transcription step. After 18-20h, the cells were again transfected with a pool containing nine RNA in vitro transcripts derived from BTV-1 RSArrrr/01 and one of the recombinant segment transcripts. The transfected cells were then incubated at 37°C for 2-3 days, before passage onto BSR cells (80-90% confluent) in T25 flasks. These cells were examined daily for the appearance of cytopathic effect (C.P.E) and further passaged onto BSR cells in T175 flasks when C.P.E was observed. Rescued viruses were verified by partial genome sequencing.

Results: Viruses containing chimeric BTV-1/BTV-26 structural proteins in a BTV-1 RSArrrr/01 backbone particle were successfully rescued. Growth kinetics for each virus were assessed in both mammalian (BSR) and *C. sonorensis* (KC) cell lines. In BSR cells, all recombinant viruses showed similar growth kinetics to the parental viruses BTV-1 RSArrrr/01 and BTV-26 KUW2010/02. However, infections in KC cells showed more noticeable differences. Most of the viruses had very similar growth profiles to that of BTV-1 RSArrrr/01, i.e. they grew exponentially from 2 days post infection (dpi) to 4-5 dpi when they reached their maximum viral titre or viral RNA load. In contrast, some viruses showed a delay in replication compared to BTV-1 RSArrrr/01.

Conclusion: BTV reverse genetics has been used to successfully generate complete BTV particles with chimeric structural proteins. Using this approach we have been able to determine protein domains that might be involved in the inability of BTV-26 to grow in KC cells or adult *Culicoides sonorensis* midges. The same approach will be used to further characterize other BTV-26 proteins.

A real-time PCR test for the detection and differentiation of porcine epidemic diarrhea virus and porcine deltacoronavirus

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Objective: Porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV) represent new threats

to the swine industry. To aid in early detection of virus, monitor shedding, or differentiate viral species, PCR has been a useful diagnostic tool. To this end, IDEXX has developed a multiplex real-time PCR test to detect and differentiate the presence of viral RNA from PEDV and PDCoV. Additionally, tests for PEDV, PDCoV and TGEV have been developed. All tests use an internal control approach based on detection of endogenous swine RNA, referred to as the Internal Sample Control (ISC) reaction.

Methods: Reaction mixes contained equal parts RealPCR™ RNA Master Mix and target-specific IDEXX detection mix for a total volume of 20 µL x number of samples tested. Samples (5ul per reaction) consisted of either synthetic oligonucleotides or nucleic acid purified from clinical samples. Clinical samples (fecal swabs and oral fluids) were purified using a commercial total nucleic acid extraction kit. The cycling program consisted of one cycle at 50°C for 15 minutes and 95°C for 1 minute, followed by 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

Results: The efficiencies and correlation coefficients for each test design were determined using serial dilutions of synthetic DNA. All test designs maintained efficiencies of 95%–105% with R2 values of $\geq$ 0.994 and detected at least 10 copies per reaction. To ensure no interference and/or competition between target and ISC reactions, multiplexed sensitivity testing was performed for all test designs. Copies of the target sequence (PEDV or PDCoV) were amplified in the presence or absence of artificially high concentrations of ISC. High levels of ISC had no impact on the detection of 10 copies of either PEDV or PDCoV. To confirm the ISC design detects swine RNA and not genomic DNA, the reverse transcriptase (RT) contained in the RealPCR RNA Master Mix was inactivated before addition of sample. Inactivation of RT resulted in complete loss of ISC signal. Test sensitivity and specificity for PEDV and PDCoV were evaluated using purified total nucleic acid from samples of known status. The PEDV/PDCoV multiplex test had PEDV sensitivity of 99.5% (n=191) and PDCoV sensitivity of 100% (n=44). Both designs had 100% specificity.

Conclusion: These results demonstrate the high sensitivity and specificity of the IDEXX RealPCR swine coronavirus tests. The tests are configured as either single target, PEDV, PDCoV, and TGEV tests, or as a PEDV/PDCoV multiplex test. All configurations include an ISC for the detection of swine RNA as an internal control.

Parallel EPIZONE Poster Presentation Session 3

Wednesday 2nd September 2015
17:00-18:00
Room Sully 2

Chair: Jean-Luc Guérin & Catherine Cêtre-Sossah

Development of a duplex real-time qRT-PCR method for detection of flaviviruses belonging to Japanese encephalitis and Ntaya serogroups

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Introduction and objective: Japanese encephalitis (JE) and Ntaya serocomplexes of the *Flaviviridae* family comprise mosquito- borne viruses associated with encephalitis in bird species, all of them transmitted by *Culex* genus mosquitoes. Viral species within the JE serogroup are West Nile virus (WNV), (which also affects horses and humans), and Usutu virus (USUV), while Bagaza virus (BAGV) and Turkey meningoencephalomyelitis virus (ITV) belong to the Ntaya serocomplex, and all being responsible of outbreaks over the last years. In fact, the incidence of flaviviral infections in animals in Europe has risen dramatically and should be regarded as a potential threat to animal health. Molecular diagnosis of flaviviral infections relies on generic RT-PCR approaches [1]

which are used routinely for surveillance. However, because of the high genetic distance between different flaviviral species, diagnostic sensitivity and specificity of these standard techniques are limited, especially when analyzing field samples containing low viral amount. The design of molecular techniques specific for the detection of each serocomplex would provide more accurate tools to obtain reliable diagnostic results in avian monitoring programs. This study describes the development and standardization of a quantitative duplex real-time RT-PCR (RRT-PCR) method for the simultaneous and differential detection of JE and Ntaya flavivirus serogroups, to be used as a screening tool in routine surveillance or in case of a bird encephalitis event.

Methods: A comprehensive selection of Ntaya and JE serocomplex viruses' full-length genome sequences available from *GenBank* was individually aligned. Primers and TaqMan probes specific for each serogroup covering all representative virus species were designed, targeting non-structural protein 2A gene and 3' end non-coding region (3'NCR) for JE and Ntaya, respectively. RRT-PCR assays were optimized individually, under the same reaction conditions, to be afterwards modified into a duplex format using the commercial *AgPath-ID one-step RT-PCR kit (Life Technologies)*. Probes were labelled with different reporter dyes, FAM for JE serocomplex and JOE for Ntaya serocomplex, to allow a simultaneous, but differential, detection of both serogroups in a single run. Because there is no available universal RRT-PCR technique for flavivirus detection, a multiplex RRT-PCR technique for WNV (lineages 1 and 2) and USUV simultaneous detection [2] and a single RRT-PCR technique for BAGV detection[3] were employed as reference methods. RNAs from a panel of 31 different arbovirus isolates was employed to develop and evaluate each designed single and duplex RRT-PCR assays: 10 WNV lineage 1 isolates, 3 WNV lineage 2 isolates, 5 USUV isolates, 7 other JE serocomplex isolates, 1 BAGV isolate, 4 ITV isolates, 5 non Ntaya or JE serocomplex RNA flavivirus isolates. Additionally, a panel of 7 avian and equine non flavivirus isolates was employed for specific studies. Finally, a collection of avian samples selected from WNV or BAGV experimental infections were used for the evaluation of the duplex test in clinical material.

Results: The analytical sensitivity of the developed duplex RRT-PCR technique was assessed by testing replicates of $\log_{10}$ serial dilutions of each serogroup reference virus, WNV and BAGV for JE and Ntaya serogroups, respectively. Results were compared with those obtained with the separated RRT-PCR tests used as reference techniques[2,3], obtaining similar or even higher sensitivity for each of the serogroups. For determination of the detection limit, in vitro-transcribed RNA standards containing NS2A and 3'NCR genome target fragments of WNV and BAGV, respectively, were produced and final RNA concentration was determined spectrophotometrically. Triplicates of serial dilutions were assayed and the sensitivity of the duplex RRT-PCR was estimated to be below 100 RNA copies for both JE and Ntaya serogroups.

To assess the specificity of the duplex RRT-PCR, a panel of RNAs from 31 different arbovirus isolates and 7 avian and equine non flavivirus isolates were analyzed. The assay was shown to be specific for either JE or Ntaya serogroups since no fluorescence reads were observed with any heterologous flavivirus and non flavivirus isolates. Fluorescence signals were obtained exclusively for JE and Ntaya serocomplex isolates, being able to clearly differentiate between them without any cross-reaction detected.

Finally, in order to assess the performance of the developed method as diagnostic tool, a panel of samples (heart, spleen, liver, kidney and brain) obtained from three house sparrows and four partridges collected in two experimental infection studies with WNV and BAGV, carried out at INIA- CISA BSL-3 animal facilities, were selected. All samples obtained from WNV or BAGV infected animals gave positive results in the duplex assay, showing similar or even lower Ct values to those obtained throughout reference techniques. No fluorescence signal was obtained when samples from non- infected control birds were tested, confirming virus absence.

Conclusions: The duplex RRT-PCR technique developed in this study provides a specific and highly sensitive tool for rapid detection and differentiation of JE and Ntaya flavivirus serogroups in either domestic or wild animals. This new technique constitutes a valuable complement to the methods currently available for routine diagnosis of WNV, USUV and BAGV in birds and in surveillance involving wild birds and mosquitoes, which can be easily implemented for high throughput screening of samples in a rapid and reproducible way. In conclusion, the main application of this molecular tool would be the epidemiological surveillance of the mosquito-borne flavivirus species that are responsible for producing encephalitis in birds.

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Validation of the ID SCREEN® FMD NSP competitive ELISA

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Objective: Foot and mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals. There are seven distinct serotypes of FMD virus (FMDV), namely, O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3. The differentiation of herds which have been infected from those which have been vaccinated is a critically important follow-up activity to protective emergency vaccination. Both infection and vaccination elicit antibodies against structural antigens, but only assays that measure levels of antibodies against non-structural protein (NSP) can differentiate infected and vaccinated animals (DIVA).

The ID Screen® FMD NSP Competition ELISA is designed to detect 3ABC non structural protein (NSP) antibodies in bovine, ovine, caprine and porcine serum or plasma samples.

This study summarizes validation data obtained for this ELISA.

Methods: The ID Screen® FMD NSP competitive ELISA includes microplates coated with 3ABC recombinant non structural protein (NSP) and an anti-NSP monoclonal antibody horseradish peroxidase (HRP) conjugate, and offers both short and overnight incubation protocols. The test was performed as per manufacturer's instructions.

Specificity was evaluated on 364 bovine sera, 296 swine sera, 141 sheep sera, and 159 goat sera from non-endemic and non-vaccinated areas (France). These sera were analyzed in parallel using both the short and overnight protocols.

To evaluate sensitivity, the international reference panel of NSP sera used for kit evaluation, comprised of 36 sera derived from vaccinated or unvaccinated and experimentally infected cattle at IAH, Pirbright (1), was tested. (Results were kindly provided by ANSES, Maisons-Alfort, France and the Pirbright Institute, UK.)

In addition, the IAEA (through the Animal Production and Health Sub-program of the Joint FAO/IAEA Division) serum panel from infected cattle, including 6 FMDV serotypes (A, O, Asia 1, SAT1, SAT2 and SAT3 was tested.

Results: Specificity was found to be superior to 99.4 %, regardless of the species tested. Agreement between the short and overnight protocols was high (99.69%).

All 13 strains present in the international reference panel for evaluation of NSP ELISA tests, were correctly detected. Test performance was equivalent to the best commercial ELISAs evaluated in the

2007 study by Parida, S. et al (1). The ID Screen® test efficiently detected experimentally- infected animals, including carrier animals.

All 6 serotypes in the IAEA panel were correctly identified as positive.

Conclusion: The ID Screen® FMD NSP Competition ELISA demonstrates high specificity and excellent performance on reference panels. The ELISA correctly identified all strains tested and efficiently detected carrier animals.

The kit offers both short and overnight protocols. These protocols give similar results, meaning that laboratories have the possibility of offering same-day results to their customers if the short protocol is used. The test is applicable to multiple species, including ruminants and swine. Easy-to-use, the kit includes colored and ready-to-use reagents

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Validation of the ID SCREEN® African swine fever indirect ELISA

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Objective: ASF control and eradication programs require accurate and reliable diagnostic tests. The ID Screen® African Swine Fever Indirect ELISA detects anti-ASFV antibodies in both domestic and wild pigs. Unique features of the ELISA include the coating of three recombinant ASFV antigens (P32, P62, and P72), and the ability to use the test with blood filter paper and meat juice as well as serum and plasma.
This study presents validation data obtained for this test.

Methods: The ID Screen® ASF Indirect ELISA kit includes plates coated with three recombinant ASFV antigens (P32, P62, and P72), and an anti-multi-species-HRP conjugate. The test was performed as per manufacturer's instructions.
The following sera were tested:

- 763 disease-free sera from domestic pigs, wild boars, and Iberian pigs;
- 100 samples from disease-free animals in France;
- 90 negative sera, tested by both the serum and filter paper protocols;
- 3 sera from vaccinated and challenged pigs;
- 8 reference sera from the ASF EURL;
- 3 positive sera, titrated and tested by both the serum and filter paper protocols. Test sensitivity for meat juice was evaluated through the analysis of spiked samples.

Results:
Specificity:

- For the analysis of 763 disease-free sera from domestic pigs, wild boars, and Iberian pigs, measured specificity was 99.61% (CI 95%: 98.96% - 99.90%).
- For 100 samples from disease-free animals from France, measured specificity was 100% (CI 95%: 96.30% - 100%), n=100.
- All 90 negative sera tested by both the serum and filter paper protocols were correctly identified by both protocols.

Sensitivity:

- The 3 sera from vaccinated and challenged pigs gave positive results with the ID Screen® ELISA.
- The 8 reference sera from the ASF EURL were correctly identified as positive.
- For the 3 positive sera, titrated and tested by both the serum and filter paper protocols, the measured analytical sensitivity was similar regardless of the sample type tested.
- All spiked meat juice samples were correctly identified as positive.

Conclusion: The ID Screen® African Swine Fever Indirect ELISA is the only commercial ELISA based on the use of three different recombinant proteins. A flexible tool, the test may be used for filter paper and meat juice samples.

It shows excellent specificity and sensitivity, correctly detecting reference sera from the EURL for ASF (INIA-CISA, Madrid, Spain).

The use of filter papers makes sampling easier, especially for wild boars. By using the elution protocol in deep well tubes with direct transfer to ELISA plates with a multi-channel pipette, sample identification errors may be avoided.

This test is a reliable tool for the detection of antibodies against the ASFV in both domestic pigs and wild boars. The serum application has been validated by the ASF European Reference Laboratory.

Preliminary validation of the ID SCREEN® PEDV indirect ELISA

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Objective: Porcine epidemic diarrhea (PED), caused by PED virus (PEDV) is an infectious and highly contagious viral disease of pigs characterized by severe diarrhea, vomiting and dehydration. Diagnostic methods to confirm PEDV infection are virus isolation, direct fluorescent antibody (FA) test or PCR. Antibodies may be detected

either by immunoperoxidase assay (IMPA), immunofluorescence assay (IFA) viral neutralization test (VNT) or ELISA. ELISA offers the advantage of being cost-effective and easy to implement for high throughput testing.

IDvet has developed an indirect ELISA based on a recombinant nucleocapsid, the ID Screen® PEDV Indirect ELISA. This study presents validation data for this ELISA.

Results: Diagnostic specificity, evaluated on 512 sera from areas where the virus has not been reported in recent years, was 99.2% (IC95% 98.0; 99.7).

Sensitivity was evaluated on 33 IFA-positive sera. 30/33 samples were found positive by the IDvet ELISA. Testing of additional positive samples to evaluate diagnostic sensitivity is underway. Global correlation with IFA was 98%. The test detected experimentally-infected animals with the CV777 strain between 14 and 21 days post infection.

Conclusion: Preliminary validation studies indicate that the ID Screen® ELISA is an efficient tool for disease surveillance and epidemiological studies.

Development of a multi-check rRT-PCR method for panFMDV detection

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Objective: The aim of the present study was to develop and validate a multi-check strategy using rRT- PCR (multi-rRT-PCR) based on SYBR-Green I for pan/foot and mouth disease virus (pan/FMDV) diagnosis.

Methods: Based on the *in silico* analyses, different primer pairs were selected and addressed in order to reduce the probability of viral escape and possible failures in the pan/FMDV detection due to the high variability of the virus. The analytical parameters were assessed on a large representative number of viral strains. The repeatability of the test and its performance on field samples were also evaluated.

Results: The multi-rRT-PCR was able to detect novel emergent strains of FMDV which had circulated in South America during the period 2006-2010 and on which the individual assays failed when they were applied independently. We demonstrate that the system proposed is a reliable and rapid diagnosis method for sensitive and specific detection of FMDV.

Conclusion: A validated multi-rRT-PCR assay based on SYBR Green I detection coupled to melting curves analysis for pan/FMDV diagnosis on clinical samples is proposed. This work also highlights the need to incorporate the multi-target detection principle in the diagnosis of pathogens with highly variable genomes as FMDV.

Development and validation of a new ASFV real time PCR

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Objective: African Swine Fever Virus (ASFV) is a notifiable, highly contagious disease that can cause enormous economic losses. The disease is widely endemic in many parts of Africa, in parts of South Europe and is increasingly becoming a threat in Eastern Europe. As there is still no vaccine or treatment available monitoring and controlling of the disease is of utmost importance. In order to improve diagnostics Thermo Fisher Scientific has developed and validated a new duplex real time PCR kit.

Methods: For the development of a reliable, sensitive, and specific rtPCR system more than 450 different ASFV sequences representing the p72 protein encoding region were aligned. The new assay is composed of a duplex

rtPCR including an internal control to ensure reliable DNA extraction. For extraction of viral DNA from field samples the MagMax Pathogen RNA/DNA Kit and the MagVet Universal Isolation Kit were validated. In order to demonstrate the sensitivity and specificity of the new LSI VetMAX™ ASFV kit different internal and field studies including animal infection experiments (INIA, Valdeolmos, Spain; CVI, Netherlands; Germany) were carried out. In total about 1600 negative samples from ASFV free regions (Germany/ Spain) and additionally 33 different pathogens were tested to demonstrate specificity of the assay. For validation of the sensitivity about 100 ASFV positive samples from Africa and Europe were tested. The Limit of detection (LDPCR) was determined by serial dilution of a plasmid carrying a specific ASF sequence (pASF). The efficiency of the PCR reaction was identified by using dilutions of 10^{-4} to 10^{-11} of the pASF plasmid.

Results: Test results of the ASFV positive samples showed 100% sensitivity in all tested sample materials (blood, serum and tissue samples). A serial dilution of the ASF target sequence led to a limit of detection (LOD) of 16 genome copies per PCR reaction. The experimental LOD was $5x10^3$ copies per ml in serum and $1x10^4$ copies per ml in blood. By testing 1600 negative samples a specificity of 100% was demonstrated. Additionally, all 33 alternately infected samples scored negative in the ASFV specific assay.

Conclusion: In conclusion, the LSI VetMAX™ ASFV kit fulfils all the validation criteria of PCR characteristics and complete method required by the U 47-600-2 standard.

Evaluation of commercial ELISA kits for the detection of BVDV-specific antibodies in serum and milk using well-characterized sample panels

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Objective: Bovine viral diarrhoea (BVD) is one of the most important viral diseases in cattle, causing severe economic losses worldwide. The causative agent of this disease is the Bovine Viral Diarrhoea Virus (BVDV), which belongs to the genus *Pestivirus* within the family of the *Flaviviridae*. Currently, Belgium is in the first phase of a National eradication program based on the obligatory testing of newborn calves using ear notch samples. Farmers are then urged to eliminate persistently infected (PI) animals as soon as possible. In a later phase of the eradication program, farmers will have the possibility to apply for a "BVDV free" herd status. To monitor this freedom from BVDV infection status at herd level a surveillance strategy will be implemented based on serologic testing of animals in these herds at regular intervals by means of a BVDV antibody ELISA. Therefore it is of utmost importance to evaluate the quality of the currently available antibody ELISAs for both available matrices: serum and milk.

Methods: In this study, several BVDV antibodies ELISA kits from different manufacturers available on the Belgian market were evaluated using a panel of well-characterized serum and milk samples, to evaluate the analytical (ASe) and diagnostic sensitivity (DSe), the diagnostic specificity (DSp) and repeatability of each kit.

To compose the panel, virus neutralization (VN) assays on serum were regarded as the gold standard for determination of the status of the samples. Milk samples were only used if the status of the corresponding serum sample was known. Samples were considered positive if their SN titer $\geq$ 10. All samples were tested in both BVDV-1 and BVDV-2 VN tests, and were considered positive when at least one assay showed a positive result. Therefore, samples from infected animals, vaccinated animals and vaccinated and infected animals were considered positive in our study, while samples obtained from naïve animals were considered negative, since neither DIVA vaccine nor DIVA assays were available. For all samples obtained in the field, the vaccination history of the animal and the history of BVDV infection at farm level were known. In addition, some of the positive and negative samples were collected from experimental infection studies.

The final serum panel consisted of 20 positive and 60 negative samples, a two-fold dilution series (14 dilutions) of a strong positive sample obtained from an animal experimentally infected with a BVDV-1 virus and one with

a BVDV-2 virus and two times 10 repeats of a strong positive, a weak positive and a negative sample. The serum panel was used in 22 protocols (14 short and 8 long incubations), to evaluate 10 blocking ELISAs (9 anti-NS3 (also known as p80) and 1 anti-E0 ELISA) and 5 indirect ELISAs (4 total antibody and 1 anti-NS3 ELISA) from 10 manufacturers in total.

The final milk panel consisted of 44 positive and 36 negative samples, two-fold dilutions series (14 samples) of two different strong positive samples obtained from a farm infected with BVDV and two times 10 repeats of a strong positive, a weak positive and a negative sample. The milk panel was used in 15 protocols (8 short and 7 long incubation), to evaluate 6 anti-NS3 blocking ELISAs and 4 indirect ELISAs (3 anti-NS3 and 1 anti-E0) from 7 manufacturers.

The samples were distributed on two ELISA plates per tested ELISA kit for each matrix. All ELISA protocols were performed and all results analyzed according to the kit manufacturer's guidelines.

Results:

Panel of serum samples

Our evaluation showed that the indirect ELISAs have a higher DSe (ranging from 85% to 100%; average 93.5% for the total antibody indirect ELISAs) compared to the blocking ELISAs (ranging from 45% to 65%; average 57.3% for blocking anti-NS3 ELISAs). However, the anti-E0 blocking ELISA scored better than all anti-NS3 blocking ELISAs with a DSe of 75%, while the anti-NS3 indirect ELISA had a lower DSe (55%) compared to other total antibody ELISAs. False negative results were mostly found for serum samples obtained from animals that were vaccinated as well as animals with a high VN titer from farms previously or currently infected with BVDV.

Our results demonstrate a good diagnostic specificity for all kits evaluated with on average 95.7% DSp for blocking anti-NS3 ELISAs and 100% for the blocking anti-E0 ELISA and of on average 96.7% for the total antibody and the anti-NS3 indirect ELISAs. It was observed that two sera from PI calves were consistently causing false positive results in many kits.

The detectability of the ELISAs was good for all indirect ELISAs with a consistent positive result for the weak positive sample and both dilution series. In contrast, the blocking ELISAs often did not detect the weak positive sample from the panel and did not consistently detect the dilution series of the BVDV1 sample, whereas this was not problem for the dilution series of the BVDV type 2 samples.

As expected, all evaluated kits scored the strong positive sample positive and the negative sample negative. Coefficients of variation (CV) were calculated from the results obtained with the 20 repeated strong and weak positive and negative samples (repeatability). From these coefficients, a mean CV was calculated. Whereas all indirect ELISAs had mean CV's below 10%, 3 (anti-NS3) blocking ELISAs had a mean CV larger than 10%. Of these three kits, 2 had individual CVs above 15%.

Panel of milk samples

Our study showed that the DSp of both blocking and indirect ELISAs approached 100%, whereas the diagnostic sensitivity of the kits was much less. Again, blocking ELISAs (average Se = 66.8%) were found less sensitive compared to the total antibody indirect ELISAs (average Se = 76.9%), whereas the anti-NS3 indirect ELISAs performed similar to the worst blocking ELISAs. Comparable to what was found for serum, false negative results were found for milk samples obtained from animals that were vaccinated as well as animals with a high VN titer for the corresponding serum from farms previously or currently infected with BVDV.

The dilution series was detected well by all ELISAs, yet only 2 out of 8 blocking and 4 out of 6 indirect ELISAs were able to detect all repeats of the weak positive sample from the panel. The strong positive and the negative sample were correctly identified by all ELISAs tested. From these results the CVs were calculated (repeatability), as well as the mean CV. One blocking and one indirect ELISA had a mean CV greater than 10%, due to individual CVs for the high positive or negative sample above 15%.

Conclusion: A thorough evaluation of commercial ELISA tests to detect antibodies against BVD was performed. It was demonstrated that the DSp for both milk and serum ELISAs was good to excellent. The analytical sensitivity was higher for the indirect ELISAs compared to the blocking ELISAs using serum, while the weak positive samples were not always detected using milk samples. Even though most kits scored well on repeatability, it should be kept in mind that some kits had greater CVs than acceptable. Overall, we showed that the DSe was the weakest point of kits evaluated in the study for both serum and milk. Indeed, blocking (anti-NS3) ELISAs were found to be less sensitive compared to the total antibody indirect ELISAs. This may partly be explained by the results obtained from samples from vaccinated, uninfected animals. It is well known that these samples may score negative in some blocking ELISAs and positive in indirect ELISAs, which lead to the false conclusion by some manufacturers that anti-NS3 blocking ELISAs may serve as DIVA tests to distinguish vaccinated from infected animals. Yet, literature shows that no true DIVA vaccine and assay is currently available for BVDV. Moreover, two samples which had a high VN titer (1/640) and which originated from vaccinated animals from farms infec-

ted with BVDV were also found negative by these blocking ELISAs, showing an unsatisfying level of sensitivity.

Our results clearly show that BVDV-specific antibodies are best detected using total antibody indirect ELISAs on serum (Se ~ 93.6%) and that serology using milk as a matrix is much less sensitive (anti-NS3 blocking ELISAs (Se ~ 66,8%); total antibody indirect ELISAs (Se ~76.9%)). Yet, the anti-NS3 blocking ELISAs are more sensitive using milk (Se ~ 66.8%) compared to serum (Se ~57.3%).

The results obtained in this study were finally used to select those kits in each category which had the best scores on all parameters evaluated for further evaluation on a large number of serum and milk samples coming from 51 herds of different size, type (dairy/beef/mixed), vaccination and BVD status, which will be described in another abstract by De Baere *et al.*

Interlaboratory comparison of assays for detection of antibodies against Porcine Epidemic Diarrhea virus

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5: National Veterinary Institute (SVA) - Sweden

Introduction: Porcine epidemic diarrhea virus (PEDV) was first identified in Europe in the 1970's. The virus causes acute diarrhea and this can result in high mortality within young piglets. PEDV is an alphacoronavirus but is antigenically quite distinct from transmissible gastroenteritis virus (TGE) which can cause a similar disease. The apparent prevalence of PEDV in Europe declined to low levels in the 1980's and 1990's. In May 2013, PEDV was identified for the first time in the USA and has spread rapidly across that country and into Canada, Central and South America. The US viruses are closely related to viruses identified from within China. Several million piglets have died following the outbreaks in the USA. Recently, outbreaks of PEDV have been reported again in Europe within a number of countries, including Germany, Italy, France and The Netherlands; the viruses are very similar to those found in the USA.

Objective: To obtain knowledge about the occurrence and prevalence of PEDV in Europe there is a need for serosurveillance and this requires serological tests with high specificity and sensitivity. These methods are also needed to rapidly detect and investigate outbreaks. However, currently there is no published data on the sensitivity and specificity of various "in-house" and commercial assays that are used within different diagnostic laboratories. The objective of this project was to increase the partner's ability to detect infected pigs by comparing, harmonizing and optimizing the diagnostic assays used within diagnostic laboratories in 5 countries.

Methods: In a collaboration between 5 laboratories (partners within CoVetLab that comprises five EU national veterinary public health institutes), a ring trial has been conducted using a single panel of sera (>50 samples) collected from the field and also from animal experiments. A variety of assays, based on either IPMA or ELISA technologies including a commercial test kit, have been used to detect anti-PEDV antibodies.

Results: The results from this ring trial will be presented.

Conclusion: Important differences were found in the capabilities of the various assays to detect anti PEDV antibodies and the specificity of some of the assays seems to be problematic. An "in-house" blocking ELISA used at DTUVet, Denmark seemed to have the overall highest sensitivity and specificity.
Further testing is under way to validate the different assays.

Evaluation of a newly developed Classical swine fever DIVA ELISA

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Objective: Emergency vaccination with live marker vaccines represents an effective control strategy in a classical swine fever (CSF) outbreak situation. The successful implementation is dependent on a reliable accompanying diagnostic assay that allows differentiation of infected from vaccinated animals (DIVA strategy). As induction of a protective immune response relies on virus neutralizing against the E2 protein of the CSF virus (CSFV), the most promising DIVA immunoassay strategy is often based on detection of E^{rns}-specific antibodies. Thus, the aim of the present study was to develop an E^{rns}-specific ELISA which may be used as an accompanying discriminatory test for marker vaccines like the CP7_E2alf.

Methods: For the detection of CSFV-specific antibodies the concept of a double antigen ELISA was applied and tested. Therefore, a representative number of serum samples (> 1000 samples) including CSFV antibody positive sera (≤ 21 days post infection and > 21 days post infection), CSFV antibody negative sera, and sera taken from animals vaccinated with the marker vaccine CP7_E2alf were analyzed.

Results: The concept of a double antigen ELISA is shown to be a reliable strategy for development of a serological DIVA assay. Moreover, detection of serum antibodies against E^{rns} from CSFV isolates belonging to relevant genotypes could be assessed and differences between serum samples taken from infected and vaccinated pigs were observed. Sensitivity and specificity of the ELISA will be presented and application for DIVA purpose will be discussed.

Conclusion: Taken together, the E^{rns}-specific ELISA reveals promising test performance compared to results obtained with other serological assays used for the detection of CSFV specific antibodies. Ongoing studies will focus on further evaluation of the assay and on statistical analysis for its application as an accompanying DIVA test for the marker vaccine CP7_E2alf.

Development and evaluation of a multiplex classical RT-PCR for simultaneous detection and typing of FMDV in West Africa

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Objective: Foot-and–Mouth Disease Virus (FMDV) causes a highly contagious transboundary disease of cloven-hoofed domestic and wild animals. The virus belongs to the *Aphtovirus* genus in *Picornaviridae* family. There are seven immunologically and genetically distinct serotypes (O/A/C/Asia1/SAT1, 2 &3) that may be further divided in several sub-types. Foot-and-Mouth Disease (FMD) is one of the most economically devastating diseases affecting livestock animals. The West African territories are considered as regions with continuous FMDV circulation where outbreaks of FMDV serotypes O, A, SAT1 and SAT2 have been reported. Early detection and typing of FMDV is crucial for implementation of the adequate outbreak management in this region.

Methods: This study describes the development of a multiplex conventional RT-PCR for both detection and typing of FMDV strains circulating in West Africa. The RT-PCR reactions were developed by using primer sets targeting the 3D coding region, the VP1 coding region (O/A/SAT1/SAT2-specific) and the β-actin gene in order to produce amplicons of different sizes that would be easily distinguished on agarose gel electrophoresis. Two FMDV strains of each targeted serotype as well as two negative samples were used to evaluate intermediate and final RT-PCR protocols. A 6-plex prototype (O/A/SAT1/SAT2/3D/β-actin) was finally developed and additionally tested with a panel of reference strains including all serotypes of FMDV. The sensitivity of RT-PCR was evaluated on 24 negative field samples and37positive field samples together with the corresponding virus isolates.

Results: The 6-plex prototype detected all FMDV strains tested and identified the four serotypes of interest (O/A/SA1/SAT2) without any improper amplification. No FMDV target amplification is produced for the negative

field samples. The field samples known positive for West Africa serotype O and A were tested by the 6- plex RT-PCR. The results showed that the FMDV was detected in 36 samples out of 37. A or O serotype was properly characterized (VP1 target) by 6-plex for 13 samples out of 37.Using a 3-plex protocol (O or A/3D/β- actin), 19 out of 37 samples were rightly serotyped. All these field samples were properly identified using simplex (O or A) RT-PCRs. Moreover, using 6-plex protocol, all the isolates from field samples were positives for the 3D target and 33 out of 37 were correctly serotyped. All of them were successfully serotyped by 3-plex and simplex RT-PCR.

Conclusion: We have developed and evaluated a 6-plex RT-PCR allowing both detection and typing of FMDV strains. We will further validate this method on a larger panel of field samples. This multiplex conventional RT-PCR developed for rapid molecular detection of O, A, SAT1 and SAT2 serotypes of FMDV in West Africa is a promising method that can be used for early detection. The conventional RT-PCR method is well known, and moreover doesn't need expensive laboratory equipment and reagents. It could be thus easily implemented in diagnostic laboratories in developing countries, providing an improvement for rapid detection and typing of FMDV strains.

Development of a Luminex assay for the serological diagnosis of Vesicular Stomatitis virus

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Introduction: Vesicular stomatitis (VS) is a viral contagious disease affecting livestock including horses, cattle and pigs. It is a zoonosis inscribed on the list of notifiable diseases established by the OIE (World Organisation for Animal Health). The causative agent, Vesicular Stomatitis virus (VSV), belongs to the genus Vesiculovirus in Rhabdoviridae family. Two serotypes have been described: New Jersey (NJ) and Indiana (IND). The latter being subdivided into 3 subtypes (Indiana 1, 2, 3). In cattle and pigs, VS cannot be reliably clinically differentiated from the other vesicular diseases, such as foot and mouth disease (FMD), vesicular exanthema of swine (VES), and swine vesicular disease (SVD) when horses are not involved. A prompt laboratory differential diagnosis is thus essential.

VS is mostly found in the Americas, where it causes heavy economic losses. Cases of outbreaks in Brazil in 2013 and in Texas in 2014, show that this disease still raises concern. Currently, serological diagnosis is based on viral neutralization test and Elisa. This study describes the development of a serological diagnostic test based on the Luminex technology. This multiplex technology allows both serotyping and differential diagnosis from small sample volumes.

Materials and methods: The Luminex technology is based on the principle of flow cytometry and relies on the use of fluorescent magnetic microspheres of a defined spectral color ("beads") which can be coupled to different biological macromolecules. For our application, a batch of beads with a unique spectral address, was coupled to the envelope glycoprotein of the VSV, serotype NJ (VSV-G-NJ), produced as recombinant antigen using the baculovirus system and harboring a C terminus tag MAT. The coupled beads were then incubated with sera samples and the antigen/antibody complexes were revealed using a biotinylated anti-species antibody and streptavidin-phycoerythrin. The results were analyzed on the Luminex200 platform. Median fluorescence intensity (MFI) values were measured and normalized.

Results: Efficiency of antigen/bead coupling was assessed using a monoclonal antibody specific to the MAT tag. Specificity of the coupling was verified using a panel of well characterized positive bovine VSV-NJ- or VSV-IND sera samples. The protocol was then optimized to be applied to the analysis of horse sera (i.e analyze dilutions and concentration of biotinylated anti-species antibody). A prototype test was thus developed, with a cut off value determined using a panel of 100 naive horse sera, in two independent experiments.

Discussion: A first step in developing a Luminex-based serological test for VS diagnostic was reached, using the VSV-G protein-NJ. Similar work is underway using a recombinant VSV-G-IND protein produced under the same conditions and a duplex test is under development. Bead sets coupled to these VSV-G proteins may also be combined to beads batches developed in our laboratory for the FMD serological diagnosis with the aim to offer a differential diagnostic SV/FMD in cattle. The sensitivity and specificity of these luminex prototypes will also be compared to those obtained with the virus neutralization test, the reference method.

OTHER POSTERS

Topic: Arboviruses

Bluetongue virus serotype 2 does not cross the placenta of late term pregnant cows

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Objectives: Vertical transmission (VT) is a well-known property of Bluetongue virus (BTV) strains adapted on cells, including attenuated vaccine strains, and of the North-European BTV-8. In this regard, our group at the IZSAM has recently demonstrated that, a wild type BTV-2 strain passaged only once on *Culicoides sonorensis* (KC) cells was able to cross the placenta and infect ovine fetuses, thus demonstrating for the first time that a single cell passage can dramatically change patho-biological features of BTV-2. Indeed, field observations during the BTV-2 outbreaks in Italy did not support such transmission mechanism. According to the study performed in sheep, the same wild type and insect cells passaged strains were employed to infect cows in order to check for vertical transmission in the offspring.

Methods: Wild-type BTV-2 originated from a spleen of a naturally infected sheep during the 2001 epidemic in Sardinia, Italy. To prepare inoculum for animals experiments, the splenic homogenate was diluted 1:9 in phosphate buffered saline (PBS) and injected subcutaneously to a serologically and virologically BTV-free sheep. Then, blood samples were daily collected and BTV positive samples were stored at -80°C until use (virus $BTV2_{blood}$). $BTV\text{-}2_{blood}$ was propagated once onto KC cells for 10 days. Cell supernatant ($BTV2_{KC}$) was harvested, titrated on African green monkey (Vero, titre 5.97 $\log_{10}$ $TCID_{50}$/mL) cells and stored at -80°C until use. Six late term pregnant cows were administered with 10 mL of $BTV2_{blood}$ (Group A) and six cows with 2 mL of $BTV2_{KC}$.

Following infection, EDTA-blood samples were collected three times a week and tested by RT-PCR and virus isolation. Serum samples were collected once a week and tested for BTV-2 antibodies by cELISA and serum neutralization. At calving, precolostral EDTA-blood and serum samples were collected from calves, and tested serologically and virologically. The lambs were subsequently sampled as scheduled for dams.

Results: By day post-infection (dpi) 17, all infected animals seroconverted and neutralizing antibodies were detected throughout the sampling period (dpi107).

BTV RNA was detected in the blood of all infected cows starting from dpi4 in Group B and dpi7 in group A. The peak was observed at 11-14 dpi and titres remained stable throughout the sampling period (dpi107) in both groups. Infectious virus was isolated in both groups from dpi11 until dpi18. At birth, calves did show neither specific BTV antibodies nor BTV RNA. Virus isolation was nonetheless attempted, but it was unsuccessful.

Two calves, both born to dams in Group A, died at birth due to severe dystocia. Necropsy did not reveal any specific sign consistent with BTV infection, and all sampled organs tested negative by molecular assays.

Conclusion: In this study, unlike what it was observed in ewes, both wild type BTV2 ($BTV2_{blood}$) and the homologous strain passaged once onto insect cells ($BTV2_{KC}$) failed to cross bovine placenta and infect offspring. Reasonably, interpretation of the results is not easy and it is open to speculations. Indeed, differences encountered between cows and ewes may be related to the diverse morpho- functional and histological properties between ovine and bovine placenta or alternatively to the different host related mechanisms involved during viral replication and clearance.

Overall no definitive conclusions can be drawn on BTV VT in cows, and further studies are reasonably required for a better interpretation of these results.

What is accepted is that BTV VT is an elusive phenomenon whose underling mechanisms are far from being fully elucidated, and many variables (host species, serotype, strain, gestational age, etc.) must be taken into account when dealing with it.

Circulation of Schmallenberg Virus in Turkey, 2013

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Objective: Schmallenberg virus (SBV) infection emerged in European domestic and wild ruminants in 2011. There is very limited information about the characterization of SBV isolates and the epidemiology of its infections in the rest of world, except for in European countries. We investigated the circulation of SBV in cow herds in Central Anatolia, Turkey, in 2013.

Methods: A total of 180 whole blood samples were analyzed using real-time RT-PCR. For phylogenetic analysis and confirmation of real-time RT-PCR results, the S gene segment was amplified, sequenced, and compared to other SBV strains.

Results: The presence of SBV RNA was detected in 6 of 180 (3.3%) samples. In addition, SBV-specific antibodies were detected in 87 (24.1%) of 360 sera collected from this region using a virus neutralization test.

Conclusion: The results of this study indicated that SBV was in Turkey in 2013. Furthermore, sequencing results suggest that circulation SBV in Turkey it is genetically identical SBV European strains.

Culicoides dewulfi and its possible role as Bluetongue vector in Italy in 2014

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Objective: Bluetongue (BT) is a vector borne disease affecting ruminants, caused by an Orbivirus (Reoviridae) and transmitted by vectors belonging to the genus Culicoides (Diptera: Ceratogonidae). Recently an extensive epidemic occurred in Italy, where Bluetongue virus (BTV) serotype 1 circulated in 2014, causing severe clinical outbreaks.

Within the subgenus Avaritia, In Italy the main BTV vector species are C. imicola and the species belonging to the Obsoletus complex (i.e. C. obsoletus and C. scoticus), whereas other species can play a role in transmitting the disease in Northern Europe, namely C. dewulfi and C. chiopterus.

Culicoides chiopterus is known to have a gradient from North to South in Europe, and particularly in Italy can be considered a rare species, whereas C. dewulfi can be collected in the whole Country, even if at low abundance, and to date has never been found infected with BTV.

Culicoides dewulfi, C. chiopterus and the species of the Obsoletus complex have a similar morphology, thus they are often grouped under the “C. obsoletus group”.

This study reports the finding of C. dewulfi in Central and Southern Italy, and its possible role as BTV vector, as results of the entomological activities within the National Surveillance Plan for Bluetongue in 2014.

Moreover the method used for C. dewulfi identification is described, based on both morphology and molecular assays.

Methods: The Culicoides collections were performed and analysed according to the protocol of the National Reference Center for Exotic Diseases.

Culicoides dewulfi was identified morphologically, based on wing pattern and abdomen morphology. Furthermore a specific RT-PCR, based on internal transcribed spacer 2 ribosomal DNA sequences (ITS2), was used to confirm the species identification. Control plasmids have been created and used as positive reaction controls.

The parous females were divided in pools, according to location and date of collection, and tested for BTV by a real time RT-PCR.

Results: Culicoides dewulfi was identified in 34 collections, from seven Italian Regions: Abruzzo, Apulia, Calabria, Campania, Emilia Romagna, Tuscany and Umbria. Overall, the relative abundance of this species was

1.1 (567/51412 midges) ranging between 0.1% and 8.3%.
A total of 55 pools (329 midges) of C. dewulfi adult parous females were sorted and tested for BTV. Two pools resulted positive, collected in August and September 2014, on a cattle farm in Abruzzo region, consisting of six and seven midges, respectively.

Conclusion: This study confirms that C. dewulfi is a low abundant species on livestock in Italy. Nevertheless, it was collected on farms where BTV was circulating, in seven Italian regions. Moreover, viral genome of BTV serotype 1 was detected from adult parous females of this species.

Further investigations on the ecology of C. dewulfi in Italy should be achieved, in order to evaluate the risk of possible increasing of local population of this important European vector.

Deciphering the tick pathobiome

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Objective: Ticks are distributed all over Europe and are the first arthropod vector of disease agents to humans and domestic animals (Jongejan and Uilenberg, 2004). In Europe the most important ticks in term of public health is *Ixodes ricinus*, the vector of the agent of Lyme Borreliosis (Hubalek, 2009; Rizzoli et al., 2011). Because of its biology and its capacity to feed on a large variety of animal species, *I. ricinus* is able to transmit a large variety of pathogens including bacteria, parasites and viruses In Europeans. The most prevalent tick-borne disease is Lyme borreliosis, transmitted by *I. ricinus*, with over 85 000 new cases each year in Europe (Hubalek, 2009; Rizzoli et al., 2011). However, patients bitten by ticks can also be infected by many other zoonotic pathogens. These include bacteria (*Borrelia burgdorferi* sensu lato complex, *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, *Rickettsia* spp., *Candidatus* Neoehrlichia mikurensis, *Bartonella* spp., …), parasites (*Babesia* species mainly) and viruses (Tick Borne Encephalitis Virus) (Charrel et al., 2004; Parola and Raoult, 2001). Some of these pathogens have been identified in ticks decades prior to their association with human disease (such as *B. miyamotoi*), whereas others have only been discovered very recently (such as *candidatus* Neorehrlichia mikurensis). Globally the incidence of tick-borne diseases is increasing worldwide and tick borne diseases has been classified in the “red list” of diseases with a high risk of emergences due to environmental changes (Githeko et al., 2000; Jore et al., 2014; Lindgren et al., 2012; Vayssier et al., 2015). However, tick- borne pathogens are still poorly understood, and it is estimated that half of all human tick-borne disease has an unknown origin. In this context, we decide to undertake a study aiming to draw a global picture of bacteria, parasites and viruses in *Ixodes ricinus*, using deep sequencing of RNA as the hallmark of identification of pathogens that replicate in ticks collected in France and to better characterize them.

Methods: RNA and DNA were extracted from 1450 *I. ricinus* questing nymphs collected by flagging in East of France. RNA was pooled and used for NGS. Following de novo assembly, bacterial, parasitic and viral contigs were assigned to the closest known taxonomy. DNA and RNA were used for real time PCR to confirm taxonomic species assignment of NGS-derived contigs for the doubtful cases, and for determination of prevalence.

Results: We have generated a global in-depth picture of tick-borne bacteria, parasites and viruses. We identified RNA from the main pathogenic bacterial species known to be transmitted by *I. ricinus*. In addition we also identified unanticipated bacterial species for which we have estimated the prevalence within those ticks inhabiting the studied areas (Vayssier et al., 2013). Sequences corresponding to parasites from two distinct genera were recovered in *Ixodes ricinus* ticks collected in Eastern France: *Babesia* spp. and *Theileria* spp. Four Babesia species were identified, three of which were zoonotic: *B. divergens*, *Babesia* sp. EU1 and *B. microti*; and one which infects cattle, *B. major*. This is the first time that these last two species have been identified in France. This approach also identified new sequences corresponding to as-yet unknown organisms similar to tropical *Theileria* species (Bonnet et al., 2014). Regarding the viral contigs, the vast majority of hits mapped to virus families known to replicate in arthropods. Most of them belong to *Bunyaviridae* (*Nairovirus*, *Phlebovirus*, *Orthobunyavirus*), *Reoviridae* (*Coltivirus*), *Rhabdoviridae* (*Vesiculovirus*, *Sigmavirus*), *Picornaviridae*- like

(*Iflavirus*, *Drosophila* virus A). Hits with close nucleotide identity (94-100%) with the Eyach virus were identified. Other hits were distant from known viruses (average 50%, range 25-72%), but the fact that most of them belong to families already known to replicate in arthropods suggest that they should rather correspond to new viral species than to background noise (Moutailler et al., in prep).

Conclusions: The data obtained from this study has proven that NGS has an enormous potential to detect the unexpected and provides the means to monitor pathogen occurrence.

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Detection of Schmallenberg Virus RNA in bull semen in Poland

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Objective: The presence of Schmallenberg virus (SBV) in bull semen may pose a threat of genital infection in female during artificial insemination and natural breading. Although the transmission of SBV from an infected male has not been proven yet, viral RNA has been detected in the bull semen with variable dynamics even after three months from seroconversion. Recent French study showed infection of transgenic mice with SBV positive semen. This discovery may implicate cost-intensive trade restrictions in bovine semen trade. The aim of the study was to evaluate the possible contamination of semen with SBV in bulls from bull insemination centers in Poland.

Methods: Semen samples from 115 bulls collected between April to August 2014 from four biggest insemination centers was tested. The seropositivity status of individual animals was unknown. In order to optimize the protocol and evaluate the efficiency and sensitivity of different RNA extraction methods, SBV DNA standard (a product of RT-PCR- DNA strand, complementary to the RNA segment of SBVS template) was prepared. Four extraction methods were assessed for level of β-actin (internal control of amplification) and recovery rate of prepared DNA standard detected in RT-PCRin semen. Initially, tenfold dilutions of DNA standard in TE buffer were prepared, subsequently standard DNA was used to prepare tenfold dilutions of SBV-spiked semen and after extraction RT-PCR was performed. Two pairs of primers designed to detect SBV S segment and gene β-actin as internal control were used in the standard real-time RT-PCR developed at FLI (Riems, Germany) using AgPath-ID One-Step RT- PCR Reagents (Ambion, Applied Biosystem) kit in StepOne Real-Time PCR system (Life Technologies). The reaction was considered positive when a Ct value < 40 was obtained both forSBV-S and β-actin.

Results: Six semen samples out of 115 (5.2%) tested were positive for the presence of SBV RNA with Ct values

between 34.5 and 37.2. Positive samples originated from two out of four breading centers and accounted for 4.1% and 13.3% of the samples tested from those two locations.

From the four tested extraction methods, Viral RNA Mini Kit (Qiagen) was selected as the most sensitive method allowing detection of 10 copies of viral DNA per reaction and PCR efficiency of104% in standard DNA-spiked semen. The extraction method resulted in the lowest levels of Ct values for β-actin in the commercially diluted semen sample which probably due to some inhibitors which affected negatively other extraction methods tested. And finally Viral RNA Mini Kit needs only 140µl of semen what is also significant when testing semen samples frozen in straws. The detection limit of 1 copy per reaction and efficiency 93,1% was established for the DNA standard diluted in TE buffer. This demonstrates that semen extraction method which was chosen and semen as a potentially inhibiting medium did not decrease substantially the detectability of SBV.

Conclusion: Detection of SBV RNA semen in Poland is not surprising considering high seropositivity found in cattle in 2014 (58.5%) and detection of SBV in some *Culicoides* spp. trapped across the country in 2014 what demonstrates active SBV circulation. The presence of viral RNA in the semen suggests quite recent SBV outbreaks in the two insemination centers, however the source has not been identified. The results obtained are consistent with the data from Germany and Netherlands from 2012 were the occurrence and Ct values were comparable to our results. The presence of virus genetic material in the tested semen does not implicate directly its infectivity. According to EFSA report from 2014, no scientific evidence of virus transmission during artificial insemination exists and in the worst case it would have led to the viraemia in female. That for any restrictions in semen batch trade are unsubstantiated.

However, the possibility of SBV transmission through artificial insemination or mating should not be ignored and the emergence of new outbreaks in naïve herds especially during midge absence periods should be investigated considering this aspect.

Multiple Culicoides species of the Pulicaris complex implicated in Bluetongue virus transmission in Italy, 2012-2014

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Objective: Bluetongue (BT) is an infectious non-contagious disease, affecting domestic and wild ruminants, transmitted by *Culicoides* biting midges (Diptera: Ceratopogonidae).

Since the first introduction in August 2000, Italy experienced BTV incursions almost constantly every year. Six different serotypes (BTV-1, BTV-2, BTV-4, BTV-8, BTV-9, BTV-16) spread in different regions, between 2000 and 2014. In 2012, the BTV-1 and BTV-4 were circulating in Sardinia. During the following year, the BTV1 spread all over the island, then the virus circulated also in Sicily and reached the Tyrrenian coast of Central Italy in late 2013. In summer 2014, BTV-1 spread toward inland territories, affecting large areas of the Country.

Within the national entomological surveillance program, *Culicoides* collections were performed in the BTV affected Italian Regions during the epidemics 2012-2014, in order to identify the possible vector species responsible of virus transmission. Here we report the detection of BTV from species belonging to the Pulicaris complex.

This complex belongs to the subgenus *Culicoides (Culicoides)*, which includes about 50 species that should probably be grouped in at least four different subgenera. The subgenus *Culicoides sensu stricto* includes, among others, some species commonly found in Italy such as *Culicoides pulicaris*, *Culicoides punctatus* and *Culicoides newsteadi*.

The taxonomy of the subgenus *Culicoides (Culicoides)* is still not clearly defined, and more than one taxa probably refer to *C. pulicaris* and *C. newsteadi*. In this study, for convenience, the species belonging to the subgenus *Culicoides (Culicoides)* will be grouped under the term Pulicaris complex.

Methods: The *Culicoides* collections were performed within the Italian Entomological Surveillance Plan for Bluetongue, according to the protocol of the National Reference Centre for Exotic Diseases.

More than 650 collections were performed from October 2012 to December 2014 in 14 Italian regions, on BTV affected farms. *Culicoides* were identified at species or complex level, basing on wing pattern, and age-graded. The adult parous females were divided into pools (maximum 50 midges/pool) and tested for virus detection by real time RT-PCR.

Results: Overall nearly 3,000 pools were sorted and analyzed for BTV virus detection. Of these, 916 pools were composed by midges of the Pulicaris complex. A total of 72 pools of *C. newsteadi* (collected in four Italian regions), 17 pools of *C. punctatus* (from five regions) and 24 pools of *C. pulicaris* (from eight regions), resulted positive. BTV serotype 1 was identified in all positive pools. In addition one pool of *C. newsteadi*, collected in Sardinia in 2012, resulted positive for both BTV-1 and BTV-4.

Conclusion: During the epidemics 2012-2014 in Italy, BTV genome was repeatedly detected in parous adult females of *C. newsteadi*, *C. punctatus* and *C. pulicaris.* This suggests that species of the Pulicaris complex could have played a role in spreading the disease, in addition to the main vectors *C. imicola* and *C. obsoletus/scoticus*. However further studies are needed to assess the vector competence of these species, particularly *C. newsteadi* and *C. punctatus*, which were never involved before in BTV transmission.

Perinatal West Nile Virus Infection in a Foal in Turkey

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Objective: West Nile virus (WNV) is a mosquito-borne Flavivirus, belonging to the Japanese Encephalitis complex and primarily maintained in nature by transmission cycles between mosquitoes and birds. Occasionally, WNV infects and causes disease in other vertebrates, including humans and horses. WNV has caused outbreaks of severe neuroinvasive disease in humans and horses in Europe, the Mediterranean Basin and American continent. WNV activity in Turkey has been identified previously in serosurveillance studies and reports of acute human and/or horse WNV cases have announced since 2009. Aim of the study was to diagnose etiological agent caused sudden death of the foal.

Methods: A foal, 15 days old from a private stable in Bursa province, was died suddenly in mid June 2014. Local veterinarian made postmortem examinations and variety of tissue samples (spleen, heart, lungs, intestine, liver) and blood were directly sent to the laboratory of Virology Department, Faculty of Veterinary Medicine, Ankara University for the diagnosis of possible viral etiology. Total RNA extraction was made from all samples using a commercial RNA isolation kit and subsequently submitted to the RT nested PCR and qRT-PCR. All tissues were also examined with histopathological and immunohistochemical techniques.

Results: The tissues and blood were found positive WNV genomic RNA (gRNA) in RT nested PCR and qRT-PCR revealed the highest virus load in lungs, which was followed by liver, spleen, intestine and heart in order of virus content. Histopathologic examinations showed no specific lesions other than pulmonary edema in organ samples studied. However, immunohistochemistry performed using polyclonal anti-WNV IgG raised in mouse, revealed intensive WNV-Ag localization in cells of vascular media layer and connective tissue cells.

Conclusion: The results indicated that the foal died because of asphyxiation caused by pulmonary edema. The wide multisystemic dissemination of the virus indicated that WNV has transmitted foal before parturition near to birth.

Topic: Clinical virology

An experimental study of bovine coronavirus shedding and the potential for transmission

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Objective: Bovine coronavirus (BCoV) is an important livestock pathogen, causing substantial losses in the beef and dairy industries worldwide. The duration of virus shedding and how long cattle are contagious are not well documented. In order to minimize spread of the virus between farms, more knowledge is needed. The aim of the present study was to investigate the transmission potential from infected calves by describing the duration of virus shedding and determining if calves transmit the virus to naïve calves three weeks after infection.

Methods: An experiment based on naturally infected calves was conducted. Six calves from a herd with an ongoing outbreak of BCoV were used to infect a group of four BCoV seronegative calves. From the day of exposure these calves were examined daily until 42 days post exposure (DPE). On 21 DPE two seronegative sentinel calves were introduced. Nasal swabs and fecal specimens were collected daily and assayed for BCoV by qRT-PCR. Serum antibody titers to BCoV were measured by ELISA prior to and three weeks post exposure.

Results: All four calves developed mild to moderate respiratory symptoms and two of them showed mild enteric symptoms during the trial. Serum samples from all four calves were negative for antibodies against BCoV before the trial and positive at 21 DPE. All four calves shed BCoV nasally from one to 17 DPE. One calf was positive for BCoV in nasal specimens until 28 DPE. The virus was found in feces from all calves from two to eight DPE. From nine to 25 DPE the calves shed virus intermittently in the feces. The sentinel calves did not seroconvert to BCoV and the virus was not detected in fecal or nasal specimens.

Conclusion: Calves infected with BCoV can shed virus for a period of four weeks despite seroconversion. However, the calves did not transmit the infection to sentinel calves at 21 DPE and the shedding of potential infectious virus after seroconversion needs further investigation.

Etiological causes of canine infectious tracheobronchitis in Swedish dogs

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Objective: Kennel cough, or canine infectious tracheobronchitis (CITB), is a well-known disease in dogs and is often easily recognized. CITB is a disease complex that can be caused by several viruses and bacteria, but the diagnosis is usually based on characteristic clinical signs, without any further laboratory diagnostics to establish the etiological agent. Therefore, the knowledge of which viruses and bacteria that causes disease outbreaks in Sweden is limited. The main contributors to the development of CITB are considered to be canine parainfluenzavirus (CPIV) and *Bordetella bronchiseptica,* for which there are commercially available vaccines. More recently, canine respiratory coronavirus (CRCoV) was detected in and isolated from dogs with upper respiratory signs. Experimental infection has confirmed that CRCoV is able to induce CITB. Other viruses and bacteria have also been considered as possible causes of CITB, either as a single infection or as co-infections. This study aims to give important information about the different viral and bacterial agents involved in disease outbreaks of CITB in Sweden. The information is important for disease prevention and control, and for giving adequate advice to veterinary clinicians and dog owners to prevent outbreaks and spread of disease.

Methods: Swedish privately owned dogs with characteristic upper respiratory signs of CITB (dry cough) up to 7 days (n=77) were included in the study, in which sample collection is still ongoing. Clinical signs were graded as mild (only mild intermittent coughing), moderate (severe frequent cough without affected general condition) or severe (affected general condition and deep coughing). As healthy controls, dogs with no upper respiratory signs for at least 6 months at time of sampling were used (n=17). No more than two dogs per household were sampled. Oral and nasal swabs for virological and bacteriological analyses were collected from all dogs. Molecular diagnostics (real-time PCR/RT-PCR) of canine adenovirus type 2 (CAV-2), canine herpesvirus (CHV), canine influenzavirus (CIV), CPIV, CRCoV and *B. bronchiseptica* were performed. Culturing of aerobic and anaerobic bacteria was also carried out. Real-time RT-PCR positive samples were further investigated by conventional RT-PCR to retrieve longer stretches of viral genomes for sequence analysis.

Results: In total, 41 out of 77 dogs (53%) with CITB signs were found virus positive by real-time RT-PCR. CPIV was detected in 29 (38% of all CITB cases) and CRCoV was found in 12 (16% of all CITB cases) of these dogs. One of the CRCoV positive dogs was also positive for CAV-2. None of the healthy control dogs were positive for any of the viruses included in the molecular diagnostic panel. Thirteen of the 29 CPIV positive dogs had been vaccinated with a live attenuated CPIV vaccine (subcutaneously n=12; intra-nasally n=1) within a year from the sampling occasion. Fifteen of the CPIV positive dogs had not been vaccinated within a year prior to sampling. Seven of the non-vaccinated CPIV positive dogs had mild clinical signs, whereas six had moderate and two had severe clinical signs. In the vaccinated CPIV positive dogs, six had mild clinical signs, three had moderate and four had severe clinical signs. Most CRCoV positive dogs (n=9) had mild clinical signs, two had severe clinical signs and the clinical signs were not graded in one dog. All dogs were culture-negative for *B. bronchiseptica*, while two dogs with CITB (3%) were PCR-positive. One recently vaccinated healthy control was also PCR-positive, indicating presence of the live *B. bronchiseptica* vaccine strain. Sequencing of detected viruses is under progress.

Conclusion: In this study, an etiological cause could be detected in more than half of the dogs with CITB with CPIV being the most common finding, whereas no viruses were detected in the healthy controls. Surprisingly, *B. bronchiseptica* was only found in two dogs with CITB and not at all detected at culture. This is in contrast to studies of CITB in other countries, where *B. bronchiseptica* was detected quite frequently. The discrepancy in findings of *B. bronchiseptica* between studies could depend on time from debut of clinical signs to sampling, or differences in geographical distribution. On the other hand, CRCoV was a common finding, further strengthening its importance in causing CITB. In most of the CRCoV positive dogs, mild clinical signs were seen, but two cases displayed severe clinical signs. Based on the limited number of animals included in the study so far, we could not see any differences in clinical signs between vaccinated and non-vaccinated CPIV positive dogs. Sequencing of detected viruses is still ongoing and those results might be helpful in understanding the spread of CPIV and CRCoV.

Topic: Diagnosis

Droplet digital PCR (ddPCR) for studying mixed infection of different NDV strain pathotypes.

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Objective: Newcastle disease and Avian Influenza are considered to be the most dangerous fowl diseases which may cause huge economic losses. Newcastle disease is caused by the enveloped, non- segmented, single-stranded RNA virus (NDV, APMV-1; belonging to *Paramyxoviridae* family), that can be further divided into sixteen different genotypes grouped into five pathotypes according to their pathogenicity. Virulence of a strain depends mainly on the short amino acid sequence of the precursor protein responsible for membrane fusion. Highly pathogenic NDV usually contains sequence: C-112R/K-R-Q/K/R-R/K-R-F117-N, while low pathogenic has: C-112G/E-K/R-Q-G/E-R-L117- N. These motifs are recognized by different cellular proteases that limit virus ability to propagate only in specific host tissue. World Organization for Animal Health distinguishes five pathotypes of Newcastle disease virus: viscerotropic and neurotropic velogenic (highest mortality rate, Intra Cerebral Pathogenicity Index > 1.5), mesogenic (low mortality but moderate signs from respiratory system, ICPI 1.5-0.7), lentogenic (mild respiratory infection with no mortality, life-vaccine strains, ICPI
< 0.7) and asymptomatic (no signs or subclinical enteric infections). It is known that vaccination with low pathogenic virus do not protect birds from secondary infection caused by highly pathogenic strains. Studies that quantify the level of viral prevalence during mixed infection cannot be found in the literature. Here, we present a method that can be employed for analysis of competition between virus strains in host organism.

Methods: In our studies we have used new and unique technology named Droplet Digital PCR (ddPCR). With outstanding precision in comparison to other molecular diagnostic methods we have performed absolute quantification of the number of NDV nucleotides in the samples collected from eggs co-infected with different virus strain pathotypes. After initial validation by standard RT-real time-PCR viral RNA isolated form allantoic fluid was used in experiments conducted on QX200 Bio- Rad System with the use of three specific hydrolysis molecular probes.

Results: Results from four experimental setups, in which LaSota, Roakin and Italy NDV strains were used for co-infection in SPF eggs will be shown. The difference in the detected virus amounts depends on pathotype and virus titer used for initial inoculation.

Conclusion: Techniques based on next generation sequencing with genetic variant analysis are used widely for establishing virus copy-numbers variations. Though the results are often excellent, the price for large number of analysis is not acceptable. We developed a method that is less expensive and still generates high quality data. Experiments done with ddPCR systems can be easily scalable, which decreases the price even more. This new tool could be the method of choice for a scientist who wishes to study the dynamics of mixed infections.

Fecal samples for PRRS virus diagnostics

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Objective: Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease of swine worldwide and is characterized by reproductive problems e.g. abortions and stillbirth and respiratory distress especially in young pigs. The causative agent of the disease, small enveloped virus with 15 kb single-stranded RNA, belongs to the family *Arteriviridae* and order *Nidovirales*.

The main aim of our study was to detect viral RNA of PRRS virus and PRRSV specific antibodies in feces of pigs vaccinated or/and infected by PRRS virus. Another aim was to prove possible relationship between the amount of shed virus and the titer of specific antibodies in fecal samples.

Methods: Total of 24 piglets divided into five groups were used in our study. Two groups were vaccinated by commercially available inactivated vaccines (In-A, In-B), another two groups were vaccinated by commercially available attenuated vaccines (MLV-A, MLV-B) and the last control group was left unvaccinated. Subsequently all groups were exposed to 10^6 $TCID_{50}$ of PRRSV (Lelystad virus). Fecal samples were collected in one week intervals. Virus RNA was detected by qRT-PCR test, N specific antibodies by ELISA test.

Results: The virus was present in feces in almost all animals vaccinated with attenuated vaccine or following experimental infection. The titer of shed virus was not influenced by the type of used vaccine and reached about 10^3-10^5 virus genome copies/g in all vaccinated and control groups of piglets. Nonetheless in some animals intermittent shedding virus was observed.

Only IgA antibodies were detected in fecal samples, no IgG were detected. All vaccines were able to induce IgA antibody response in the gut of piglets. The titer of IgA antibodies didn´t significantly correlated with the amount of shed virus.

Conclusion: In conclusion, feces can be used as a sample for individual swine testing of PRRS infection. Fecal samples are suitable for qRT-PCR without major negative effect to reaction. We designed the method for treatment of fecal samples to serological testing and we optimised ELISA test for this type of sample.

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MediLabSecure: Implementing a network of animal virology, human virology and entomology laboratories for a one health approach of vector-borne viruses in the Mediterranean and Black Sea regions

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Objective: As (re-)emerging viruses are threatening global health, the EU-funded MediLabSecure project (2014-2017) aims at enhancing the preparedness and response to viral threats by establishing an integrated network of animal virology, human virology and entomology laboratories in 19 non-EU countries of the Mediterranean and Black Sea areas in partnership with 4 Institutes in France, Spain and Italy. The MediLabSecure project is reinforcing the public health laboratory and epidemiology networks previously established by the EpiSouth Plus project (2010-2013) by additionally involving animal virology and medical entomology laboratories in a fully integrated "one health" approach for surveillance and control of emerging arboviral diseases.

Methods: One laboratory per field of study (human virology, animal virology, medical entomology) and per country was selected in 2014. A first meeting involving the heads of laboratories was held in Paris in January 2015 with the aim of defining priorities and adapting upcoming activities of the project to the needs and interests of participating countries. A "Needs assessment" questionnaire was implemented to assess laboratory capacities and needs regarding biosafety, diagnostic methods and integration of laboratory and epidemiological surveillance for emerging vector-borne viruses.

Results: Forty-seven laboratories were selected to actively join the project. The January meeting allowed the project partners and head of laboratories to meet and exchange on the objectives and future steps of the project as well as on their experiences, needs and expectations. Based on these discussions and on the responses to the "Needs assessment" questionnaire, the first tailored training sessions will be organized in June 2015, enabling laboratories to implement harmonized and up-to- date techniques to perform (1) laboratory diagnosis of relevant vector-borne viral diseases such as West Nile, Chikungunya and Rift Valley Fever in humans and animals and (2) mosquito species determination and entomological field surveys.

Conclusion: By enhancing diagnostic capacities and regional multidisciplinary cooperation, the Medilabsecure network could represent the cornerstone of a corporate preparedness and response to vector-borne viral threats in the Mediterranean and Black Sea regions based on a One Health.

Prevalence of Aujeszky's disease virus infection in wild boar in Poland in 2012-2014

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Objectives: Aujeszky's disease (AD) is one of the most economically important diseases of swine. The disease is caused by Suid herpesvirus 1 (SuHV1) for which domestic pigs (DP) as well as wild boar (WB) are the only natural hosts (1). Although successful eradication of AD in DP populations in many parts of the world was achieved (1), the potential risk of SuHV1 trans- mission from infected WB has become a growing matter of concern. Currently, Poland is in a last stage of AD eradication program and therefore the prevalence of SuHV1 infection in Polish WB population is of great importance.

Materials & methods: In the study a total of 17308 blood samples taken from WB shot in all 16 voivodships of Poland was used. The samples were collected during 2012, 2013 and 2014. A commercially available ELISA was used for screening of antibodies to ADV according to manufacturer's instruction (HerdChek Anti-PRV gpI, IDEXX, Inc., USA).

Results: From 17308 blood samples that have been received for testing purposes 17173 were used. Antibodies to ADV were found in 5214 (30.4%) of the tested samples and the prevalence dif- fered among voivodships as shown in Table 1.

Conclusions: Presented data indicate that SuHV1 is endemic in the WB population in Poland. In western part of Poland the percentage of seropositive WB was higher than calculated for the whole country and ranged from 32.3% to 42.7%. What is interesting is that similar studies conducted in Poland in the years 1997-2000 revealed in average 14.52% seropositive WB (2) and now the average ADV seroprevalence amounted 30.4%. It means an important increase of SuHV1 infected WB in Poland. Similar observations were made also in many European

countries and USA as well (1, 3, 4, 5). Taking into account that the overall seroprevalence of ADV in WB in neighboring countries is on similar level as was detected in Poland it can be concluded that together with increasing WB population observed across Europe (1), the number of infected with SuHV1 WB arose also. What is also important from practical point of view is that in countries free of AD or in those that are close to finish the AD eradication program, WB should be taken into consideration as a potential source and reservoir of SuHV1 for DP. Therefore, a continuous monitoring of this disease in WB population would be advisable (6).

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	Number of samples				
Voivodship	**received**	**tested**	**positive (%)**	**doubtful (%)**	**negative (%)**
DS dolnośląskie	1721	1704	671 (39.4%)	58 (3.4%)	975 (57.2%)
KP kujawsko-pomorskie	1261	1255	372 (29.6%)	31 (2.5%)	852 (67.9%)
LB lubelskie	1248	1247	342 (27.4%)	31 (2.5%)	874 (70.1%)
LU lubuskie	1486	1484	447 (30.1%)	39 (2.6%)	998 (67.3%)
LO łódzkie	582	578	168 ((29.1%)	8 (1.4%)	402 (69.5%)
MP małopolskie	508	506	104 (20.6%)	8 (1.6%)	394 (77.8%)
MA mazowieckie	591	591	169 (28.6%)	17 (2.9%)	405 (68.5%)
OP opolskie	788	780	330 (42.3%)	23 (3.0%)	427 (54.7%)
PK podkarpackie	835	832	189 (22.7%)	22 (2.6%)	621 (74.7%)
PD podlaskie	796	796	225 (28.3%)	24 (3.0%)	547 (68.7%)
PO pomorskie	2151	2132	500 (23.5%)	39 (1.8%)	1593 (74.7%)
SL śląskie	833	831	181 (21.8%)	25 (3.0%)	625 (75.2%)
SW świętokrzyskie	211	207	17 (8.2%)	7 (3.4%)	183 (88.4%)
WM warmińsko-mazurskie	1499	1473	372 (25.3%)	37 (2.5%)	1064 (72.2%)
WI wielkopolskie	492	489	158 (32.3%)	5 (1.0%)	326 (66.7%)
ZP zachodnio-pomorskie	2306	2268	969 (42.7%)	141 (6.2%)	1158 (51.1%)
Total	**17 308**	**17 173**	**5 214 (30.4%)**	**515 (3.0%)**	**11 444 (66.6%)**

Table 1. Results of ADV antibody detection in wild boar in Poland in 2012-2014

Spike and ORF3c genetic analysis of feline coronavirus from cats suffering or not from feline infectious peritonitis

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Introduction: Coronaviruses are known to evolve through accumulation of point mutations, insertions, deletions or recombination events within their genome that led sometimes to change in virulence, tissue and/or species tropism. Feline coronaviruses (FCoV) are common pathogen among cat population. For many years, FCoVs have been classified into different biotypes on the basis of their pathobiology. Avirulent strains, which usually induce mild or subclinical symptoms, are referred to as feline enteric coronavirus (FeCV). Virulent strains cause a fatal disease and are called feline infectious peritonitis viruses (FIPV).The molecular determinants that may account for the dramatic difference in pathogenesis between FeCV and FIPV have been extensively investigated. Today, FIPV is considered to be a genetic variant of enteric FECV and recent genetic investigations pointed the envelope S and the non-structural ORF3c proteins as hypothetical determinants of virulence.

Objectives: The main objective of this project was to investigate the genetic sequences of the spike and ORF3c gens from cats suffering or not from feline infectious peritonitis and to determine whether the mutations supposed to be associated to FIPV strains only could be effectively identified only in sick cats. Moreover for FIP cats, different samples were recovered per cat, at least rectal swabs and samples from fluids or affected organs to determine whether FIP strains could be excreted in feces.

Methods: Clinical specimens: Clinical samples from 30 cats having clinical signs of wet or dry form of FIP were recruited in France and Romania. As far as possible, cats were autopsied and different organs were recovered as well as rectal swabs. In parallel, 30 rectal swabs from healthy cats were also analyzed.

RNA isolation: Viral RNA was extracted from clinical samples using the QiAamp virus RNA mini kit (Qiagen).

Amplification by RT-PCR: Coronavirus detection was performed by using primers previously described. For the amplification of the ORF3c, and S genes, PCR primers were chosen for conserved sites on the basis of mismatch to other FCoV strains and according previous publications.

Sequence analysis: Nucleotide sequencing was performed by automated sequencing at Eurofins Company. Deduced viral protein sequences were analyzed and phylogenetic trees were constructed by the neighbour-joining method in MEGA 5 program.

Results: In total, 93 sequences of the spike gene and 71 of ORF3c were analyzed. Phylogenetic trees will be presented as well as the genetic diversity of sequences in samples recovered at different locations for each cat.

Conclusion: In conclusion, our results suggest that the sequence of S and ORF3c are relevant for the discrimination of FIPV and FeCV strains.

Surveillance of African Swine Fever virus in domestic pigs in Republic of Korea: 2010-2015

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Objective: Classified as a notifiable disease by the World Organization for Animal Health (OIE), African Swine Fever (ASF) is a highly contagious disease of domestic and wild pigs. Given its extremely high potential for transboundary spread, the disease has spread from Africa to Asia (Georgia, Armenia) and Europe (Russia, Ukraine), and is a threat to as yet unaffected countries including the Republic of Korea. Therefore surveillance is required for such countries to quickly identify new incursions and to demonstrate free status. The OIE Terrestrial Animal Health Code requires surveillance of domestic and wild pigs to be in place that finds no evidence of infection to be classified as an ASF free country. Also, there should be no evidence of tick involvement in the epidemiology.

Methods: Virological surveillance: Real time PCR in accordance with OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Serological surveillance: ID Screen® African Swine Fever Indirect ELISA Kit from ID.vet and INGEZIM PPA Compac African Swine Fever Blocking ELISA Kit from INGENASA

Results: In the Republic of Korea, a serological surveillance has been conducted in domestic pigs from 2010 to 2014. A total of 4,548 domestic pigs were tested and confirmed as serologically negative by commercial ELISA kits (2010: 864 pigs, 2011: 866 pigs, 2012: 77 farms/808 pigs, 2013: 132 farms/867 pigs and 2014: 96 farms/1,143 pigs). In addition, as a result of athropod vector monitoring (total of 22,894 ticks – 2013: 19,595 ticks and 2014: 3,299 ticks), no known arthropod vectors (ticks of genus *Ornithodoros*) of ASFV were found.

Conclusion: Current evidence suggests that the Republic of Korea is free from ASF and in 2015, the surveillance will continue to be conducted and strengthened through the following. Firstly, educational brochures on clinical signs will be distributed to farmers, veterinarians and hunters to strengthen passive surveillance. Secondly, virological surveillance will be conducted on dead domestic and wild pigs when samples are submitted for differential diagnosis on suspect cases of classical swine fever. In addition, smuggled pork and related products confiscated at borders will be tested for ASFV. Thirdly, serological surveillance will be expanded from domestic pigs to include wild pigs (*Sus scrofa coreanus HEUDE*). Finally, athropod vector monitoring will continue to be conducted to identify possible vectors for ASFV.

Development and validation of complete workflow solution for SIV testing

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Objective: Swine influenza virus (SIV) is a highly contagious viral infection of pigs, resulting in significant economic losses in the swine industry and posing a significant threat to human health through zoonotic transmission. SIV subtypes are defined by the surface glycoproteins: hemagglutinin and neuroaminidase, with H1N1, H3N2, and H1N2 representing the predominant subtypes in swine.

Methods: We have validated an SIV testing workflow consisting of high throughput nucleic acid purification, SIV detection, and SIV subtyping from porcine nasal swab samples. SIV can be detected using the USDA- licensed VetMAX™-Gold SIV Detection Kit, a single-tube one-step real-time RT-PCR kit for the rapid and accurate screening of influenza A. The assay targets three independent regions of the SIV genome to dramatically limit the number of false-negatives due to mutation of the viral genome. The VetMAX™-Gold SIV Detection assay also incorporates multiple degenerate primers and probes designed to detect all known strains of SIV. It is multiplexed with an internal positive control (IPC) to monitor for nucleic acid recovery and PCR inhibition. Laboratories wishing to obtain more information about SIV-positive samples can utilize the VetMAX™-Gold SIV Subtyping Kit to further characterize their samples and confirm positive results. The VetMAX™-Gold SIV Subtyping Kit is a pair of single-well real-time RT-PCR assays to detect and differentiate the H1, H3, N1 and N2 alleles.

We validated the screening and workflow by testing >100 SIV-positive and >100 SIV-negative porcine nasal swab field samples and virus isolates originating from diverse geographic regions in the US with the screening and subtyping kits. The SIV status and subtype of each sample was confirmed prior to the start of the study with Virus Isolation (VI) and/or whole genome sequencing. Collaborator laboratories purified the viral nucleic acid using the MagMAX™-96 Viral RNA Isolation Kit (AM1836) and MagMAX™ Express-96 Magnetic Particle Processor. Extracted nucleic acid (8ml) was tested with the VetMAX™-Gold SIV Detection Kit and VetMAX™-Gold SIV Subtyping Kit on the AB 7500-Fast Real-Time PCR System.

Results and Conclusion: Results of validation testing were used to determine diagnostic sensitivity and specificity for each kit. Detection with the VetMAX™-Gold SIV Detection Kit resulted in calculated diagnostic sensitivity and specificity values of 98.4% and 99.1%, respectively. The VetMAX™-Gold SIV Subtyping Kit produced >97% sensitivity and specificity for identifying the SIV subtype from nasal swab samples. This study indicates that RNA isolated from diagnostic porcine nasal swab samples, tested with the VetMAX™- Gold SIV Subtyping Kit in conjunction with the VetMAX™-Gold SIV Detection Kit, provides an economical and rapid solution for SIV screening and subtype identification.

Topic: Domestic & wild life virus interaction

Bat Coronaviruses circulating in Danish bats

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Objective: Coronaviruses (CoVs) are a diverse group of large positive-stranded RNA viruses. Several CoVs are known to cause severe diseases in animals (e.g. PED and TGE) and humans (e.g. SARS and MERS). Bat populations worldwide are considered to be natural reservoirs for many diverse CoVs. However, incomplete information exists about the bat species hosting specific CoVs and also the nature of bat CoVs circulating in Europe. In this study, we investigated Danish healthy bat populations for the presence of CoVs.

Methods: Fecal samples were collected during the autumn of 2013 and 2014 from 10 out of 17 Danish species of bats caught alive and released after sampling. The samples were screened for the presence of CoV RNA using pan-CoV RT-qPCRs targeting conserved regions of the ORF1ab that encodes the viral RNA polymerase. Amplicons that were generated were sequenced to confirm the presence of CoV and to determine the type and diversity of CoVs among Danish bat species.

Results: CoV RNAs were detected in 4 of the 10 Danish bat species: M. daubentonii, M. nattereri, P. pygmaeus and E. serotinus. The nucleotide sequencing revealed distinct CoV sequences within each of the four bat species indicating that specific CoVs are confined to single host species.

Conclusion: Our results show, for the first time, that several different bat CoVs are present in the Danish bat population.

Prevalence of Avian Corona Viruses in samples collected for passive avian influenza surveillance on wildbirds in Sweden

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Objective: The knowledge of the prevalence of AvCoVs in wild bird's species is limited. With exception of isolates from chickens, turkey, guinea fowl and peasant, only a small number of experimental studies have been carried out to study host range of avian CoVs in Europe. Here we will discuss the prevalence of AvCoVs in 407 samples collected from 87 different bird species in 2013.

Methods: The survey in wild birds in Sweden is consists of both active surveillance on living birds and passive surveillance on birds found dead or diseased. The active surveillance was primarily targeting high risk species for avian influenza in accordance with Commission decision 2007/268/ EC, Annex II. However within the passive surveillance birds from different species where sampled which was carried out by national veterinary institute. From dead birds that were autopsied, swab samples (mostly both cloacal and tracheal) were used for PCR analyses. The samples were analyzed for the detection of avian corona virus genome by using pancoronavirus qPCR targeting a 179-nt stretch of the RNA-dependent RNA polymerase (RdRp) gene. Positive samples were further analyzed with a second PCR targeting a 440 bp stretch of the CoV RdRp and the PCR product were analyzed by sequencing.

Results: Within the passive surveillance 407 birds of 87 different species were sampled and five birds, one Common Guillemot (*Uria aalge), three mallards (A. platyrhynchos)* and one Great Black-backed Gull (*Larus marinus*) were PCR positive for avian corona viruses, but no virus could be isolated. All the coronaviruses identified in this study could be phylogenetically classified as gammacoronaviruses and deltacoronaviruses.

Conclusion: Our data suggest the circulation of coronaviruses in diverse populations of wild aquatic birds. Additional coronavirus surveillance in birds of different orders and a full genome analysis of avian coronaviruses may help us to better understand the evolution of coronaviruses.

Topic: Emerging virus & diseases

Absence of serological evidence of Pteropine Orthoreoviruses (genus Orthoreovirus, family Reoviridae) from domestic and wild animals in Italy

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Objective: According to the International Committee on Taxonomy of viruses the family *Reoviridae* is composed by two subfamilies, *Sedereovirinae* and *Spinareovirinae*. This latter includes nine genera including genus *Orthoreovirus* which is composed by five different species including *Mammalian Orthoreovirus*, *Avian Orthoreovirus*, *Baboon Orthoreovirus*, *Reptilian Orthoreovirus*, and *Nelson Bay virus* also known as *Pteropine Orthoreovirus*. Mammalian orthoreoviruses are non-fusogenic whereas the remaining members of the genus are fusogenic. The first detection of a *Pteropine Orthoreovirus*(PRV) viral species from fruit bats (*Pteropus*spp), also called flying foxes, dates back in 1968 in Australia. The prototype virus was called Nelson Bay virus. Interestingly, the zoonotic potential of PRVs was evidenced in 2006 when a man suffered from fever and respiratory symptoms after exposure to a flying fox. The human isolate was called Melaka virus, a strain strictly related to Nelson bay virus. Afterward, other viral isolates genetically and antigenically related to the prototype Nelson Bay virus such as Kampar, Miyazaki-Bali/2007, HK23629/07 and Sikamat have been isolated from patients with respiratory tract infections. All these viruses have been shown to have limited human-to-human transmission. Together with Pulau and Xi river viruses, isolated from flying foxes in 1999 in Malaysia and in 2010 in China, respectively, they form the *Pteropine Orthoreovirus* species. Furthermore, we recently discovered a novel member (Indonesia/2010) of the *Pteropine Orthoreovirus* species from fruit bats legally imported to Italy from Indonesia whereas a further isolate named Cangyuan virus has been described from the intestinal content of one fruit bat residing in China's Yunnan province. It is not known whether these bat orthoreoviruses are capable of infecting and causing disease in animals. In this study we decided to investigate whether PRVs had the chance to circulate within domestic animals in Italy. Therefore we recruited a total number of 769 serum samples from different animal domestic and wild animals including cattle (N=470), buffalo (N=9), horse (N=47), dog (N=28), goat (N=2), deer (N=12), cat (N=8), Marsican brown bear (N=1) and sheep (N=64) collected in 2014 and 2015. Furthermore 128 serum samples of camels from Morocco were also tested. Serum-neutralization (SN) was employed as read-out assay.

Materials and Methods: Inactivated passage 2 onto African green monkey (Vero) cells of Indonesia/2010 was used to produce hyper-immune serum in rabbits and employed as positive control serum throughout the entire retroactive serological surveillance. Field serum samples were inactivated at 56°C for 30 minutes. Starting from a dilution titer of 1:2, serial 2-fold dilutions were made in microtiter plates, and 100 tissue culture infective doses ($TCID_{50}$) of antigen were added to each dilution. Thereafter, the mixtures were incubated at 37°C for 1 h, and 10^5 Vero cells were added to each well. The plates were incubated at 37°C for 5 days. Starting from the third day after incubation, the plates were checked for cytopathic effect, and the antibody titer was defined as the reciprocal of the highest dilution of the serum that showed 100% neutralization. Positive and negative control sera were included in each plate. Furthermore, in order to investigate the presence of serological cross-reactions with Mammalian Orthoreoviruses (MRVs) which are known to circulate in Italy within animals and humans, Indonesia/2010 was tested against hyper-immune sera produced for MRV1 Lang, MRV2 Jones and MRV3 Abney isolates. Accordingly, MRV1 Lang, MRV2 Jones and MRV3 Abney viruses were tested by SN against the hyper-immune serum produced in rabbits for Indonesia/2010.

Results: All field serum samples tested turned serologically negative for PRVs. No cross-reactions have been detected between isolates and sera of PRV and MRVs employed in the study.

Conclusion: To the best of our knowledge Pteropine Orthoreoviruses have never been described to circulate in Europe and bats involved in their transmission cycle do not naturally occur in Europe. Indonesia/2010 is the ninth member of this viral species which has been described since the first description of these viruses in 1968, the fourth isolated from flying foxes, the first described in Europe although from healthy and legally flying foxes imported from Indonesia. In this study we decided to investigate whether PRVs had the chance to circulate within domestic and wild animals in Italy. We tested by a specific SN assay for PRV a total number of 769 serum samples from different domestic and wild animal species mostly coming from the Abruzzi region (central Italy). Moreover, 128 serum samples were from camels collected in Morocco during previous serological surveillance activities for other viral pathogens including West Nile and Bluetongue viruses. As expected, all samples turned out to be serologically negative for PRVs and no serological cross reactions were showed between PRV and MRVs used in this study. On the other hand, strong serological cross-reactivity has been previously described to exist within PRV isolates. This report describes a pilot study and certainly has some drawbacks. First, serum samples from domestic and wild animals were randomly selected and samples from bats and humans were lacking. Second, samples mainly originated from the Abruzzi region and from camels from Morocco. Therefore, the geographical origin of samples is not representative of the entire Italian territory. Indonesia/2010 reached Italy in 2010 with flying foxes legally imported from Indonesia. At the time of their arrival, the animals were straightly transported under sanitary conditions from the quarantine station of the Fiumicino airport (Rome) to a quarantine center in the municipality of Gatteo (Emilia Romagna Region). They remained in quarantine for 30 days before being shipped to an animal facility in Northern Europe. However, the chance of a spill-over event and infection of other animals or humans during their stay cannot be excluded. Moreover, PRVs may potentially already be present in Italy and Europe as for the chance of acquired infections during tourism in areas where flying foxes densely live. This event has been described indeed in a Japanese man who travelled to Bali in 2007. Epidemiological investigation by screening of 109 sera collected from human volunteers on the Pulau Island in Malaysia (where Pulau virus has been isolated from flying foxes) revealed that 14 of 109 (13%) were positive for Pulau virus, indicating that this group of viruses may be able to switch host and infect humans more frequently than other bat-borne viruses, such as the Nipah virus. Therefore, whereas human infection by PRV has been shown to occur after exposure to a flying fox, we do not have data supporting the infection in other animals. Overall, we investigated whether PRVs circulated within domestic animals in Italy and, based on the samples tested so far, there is no evidence of previous PRVs circulation. Certainly, more serum samples from different animal species and from different areas need to be tested and more importantly, human serum samples need to be included in the analysis. Pteropine Orthoreoviruses are able to cause respiratory tract infections in humans, therefore physicians should consider these pathogens in the diagnostic workflow particularly with patients coming from areas where flying foxes live or with patients who had contacts with them.

Application of silver nanoparticles to control Rift Valley fever virus infection in vitro and in vivo

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Objective: In this work we have tested the potential antiviral activity against Rift Valley fever virus (RVFV) of silver nanoparticles formulated as Argovit. Different formulations of silver nanoparticles have already been reported to display antiviral activity against several viruses belonging to different families. Compared to the classical antiviral strategies, the use of metal nanoparticles is a novel approach that poses many advantages, such as the non-emergence of resistant variants, as well as its safety and low cost. RVFV is a mosquito-borne pathogen causing an important disease in ruminants often transmitted to humans after epizootic outbreaks. Since there is no available treatment or licensed Rift Valley fever vaccine for human use, the development of new approaches able to inhibit viral replication and transmission for an efficient control of the disease is a must.

Methods: Argovit is a commercial formulation of silver nanoparticles provided by Vector-Vita Ltd (Russia) consisting of spheroid silver nanoparticles 35 nm-average-sized functionalized with polyvinylpyrrolidone (PVP) with

specific properties that increase silver nanoparticles stability and biocompatibility. The antiviral potential of Argovit against RVFV has been tested on Vero cell cultures and in an IFNAR (-/-) mouse infection model. In both systems, two different approaches have been assayed: (i) different dilutions of Argovit were added to previously infected cells or were administrated at different doses, routes and schedules to animals infected with a lethal dose of virus; (ii) virus was pre-incubated with different dilutions of Argovit before inoculation in mice or cultured Vero cells.
The effect of Argovit on viral infectivity was estimated by comparing virus production in cell cultures at the different Argovit conditions assayed; in the in vivo infection model clinical disease and death were compared between groups of mice receiving different treatments.

Results: In cells infected with RVFV, the presence of Argovit silver nanoparticles into the medium was able to control viral production in a limited manner, with a 50% reduction of the total virus yield. In contrast, preincubation of RVFV with Argovit concentrations near the citotoxicity threshold (0.2 mg/ml) abolished almost completely viral propagation, leading to a reduction of infectivity of 98%.

On the other hand, daily administration of Argovit by oral gavage to lethally infected mice, beginning one day after infection, slightly reduced the viral load in infected animals but this reduction was not enough to prevent their final death. In contrast, mice inoculated with a lethal dose of virus previously incubated with 20 mg/ml of Argovit silver nanoparticles showed a delayed-onset clinical disease and mortality, with a survival rate of 60%.

Conclusion: Although the ability of silver nanoparticles to control an ongoing RVFV infection in the conditions tested seems to be limited, the incubation of virus with Argovit before the infection leads to a clear reduction of its infectivity bath in vitro and in vivo. These results reveal the potential application of the microbicidal properties of silver nanoparticles to control the infectivity of this important zoonotic pathogen.

Application of viral metagenomics to investigate viruses circulating in wildlife-livestock interface in Mozambique

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Objective: Emerging infectious diseases (EIDs) are a major threat to human and veterinary public health. Majority of the viral EIDs originate from vector-borne viruses causing severe problems to the animal and human populations. Based on socio-economical, environmental and ecological factors different regions have been identified as so called hot spots where new EIDs are most likely to originate. One such hot spot is Mozambique, which is rich in arthropod vectors and wild life species: one of the predictors for originating new EIDs. In the study regions in Mozambique there is also a close proximity between wildlife, domestic animals and humans making the viral transmission from one species to another more likely to occur. Thus, it is important to identify and characterize the viruses circulating in these areas to prepare for future introductions of new pathogens and combat these infections. In this regard, the current aim of the study is to identify and see the prevalence of vector-borne viruses circulating in wildlife-livestock interface in Mozambique to be better prepared for upcoming emerging infectious diseases.

Methods: Viral metagenomics, broad-range PCRs and serological methods are used to investigate unknown and known viruses in Mozambique. Arthropod vectors (mosquitos and ticks) and serum samples from ruminants were collected from different farms in Mozambique during October/November 2014. Extraction of RNA from mosquitos and ticks was done after homogenization and DNAse treatment and these was then prepared for metagenomic analysis through high-throughput sequencing. The sequence data was analysed with bioinformatic tools for quality check, assembly and homology search to identify viral nucleic acids in the sample. Follow-up studies will be done with real-time PCR or conventional PCR to confirm the presence of viral nucleic acids in the sample and further characterize the identified viruses. In parallel, competitive ELISA has been performed to detect antibodies against Schmallenberg virus nucleoprotein in serum samples for cattle, sheep and goat. Nucleic acids has been extracted from the ruminants and arthropod vectors to be used in more specific broad-range PCRs.

Results: Metagenomics is a powerful method that can characterize the virome of the investigated sample/s and identify novel viruses that may be overlooked by classical virology methods. The metagenomic analysis of the different vectors (mosquitoes and ticks) is currently on-going but the broad-range PCRs has identified a flavivirus in one of the mosquito samples and further genetic characterization is on the way. The SBV ELISA results show that all the species and farms have animals that are seropositive. All cattles from the different farms were seropositive and the observed mean prevalence was 90 %. The seroprevalence observed for sheep and goats was 60-80%. To determine if SBV is truly circulating in Mozambique, or if a related virus from the simbu-serogroup is cross-reacting giving the antibody positive response, further genetic studies will be performed.

Conclusion: The development of viral metagenomics, i.e. the characterization of the complete viral composition of an organism or an environment using high-throughput sequencing technologies, is a promising tool for the surveillance of pathogens that may cause EIDs and can be accomplished by analysing the viromes of selected animals and arthropods.

Detection and characterization of emerging HoBi-like viruses in commercial foetal bovine serum batches

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Objective: BVDV is major pathogen of cattle, and infection results in significant economic loss worldwide. Pestivirus genomes consist of a single-stranded, positive sense RNA approximately 12.3 kb in length. A single open reading frame that encodes 11-12 structural and nonstructural proteins is flanked at either end by 5' and 3' untranslated region (5'UTR and 3'UTR). Of these, the non-structural amino terminal autoprotease N^{pro} and the major envelope glycoprotein E2 have most commonly been used for genetic classification of new virus isolates together with the 5'UTR region which represents the most important and useful region both for diagnostic purpose and sequence analysis Besides the established BVDV-1 and BVDV-2 species, an additional group of unclassified pestiviruses consisting of "atypical" pestiviruses of bovine origin has been identified as a putative new species of pestivirus. Tentatively called "HoBi-like," or "atypical pestiviruses", the type virus of this putative species is atypical pestivirus strain D32/00_'HoBi', which was isolated from batch of foetal bovine serum (FBS) imported from Brazil. A number of atypical pestiviruses have been described as contaminants in FBS or cell culture, as well as in live ruminants, such as buffalo and cattle. Natural outbreaks of severe respiratory disease in calves have been reported in Italy. Recently, HoBi-like viruses have been identified from naturally infected cattle also in India and Bangladesh. Because the clinical presentation in cattle is indistinguishable from that seen following BVDV1 and BVDV2 infection, BVDV-3 has been proposed as a name for this species. However, to date the International Committee on Viral Taxonomy has not yet arrived at a decision to recognize HoBi-like viruses as an official species within the Pestivirus genus. The earliest samples from which HoBi-like viruses have been isolated were collected in 2002. However it is likely that emergence was earlier. Because the HoBi-like viruses isolated from FBS, originating from South America, and the HoBi-like viruses isolated from cattle in Italy as so similar it has been proposed that HoBi-like viruses were introduced into Europe via the use of contaminated FBS. Although the genetic diversity of HoBi-like viruses is not fully known, it is probable that, like other member of the pestivirus genus, they will be genetically diverse. The objective of this study was to investigate by RT-PCR and DNA sequencing, the genetic diversity of HoBi-like viruses detected in commercial FBS batches from major producers.

Methods: The current study included samples from 26 batches of FBS obtained from commercial undisclosed suppliers. Samples were from filtered, gamma-irradiated and non-gamma-irradiated batches of FBS. Viral RNA was extracted from 1 ml of serum using the QIAamp UltraSens Virus Kit (Qiagen).RT-PCR targeting a 221 bp segment of the 5'UTR was performed using the primer pair B5/B6. In addition to the above described method, all the samples were tested by the panpestivirus specific RT-PCR using primers 324/326 flanking a 288-bp fragment of the 5'UTR.The generated products were purified and sequencing performed on an ABI PRISM 3130 sequencing device (Applied Biosystems, Foster City, CA). The sequencing reads were assembled using SeqMan software within Lasergene package (DNASTAR Inc., Madison, WI). For phylogenetic analysis, 59 additional sequences of atypical pestiviruses were retrieved from GenBank. Multiple sequence alignment was done using program

Bioedit v.7.0.5.2. Phylogenetic analysis was performed twice with the software MEGA6 and the phylogenetic tree is presented in this paper.

Results: All 26 batches of FBS were found containing at least one species of bovine pestiviruses. Fifteen batches were positive for HoBi-like viruses. Of these 15 batches, 7 were labeled as originating in South America, one from Australia. The country/region of origin was not identified for the remaining 7 batches. The neighbour-joining analysis revealed that the 5'-UTR sequences formed a well supported group together with viruses from South America, Australia, North America, Europe and Thailand. The Thai isolate Th/04_KhonKaen, is the most divergent member of this group displaying 90.7-92.5% intra-group similarity. All the newly determined sequences have been deposited in GenBank.

Phylogenetic analysis of the 5'-UTR sequences of 15 newly identified HoBi-like viruses combined with analysis of additional 59 sequences from GenBank, identified 4 genetic groups tentatively named 3a-3d. Group 3a includes the type virus D32/00_'HoBi' strain together with viruses originating from South America, Australia, North America, Europe, China and Thailand. Groups 3c and 3d contained viruses co-circulating in India; viruses originating from Bangladesh were mainly grouped in a separate genetic group 3b, with the exception of BGD/ZS5 strain which clustered within genetic group 3c.

Conclusion: In the current study 26 batches of FBS were screened for the presence of pestiviruses. The 5'UTR genetic region of the pestiviral genome was PCR amplified and sequenced for genetic analysis, 59 additional sequences were retrieved from GenBank. The results indicated that there were 4 distinct genetic subgroups3a-3d.As some batches of the tested FBS were manufactured within European Union, there is the possibility of contaminating FBS from different source during manufacturing steps, as a consequence we cannot speculate on the genuine country of origin. Nevertheless, the grouping obtained by our genetic analysis revealed a particular clustering pattern consisting in three distinct genetic groups originating from the Indian subcontinent(3b, 3c and 3d) and a fourth group 3awhich includes all the Hobi-like viruses isolated from samples collected outside the Indian subcontinent. Interestingly, strain 228/21123/92was detected during analysis of FBS of unknown origin dating to 1992, indicating the presence of HoBi-like virus many years earlier than previously reported. This study demonstrated that FBS products labeled with different geographic origins are contaminated not only with BVDV-1 and BVDV-2, but also with the emerging HoBi-like viruses. This finding has implications for the safety of biological products, such cell lines and vaccines. The regulation and testing of FBS should be thoroughly investigated from several aspects including veterinary and human medicine. In addition, the emergence of the new pestivirus strains could represent newly recognized risk factors for BVDV control programs.

Highly genetically distant and recombinant Swine Enteric Coronaviruses from Italy

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Objective: Porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) causes watery diarrhea, dehydration and a high mortality among suckling pigs. Belonging to the family Coronaviridae, genus Alphacoronavirus, PEDV and TGEV are single-stranded, positive-sense RNA, enveloped virus. Coronaviruses (CoVs) are prone to genetic evolution through accumulation of point mutations and homologous recombination. The emergence of new swine enteric coronavirus with potentially different pathogenic features is of great concern for pig health. Recently, PEDV spread to the United States, Canada, and Mexico causing severe economic losses to the global swine industry. In Europe, the last important outbreak occurred in 2005-2006 in Italy and, since then, only sporadic outbreaks were observed in Northern Italy. Recently, PEDV was also detected in a German pig herd. The objective of this study was to genetically characterize swine enteric coronaviruses circulating in Italy from 2007 to 2015.

Methods: Swine feces and intestine samples from pig farms were collected as suspected of PEDV. Samples were submitted for electron microscopy, PEDV specific ELISA, culture isolation and a pan-CoV RT-PCR for RNA-dependent RNA polymerase (RdRp) gene. If samples were positive, a larger fragment of the RdRp (349 nt), the spike

(553 nt), and M (439 nt) gene were also amplified. Whole genome sequence was obtained from two representative strains by using NEXTERA-XT kit and a MiSeq sequencer. The phylogenetic trees of S1, M and RdRp partial genes, complete S1 gene and full-length genomes were built by the neighbor-joining method in MEGA5. Determination of recombinant breakpoints was performed using the Recombination Detection Program (RDP) 3.0.

Results: On the base of RdRp, S1 and M partial sequences, Italian strains clustered in 3 different clades temporally subdivided suggesting that 3 independent entries occurred in Italy: clade I, included strains circulating until mid-2009, clade II with strains present from mid-2009 to 2012, and clade III from 2014 and
2015. The complete S1 sequence of strains of clade III shared 99.7 % nt identity with the strain PEDV L00721/GER/2014 from Germany and 99.3-99.5 % with OH851 from USA. The complete genome was obtained only from two strains, PEDV/Italy/7239/2009 and SeCoV/Italy/213306/2009, representative of clade I and clade II respectively. Recombination points within the complete genome of the SeCoV/Italy/213306/2009 were detected, showing a recombination event between a PEDV and a TGEV ancestor occurred to generate this new swine enteric coronavirus. Interestingly, all the swine enteric CoV Italian strains from this study (2007-2015) contain characteristic insertions and deletions in the S gene (S INDELS) common to some PEDV strains that have reported decreased virulence.

Conclusion: Between 2007-2105, swine enteric coronavirus strains circulating in Italy clustered in 3 different temporal clades with different genetic features. The detection of a PEDV/TGEV recombinant virus confirms the high ability of natural recombination among coronavirus and the likelihood of continued emergence of novel CoVs with distinct pathogenic properties. In 2014-2015, a new entry of PEDV occurred in pig farms from Northern Italy, suggesting this variant, detected first in USA, is now spreading in Italy.

Isolation and identification of a novel serotype of avian paramyxovirus

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Objective: Twelve serotypes of APMVs (APMV-1 to -12) have been reported worldwide. We isolated a novel APMV from coot that has never been reported yet in birds. This study is to provide information about virus serotype, biological and genetic characterization of the isolated virus.

Methods: For virus isolation, fecal droppings from wild birds were inoculated onto SPF chicken eggs. Resulting HA positive agent was tested by RT-PCR assays for AIV and NDV. Cross HI tests were performed for serotyping of AIV- and NDV-negative agent. The virus morphology was examined by electron microscopy. The virulence was determined by ICPI test. Virus genome was fully sequenced and the sequence was compared with that of known APMV serotypes.

Results: Unknown HA positive virus was isolated from fecal samples collected at Upo wetland in 2014. The virus was diagnosed neither AIV nor NDV by RT-PCR assays. Electron microscopy revealed that the virus particles showed characteristics of APMV but the virus was not classified into any known serotype of APMV by cross HI test. The virus was determined to be avirulent by ICPI test. The virus had the genome of 15,180 nucleotides (nt) long in agreement with the rule-of-six of Paramyxovirus. Phylogenetic analysis revealed that the virus was placed in sole member distinct from other APMVs. In addition, the calculation of evolutionary distance at the nucleotide level indicated that the virus was divergent from other APMVs.

Conclusion: Based on biological and genetic characterization, we propose that the virus, designated wild birds/Korea/H216/2014, could be the prototype for new APMV serotype 13, although it is still needed to clarify which bird species play a role of reservoirs for this virus.

Serological evidence of continuing spread of peste des petits ruminants in Tanzania

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Objective: Peste des petits ruminants virus (PPRV), a Morbillivirus closely related to the recently eradicated rinderpest virus, is the causative agent of the highly contagious and deadly disease, peste des petits ruminants (PPR). PPR affects primarily sheep and goats, but small wild ruminants and camels are also susceptible to infection. Infectious diseases, such as PPR, are of great socioeconomically importance as they affect the household economy of people living in rural Africa and Asia who may depend on their sheep and goats for food and income. Sheep- and goatherds are often held in close proximity to wild animals and thereby transmission of disease between the two is possible. As it has been shown that wild ruminants are susceptible for PPRV, they may play an important role in the PPR epidemiology, however, the definite contribution is yet to be identified. In this study, we performed a serological screening of sheep and goats as a first step to understand the epidemiology of PPR in Tanzanian wild small ruminants. Samples were taken from animals that intermingle and share pastureland with wild small ruminants on a daily basis. By screening for antibodies against PPRV, and focusing on the results from animals younger than one year of age, we can determine if the virus had been circulating in Tanzania since the last vaccination campaign in 2013. Further analysis of the samples from domestic ruminants is on-going and includes screening by RT-PCR for PPRV and full genome sequencing. Results from screening of domestic ruminants will in a next step be compared with results from wild small ruminants from the same areas.

Methods: Blood samples were taken from 478 sheep and goats from 39 different herds that intermingle with wild animals in varying degree. In addition, nasal swabs were retrieved from 315 of the 478 animals. Sampling sites were located in three different areas in Tanzania: Ngorongoro in the north, Mikumi in the east and Mahenge in the south. Sites were selected because of presence of interface between domestic and wild ruminants. Another reason, in the case of Mikumi and Mahenge, were that no studies of PPRV had been performed in these areas. Blood samples were analysed for presence of antibodies against PPRV with a competitive ELISA (ID-Vet Innovative Diagnostics) according to manufacturer's instructions. Samples with doubtful result according to the calculations provided by the manufacturer of the cELISA was not used in the further downstream analysis of the samples. Screening of the blood samples and nasal swabs for presence of PPRV by RT-PCR is in progress.

Results: The serological screening showed that 47.2 % (108 of 229) of the sheep and 49.1 % (115 of 234) of the goats were positive for antibodies against PPRV. Highest overall prevalence was seen in Mikumi with 65.9 % (114 of 173), followed by Ngorongoro with 40.0 % (60 of 150), and Mahenge with 35.0 % (49 of 140). In herds with no report of vaccination, 37.4 % (80 of 214) of the animals had antibodies against PPRV. Among individuals < 1 year of age, 17.8 % (24 of 135) were seropositive. When separating this group by area, to see where the spread of PPRV is the most active, 34.5 % (10 of 29) in Mikumi, 13.6 % (9 of 66) in Ngorongoro, and 12.5 % (5 of 40) in Mahenge were positive. Chi-square test showed statistical significance when comparing seropositive individuals < 1 year of age in Mikumi to Ngorongoro ($p=0.012$) and Mikumi to Mahenge ($p=0.012$). No statistical significance was observed between samples collected from Ngorongoro and Mahenge ($p=0.215$).

Conclusions: Our serological results indicate that PPRV is circulating in the sheep and goat population in the studied areas in Tanzania. Highest prevalence was seen in Mikumi, where 10 of 12 herds were vaccinated against PPRV according to owners. Because one dose of vaccine or survival of disease leads to lifelong immunity, and because no DIVA-vaccine is available for PPRV, it was not possible to discriminate between serological response due to vaccination or natural infection. Animals under one year of age, however, were not present during the last vaccination campaign in 2013 and therefore represent the current situation of PPRV. Overall, 17.8 % (24 of 135) of animals under the age of one year were seropositive. In Mikumi 34.5 % of animals <1 year of age had encountered the virus, which makes Mikumi the area with the highest proportion of seropositive animals. The high percentage of young animals (<1 year of age) indicates that PPRV was present and actively spreading in the ruminant population during the last year. We conclude that despite of efforts to vaccinate the sheep and goat populations in eastern Tanzania the spread of PPRV is on-going.

Spatiotemporal distribution of novel Russian isolates of African Swine Fever Virus with and without the additional tandem repeat insertion in the intergenic region between genes I73R/I329L

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Objective: African swine fever (ASF) is the most devastating swine disease characterized by very high mortality. It makes it a great disincentive to the development of the pig industry in Russia. In 2007 African swine fever (ASF) entered Georgia and in the same year the disease entered the Russian Federation. From 2007 to 2012 ASF spread throughout the southern region of the Russian Federation.

Extremely high variability of ASFV contributes to its fast emergence, adaptation and persistence in various swine populations. Variations in genome size are associated with gain or loss of genes in multigene families and the number of tandem repeats (TR) located in various genomic positions both within coding regions and in intergenic regions.

In the present study we report the results of sequencing analysis of Russian ASFV isolates identified in 2013-2015 in Tula, Voronezh, Belgorod, Moscow, Kaluga, Pskov, Vladimir, Smolensk and Tver regions.

Methods: After the presence of ASFV was confirmed at FGBI ARRIAH, Vladimir, Russia, sequencing of the intergenic region between the I73R and I329L genes at the right end of the genome was performed using virus DNA extracted directly from homogenized tissues. The newly generated sequences were compared to the nucleotide sequences obtained in previous studies.

Results: Our results demonstrate that isolates Lazarevskoe 01/14, Nikolaevsky 08/14, Grafskoe 06/14, Kurtnikovo 02/15, Gribovo 06/14, Antonovo 07/14, Odintsovo 02/14, Voronezh 12/14, Kaluga 02/15, Boguchary 06/13, Alekseevsky 06/14 had a TR insertion identical to that present in European ASFV isolates from Belarus and Ukraine. This TR insertion was absent in the remaining isolates Shihobalovo10/13, Kashino 04/13, Kashino 06/14, Vasilkovo 09/13, Karamzino 06/13 and Vyazma (see poster).

Conclusion: These findings allow analyzing spatiotemporal distribution of novel ASV isolates. This particular TR insertion was initially identified in Ukranian isolate ASFV_Ukr12/Zapo (2012) by Gallardo in 2014, earlier than in any of analyzed Russian isolates. According to our data, in Russia this insertion was identified for the first time in Boguchary 06/13 isolate. However, it was absent in the genome of isolates identified after June 2013 including Shihobalovo10/13, Vasilkovo 09/13, Vyazma 08/13. Next time, the presence of the insertion was confirmed in Lazarevskoe 01/14 isolate (Tula region, 01/2014).

Thus, nearly all recent ASF outbreaks in Russia were caused by the isolates containing this insertion, with the exception of Kashino 06/14. Our data provide the evidence that there are two variants of ASFV isolates currently co-circulating in Russia: with the TRS insertion (detected in 2013-2015) and without it (detected in 2007-2015). Such genetic data are essential to study the origin, spread and evolution of ASFV isolates.

The characterization of nucleoprotein and glycoprotein genes of bovine ephemeral fever virus isolated from two different outbreaks in Turkey

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Objective: Bovine ephemeral fever (BEF) is caused by the arthropod-borne bovine ephemeral fever virus (BEFV), which is classified in the family Rhabdoviridae and the genus Ephemerovirus. Sporadic cases of BEF were documented in Turkey. The last two outbreaks of BEFV were reported in 2008 and 2012 years in Turkey. BEFV causes low mortality (3-4%) in 2008 outbreak in South-East Anatolia and high mortality (15-20%) in 2012 outbreak in East and West Anatolia in Turkey. This study is aimed to find any relationship BEFV evolution and high mortality rate. For this reason, we analyzed the nucleotide and amino acid sequences of nucleoprotein (N) and glycoprotein (G) genes of the field viruses (three isolates for each year) isolated from 2008 and 2012 outbreaks.

Methods: The G and N genes of BEFV Turkey isolates were amplified by RT-PCR and cloned into pGEM®-T Easy Vector. Then, the recombinant vectors including the genes were sequenced and analyzed.

Results: Some amino acid differences (two amino acid differences in N gene and three amino acid differences in G gene) were found in between N and G genes of BEFV isolated in 2008 and 2012 outbreaks.

Conclusion: We speculate that these amino acid changes may cause high mortality although more extensive studies are required to elucidate the role of virulence and transmission potential of the virus of these amino acid changes. Moreover, if effective control measures do not take, BEFV may spread to Europe countries such as Greece and Bulgaria in the next years.

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Topic: Epidemiology, risk assessment & modeling

A prospective study investigating transplacental transmission of Babesia caballi in thoroughbred foals in Trinidad

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Objective: This study aims to investigate transplacental transmission of *Babesia caballi* from thoroughbred mares naturally infected via the tick vector and the risk factors associated with *B. caballi* infections.

Methods: This research involved a cross sectional study of 117 mares in the fifth month of pregnancy from ten different farms throughout Trinidad. A questionnaire of four different sections was filled out for each of the mares in this study. The first section on bio-data included questions such as name of the mare, breed, date of birth and breeding date of the mares. The section section included the medical history of the horse such as previous pregnancies, history of resoptions, abortions and stillbirths as well as previous diagnosis with equine piroplasmosis. The third section of the questionnaire had questions pertaining to the health of the previous foals during the first month after parturition. Section four included questions pertaining to the methods of tick control and prevention used on the animals, stalls and pastures on the farm.

Whole blood and serum samples were collected from 117 mares in the fifth month of pregnancy. The mares were monitored for any resorptions, abortions or stillbirths until parturition. Samples were also collected from each of their foals within the first 36 hours after parturition. All whole blood samples were analyzed microscopically for piroplasms. If an aborted foetus was recovered a post mortem examination was carried out and splenic samples were taken for molecular detection of *B. caballi*. Samples were analyzed for the presence of *B. caballi* by serum ELISA, conventional and real time PCR amplification of the 18S rRNA gene from DNA extracted from whole blood or spleen.

The χ^2 test for independence was used to determine if there were any association between PCR or ELISA results of the mares and foals for *B. caballi* and risk factors such as history of tick exposure, purpose of horse, local or imported mares, previous history of abortions or stillbirths, tick fever, previous foals with medical conditions during the first month of its life, contact with other species of animals, outcome of current pregnancy as well as tick control on the pastures. Data were analyzed using SPSS (version 20). The level of significance was set at $p < 0.05$. Fisher's Exact test was used if one or more of the cells had an expected count of less than five. Otherwise the Yates continuity correction was used. A mixed model linear regression analysis was conducted on the risk factors and foals being serologically positive for *B. caballi*. A random effect for foals was included in the analysis. The intraclass correlation coefficient (ICC) was calculated to determine the degree of relatedness among the different risk factors. The log likelihood ratio test of the constant model vs the full model was used to determine the random effect parameter, foal into model. Statistical significance was set at $p < 0.05$.

Results: Data was obtained for 111 mares as two of the mares died and four mares were relocated to farms that were inaccessible. All the mares in the study were used for breeding purposes and had access to pasture once

a day. The ages of the mares in this study ranged from four to seventeen years of age. Sixty-six (56%) of the mares were local horses. Sixteen (14%) of the mares were maiden mares. Twenty-one (18%) of the mares in this study had a previous history of reproductive losses with the majority of losses were due to resorptions, as the majority of losses occurred during the second and third months of pregnancy. Only six (5%) of the mares had previous foals that developed medical conditions during the first month of its life. Some of these medical complications included conformational problems, foal too weak to suckle, fever and some of the complications remained undiagnosed. Sixteen (14%) of the mares had been previously suspected to have equine piroplasmosis. However this diagnosis was based on results obtained from complete blood counts in 12.5% of the mares. There was no differentiation between *T. equi* or *B. caballi* as the causative agent. There was an abortion rate of 19.8% in this study as 22 of the mares lost their foetuses between the second and eleventh month of pregnancy. Eighty three (74.7%) mares and 40 (44.9%) of their foals were seropositive for *B. caballi*. Nineteen of these seropositive mares aborted between the third and tenth months of pregnancy. Four (3.4%) mares were positive for *B. caballi* by conventional PCR. One of the conventional PCR positive mares also had piroplasms on microscopic examination of the blood smear. This mare had a stillbirth at the 310th day of gestation. Partial sequencing of B. caballi DNA from this mare showed 99% homology to various B. caballi genomes on GenBank from South Africa (EU642512.1), Spain (AY309955.1) and Mongolia (JQ288736.1). Three of the four conventional PCR positive mares for *B. caballi* either had resorptions, abortions or stillbirths for that pregnancy. The one foal that was born to a conventional PCR positive mare was PCR negative for *B. caballi* by conventional and real time PCR. Real time PCR analysis revealed that two of the foals were positive for *B. caballi* while another three had Ct values that were borderline positive. The samples that were analyzed for these five foals were DNA extracted samples from whole blood. Splenic samples of an 8.5 months old aborted fœtus were also borderline positive for *B. caballi* by real time PCR analysis.

A pregnant mare being serologically positive for *B. caballi* and imported mares were the only two risk factors associated with the foal being seropositive for *B. caballi*. Pregnant mares that were serologically positive for *B. caballi* were 18 times more likely to have the foal also serologically positive for *B. caballi*, (χ^2_{1df} $p < 0.01$), OR = 18.24, (95% CI = 3.96 – 84.09). This is due to the ingestion of colostrum containing *B. caballi* antibodies as antibodies cannot cross the epitheliochorial placenta to the fœtus during pregnancy. Imported mares were 2.7 times more likely to have a positive ELISA result for *B. caballi*, (χ^2_{1df} $p = 0.04$) OR = 2.77, (95% CI = 1.16 – 6.61) as 22 of the 37 imported mares in our study had a positive ELISA result for *B. caballi*. A logistic regression model was developed for foals being serologically positive for *B. caballi* using these two variables. The overall model was significant however when farm was introduced as a random effect, imported mares did not have a significant effect on the ELISA results of the foal for *B. caballi*. The intraclass correlation coefficient of farm applied as a random effect was 0.14. This indicated that the foals within farms were different in comparison to each other. Therefore if the mare was seropositive for *B. caballi* by ELISA testing, the foal had an odds of 34.3 times more likely to also be ELISA positive for *B. caballi.* There was no significant effect observed for foals being PCR positive for *B. caballi* in this study.

Conclusion: Transplacental transmission of *B. caballi* can occur and this parasite can be implicated in fœtal losses. The only risk factor that had a significant effect on the foal being seropositve for *B. caballi* was the mare also being seropositive for *B. caballi* by ELISA testing during the fifth month of pregnancy.

Antibodies to West Nile and Avian Influenza Viruses in domestic bird species in the Samsun Province of Turkey

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Objective: The objective of this study was to investigate the AIV and WNV infections as serologically in domestic birds (chicken, duck, goose and turkey) reared in rural areas of the northern Turkey.

Methods: In this study, blood samples randomly collected from domestic birds (chicken, duck, goose and turkey). The material consisted of 736 domestic birds, including 394 geese, 129 ducks, 117 turkeys and 96 chickens, from Samsun province in northern Turkey. The serum samples were analyzed for the presence of antibodies to WNV and AIV using a competitive enzyme-linked immunosorbent assay (C-ELISA).

Results: Seropositivity rates in chickens, ducks, geese and turkeys were detected as 3.13%, 0.78%, 1.78% and 17.95% for WNV and 4.17%, 9.30%, 0.51% and 1.71% for AIV, respectively. Out of 736 serum samples examined,

32 (4.3%) were positive for WNV, 20 (2.7%) were positive for AIV.

Conclusion: The results, recorded for the first time in Turkey, supported the hypothesis that domestic bird act as a potential reservoir of selected viruses, and thus have a role in the epidemiology of these diseases

Bluetongue serotype 1 epidemics in the Italian Central Regions of Latium and Tuscany in 2014

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Objective: Bluetongue (BT) is a viral disease of ruminants transmitted by *Culicoides spp.*. In Italy, first BT outbreaks occurred in 2000, introduced by wind-dispersed infected vectors from Northern Africa, and are still occurring, even if the serotypes involved and the magnitude of the annual episodes varied throughout the years and different areas.

In Latium and Tuscany, regions on the tyrrhenian coast where at least two BT vectors (*Culicoides imicola* and *Culicoides obsoletus sl*) are widely present, serotype 2 (BTV2) was initially detected in 2001, in the coastal areas facing Sardinia, where numerous outbreaks were being registered. In these two Regions, during the first years of BT virus (BTV) introduction, virus circulation was principally diagnosed by seroconversion of sentinel animals during the summer-autumn period. Furthermore, within the sporadic clinical outbreaks due to BTV2, only a small number of animals were clinically affected, mostly with mild signs of the disease. The incidence of BTV2 outbreaks dropped in the following years with its disappearance in 2009. Further to this, between 2004 and 2012, serotype 16 (BTV16) circulation was also registered in Latium and Tuscany with its typical characteristics of low contagiousness and virulence.

In autumn 2013, BT re-emerged after a long period of sporadic circulation, leading to a massive epidemics caused by serotype 1 (BTV1) that continued even during 2014. Between September 20th and December 18th 2013, 74 outbreaks were notified, starting from the coastal areas and spreading inland.

In Italy, a National BT surveillance and control program is ongoing, based on three main activities: sentinel animals are tested monthly by an officially approved ELISA, clinical outbreaks are reported by the Veterinary Authorities and vectors are captured weekly, using black light traps.

The aim of this paper is to describe in particular the 2014 BT epidemics in Latium and Tuscany by estimating the incidence of outbreaks (infected farms) along with the morbidity, mortality and case fatality rates, using the data obtained from the reports of the surveillance activities. The general objective of the study is to measure the impact of 2014 BTV1 epidemics in Latium and Tuscany and to compare it with past epidemics.

Methods: A case definition of BT was identified within the surveillance plan. Suspect case: a) clinical signs in sheep; b) ELISA seroconversion of a sentinel animal (sheep, goats, and cattle). Confirmed case: a) clinical signs in sheep in a Municipality with a confirmed previous case ; b) Serum- neutralization positive test of a sentinel animal: c) ELISA seroconversion of a sentinel animal in a farm or in a Municipality with a confirmed previous case; d) PCR positive test in animals or vectors. Morbidity, mortality and case fatality rates were calculated only within sheep farm outbreaks, as the infection does not cause clinical disease in cattle. Data of the sheep herds were obtained from the National Animal Disease Informative System (Siman), where, on BT confirmation, Veterinary Officials record the number of animal present and the number of diseased and dead animals.

The cumulative incidence of BT outbreaks was calculated as number of farm outbreaks/number of total farms, relative to 2014. Outbreak incidence was estimated separately for clinical outbreaks in sheep, seroconversion outbreaks in sheep and in cattle.

Data sources: outbreak records of the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana, National Animal Disease Informative System (Siman) and National Animal Registry (BDN).

Results: A total of 449 outbreaks of Bluetongue were recorded in 2014, 361 in Latium and 88 in Tuscany. In Latium, the bovine farms are 10,074 and the sheep-goat farms are 7,029; in Tuscany 3,390 and 5,868 respectively. The cumulative incidence was 2.51% for clinical outbreak, 0.002% for serological outbreak in sheep, 0.24% for total outbreak in sheep, 0.70% for serological outbreak in bovine. The incidence peak was in September.

Clinical outbreak was 324 (98% sheep, 2% goat), seroconversions of sentinel animals were 125 (75% cattle, 25% sheep). The total number of diseased animals was 3045 in Latium (min 1-max 140) and 264 in Tuscany (min 1-max 75), the total number of dead animals was 1935 (min 0-max 80) and 60 (min 0-max 15), respectively.

The calculated morbidity was 4.91% (min 0.13%-max 100%) in Latium and 2.26% (min 0.19%-max 50%) in Tuscany, the mortality was 3.12% (min 0%-max 50%) and 0.51%% (min 0%-max 20%) and the case fatality rate was 63.55% (min 0%-max 100%) and 22.73% (min 0%-max 100%), respectively.

Conclusion: In 2014, BT widely spreads in Latium and Tuscany, involving the whole territory of the two regions, from the coastal to the inland mountainous areas. Morbidity and mortality rates recorded during 2014 BTV1 epidemics in Latium and Tuscany were lower than those observed in Sardinia and in other European Union countries, affected in recent years by several BT serotype epidemics, including BTV1. The high case fatality rates observed in Latium resulted similar to those estimated in other BTV epidemics, nevertheless few animals presented the clinical disease in the notified BT outbreaks. The probability that a sheep farm could be infected with clinical disease in 2014 was very low, 2.51%. Thus, the huge losses suffered by the farmers were mainly due to animal movement restriction from infected zones rather than from direct losses.

The 2014 BTV1 epidemics presented different features compared to that of BTV2. The earliest outbreaks were notified in January, at the peak of the local cold temperatures, starting from the inland areas in southern Latium. In the following months, the infection progressively spread north along an inland route. Outbreaks along the coastal areas were indeed sporadically observed only from late September 2014. The expected winter epidemiological interruption, due to vector inactivity was almost absent between 2013 and 2014. All these features make unlikely the hypothesis of an “airborne” seasonal introduction of BTV1 in Latium and Tuscany from the 2013 high BTV1 incidence in Sardinia, which was the hypothesized introduction in the 2001 BTV2 epidemics. In contrast, an introduction of BTV1 from Italian Southern regions through infected animal movements is more likely to have occurred and fits better with the observed spatial and temporal dynamics of the 2014 epidemics.

The composition, distribution and abundance of the population of vectors are known to be the key factor for BT virus (BTV) amplification and spread. Unlike in 2001-2003, when *Culicoides imicola* was thought to have been the main vector of BTV2, the majority of BTV1 outbreaks in 2014 were notified in the inland, including mountainous areas where *Culicoides obsoletus* Complex was previously demonstrated to be the main or the only vector present. The almost exclusive detection of BTV1 outbreaks where *C. imicola* is rare or completely absent confirms that at least one species included in the *C. obsoletus* Complex is an efficient vector for BTV1 circulation in Italy.

The efficient BTV1 transmission by the described vector demography is coherent with the high number of BTV1 outbreaks notified in 2014, that was much higher than in the 2001-2003 BTV2 epidemics, suggesting an intense virus circulation and amplification capable of increasing the infectious pressure on the susceptible animals population and cause clinical disease in sheep.

An ongoing survey is in progress in Latium, to assess the immunity level of ovine population within the non-vaccinated herds following the 2013-2014 epidemics. The preliminary results show a prevalence of about 30%. This herd level immunity is likely to be insufficient to mitigate future BTV1 circulation and suggests the need of a vaccination strategy aimed to limit both the spread and the clinical impact of the disease. An extensive vaccination campaign performed during the winter months would increase the population immunity in addition to the natural virus circulation.

Nevertheless, the use of the present available inactivated vaccine induces a short-term immunity and does not give cross-protection against different BT serotypes that are currently circulating in Italy and Europe. The need to yearly re-boost the immunity makes the cost of a mass vaccination campaign with this kind of vaccine even more remarkable.

After almost 10 years from the epidemics caused by BTV2, another serotype has been able to spread in the same area, similarly to what observed in other part of Mediterranean Basin (southern Italy, Corsica, Sardinia, Greece, Spain, Portugal) and to involve a high number of animals. In view of the results presented, Latium and Tuscany appear to have ecological conditions fit for recurrent epidemics.

In addition to traditional *Culicoides imicola* habitats close to coastline, the presence of *Culicoides obsoletus* Complex, well adapted to temperate climate and distributed in the whole territory, explains the wide observed spread of the infection and represents a risk factor for periodic BT epidemics in the future.

Detection and preliminary characterization of Equine infectious anemia virus from Russia.

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Objective: Equine infectious anemia (EIA, swamp fever) is a contagious viral disease of equines. The causal agent of equine infectious anemia is an RNA virus of the genus Lentivirus of the *Retroviridae* family. EIA virus can cause a clinical disease or persistent infection. EIA is worldwide distributed and was reported in such countries as Germany, France, Italy, Japan, Canada and many others. Several EIAV isolates have been described from different countries but no information concerned the genetic diversity of equine infectious anemia virus in Russia is available.

This study was performed to provide molecular characteristics, genetic diversity and the phylogenetic relationship of EIAV strains causing an outbreak of this disease in Russia.

Methods: 58 blood and serum samples from animals suspected for EIA were examined in this study. Extraction of viral RNA was performed using a modified method of silica adsorption described elsewhere. Real-time reverse transcription PCR developed earlier was used to detect EIAV RNA. The primer sets spans part of EIAV gag-gene were applied for nucleotide sequencing on Applied Biosystems Genetic Analyser 3130.

Results: We investigated samples from horses from different regions of the Russian Federation from different years: Zaporizhia – strain 3-K-VNIITIBP-VIEV (1967, KM248275.1), Nizhniy Novgorod (2011, KM202106.1), Omsk (2012) and Buryatia (2014).

We have found 10 positive samples containing the EIA virus sequences. Then gag-gene fragments sequenced and subsequently phylogenetic analysed. The obtained EIAV sequences were compared with others genome available in the GenBank database using the program BLAST.

Phylogenetic analysis based on the sequence of a ± 1018 bp fragment of the EIAV gag-gene showed that the isolates from the central region of Russia (from Nizhniy Novgorod and Omsk) are closely related to those of European strains and isolates. Nucleotide sequence homology was 83% - 82%.

However, these EIAV isolates were significantly distinct from previously characterized EIAV strain 3-K-VNIITIBP-VIEV (Zaporizhia, 1967), which is closely related to field strains originating from North America. Nucleotide sequence homology was 98% with EIAVWYOMING virus strain and strain V70 and V26.

Sequence analysis of part of the gag-gene of EIAV from Buryatia showed that this isolate (Vitim-14) genetically closely related to Asian EIAV strains and isolates. By comparison of nucleotide sequences was observed homology 83% - 80% respectively.

Conclusion: The results indicate that there are at least two different genetic variants of EIAV currently circulating in Russia among equines. These findings are essential in expanding the data of molecular epidemiology of local EIAV strains.

Enzootic Bovine Leucosis in Suspected Herd

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Objectives: A dairy herd of private farm in Samsun, Northern Turkey was observed clinically enzootic bovine leucosis like symptoms (lymphadenopathy, descreased milk yield, inappettite and loss of weight). Aim of this study, to detect of BLV in dams and their calves in EBL suspected a herd.

Methods: To determine of Bovine Leukemia Virus (BLV), All of the animals (50 dams and 11 calves of them) in the herd, serum and leukocyte samples were collected in Enzootic Bovine Leucosis (EBL) suspected herd. All of the serum samples were tested for the detection of antibodies against BLV using by commercial ELISA kit (Institut Pourquier, France) and leukocyte samples were investigated by nested-PCR.

Results: In this study, 5 of 50 dams (10 %), 5 of 11 calves (45.4 %) (two of four calves were 1-6 months old and three of seven calves were 0-30 days old) were found seropositive by ELISA against BLV. Also, five cows (10 %), three calves (27.2 %) (one calf was 1-6 months old and 2 calves were 0-30 days old of them) were detected positive for BLV nucleic acid by nested RT-PCR.

Conclusion: The seropositive dams' calves (2 calves were 1-6 months old and 3 calves were 0-30 days old) were found seropositive by ELISA. If the investigation is performed only used by ELISA test, these positivities are considered as maternal immunity. However, in this study all of the seropositive calves were investigated by PCR. Seropositive three of five calves were detected PCR positive. So, detection of BLV nucleic acid in three calves was

interpreted as these calves were infected with BLV. As a result of, all of the new borns should be tested by PCR in seropositive herds. Thus, the decision of infected calves or maternal immunity may be more accurate. The early detection of BLV can be performed confidentially in this way and virus positive calves can be removed from the herd earlier by PCR assay. This is one of the important strategies for EBL eradication.

GIS tools to define at-risk areas for diseases prevention and control in rabbit farms

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Objective: Rabbit hemorrhagic disease (RHD) cases have been observed in Italy since early 90s and control measures have been enforced to avoid the disease spreading to unaffected farms, including vaccination toward RHD virus (RHDV) and RHD virus strain A (RHDVA). Despite the efforts to avoid uncontrolled outbreaks of RHDV, a novel strain of RHDV started spreading between rabbit premises since June 2011. The new virus was originally denominated RHDVFra10, as it had been firstly identified in France in 2010; it showed antigenic, molecular, clinical and epidemiological characteristics to be classifiable as a completely new serotype of RHDV (RHDV2). Due to the differences with the virus classically circulating until 2011, vaccination measures enforced at the time were not completely effective in limiting the spread of the disease. Since the bivalent vaccine against RHDV and RHDVA resulted not sufficiently protective toward the novel strain, an auto-vaccine was prepared at the Istituto Zooprofilattico Sperimentale of Umbria e Marche from the RHDV2 isolated from the outbreak. In compliance with the Italian Ministry Decree 287/1994, the vaccine administration was only allowed in the holding where the positivity was confirmed and in an area considered epidemiologically relevant, to decrease the risk of disease spread. The principal factors to identify the population to be subjected to vaccination included: proximity of rabbit holdings, contacts between farms, belonging to the same company, and level of biosecurity measures. Nevertheless no detailed provisions were given in term of geographical extension of the vaccinated area, location, and minimum population included. Hereby we describe the procedures adopted to delineate a Densely Populated Rabbit Area (DPRA) to be subjected to vaccination campaigns, using the methodologies already applied in the territorial management of poultry holdings in north-eastern Italy to control Avian Influenza infections. The methods have been applied to the rabbit sector in Veneto region, which accounts for about the 40% of the whole national production of rabbit meat. However, the delineated procedure could also be used in the drafting and management of surveillance programmes for other diseases of livestock, to identify territorially continuous areas with high density of animal holdings, to be subjected to surveillance activities and control measures.

Methods: Geographical Information Systems (GIS) and Exploratory Spatial Data Analysis (ESDA) techniques were used, taking into consideration the spatial location of rabbit premises in Veneto region. In a first analytical step, the geographical distribution of holdings was firstly analyzed on a global scale to investigate the presence of a general spatial pattern. Ripley's *L (d)* function was used to assess whether and at which level farms were spatially aggregated, gaining a first picture on the existence of areas with high density of rabbit holdings. A global spatial autocorrelation index, Moran's *I*, was then used to detect if there was any pattern in the distribution of farms with similarly great size. In a second step the territorial extension of the region was then partitioned using the Thiessen polygon method, allowing to univocally assign a certain area to each holding, basing on the geographical location of neighbouring farms. Therefore, the closer the holdings were, the smaller was the size of the surrounding area, and vice versa. A density value was calculated for each polygon as the ratio of the number of animals reared to the area of the associated polygon. Local levels of autocorrelation were assessed using the $G_i^*(d)$ statistic. This allowed to define areas where polygons with similarly high values of rabbit densities were significantly close to each other. The spatial relation between polygons was conceptualized as Queen Contiguity (i.e. edges and vertices contiguity), considering a fifth order of contiguity (i.e. up to an unbroken chain of 5 contiguous polygons). Densely Populated Rabbit Areas were thus designed as the spatially continuous set of polygons showing a significant level of autocorrelation.

Results: A total of 1179 rabbit holdings were included in the analysis, accounting for all of the premises in Veneto region with the exclusion of backyard farms. The rabbit holdings resulted not clustered on a global scale, with Ripley's *L (d)* functions indicating a general overdispersion of points. The Moran's *I* function resulted in a very low level of autocorrelation ($I = 0.045$, $p < 0.001$). Although significant the coefficient autocorrelation indicated that the distribution of holdings with large number of animals did not follow any appreciable pattern.

The $G_i^*(d)$ function applied to the animal density per Thiessen polygon resulted in the identification of a continuous area where polygons resulted having similarly high values (hot spot). The area is located in the north-eastern part of Veneto region. The higher order of contiguity used allowed to account for the effect of farm density.

Conclusion: RHDV has been circulating in Italy since its appearance in Europe, the disease control methods applied so far have proved to be effective in limiting the disease spread in areas with the highest density of rabbit farms. Nevertheless the introduction of RHDV2 strain in 2011 raised concerns due to the inefficacy of the vaccine strain in use toward the novel virus. The administration of an auto-vaccine was therefore consented in an epidemiologically relevant area. GIS and spatial analysis methods were used to define such area in Veneto region. Although preliminary analyses showed that rabbit farms were not clustered within the region of study, further elaborations allowed to identify an area with high density of reared rabbits. The identified hot-spot also coincided with the area where most of the RHD cases occurred in 2013, and was therefore considered suitable for vaccine administration. All of the rabbit farms included in the identified area were vaccinated and no further RHD cases were detected.

The use of GIS and spatial analysis methods represent highly effective tools for managing and optimizing diseases control measures. Although the analyses only accounted for the location of rabbit premises and density of animals in a single region, they could easily apply on a National level. The methods proposed in the study could also be used on a wider context, to assess the presence of areas with high animal density for the management of different reared species, their diseases, and related control measures. Moreover further layers of complexity could also be added such as other environmental factors (such climate variations, elevations, land use, and connecting routes), which may be considered as influencing the management of the farms and/or the ecology of the disease.

Herd-level prevalence and risk factors for bovine viral diarrhea virus infection in cattle in the state of São Paulo, Brazil

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Objective: Bovine viral diarrhea virus (BVDV) is one of the most important viruses responsible for production losses in cattle and is endemic in Brazil. This study aimed at estimating the seroprevalence and the risk factors for BVDV in the state of Sao Paulo, Brazil, with a view to provide a knowledge base for the state veterinary services' planning.

Methods: A total of 12.854 serum samples from 1.732 farms were tested against BVDV antibodies using the virus neutralization method. Only females older than 24 months and unvaccinated for BVDV were tested. A herd was considered reagent when at least one seroreagent animal was detected.

Results: The herd-level and the animal-level prevalences in the State of São Paulo were 78.21% (76.23-80.19-IC 95%) and 47.08% (43.46-50.70 -IC 95%), respectively. The risk factors ($p<0.05$) identified through a logistic regression multivariable model were: a-) animal purchasing b-) rental of pastures, C-) herd size (more than 11 animals) and d-) region within the state.

Conclusion: Our findings suggest that the implementation of control and prevention measures among farmers, with the aim of preventing dissemination of the agent in the herds, is necessary. Special attention should be given to addressing the identified risk factors, such as sanitary control prior to animal purchasing and to discourage the rental of pastures, as well as to encourage the vaccination in the herds. Considering that type of cattle breeding in Brazil is huge (beef and dairy, small and large farms), different strategies or combination of strategies of control must be evaluate. In case of small properties may adoption of identification and removal of PI animals in association of strict biosecurity measures in order to establish herds completely free. Otherwise, in large farmers may adoption control with removal of PI animal in association with vaccination as part of the con-

trol. Also as fundamental step towards an efficient reliable diagnostic strategy is the implementation of the ear notch testing in order to PI detection at a very early stage (newborn and young calves). These data on the prevalence of infection in cattle in Sao Paulo, will allow an open discussion on what are the best alternatives to start a control program in Brazil.

Keywords: cattle, BVDV, serology, virusneutralization, control, epidemiology.

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Identification of viruses affecting swine in Trinidad and Tobago, West Indies

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Objective : The purpose of this study was to gather information on the presence/absence of selected viruses potentially affecting pig populations in Trinidad and Tobago (T&T). There is currently little or no information on the status of these viruses in the pig populations of T&T, Viruses studied include, Porcine Parvovirus (PPV), Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Transmissible Gastroenteritis Virus (TGEV), Porcine Respiratory Coronavirus (PRCV), Classical Swine Fever Virus (CSFV), Porcine Circovirus Type 2 (PCV2) and Swine Influenza A (SIV).

Methods : A combination of serological sampling, to gain knowledge of the seroprevalence of antibodies to these viruses in pigs and clinical surveillance to identify the viruses that are circulating in the pig populations was carried out.

1. Serological surveillance – A total of 442 serum samples were collected from 23 farms across Trinidad and Tobago. This number was achieved by using the Cannon and Roe (1982) formula where expected prevalence was set at 50% and desired absolute precision at 5%. For each ELISA kit used, a second formula developed by Cameron and Baldock (1998) was used, which took into consideration the specificity and sensitivity of the ELISA tests. Both formulae indicated that the minimum number of samples needed to be collected was 384. Farms that had more than 200 pigs were identified as major farms and farms where the number of pigs was less than 200 were identified as small scale farms.

2. Clinical surveillance – Active field surveillance for signs of clinical disease where virus infection was suspected.

3. Slaughterhouse surveillance – Testing animals with signs of disease consistent with a viral infection upon ante mortem examination and testing samples from animals with suggestive lesions upon post mortem examination.

Serum samples were taken from both symptomatic and asymptomatic swine herds for antibody detection. Nasal swabs were taken from healthy as well as acutely ill pigs exhibiting respiratory disease and/or fever. Both tracheal swabs and lung samples were taken from dead pigs.

Laboratory testing:

Testing for antibodies: Standard ELISA procedures were used to test for antibodies to the selected viruses in pigs. ELISA kits used were LSIVet™ Porcine Parvovirosis – Serum, LSIVet™ Porcine PRRS/US – Serum, ID.vet ID Screen Influenza A Antibody Competition Multi-Species and SVANOVIR TGEV/PRCV-Ab. Biocheck CSFV E2 Antibody test kit and Biocheck PCV2 Further ELISA test kits will be used to test for antibodies to CSFV and PCV2.

Testing for virus: Real-time Reverse Transcription Polymerase Chain Reaction (real time- RT-PCR) was carried out for the detection of swine influenza using primers targeting the matrix protein (M) gene (generic for influenza subtypes) using Primerdesign™ Ltd Human Influenza A Virus (M1) genesig Standard Kits.

Results: Porcine parvovirus (PPV) was detected in approximately 42% (10 out of 24 farms) of the sampling locations including two major farms. Of the total number of pigs tested, 15% (57 out of 392 pigs) were serologically positive for PPV antibodies. Approximately 80% of PPV positives corresponded to an HI titre of 10240 to 20480 according to the test kit insert, suggesting PPV infection. Swine influenza A virus (SIV) was detected in 50% (12 out of 24 farms) of the sampling locations and was found in all five of the largest farms in Trinidad. Of the total number of pigs tested, 37% (146 pigs out of 392) were serologically positive for SIV antibodies.

Antibodies to porcine reproductive and respiratory syndrome virus (PRRSV), transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) were not found to be present in pigs in T&T.

Serological testing for antibodies to CSFV and PCV2 is ongoing and results from this testing will be presented.

Conclusion: A seroprevalence study for antibodies to various priority viruses potentially affecting pig populations in T&T was carried out. There was no evidence for antibodies to PRRSV or TGEV/PRCV. Antibodies to PPV and SIV were detected in pigs from Trinidad but not Tobago. Further testing for antibodies to CSFV and PCV2, as well as identification and characterization of the PPV and SIV viruses circulating, is ongoing.

Identifying pig herds at risk for Toxoplasma gondii: prevalence and test characteristics

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Toxoplasma gondii has repeatedly been named as one of the most important zoonotic infections in Europe, in terms of its impact on human health. EFSA advised to include serological testing of pigs on T. gondii infections and audits of pig farms on risk factors for T. gondii infection (EFSA, 2011). In order to generate knowledge about the epidemiology and prevalence of T. gondii infections in pig herds we studied the long term seroprevalence on farms, persistence of infection and variation in results between and within herds. Sera which were routinely taken in slaughterhouses of VION in the Netherlands for the serological monitoring of Mycobacterium avium infections in pigs (Hiller 2013) were also tested for anti T. gondii antibodies.

Sera were tested using the PrioCHECK Toxoplasma Ab porcine ELISA (Thermo Fisher Scientific Prionics Lelystad B.V.), using a test cut off value of 20 percent positivity (PP). It was also analyzed if the suggested cut off was appropriate for our goals, which was detecting (high) positive farms. A high test specificity was needed to avoid detecting false positive farms.

Results of 120,666 sera, collected from January 2012 until August 2014, showed an average of 2% serological prevalence in pigs. Pigs from organic farms had a prevalence of 3.6%. Farm prevalence was much higher, ranging from approximately 30% for conventional farms to 90% for organic farms. Pigs delivered to the slaughterhouse during winter months had a higher prevalence than pigs delivered during summer months.

It could be concluded that serological monitoring can be very useful in detecting farms infected with T. gondii. A test cut off of 20PP was the most appropriate.

***Identifying pig herds at risk for Toxoplasma gondii*: prevalence and test characteristics**

Toxoplasma gondii has repeatedly been named as one of the most important zoonotic infections in Europe, in terms of its impact on human health. Therefore, monitoring of T. gondii in the pork supply chain was initiated by Vion, a pork slaughter company in the Netherlands and Germany. The monitoring was aimed at detecting risk herds and quantifying the prevalence of the infection in pigs.

Active serological monitoring on pigs entering their slaughterhouses was initiated, using the PrioCHECK Toxoplasma Ab porcine ELISA. The collected data were evaluated to quantify the prevalence of *T. gondii* infections in pigs and to identify herds at risk. An analysis of appropriate cut offs of the ELISA test for active field monitoring was performed, since knowledge on this issue was limited.

We found that for the studied population, a very high test sensitivity can be obtained with a cut-off value around 10 percent positivity (PP). A high specificity can be obtained with a cut-off value around 20 PP. Using a cut-off value of 20PP, we found that 2% of the pigs at slaughter are infected with *T. gondii*. The seroprevalence on organic farms (with outdoors areas) was twice as high. Furthermore we found a clear seasonality in the data, with a higher *T. gondii* risk for pigs that go to slaughter in the first quarter of the year, as compared to the third quarter.

The prevalence of T. gondii in Dutch pigs is limited but existing. A further study into risk factors and the sources of the seasonality may help inreducing the prevalence in pigs or in pork. When more is known about its prevalence, intervention measures can be developed and implemented.

Investigation of Ovine Herpes Virus-2 in Sheep

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Malignant Catarrhal Fever (MCF) is a lymphoproliferative, generally fatal disease of cattle, water buffalo, American bison, various breeds of goat and other wild ruminants. The disease is characterized with lymphoid cell accumulation in non-lymphoid organs, vasculitis and lymphocyte hyperplasia in lymphoid organs.

The disease agents are members of the MCF virus group which are related to each other and are in the gamma-herpesviriane sub-family of Macavirus genus. Presently, there are 10 known viruses in the MCF virus group. While six of them can cause natural infections (alcelaphine herpesvirus-1 (AIHV-1), alcelaphine herpesvirus-2 (AIHV-2), ovine herpesvirus-2 (OvHV-2), caprine herpesvirus-2 (CpHV-2), malignanat catarrhal fever virus white-tailed deer (MCFV-WTD), and Ibex-MCFV), one (hippotragine herpesvirus-1 (HipHV-1)) can cause experimental infection in rabbits and the remaining three's connection to disease is unknown.

OvHV-2 and AIHV-1 have been reported to be the major and economically more important agents of this disease. While AIHV-1 is not an infection agent for wildebeest, its natural host; it causes disease in cattle in the African region and various ruminant species in zoos around the world. OvHV-2 causes subclinical infections in sheep and disease in cattle. AIHV-1 causes transplacental transmission whereas it is rare in OvHV-2 which generally shows horizontal transmission. Still, disease freedom in herds can be achieved with removal of newborns from the herd.

With this work, molecular investigation of OvHV-2 in region's sheep was aimed. For this purpose; blood, internal organ and fetal samples taken from different provinces of the Aegean region were evaluated with hemi-nested PCR. 59 blood samples within a total of 37 were found to be positive. 3 were found to be positive in 18 abort samples. 27 internal organ samples within a total of 7 were found to be positive. Results of this work have revealed the existence of OvHV-2 in sheep in the Aegean region and the necessity of investigating cases in cattle.

Molecular characterisation of infectious pancreatic necrosis viruses isolated in Finland during 2000-2014

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Objective: Infectious pancreatic necrosis (IPN) is a highly contagious viral disease of salmonids that has a serious economic impact on aquaculture worldwide. Virulent IPN virus (IPNV) strains can cause high mortality in fry, whereas survivors of infection may remain asymptomatic carriers for their entire life. IPNV is the type species of genus Aquabirnavirus in the family Birnaviridae. It is a non-enveloped double stranded RNA virus with a bi-segmented genome that encodes five viral proteins. IPN viruses and other marine aquabirnaviruses can be categorized into seven genogroups based on their genetic properties (Blake et al. 2001, Nishizawa et al. 2005).

Until 2012, the inland areas of Finland were officially free of IPNV-infections, whereas in the coastal area, IPNV had been a regular finding for decades without causing clinical disease or increased mortalities. In 2012, and again in 2013 and 2014, IPNV was detected in six inland farms from three different freshwater systems. The aim of this study was to investigate genetic relationships of the 2012 -2014 inland isolates together with other Finnish IPNV strains isolated in the past 14 years. The results of the genome analysis will be presented and discussed.

Matherials & Methods: Altogether more than 120 IPNV isolates were analyzed based on partial viral capsid protein (VP2) gene sequences. Additionally, complete viral genome was sequenced from selected isolates.

Based on the VP2 sequences, representative isolates were selected for a complete viral genome analysis. The results of the genome analysis will be presented and discussed.

Results: All isolates from the inland farms belonged to IPNV genogroup 2 (serotype Ab), whereas the coastal isolates were from the isolates from the coastal fish farms belonged to genogroups 2, 5 (serotype Sp) and 6 (serotype He). Low or negligible mortality was observed at the infected farms, but the fish exhibited histological changes typical for IPNV infection. In 2013 and 2014, the number of IPNV findings remained relatively high in both inland and coastal farms.

Conclusion: It has been shown that a low virulent IPNV strain can revert into a virulent one in persistently infected fish subjected to stress. Moreover, IPNV is a very robust virus that can survive free for days in aquatic environments, making it difficult to eradicate from the infected farms. The recent introduction and rapid spread of genogroup 2 IPNV in the Finnish inland farms might be a cause for future disease outbreaks and pose a possible threat to the fish farming industry in Finland.

Molecular characterization of the HAH-2 infectious pancreatic necrosis virus (IPNV) from Turkey

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Objective: The Turkey salmonid industry was developed by importing breeding materials, a practice still in effect due to deficits in the national supply of roe. Importation of breeding materials is often associated with the transmission of pathogens. Infectious pancreatic necrosis virus (IPNV) is a bisegmented double-stranded RNA virus belonging to the family *Birnaviridae*, genus *Aquabirnavirus*, which is a major viral pathogen of salmonid fish. The virus infects wild fish and cultured salmonids, causing high mortality in juvenile trout and salmon. This virus causes economically significant diseases of farmed rainbow trout, in Turkey, which is often associated with the transmission of pathogens from European resources. This study describes molecular characterization of the HAH-2 IPNV strain (KM972673), and its phylogenetic relationships with selected previously published sequences.

Methods: IPNV strain was isolated from wild turbot (*Scophthalmus maximus)* in Trabzon province of the Black Sea region of Turkey during field survey. Virus propagated in RTG-2 cell line.Viral RNA was extracted directly from IPNV infected RTG-2 cell culture supernatants by using the QIAamp viral RNA extraction kit, as per manufacturer's instructions. RT-PCR was performed with the Titan One-tube RTPCR system (Roche) as per manufacturer's instructions. PCR products were electrophoresed and analysed on 1% agarose gels. DNA bands were purified by using the QIAquick gel extraction kit. PCR products were directly sequenced in both directions to generate consensus sequences. In some cases where RT-PCR amplification was insufficient, products were cloned into the pGEMT (Easy) vector (Promega), and the cDNA clones for each were sequenced in both directions. Sequences were assembled and analysed using the Vector NTI suite of programs (Invitrogen) and SeaView v4.2. Genomic sequences of 20 IPNV isolates were downloaded from GenBank. Nucleotide sequences were aligned and edited using the MUSCLE algorithm in Seaview v4.2 keeping gaps consistent within the reading frame. NJ trees were constructed using PHYLIP and Seaview v4.2.

Results: We have sequenced the full length VP2 genome of HAH-2 IPNV isolate (KM972673), compared them to 20 previously published genomic seguences. Based on a full length VP2 gene nucleotid and amino acid sequences, HAH-2 IPNV isolate falls within genogroup 5, serotype A2 strain SP, having 100 % nucleotide and amino acid identities with the strain NVI-015 (AY379740) exhibited mortality of approximately 90% of susceptible Atlantic salmon fry and induced severe pathological lesions. At positions 149, 213, 427, 433, 564, 731, 851, 1047, 1261, 1411, chromatograms often displayed double peaks.

Conclusion: Turkey has imported rainbow trout roe from Norway. Both isolates have same virulence motifs. These results suggest that the Almus isolate may have originated from Norway and IPNV which is highly virulent for Atlantic salmon caused high mortality in rainbow trout.

Molecular detection and seasonal distribution of lumpy skin disease virus in different cattle breeds from provinces in northern Turkey

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Objective: The aim of the present study was to investigate the prevalence and distribution patterns of LSDV infection in the provinces of northern Turkey, and to detect the factors influencing the epidemiology of LSD virus infections (age, breed, season, climate, geography, population dynamic, animal movement), and to assess the diagnostic values of the sampled materials in the diagnosis of LSDV infections.

Methods: In this study, a totally 564 materials (skin, blood and lung samples) from different cattle breeds (Jersey, Holstein, Anatolian Black, Simmental and Brown Swiss) (n=465) in the many herds suspected the LSDV infection as clinically and macroscopic pathologic remarks, housed in the 7 different provinces of northern Turkey were used for PCR.

Results: LSDV nucleic acid was detected in 259 of 564 (45.92%) materials by PCR. According to the result of PCR, the LSDV infection was diagnosed in 54.62% (254/465) of the sampled animals. Diagnostic value of necropsy and clinical materials such as skin and lung were determined more valuable diagnostic materials in the diagnosis of LSDV infection by PCR.

Conclusion: Data showed that LSDV infection was widespread in the provinces of northern Turkey and that the prevalence of the infection in the region varies in accordance with the factors such as geographical conditions (climate, season, location etc.) and the method of breeding. Additionally, it is determined that PCR is sensitive and reliable method in the diagnosis of LSDV infection.

Molecular Epidemiology of Peste des Petits Ruminants Virus (PPRV) Infection in Sheep and Goats in Iran

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Objective: Peste des Petits Ruminants Virus (PPRV) infections has been recognized as one of the major factors in limiting of small ruminant production due to high veterinary costs and deaths in young small ruminants. However, PPR infection continues to spread in the Africa, Asia, Middle East and Turkey.

In this study, PPRV infections suspect sheep and goats specimens collected from different provinces of Iran in the years 2009-2012 and current data obtained to the molecular epidemiology of the PPRV infection by using RT-rtPCR, sequence and phylogenetic analysis to the N protein-encoding gene region of virus.

Methods: Taqman RT-real time PCR assay based on N protein coding gene region which is the rapid and sensitive for diagnosis of infection was used for detection of PPRV nucleic acid. For this purpose, a total of 341 field specimens (296 blood, 5 nasal swap, 40 tissue) from sheep and goats with suspicious PPRV infection has been tested by RT-rtPCR technique at 2009-2012 years.

Results: Resulting out of RT-rtPCR, 29.91% (102/341) was detected as positive for PPRV nucleic acid. Sequence analyses were performed with the total of 16 samples from four different provinces of Iran. The phylogenetic tree based on the N partial gene sequences, PPR viruses circulating in Iran were seen in lineage IV and closely related to Turkey00 isolate as previously determined by the molecular studies in Iran. The data sequence analysis of 16 samples at nucleotid homology showed that 96.8-100% between them, 97.6-100% for reference isolate Turkey00 and 88.2-89% for strain Nigeria 75/1 used as a vaccine strain in Iran. The assessment of N partial sequences obtained with the same lineage reported in Iran1998, at nucleotid level showed 95.2-96% homology.

Conclusion: Classification of PPRV into lineages based on the N gene sequences appeared to yield better picture of molecular epidemiology for PPRV. PPRV infection once again emphasis to be endemic in the Iran; attention to the difficulty of limiting animal movement, it need to focus on the most effective way about vaccination of susceptible population for prevention of infection.

Network analysis applied to Classical Swine Fever epidemiology in Cuba

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Objective: Classical swine fever (CSF), is one of the most important viral disease of swine. In Cuba, CSF is an endemic disease, causing significant economic losses annually in the swine industry. The Cuban Veterinary Authority with the collaboration of research centers and pigs producers, arranged a strategy of eradication by zones, in agreement with CSF Eradication Plan for America projected by Food and Agriculture Organization (FAO). Contagious diseases can spread between holdings and one of the most important ways is the movement of live animals.

Network analysis has previously been used to provide summaries of animal movements, and to improve the understanding of interconnectivity among farms. Those methods can help to explore the potential of speed and range of spread of an infectious agent.

In Cuba, the qualitative studies of animal movements as part of livestock production has been mainly applied to explain the past or ongoing epidemics, but the statistical assessment has not been applied to the disease spread analysis and management of risk.

The network analysis is an important tool for a better understanding of the structure, links and animal flow, and to identify nodes with higher or lower risk of introduction and/or dissemination of diseases through the commercial relationships, which help to developing strategies for risk based surveillance.

The aim of our study was to identify higher-risk districts for CSF outbreak occurrences and those districts with high potential to spread the disease on the pig movement network. The study can make a decisive contribution to the surveillance activities, implementation of control measures and hence to disease eradication.

Methods: The study was developed in a western province of Cuba, Pinar del Río province are divided into 11 municipalities and these municipalities have 97 districts (in Spanish *consejos populares*). Pinar del Río is one of the selected provinces for starting the CSF eradication campaign by zones because the geographic location and the epidemiological situation of the disease could make it easier than other provinces. Seventeen eight (78) districts have registered pig movements from or to others districts. We used data of pig movements from July/2010 to December/2012 recorded by the Veterinary Authority. During the studied period occurred 1121 pig movements. Network parameters In-going (ICC) and Out-going (OCC) contact chain, In-degree, Out-degree and PageRank were calculated.

A logistic regression was used to identify risk factors (ICC, In-degree and PageRank) for CSF outbreak occurrence. The variables that results significant were categorized to facilitate the identification of higher-risk districts.

A network disease spread was simulated to identify the districts with higher impact of CSF dissemination in our scenario, using a SI (Susceptible + Infected) model where spread process starting from each node in the network. The visual graphs of the network were made with Gephi 0.8.2, and was geographically represented using ArcGIS 10.2.

Results: Pinar del Río has received pigs from three municipalities of the neighbor province. Those represent the 2.3% (26 movements) of the analyzed movements and were introduced 4431 animals by this way. It just occurs from July 07, 2010 to June 2011, because according to the implemented policies to eradicate the disease in Pinar del Río the introduction of live animals from other provinces has been forbidden since 2011.

Our network has 1121 animal movements and 212 links among districts. In the studied period were moved 127 653 animals, an average of 41 (±11) shipments and 4 255 animals per month. The most animals moved in the province are intended to fattening.

Only the ICC showed a significant association with the occurrence of CSF outbreaks (p = 0.045). Districts with ICC between 8 and 11 have higher risk (OR = 5.1, 95% CI: 1.04 – 25.04, p=0.036) than nodes with ICC (baseline category), and nodes with ICC between 1 and 7 showed no significant difference with the reference category.

Through the performed SI simulation model were identified 11 districts with high potential for disease spread (epidemic nodes) in the province, the district with greater potential could spread the disease affecting the 70% of districts, and the district with lower potential only achieved to spread to 4% of the districts.

Conclusion: The network analysis to identify districts with more probability of outbreak occurrence and where is more likely to disease spreading , brought useful results for understanding the CSF dynamic , and getting support to policy makers, producers, veterinary authority and researchers. It contributes to improve the decision-making process for the CSF control an eradication strategy. . Future studies will include community detection, other variables and a link to geostatistics results, improving the approach.

Preliminary analysis on the role of biodiversity of arthropod vectors in the epidemiology of West Nile Disease

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Objective: West Nile Virus (WNV) has widely spread in Italy since its reappearance in 2008, and the yearly recurrence of WNV in the north-eastern regions suggests the possibility that the virus may have become endemic in that area. The wide extant of WNV spread throughout Italy has been related to environmental and demographic changes that ultimately led to increases in abundances and distribution of mosquito vector populations. Nevertheless, the occurrence of WNV infections in horses and humans in recent years has showed a non-predictable pattern, which indicates the presence of factors not being accounted for yet that may affect the ecology of the disease.

Recent researches have approached the risk of vector borne diseases (VBD), such as WNV, by gaining deeper insight on the biodiversity of hosts and vectors, pointing to their importance in the VBD dynamics. Biodiversity is the study of the composition of communities, producing information on both the number of species present in an area (species richness) and their relative abundances (which is expressed as the dominance of a few species, or the evenness of several species).

The ecology of WNV mosquito vectors has been investigated in detail in Italy; however data on the population diversity are still scant, although the results of the entomological surveillance indicate *Culex pipiens* as the largely dominant mosquito species in the north-eastern regions, thus suggesting an overall low level of biodiversity in the area.

Hereby we describe the preliminary study of mosquito vector biodiversity, exploiting the results of the WNV entomological surveillance activities. The principal aim is to explore whether biodiversity might be related to the occurrence of WNV infections, therefore permitting to generate more complex hypotheses on how to include such factors in the delineation of a more focused control program.

Methods: Mosquitoes were captured in the context of the 2011 – 2014 WNV entomological surveillance plan in Veneto and Friuli Venezia Giulia (FVG) regions, north-eastern Italy. Sampling sites were located in a variety of settings including livestock farms, protected natural areas, and at private houses. The number of sites surveyed each year varied in accordance to the routine surveillance programme operating during the period of reference. At each site mosquito collection was conducted using CDC traps baited with carbon dioxide. As provided in the surveillance plans, one overnight sample was conducted every 15 days from the first week of May until two consecutive negative mosquito captures (usually occurring between the end of October and first week of November), which was assumed to indicate the end of the mosquito season. Temporary sampling sites located in proximity of areas where WNV infections were detected were also considered, albeit a minimum of five captures were required for being included in the analysis. Mosquitoes were counted and taxonomically identified, allowing to obtain a measure of abundance and species richness for each of the captures.

Shannon-Wiener's biodiversity index (H) was calculated considering the cumulative number of mosquitoes per year per site. Shannon index provides a simple synthetic summary of both components of the biodiversity of a community, taking into account both the species richness (the number of species observed in a sampling point) and evenness (i.e. how equally the observed species are represented in the sample). Nevertheless, this is also seen as a weakness as it complicates the comparison between communities that differs greatly in richness. The index is expected to increase when both richness and evenness in the community increase. The values of Shannon-Wiener's H index were subsequently transformed into the Effective Number of Species (ENS), which represents the number of equally-common species required to give a particular value of H, allowing an easier comparison between capture sites. ENS measures were then spatially interpolated to obtain a continuous map showing the spatial trend of mosquito biodiversity for each year of reference. The locations of the equine farms and of the municipalities where infections in horses and humans were respectively observed were overlaid on the interpolated biodiversity map. A preliminary visual analysis was performed to assess the likely relationship between biodiversity levels and WNV occurrence.

Results: A total of 61, 36, 59 and 39 capture sites for 2011-2014 respectively were included in the analysis. The average species richness per year resulted almost overlapping in the period of reference, with an average of 5 species observed in 2011, and 6 species observed in 2012-2014. The average number of mosquitoes per capture significantly varied in the reference period ($F_{3,191}$=8.776, MSE=57697, $p < 0.001$), ranging from 106 in 2011 (95% CI: 102.46 – 109.56) to 325 in 2013 (95% CI: 313.20 – 336.92). Captures observed in 2013 being significantly more abundant than in 2011 and 2014 (post-hoc Tukey $p < 0.001$ and $p = 0.043$ respectively), while captures in 2012 resulted sensibly higher than 2011 only (post-hoc Tukey $p = 0.014$). In all of the observed years the most abundant species resulted being *Culex pipiens*, accounting for the 88.64% of the total mosquitoes captured in 2011-2014, followed by *Ochlerotatus caspius*, *Aedes vexans* and *Ae. albopictus* representing the 8.09%, 1.03% and 1.02% of the overall collected mosquitoes respectively. As suggested by these proportions, the overall Shannon-Wiener's H indexes calculated per year were very low throughout the whole study period, ranging from H = 0.319 in 2013 to H=0.657 in 2012 (average: 0.525; 95% CI: 0.453 – 0.600). The lower value of H observed in 2013 indicates that, although the captures were more abundant on average, the number of dominant species were lower.

Nevertheless, some differences were observed when considering the indexes calculated for each separate capture site in each year, with different geographical pattern of biodiversity throughout the study area in the years (as showed by the variations of ENS per site per year). The locations where WNV infected horses and humans were detected, visually appeared mostly concentrated in areas where the lowest levels of biodiversity were observed, as indicated by overlaying the location of WNV infections over the interpolated biodiversity map.

Conclusion: The wide spread of WNV in Italy, and the so far unpredictable pattern of occurrence of WNV infections, has prompted for an approach that include the ecology of the virus and of the mosquito vectors to get deeper insights in the disease dynamics. In this context, the community structure of mosquito populations could likely provide useful information as it may point to unexpected effects of biodiversity, permitting to generate hypothesis on the role of overlooked species in the disease transmission. Nevertheless, quantifying the species diversity of ecological communities can be complicated. Issues may rise on how to obtain samples that are representative of an area, the rather arbitrary nature of delineating an ecological community, and the difficulty of positively identifying all of the species present.

The data gathered in the context of the WNV entomological surveillance were used in the analysis, therefore presenting a possible bias in the final estimation of the community structure, due to the geographical position of the capture sites and the period of trapping. Nonetheless the analyses provided interesting results on the possible relationship between biodiversity and WNV occurrence. Although small differences were observed, the variation in levels of biodiversity observed throughout the study area may be related to several factors. Hypotheses could range from different community structures of the preferred host, to small differences in the biotopes where capture sites were located. A small index of biodiversity may mean that low to no competition exists between the species, with a single one being dominant over the other. The likely association between cases and low biodiversity indexes could lead to hypothesise that very few species are competent for WNV transmission in the observation area, although such inferences may be excessive without further information.

The present study is a very preliminary attempt to reconstruct the ecology of WNV vectors and hosts. Further steps would include a more focused sampling design for mosquitoes, in order to include a wider array of biotopes, and the study of the community structure of residential wild birds in north-eastern Italy, to detect any possible dilution effect in WNV transmission.

Serological Evidence of Foot-and-Mouth Disease Virus Infection in Randomly Surveyed Goat breeds in the Samsun province of Turkey

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Objective: The objective of this study was to investigate the foot and mouth disease (FMD) infection serologically in different goat breeds (Anatolian black goat, Maltese and Saanen) reared in rural areas in the Samsun province of Turkey, and to detect the factors influencing the epidemiology of FMD virus infections (age, breed, climate, geography, population dynamic, animal movement).

Methods: In this study, blood samples randomly collected from different domestic goat breeds (Anatolian black goat, Maltese and Saanen) in two towns (Tekkeköy and Havza) of Samsun province. Maltese goat breed blood samples were collected in Havza town, the others were collected in Tekkeköy town. The material consisted of 368 domestic goats, including 121 Anatolian black breeds, 125 Maltese breeds and 122 Saanen breeds from Samsun province in northern Turkey. The serum samples were analyzed for the presence of antibodies to foot and mouth disease virus using a NSP competitive enzyme-linked immunosorbent assay (ELISA).

Results: Seropositivity rates in Anatolian black, Saanen and Maltese breeds were detected as 0.83%, 0.82% and 8.00% for FMD, respectively. Out of 368 serum samples examined, 12 (3.26%) were positive for FMD.
Conclusion: The results of the investigation indicate that FMD is less widespread in goats than sheep and cattle in Samsun province. Although seropositivity rate in Maltese goat breed is higher than others, this result is not related to breed susceptibility. The main reason of this is related to the FMD exposure times. Since 2008, a total of 39 FMD outbreaks have been documented in Havza town, but only 4 FMD outbreaks have been documented in Tekkeköy town. The prevalence of antibodies did not differ between male and female goats for FMD.

Seroprevalence and distribution of pestiviruses in goat breeds in the Samsun province of Turkey

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Objective: The objective of this study was to investigate the pestivirus infection serologically in different goat breeds (Anatolian black goat, Maltese and Saanen) reared in rural areas of the provinces of northern Turkey, and to detect the factors influencing the epidemiology of pestivirus infections (age, breed, climate, geography, population dynamic, animal movement).

Methods: In this study, blood samples randomly collected from different domestic goat breeds (Anatolian black goat, Maltese and Saanen). The material consisted of 368 domestic goats, including 121 Anatolian black breeds, 125 Maltese breeds and 122 Saanen breeds from Samsun province in northern Turkey. The serum samples were analysed for the presence of antibodies to pestiviruses using a enzyme-linked immunosorbent assay (ELISA).

Results: Seropositivity rates in Anatolian black, Saanen and Maltese breeds were detected as 4.13%, 1.64% and 6.40% for pestiviruses, respectively. Out of 368 serum samples examined, 15 (4.03%) were positive for pestiviruses.

Conclusion: The results of the investigation indicate that pestivirus is less widespread in goats than sheep and cattle in Samsun province. The Anatolian black and Maltese breeds were more susceptible for pestivirus infections than Saanen goat breed.

Temporal and geographical variation of pestivirus and alphaherpes virus infection in Swedish semi-domesticated reindeer (Rangifer t. tarandus)

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Objective: The overall prevalence of virus infections in semi-domesticated reindeer and their impact on reindeer health and reproduction remains to a great extent unknown. Mortality in reindeer is mainly connected to predators, but during the recent decades health problems arising from different pathogens have been recognised as both direct causes of mortality and as indirect factors contributing to other types of mortality, such as predation. It is likely that the clinical and subclinical effect of these pathogens cause economic loss for reindeer herders. Some specific viruses have been identified as causes of disease outbreaks in reindeer over the years. Parapoxvirus has been known for a long time to cause contagious ecthyma. A more recent finding is the reindeer alphaherpesvirus, cervid herpesvirus type 2 (CvHV-2), associated to infectious keratoconjunctivitis (IKC), foetal and respiratory infections. Furthermore, pestivirus-specific antibodies have been detected, indicating the circulation of a yet unidentified pestivirus in the reindeer population.

In cattle, the pestivirus bovine viral diarrhoea virus (BVDV) and the alphaherpesvirus bovine herpes virus type 1 (BoHV-1) are considered as major causes of economic losses. Because of the major impact on farmers' economy, control programmes for BoHV-1 and BVDV were launched in the Scandinavian countries during the 1990s, which resulted in successful eradication of these pathogens. Only one pestivirus strain has been isolated from reindeer, originating from a captive animal in a German zoo. This strain is most similar to the ovine pestivirus known as Border disease virus (BDV) type 2, which has never been isolated in Sweden or Norway. Recent serological data in Sweden points to the presence of pestivirus circulating among reindeer populations in areas considered BVDV-free, where reindeer have little contact with cattle and sheep. In a study using samples collected in 2001-2002, the prevalence of pestivirus- and CvHV-specific antibodies varied in Swedish reindeer herding districts, where some districts seemed to be free of infection. An important factor behind this variation could be self-clearance of infection, a phenomenon previously reported in both herpes- and pestivirus infection.

The objective of the current study was to investigate the temporal and geographical variation of pesti- and herpesvirus infection by comparing the seroprevalence in herding districts studied 10 years apart.

Methods: Serum samples were collected at slaughter in 2001-2002 and 2012-2014 in reindeer from Swedish herding districts. For serological analyses, two commercial ELISAs were used: IBR GB X2 (Idexx) for CvHV-2 antibody detection and SVANOVIR BVDV p80-Ab (Svanova) for detection of antibodies to pestivirus. Both methods have been previously evaluated for the use in reindeer.

Results: Temporal variation in seroprevalence of pestivirus and CvHV-2 antibodies was seen within herding districts and geographical variations between herding districts analysed the same years were also seen. In one of the districts (V), the seroprevalence of pestivirus was significantly lower in 2012 compared to in 2002 (60.2% in 2002, 27.6% in 2012, $p<0.01$ in Fisher's exact test). The CvHV-2 seroprevalence was showing a similar significant decrease in the same herding district (42.6% in 2002, 13.8% in 2012, $p<0.01$). In another herding district (R), significant differences in seroprevalence were only seen for pestivirus (26.8% in 2002, 13.9% in 2012, $p<0.01$), but not for CvHV-2 (46.8% in 2002, 33.6% in 2012, $p=0.09$). The seroprevalence of pestivirus in herding district M was significantly lower compared to district V for year 2002 (14.3% vs. 60.2%, $p<0.01$) and 2012 (6.0% vs. 27.6%, $p<0.01$), but there was no significant decrease in seroprevalence between years in herding district M ($p=0.18$). All data is however not yet analyzed.

Conclusion: In this study, we show geographical and temporal variation of a yet unidentified pestivirus circulating in the Swedish reindeer population. Whether this pestivirus is inducing clinical signs and potential economic losses due to abortions and immunosuppression of infected animals has not been apparent within the reindeer herding communities, and warrants further studies. The reindeer herpesvirus CvHV-2, linked to infectious keratoconjunctivitis, is also continuously circulating in the reindeer population. There seems to be an at least partial self-clearance, or annual variations, of the two viruses in the reindeer populations of studied herding districts, indicated by the variation and decreased seroprevalence in 2012 compared to 10 years earlier. The geographical variation in seroprevalence between herding districts could indicate different management routines, such as animal trade habits and biosecurity measures.

Virological Investigations in Clinical Infected Wild Goats (Capra aegagrus aegagrus) in Western of Turkey

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The aim of this study is the investigation of the presence of some clinical and persistent virus infections are in wild goats (Capra aegagrus aegagrus). For this purpose, virological investigations have been carried out in wild goats in western Turkey in 2014 when cause of death was unknown.

Seven whole bloods and internal organ samples with a total of 14 samples, supplied from 12 different wild goats which died within a week with nonspecific clinical symptoms have been investigated because of the probability in wild life the viral infections circulating in the region. Blutongue virus (BTV) and Epizootic Hemorrhagic Disease (EHDV) were investigated by quantitative RT-PCR, Peste des Petits ruminant virus (PPRV) and Border Disease Virus (BDV) by RT-PCR, Ovine herpes virus-2 (OvHV-2) by hemi nested PCR and caprine herpes virus- 2 (CpHV-2) by Touch Down PCR.

All samples have been found out to be negative for BTV, EHDV, PPRV, BDV and OvHV-2. But, five samples have been found out positive for CpHV-2. The prevalence of CpHV-2 in wild goats was found to be 41.5%.

As a result, these findings were found to be relevant in displaying the risk caused by MCF infections related to CpHV-2.

Topic: General Virology

An experimental study of bovine respiratory syncytial virus shedding and the potential for transmission

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Objective: Bovine respiratory syncytial virus (BRSV) is an important respiratory pathogen in livestock, causing substantial losses in the beef and dairy industries worldwide. The duration of virus shedding and survival of the virus on surface material are not well documented. In order to minimize spread of the virus between farms, more knowledge is needed. The aim of the present study was to investigate

the transmission potential from infected calves by describing the duration of virus shedding and the indirect transmission potential of virus via contaminated equipment and persons in contact with the calves.

Methods: An experimental pilot study was conducted. Two calves negative for antibodies against BRSV from three Norwegian dairy herds were included. One calf was inoculated with BRSV and one was naturally infected. From the day of infection the two calves were examined daily until21 days post infection (DPI).Nasal swabs from the calves were collected daily. From 7-9 DPI coats and boots from six persons handling the animals for at least ten minutes were swabbed prior to the handling, 0.5, 2 hours and the day after. The boots were flushed with luke warm water after handling of the calves. Nasal swabs were also collected from the same persons prior to contact with the calves, after 0.5, 2, 4 and 6 hours and the day after. All swabs were assayed for BRSV by qRT-PCR.

The main experiment was conducted, repeating the investigations performed in the pilot study. In the main experiment, eight calves were included.

Results: The calves developed moderate to severe respiratory symptoms during the trial of the pilot study. Serum samples from all calves were negative for antibodies against BRSV before the trial and positive at day 14 DPI. Both calves shed BRSV nasally from two to 16 DPI. The swabs from coats were all positive for BRSV until the day after. For the boots three out of six were positive after 0.5 hours and one was positive after 2 hours and the day after. All the nasal swabs obtained from humans were negative.

The results of the main experiment will be presented.

Conclusion: Calves infected with BRSV can shed virus for a period of two weeks. Virus can be detected on equipment until the day after contact with infected calves. These results show that the potential of spreading the virus directly and by indirect transmission is longer than previously shown. This knowledge is of importance in order to minimize spread of the virus between farms.

Biobank of veterinary resources and potential applications in Veterinary Medicine

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Objective: The European Technology Platform for Global Animal Health (ETPGAH) identified the lacking of reference biological material as one of the main gaps in the development of new effective tools for the control and prevention of animal diseases. To cover this gap the Biobank of Veterinary Resources has been developed, with the aim to optimize storage of reference reagents and cooperation in sharing biological resources that have been controlled and characterized by standard procedures and production.

Methods: In Veterinary Medicine wide availability of biological samples provide the basis for research leading to better understanding animal disease biology and the development of new diagnostic tests that require the use of reference reagents. Nevertheless, it is difficult to obtain biological samples with known characteristics except for those that are received from certified Centers. The availability of biomaterials and associated data a have pivotal role to improve epidemiological research allowing large scale screening studies. In particular, for retrospective studies and longitudinal designs to evaluate the course of diseases, the requirements for obtaining time-specific data are even stronger. Furthermore, biological materials are a critical resource for genetic research to improve the efficiency of selection for healthier animals with disease resistance properties. The quality of biologicals is crucial in experimental investigations and the development of diagnostic tests and vaccines for the success in terms of standardization and reproducibility of scientific methods and results that could be transferred to the national and international industrial branches. As a matter of the fact, materials collected at IZSLER Biobank are processed respecting high-quality standards, based on Standard Operating Procedures (SOPs), according to UNI EN ISO/IEC 17025 as well as ISO 9001:2008 quality systems (http://www.ibvr.org).

Results: The experience of IZSLER has been shared with other laboratories, in order to increase the types of biological resources and to implement the Italian network, each one with the maintenance of its specific identity and autonomy. The main challenge of the network was balancing the need to centralize specimens and resources with the reality of delocalized collection activities. The material is produced using a monitored process of production, controlled in the quality features and stored in a safe environment. Currently, five IZS contribute to the network with biological resources collected during their activity performed as National Reference Centres and OIE Reference and Collaborating Laboratories. In particular, they are represented by the following: *IZS della Lombardia e dell'Emilia Romagna*, *IZS delle Venezie, IZS del Piemonte, Liguria e Valle d'Aosta, IZS dell'Abruzzo e del Molise, IZS della Sicilia* (http://www.biowarehouse.net).

Conclusion: The Italian Biobank network of Veterinary Resources plays a key role in the field of research and in practice and will contribute to the scientific excellence in Europe because it allows to preserve and provide biological specimens and data for scientific and industrial application, and to increase comparative researches and studies on pathogens isolated from animals and humans. The aim of IBVR network is to gradually increase types and number of stored samples to develop an International Veterinary Biobank network and harmonize and standardized quality biobanking operating procedures, according to the OIE standard requirements and to international guidelines. Moreover, the *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna* (IZSLER) Biobank has been recognized as an OIE Collaborating Centre, an "International Depositary Authority" (IDA) for microorganisms according to the Budapest Treaty and it has been included in BBMRI (Biobanking and Biomedical Resources Infrastructure).

Distribution of deformed wing virus in colonies of honey bees Apis mellifera intermissa in southern of Algeria

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Objective: The honey bee is threatened by many pathogens. Among these virus deformed wings (DWV) is one of the most prevalent and could be responsible for deaths of colonies The aim of this work was to determine the prevalence of this virus in some apiaries in southern Algeria level and to highlight the relationship between mortalities in these apiaries and the presence of the virus.

Methods: Sampling was carried out in the southern regions of Algeria represented by Djelfa, El Bayadh, Laghouat, and Ain seffra Bechar on bee colonies of *Apis mellifera intermissa* race. 45 samples of adult bees have been made in all these areas in apiaries whose mortality rate is higher than 10%. . For the detection of the virus RNA is extracted using the NucleoSpin® RNA II kit (ACHEREY-NAGEL). Reverse transcription of RNA and DNA amplification is performed using a continuous process by the RT-PCR method with the RT-PCR Kit (Qiagen) according to manufacturer's recommendations. RT-PCR program comprises a reverse transcription step at 50 ° C for 30 minutes, followed by an initial activation phase PCR at 95 ° C for 15 minutes. This is followed by 40 cycles at 94 ° C for 1 minute, at 55 ° C for 1min, and 72°C for 1 minute. An extension step at 72 ° C for 10 minutes occurs. The products were visualized by electrophoresis in 0.6% (w / v) agarose gels stained with ethidium bromide.

Results: The results show a variation in the prevalence of the virus between apiaries and study areas. Apiaries the Bechar region recorded the highest rate of infection (45%). The least contaminated zone is that Djelfa with a rate of 25%. No correlation was detected between recorded deaths and prevalence of this virus. Other causes may be the source of the colonies marked losses in these regions.

Conclusion: This study is the first in Algeria on viruses, the results show the detection of the virus for the first time in Algeria in the southern region. Further studies are needed on other viruses and other pathogens in order to accurately determine the possible causes of mortality of bee colonies.

Full genome analysis of A(H5N1) from a human during a surge season in transmission of avian influenza in Egypt, 2014-2015

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Objective: Since its first introduction to Egypt in 2006, highly pathogenic avian influenza HPAI A (H5N1) virus has caused many outbreaks in poultry and 177 cases in humans. Starting mid November 2014 until mid March 2015, Egypt has experienced an unprecedented increase in poultry die off and 141 confirmed human cases with 49 fatalities (CFR=34.7%). Full genome analysis was performed from an H5N1 human clinical specimen of the first reported case of the investigated wave to detect any possible reassortment and/or new mutations in the circulating strains that could reveal substantial genetic drift.

Methods: RNA was extracted from the patient's oropharyngeal swab using Qiagen Viral RNA MiniKit.

Overlapping DNA fragments covering each of the 8 genes were generated using specific primers. Purified amplicons were sequenced using Sanger's method. Resulting sequences were edited using Sequencher software

v4.10.1. Sequences were aligned with all available avian and human H5N1 sequences from Egypt using BioEdit software-Muscle Wrapper application. Phylogenetic trees were constructed in MEGA5 software using Neighbor-Joining algorithm with Maximum Composite Likelihood evolutionary model. The closest nucleotide match using Nucleotide Basic Local Alignment Search tool (blastn) on GenBank was identified.

Results: Full genome analysis showed that internal genes (PB2, PB1, PA, NP, MP and NS) as well as the surface genes (HA and NA) are closely related to clade 2.2.1 viruses and are 99% similar to avian strains that were isolated from Egypt in 2013 (according to the latest published sequences). HA and NA genes kept the same signature substitutions of clade 2.2.1-C subgroup that has dominated since 2010. All sequenced genes contained well established markers of mammalian adaptation of H5N1 virus; e.g. E627K in PB2, P598L in PB1, S409N in PA, A184K in NP, ESEV motif in NS1, T215A in M1, T156A in HA and many others. Numerous novel changes were recorded that have not been previously investigated; e.g. M66I in PB2, V640A in PB1, S321G in PA. None of the markers known to confer resistance to antiviral drugs were observed in NA and M genes sequences.

Conclusion: Clade 2.2.1 H5N1 viruses continue to circulate in Egyptian poultry causing increased human infections. The characterized full genome indicates that all genes were closely related to avian H5N1 strains circulating in Egypt in 2013. H5N1 from Egypt continues to evolve and carries numerous markers in all the genes that may enhance mammalian host adaptation, increase replication efficiency and antagonize host immune response. Observed markers were previously found in avian or human strains from Egypt.

No evidence of viral reassortment was found and the virus is expected to be sensitive to antiviral drugs based on the analysis of NA and M genes sequences. Unique markers were observed in PB2, PB1, PB1- F2 and PA proteins that were not previously recorded in the Egyptian strains. Monitoring novel changes through further investigation and gain-of-function studies would help identify the significance of these mutations and interpret the drastic increase of human H5N1 infections.

Genetic and phylogenetic analysis of avian infectious bronchitis virus nephropathogenic strain IZO 28/86

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Objective: Avian infectious bronchitis (IB) is an acute, contagious and ubiquitous disease caused by the gammacoronavirus infectious bronchitis virus (IBV). IB affects chickens of all ages and it is characterised primarily by respiratory signs (mainly in growing chickens) and decreased of egg production and quality due to infection of the oviduct. Some strains of the virus (nephropathogenic strains) produce interstitial nephritis and mortality. IBV has also been reported to produce disease of the proventriculus. The severity of IBV-induced respiratory disease is enhanced by the presence of other pathogens, including bacteria, leading to chronic complicated airsacculitis.

The disease is transmitted by the air-borne route, direct chicken to-chicken contact and indirectly through mechanical spread. Due to a high rate of mutation and recombination in the viral genome, new types of the virus continue to arise

Economic impact of IB is mainly due to: poor growth performance, mortality due to the respiratory disease in broilers, egg production losses in layers and breeders. Losses caused by renal damage may be seen in broilers, layers and breeders.

The S1 subunit of the spyke glycoprotein on IBV viral surface is involved in host cells adhesion and induces neutralizing antibodies. For this reason, analysis of S1 subunit sequence is widely used to serotypization and its variability causes a decrease in cross-protection between serotypes. Ladman and colleagues (2006) studies showed that the level of homology of the S1 subunit, or part of it, can predict cross-protection: an homology greater than 90% is related to and higher chance of cross- protection.

Methods: IBV strain IZO 28/86 was originally isolated in 1986 during an outbreak in Northern Italy from chickens with nephritis and successfully attenuated after 110 passages in SPF chicken embryo eggs. Its characteristic is to be able to spread in the lungs, intestine and kidney. The complete sequence of IBV IZO 28/86 spike glycoprotein S1 subunit gene was obtained and included in GenBank (KJ941019). Data were compared with all publicly available IBV sequences.

Results: IBV strain IZO 28⁄86 shows an homology (97%) with an IB virus isolated in Italy from chicken in 2011: isolation was performed from kidney and genetic analysis showed that it belong to the Q1 genotype (Toffan A et al, 2011).

A genetic study was performed starting from data obtained by Ababneh and colleagues (2012): a phylogenetic tree was built using different amino acid sequences from isolates characterizing the typical symptoms of the disease (i.e. respiratory, proventriculus damages and nephritis) to highlight homologies between gene sequences and strain characterics. As result, IBV strain IZO 28⁄86 belong to a branch formed from several QX-like isolates characterized by nephropathogenic symptoms. A wider comparative analysis of the S1 gene sequence showed homologies greater than 90% with several nephropathogenic strains isolated in Asia and in middle-eastern countries. In particular, IZO 28⁄86 showed an homology with several CK/CH/LDL⁄97I-like isolates (91-92%), with DY09 (92%) and several QX-like isolates, as Q1, J2 and T3 strains (92%). Furthermore, IZO 28⁄86 amino acid sequence showed an homology (92%) with an IBV strain isolated in Italy in 2011 and identified as Q1-like strain. Moreover, common vaccine strains showed an homology lower than 80% with IZO 28⁄86 amino acid sequence.

At protein level, sequence from amino acid 99 to 127 in S1 protein sequence play a role in IBV tissue tropism and virulence (Li et al, 2001). IZO 28⁄86 showed an homology of 97% comparing this region with several isolates displaying kidney tropism, as confirmed by strain characteristics showed during the isolation process.

Conclusion: In literature is described the isolation in Middle-East in 2011 of several IBV nephropathogenic strains, originally isolated in China in 1995 from tracheal samples of infected chickens (Ababneh et al, 2012). These strains were isolated in flocks vaccinated with classical vaccine strains (H120 and 4⁄91), which implies insufficient cross-protection against these isolates as result of a poor relationship in S1 sequence between these strains and common vaccine strains. Recent papers show that a higher homology in S1 amino acid sequence is related with a higher chance of cross-protection (Ladman et al, 2006). IZO 28⁄86 vaccine strain show an homology greater than 90% with these middle-eastern strains and others Chinese isolates. This high rate of homology permits to postulate a sufficient cross-protection to nephropathogenic strains isolated in middle-eastern countries using IZO 28⁄86 vaccine strain.

To conclude, a combination of more vaccine strains is usually used to confer a widespread protection against different pathogens and different variants of the same pathogen. IZOVAC CHB, which contains both IBV H120 vaccine strain and IBV 28⁄86 vaccine strain, can confer a complete protection against IBV strains actually circulating in Europe and Middle-eastern countries.

Inactivation of classical swine fever virus by reagents commonly used in research and diagnosis and the effect of inactivation treatments on ELISA detection of antigen and antibody.

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Objective: The impact of classical swine fever virus on the swine industry obliges laboratories handling the virus to use appropriate measures to prevent its release into the environment. Various reagents and treatment are used to inactivate viruses prior to handling of samples outside of high containment facilities. However, data demonstrating efficacy of CSFV inactivation under the particular conditions used is often not available. The objective of this study was to demonstrate how efficacious certain laboratory reagents and treatments are in inactivation of classical swine fever virus (CSFV). The effect of heat and chemical inactivation treatments on the subsequent detection of CSFV antigen and antibody was also assessed.

Methods: Reagents commonly used for RNA extraction, cell fixation prior to flow cytometry or immunoperoxidase staining, chemical inactivation or disinfection were incubated with CSFV or sera from CSFV infected animals for times and temperatures relevant to the treatment in question. The amount of viable virus remaining was determined by titration on PK15 cells followed by immunoperoxidase staining and the viral reduction factor determined by comparison to untreated control virus. For assessment of toxic cell fixative treatments, PK15 cells infected with CSFV were treated and then washed 3 times in PBS prior to titration of virus on PK15 cells. To assess the effect of heat or chemical inactivation treatments on subsequent detection of CSFV antibody or antigen, sera from pigs vaccinated with C-strain and inoculated with CSFV UK2000/7.1 were either heated to

56°C for 90 minutes or treated with β-propiolactone (0.1% v/v) for 2 hours at 37°C. Detection of antigen and antibody, compared to untreated sera, was assessed by ELISA.

Results: No viable CSFV was detected after treatment of samples with the commercial RNA extraction lysis buffers; Buffer AVL, Buffer RLT (Qiagen) or Tri Reagent (Ambion). However, the toxicity of these reagents, and some cell fixatives used for flow cytometry and immunoperoxidase staining, limited the ability to demonstrate high viral reduction factors. An alternative strategy of treating CSFV-infected cells and washing prior to titration was therefore applied for those agents that did not lyse cells. Reduction factors of > 6 $\log_{10}$ were demonstrated for BD FACS™ Lysing Solution, BD CellFIX™ solution, BD CytoFix/Cytoperm™, 4% paraformaldehyde and 100% and 80% acetone treatments. Notably incubation in 20% acetone at room temperature for 10 minutes, which is included as a fixative method for OIE prescribed serum neutralising tests for CSFV, resulted in little or no reduction in the amount of viable CSFV. Whilst a 1:1 TMB ELISA substrate/ 1M sulphuric acid stop solution mixture reduced virus by >3.3 $\log_{10}$, viable virus was occasionally detected after treatment at a level below the quantifiable limit of the assay. FAM30, an approved disinfectant for CSFV when used at a contact time of 30 minutes, resulted in >3 $\log_{10}$ reduction in virus even after short contact times of 1 to 5 minutes, although virus was occasionally detected if pig faeces was spiked into the virus disinfectant mixture.

In contrast to heat treatment of sera at 56°C, which is not a reliable method of inactivating CSFV, β- propiolactone treatment resulted in >4 $\log_{10}$ viral reduction factor. Both heat treatment and β- propiolactone had minimal effect on antibody detection but both treatments reduced OD values obtained in CSFV antigen ELISA compared to untreated sera.

Conclusion: The results of this study provide useful information for laboratories handling CSFV for research and diagnosis and will facilitate improved protocols that minimize the risk of accidental release of virus. For example, use of 20% acetone as a cell fixative must not be considered as a method that inactivates CSFV. The demonstration of efficacy of disinfectants after short contact times will facilitate the rapid transfer of samples between high containment animal and laboratory facilities. Effective inactivation protocols will allow handling of samples in lower biosafety level facilities that will provide substantial cost savings and facilitate analyses, such as flow cytometry, not available or possible within existing containment facilities.

Molecular characterization of myxomavirus isolated in Italy

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Objective: Myxomatosis is a lethal, generalised viral disease of the wild and domestic European rabbit caused by the Myxoma virus (MYXV), a member of the Poxviridae family. MYXV is an enveloped virus with a large, linear double-stranded DNA genome, contained in a brick-shaped virion; viral replication occurs solely in the cytoplasm of infected cells.

MYXV has a worldwide distribution, is endemic in wild European rabbit populations, and can spill over into farmed and pet rabbits. Naturally transmitted via arthropod vectors during summer time, MYXV was firstly introduced as a biological control agent against the European rabbit population of Australia in 1951 and it was subsequently released in France and United Kingdom. Due to its high contagiousness, now the virus is a major problem for rabbit farmers throughout Europe, causing severe economic losses.

There is no age or sex predilection and the mortality rate ranges between 20 and 100%, according to the grade of virulence of the viral strain. Secondary bacterial infections (in particular Pasteurella sp. and Bordetella sp.) are typical in rabbits that survive to infection and may be the major cause of death in rabbits infected with subacute strains of MYXV.

Myxomatosis is essentially a disease of European rabbits and wild rabbits act as reservoirs. Two forms of the disease are observed in rabbits, the nodular and the respiratory forms. The nodular (classical) form, characterised by skin lesions, is caused by virulent MYXV strains. This form is naturally transmitted by insects (mainly mosquitoes and fleas) or direct contact, and is mainly observed in wild and pet rabbits and in small-scale rabbitries. The respiratory form, characterised mainly by respiratory clinical signs and skin nodules are few and small. This form is caused by mild and attenuated strains, especially in farmed animals.

In order to provide and efficient and effective protection against virulent MYXV, a live-attenuated vaccine has been developed and complete sequence of DNA polymerase and a unique cytoplasmic protein genes were

obtained and compared with all publicly available MYXV sequences.

Methods: Originally isolated during an outbreak of the disease Northern Italy, MYXV IZO vaccine strain was successfully attenuated after serial passages in Rabbit Kidney Epithelial Cells (RK-13) to obtain an efficient immunity but no disease manifestation.

Molecular characterization was obtained using specific primers in order to amplify the open reading frame (ORF) M034L, corresponding to DNA polymerase sequence, and the ORF M130F, that encodes a unique 15 kD cytoplasmic protein. The PCR products were sequenced and the identity of MYXV confirmed by submission to the GenBank database and compared with published MYXV sequences using a BLAST search.

Results: MYXV IZO vaccine strain propagation is able to produce plaques in RK-13 monolayers and subsequent titration showed an average titre of 5-6 Log_{10} $TCID^{50}$. MYVX IZO vaccine strain can be easily propagated on RK-13 cells forming typical foci of infection. A live virus antigen was produced in order to simulate a live vaccine production and a stress test was performed to verify stability of virus. A titration on RK-13 cells, performed on samples obtained before and after a lyophilisation process, showed a high stability with no decrease of virus titre.

The BLAST analysis of M034L and M130F sequences revealed that MYXV IZO vaccine strain is homologous (98-100%) to the corresponding fragment of several MYXV. Both sequenced regions are highly conserved in several MYXV strains, thus it is not possible to obtain a precise identification of the IZO vaccine strain at nucleotide level. Using amino acid deducted sequences is possible to obtained a more specific identification of the IZO vaccine strain as a Lausanne-like strain (homology rate greater than 95%).

Data obtained at amino acid level are confirmed by the phylogenetic analysis (performed using Neighbor-Joining method, bootstrap resampling performed on 1,000 replicates): the nucleotide sequences from MYXV IZO vaccine strain, aligned with the corresponding sequences available in GenBank, showed that it is close to a branch formed from several different MYVX strains isolated in Europe and Australia.

Conclusion: MYVX IZO vaccine strain, originally isolated in Northern Italy during an outbreak of the disease, shows an homology with several MYVX strains isolated in Europe and Australia. Sequence analysis performed at amino acid level showed that IZO vaccine strain is related to Lausanne strain. These are confirmed by the phylogenetic analysis performed using nucleotide sequences from vaccine strain.

Propagation of vaccine strain and stress-test performed on an experimental batch of vaccine confirmed the stability of virus to a normal process of live vaccine production.

A complete efficacy, safety and challenge studies will be carried out in order to produce a live vaccine able to confer protection against MYVX strains.

Multitemperature Single-Strand Conformational Polymorphism (MSSCP) Analysis of A/H1N1 Strain Variants Co-infections Among Pigs in Poland

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Objective: Monitoring and control of infections are key parts of surveillance systems and epidemiological risk prevention. In the case of influenza A viruses (IAV), which show high variability, a wide range of hosts, and a potential of reassortment between different strains, it is essential to study not only people, but also animals living in the immediate surroundings. If understated, the animals might become a source of newly formed infectious strains with a pandemic potential. We describe a method based on multitemperature single strand conformational polymorphism (MSSCP), using a fragment of the hemagglutinin (HA) gene, for detection of co-infections and differentiation of genetic variants of the virus, difficult to identify by conventional diagnostic, and phylogenetic analysis of these variants.

Methods: In years 2011-2013, the National Veterinary Research Institute collected five environmental samples (lung tissue) on farms in Poland, from pigs with influenza-like symptoms. The samples were positively diagnosed as H1N1 of avian origin using RRT-PCR and MRT-PCR. The RNA isolated from the samples was analyzed using an

MSSCP-based minor variant enrichment procedure, an electrophoretic technique separating denatured PCR products according to their single-stranded secondary structure, which utilizes a short *HA* gene fragment and allows easy detection and distinction of infections and co-infections with the various influenza strains. The analyzed fragments of HA gene were sequenced using the Sanger method and subjected to phylogenetic analysis where the evolutionary history was inferred.

Results: The presence of swine IAV in collected samples was confirmed with RRT-PCR, and the genotype of the virus was determined by MRT-PCR. All isolates were characterized as swine "avian-like" H1N1. To check whether the IAVs from the collected isolates were of human origin and/or to verify the possibility of mixed infections, al samples were subjected to MSSCP. The result of first MSSCP analysis suggested multistrain and/or quasispecies infections. The direct sequencing of analyzed HA gene fragments confirmed that the genetic material was not uniform within the isolates. Differences in the nucleotide sequence might indicate the presence of at least two genetic variants isolated from a single individual. To address this issue, we performed clonal selection in bacterial cells. After MSSCP performed on all the clones, we distinguished six unique band patterns, that occurred repeatedly during separation. To evaluate the differences in the nucleotide sequence between the individual genetic variants, all clones showing distinct band patterns were sequenced and used in phylogenetic analysis. Based on the phylogenetic relationship, the clones were assigned to three groups. The generated phylogenetic tree shows a close relationship between group I and the strains isolated in years 2006-2008, which included a classical swine A/H1N1 virus, prevalent in North America, and a human A/H1N1 lineage, predominant at the time. Group III showed the closest relationship with strains isolated after 2009, corresponding to the pandemic A/H1N1 lineage, which has been reported in pigs since its introduction in 2009. The results for group II were surprising, because the genetic variant assigned to this group was closely related to the A/Puerto Rico/8-KV/34 strain. All the analyzed genetic variants were distant from the A/Swine/Belgium/WVL/79 and A/Swine/Finistere/2899/82 strains representing the European swine A/H1N1 lineage of avian origin.

Conclusion: The constant genetic evolution of IAV is a major risk for public and animal health. An ongoing reassortment process and small changes in sequence, give rise to new IAV strains, capable of overcoming species barriers. Therefore, we need to investigate the genetic variability of the viruses circulating among humans and animals, in order to prevent, or detect, the emergence of highly pathogenic viral strains. Many routine diagnostic methods fail to detect quasispecies or multistrain infections, due to limited sensitivity or false negative results which we show in phylogenetic analysis. We describe results proving that a native electrophoretic separation of PCR products at strictly controlled temperature (MSSCP) allows detection of co-infections and differentiation of novel genetic variants of IAV in animal samples. This method is an inexpensive, sensitive and convenient way to determine variations of influenza strains and can be a very useful screening tool for epidemiological studies.

NGS as regular tool for viral epidemilogical studies

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Objective: The majority of known humans' infectious pathogens are of animal origin, namely zoonoses; both domestic and wild animals act as host reservoirs of such pathogens. Surveys of the pathogens community of wildlife may help to predict, prevent and control putative episodes of emerging diseases. The diversity and interactions of wildlife's microbiota at both the individual and population levels influences the epidemiology of infections. Hence, developing new approaches for multiple pathogens detection without any awareness on their existence becomes essential. The detection of prokaryotes in multiple reservoirs simultaneously is already established. Nevertheless, the coetaneous detection of (unknown) viruses in various reservoirs remains obscure. Accordingly, we are developing an innovative and economically competitive experimental protocol using a next-generation sequencing approach that allows to sequence from several reservoirs at the time different viruses without any consciousness of their presence and later being able to relate each virus to a studied reservoir.

Methods: In brief , our method consists in: (1) disruption of the tissue of interest; (2) clarification of the homogenized product; (3) filtration of the supernatant through a 0.45 µm filter; (4) ultracentrifugation of the filtrate to precipitate viral particles; (5) suspension of the pellet to perform an extraction of nucleic acids (DNA/RNA); (6) synthesis of the complementary DNA with random primers being able to recover any virus; (7)

klenow polymerization to generate the double strand DNA; (8) PCR-amplification of the products generated; (9) joining Illumina adapters and labeling the amplicons; (10) multiplexing of the labelled amplicons creating a library; (11) purification and quantification of the library; (12) sequencing with MiSeq from Illumina; and (13) bioinformatics analyses to obtain viral sequences attributed to each of the host, each of the sequences obtained will be assigned to a known taxa or otherwise will be analyzed as a putative novel virus.

Results: This approach will grant us with the ability to: (i) characterize newly-discovered viruses; (ii) describe microbial communities in the selected reservoirs; (iii) estimate the prevalence of circulating pathogens in the reservoir population; and (iv) evaluate microorganisms interaction (either negative or positive associations), probably relevant for their epidemiology.

Conclusion: The results will allow us to generate an almost complete inventory of potentially zoonotic known virus in the analyzed samples without any a priori on their presence. In addition, the use of multiplexing techniques grants us the ability to handle many samples at the time and screen any virus in each of the individuals analyzed, while the experimental costs remained compatible with cohort studies. To predict and control the etiological agents of diseases in natural populations it is essential not only to understand host-parasite interactions but also the entire interactions of microorganism communities. Thus, we believe that having the whole pathobiome picture is essential for the epidemiology and emerging diseases field and NGS techniques will pave the way for greater understanding of this line.

Novel antibody binding determinants of serotype O Foot-and-mouth disease virus

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Introduction: Foot-and-mouth disease virus (FMDV) displays various epitopes on the capsid outer surface. Five neutralising antigenic sites have been identified in serotype O FMDV using murine monoclonal antibodies. In addition, there is evidence of the existence of other, yet unidentified epitopes, which are believed to play a role in antibody-mediated protection. However, the relative importance of different epitopes in FMD vaccine induced-protection has not been ascertained to date in great details.

Materials and methods: Comparison of the ability of bovine antisera to neutralize a panel of serotype O FMDV identified three novel putative sites at VP2-74, VP2-191 and VP3-85, where amino acid substitutions correlated with changes in sero-reactivity. The impact of these positions was tested using site-directed mutagenesis to effect substitutions at critical amino acid residues within an infectious copy of serotype O FMDV.

Results: Using reverse genetics technique a series of recombinant viruses were generated in this study out of which two recombinant viruses, (1) by substituting the critical amino acid residues of the five neutralising antigenic sites of a FMDV type O cDNA clone (5M), and (2) by adding two additional substitutions at position VP-74 and VP2-191 (M6), are noteworthy. Discussion: Serological characterisation of 5M and M6 recombinant viruses revealed 56% and 74% reduction in neutralising antibody titre reflecting the significance of these residues in the antigenicity of the virus. However it is possible that more unidentified epitopes may exist as 100% reduction in neutralization was not observed. Work is on-going in our laboratory to identify additional capsid amino acid residues that could have an impact on the antigenic nature of the virus.

Productive replication of the peste des petits ruminants virus Nigeria 75⁄1 vaccine strain in Vero cells correlates with inefficient maturation of the viral fusion protein.

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Objective: PPRV, a member of the *Paramyxoviridae* family expresses two membrane glycoproteins, the haemagglutinin (H) and the fusion (F) proteins which are involved in initiation of the viral infection and in membrane fusion processes leading to the induction of syncytia in PPRV infected cells in vitro and in vivo. In the context of the characterization of the virulent lineage 4 strain PPRV Kurdistan 2011, isolated from wild goats from the Kurdistan region in Iraq ,we observed that both PPRV Kurdistan 2011 and PPRV Nigeria75⁄1 vaccine strain led to induction of large syncytia in Vero/dog-SLAM cells within 24 h hours whereas both failed to induce detectable cell-cell fusion events in two tested normal Vero cell lines of different passage histories. Surprisingly, on Vero/dog-SLAM cells the vaccine strain grew to titers of $10^{5.25}$ $TCID_{50}$/ml whereas infectious virus yield was about 200-fold higher on the non-transgenic Vero cells. The virulent strain Kurdistan 2011 behaved, with titers of $10^{7.0}$ $TCID_{50}$/ml on Vero/dog-SLAM cells and only $10^{4.5}$ $TCID_{50}$/ml on normal Vero cells, contrary.

The latter result was as expected since Vero cells lacking a receptor for PPRV are regarded as not permissive for infection.

To elucidate the divergent productive replication behaviour of PPRV Nigeria75⁄1 vaccine strain on the differing Vero cells, we asked whether the cause might be associated with intracellular transport and/or maturation of the viral envelope glycoproteins F and H.

Methods: Due to the lack of antibodies reactive in Western blotting und immunoprecipitation experiments, monospecific sera directed against F and H were raised in rabbits. The sera were used in pulse-chase experiments, immunoblotting to analyze steady state levels of F and H in cells infected or transiently transfected with respective expression plasmids, and to monitor cell-cell fusion after infection or transient infection.

Results: Growth kinetics confirmed the results mentioned above with regard to differential productive replication of the virulent and vaccine strains on the different cell lines. Monitoring of cell-cell fusion by indirect immunofluorescence revealed that both strains induced syncytium formation in Vero/dog-SLAM cells but not in normal Vero cells. In contrast to several reports in the literature, we were not able to detect syncytium formation in transiently transfected cells expressing F alone. Also G alone, as expected, was negative in this respect whereas co-expression of F and G induced large syncytia – in Vero/dog-SLAM cells only. In normal F and H expressing Vero cells fused cells were rarely detectable and they contained only a few nuclei. Monitoring the steady state levels of F and H after infection and transient transfection and transport/maturation in pulse chase experiments after infection demonstrates that F, in contrast to H, does mature only inefficient in normal Vero cells which may be due to hampered intracellular transport since the pulse/chase experiments gave no indication for a timely processing of the putative F_0 protein and also steady state levels in infected or transfected cell revealed mainly uncleaved F0. In contrast, steady state levels of F0 and the large subunit F1 appeared as to be expected in infected or transiently transfected Vero/dog-SLAM cells.

Conclusion: Our results provide good evidence that the inefficient processing of the PPRV fusion protein is beneficial for the productive replication of the PPRV Nigeria75⁄1 vaccine strain in normal Vero cells. The molecular mechanism leading to processing hindrance in cells which apparently process normally type I glycoproteins of other viruses needs to elucidate.

The expression of a truncated Hendra virus attachment protein in the trypanosomatid protozoan host Leishmania tarentolae

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Objective: Hendra virus (HeV) is an emerging zoonotic paramyxovirus of the genus Henipavirus that has caused several outbreaks in Australia since its first discovery in 1994 with a total of 83 fatalities in horses and four in humans. In experimental challenge studies carried out under BSL-4 conditions HeV infected a wide variety of mammalian species. This wide host spectrum may result from its binding to EphrinB2 as viral entry receptor, which is highly conserved among different species. HeV infection of host cells is mediated by two structural membrane glycoproteins: the attachment (G) and the fusion (F) glycoproteins. Vaccine strategies for HeV infections mainly focus on attachment (G) protein which elicits a potent neutralizing antibody response. The eukaryotic parasite *Leishmania tarentolae* (*L.tarentolae*) which is apathogenic to humans has recently been introduced as a useful tool to express recombinant proteins with mammalian-type glycosylation. So far, just a very few viral proteins have been expressed in this system. Here we describe the expression and purification of a truncated

HeV attachment protein.

Methods: *L. tarentolae* host strain p10 was stably transfected with cDNA encoding a transmembrane/cytoplasmic tail deleted HeV attachment (G) protein. After clonal selection and cultivation, protein expression and purification were conducted using *Strep*-tag affinity chromatography under native conditions resulting in approximately 500µg of protein per litre of Leishmania culture. Leishmania-expressed recombinant G protein was further characterized by silver and Coomassie staining. Immunoblots with monoclonal α*Strep* antibody and anti HeV IgG positive pig sera were performed as well as mass spectrometry to confirm the authenticity of the protein. To further investigate functional characteristics, a cell adhesion assay was conducted using HeV susceptible cell lines.

Results: Stable transfection of *L. tarentolae* with pLEXSYsat2-HeVG resulted in a cytosolic expression of the target protein, which is already detectable by immunoblot analysis of a cell lysate with monoclonal anti-*Strep*tag antibodies. After purification via *Strep*-tag under native conditions the protein was visualized in SDS-PAGE followed by silver staining resulting in a molecular mass of approximately 63kDa. The protein could also be detected in immunoblot by both, monoclonal anti-*Strep*tag antibodies and anti-Hendra virus IgG positive pig sera. The authenticity of the recombinant protein was furthermore confirmed by mass spectrometry. In the functional cell adhesion assay the protein bound to HeV susceptible cells in a dose-dependent manner.

Conclusion: Successful detection of recombinant truncated HeV G in immunoblot by anti-HeV IgG positive sera and the functional cell adhesion assay revealed a folding structure and conformation of the recombinant HeV G protein which is comparable to that of the wild-type protein. From the dose-dependent binding of Hendra virus susceptible cells to the recombinant protein, we assume an interaction with ephrin-B2, the cell entry receptor of Hendra virus. Taken together, these experiments confirm the suitability of *Leishmania tarentolae* for the expression of viral glycoproteins.

Use of H-Index to evaluate research productivity outcome on viral diseases of swine

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Objective: The assessment of research interest areas has been traditionally a controversial issue, many times driven by subjective measures. The bibliometric indicator H-index (or Hirsch index) attempts to measure both the productivity and the citation impact of published work on a subject. It is based on the set of most cited papers and the number of citations they have received: number of published papers (N) that have been cited N or more times. The present study aimed to calculate, compare and analyse the H-Index of publications of swine viruses.

Methods: The H-index for 54 swine virus was calculated using the bibliographic software package Institute Scientific information's Web of Science (WOS) v.5.16.1.All searches were done related to the terms "porcine", "pig" or "swine" using each virus full name, acronyms, colloquial names and their synonymous for them and their associated disease/s. Selected papers were reviewed one by one to ensure database accuracy. H-indices were measured during March, 2015.The ten viruses with the highest H-indices were considered and classified by their zoonotic potential, emerging (less than 25 years from first description) or non-emerging associated disease, OIE-listed diseases and the distribution of the disease (global/regional). Besides, country of origin of each paper included in the H-indices dataset was investigated. Quartiles ranking of journals (2013 JCR by Thomson-Reuters) where papers were published were also determined.

Results: In decreasing order, the ten highest H-indices were: 1-Porcine reproductive and respiratory syndrome virus (PRRSV) (H=95);2-Porcine circovirus type 2 (PCV2) (H=85);3-Swine influenza virus(SIV) (H=79);4-Classical swine fever virus (H=72);5-Aujeszky's disease or pseudorabies virus (H=58), 6-Foot-and-mouth disease virus (CSFV) (H=56), 7-African swine fever virus (ASFV) (H=56), 8-Pig endogenous retrovirus (PERV)(H=55), 9-Porcine rotavirus (PRoV)(H=49) and 10- Hepatitis E Virus(H=49).Regarding the number of total citations for the publica-

tions included in each H-index, PRRSV summed16398, PCV2 14068, SIV 16008 and the following seven viruses from 4498 to 8689. The mean number of citations for the publications included in the H-index of these ten viruses ranged from 85.75(CI95%:73.23-98.27) (ASFV) to 202.63 (CI95%:137.44-267.82) (SIV). The mean quartile ranged from 1.60 to 1.77 for 6 these viruses, except for CSFV1.26 (CI95%:1.12-1.40), SIV 1.28 (CI95%:1.12-1.43), PERV 1.40 (CI95%:1.20-1.59), and PRoV1.408 (CI95%:1.23-1.58). H-index for non-zoonotic viruses was higher compared to zoonotic ones: 70.33 (CI95%:46.85-93.81) and 58.00 (CI95%:45.63-70.37), respectively; H-index for emerging viruses were slightly higher than non-emerging ones: 67 (CI95%:41.55-92.45) versus65 (CI95%:49.36-80.64). Viruses associated with OIE listed diseases had also slightly higher H-indices than viruses who were not: 67.40 (CI95%:50.59-84.21) versus 63.40 (CI95%:46.1-80.68). All ten viruses were widely distributed (reported in more than two continents). Finally, location of first author affiliation for the 654 publications included in the H-indexes was heavily skewed: 50.3% Europe, 39.8% North America, 7.3% Asia, 1.1% Oceania, 0.9% South America and 0.6% in Africa.

Conclusions: The use of the H-index allowed assessing the importance of different viruses in porcine disease research, identifying PRRSV, PCV2 and SIV as the swine pathogens with the highest scientific productivity. In addition, obtained results pictured the economic efforts spent in swine research. These data may help objectivizing the assessment of research investment policies and definition of research interest areas.

Use of lateral flow device for safe and low cost shipment of FMDV suspected sample

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Objective: Foot-and-mouth disease virus (FMDV) belongs to the *Aphtovirus* genus in *Picornaviridae* family. This virus causes highly contagious disease of domestic and wildlife cloven-hoofed animals, characterized by appearance of vesicular lesions on hooves, mouth and udder of infected females. The Foot-and-Mouth disease (FMD) is considered to be of socio-economic importance and of significance for international trade and safety by the OIE. Therefore, rapid and accurate detection of FMDV is essential for immediate implementation of outbreak control measures. An essential step towards the global control and eradication of FMD is to identify circulating strains in endemic areas. However, the cost of sending samples due to the biological risk and shipment under freeze conditions is one of major obstacle to submission of suspected samples to Reference Laboratories. Penside tests based on immune-detection method on strip are used on field to detect FMDV. In this study, we aim to develop a low cost and safe method for shipment of FMD samples, based on the inactivation of FMDV on the lateral flow device, allowing its subsequent detection by real-time RT-PCR and recovery of live virus upon RNA transfection into cells.

Methods: FMDV strains were deposited onto penside tests (Svanodip® FMDV-Ag). Different concentrations of citric acid and sodium hydroxide solutions were tested to inactivate virus on the strip. Strips were then disassembled and grounded. Monolayer cells (IBRS-2 and ZZ-R127) were then incubated with grinding supernatant for 48 hours. A second passage in cell culture was realized in the same conditions. In parallel, viral RNA was extracted from grinding supernatant by using the QIAamp Viral RNA mini kit. Real-time RT-PCR targeting FMDV genome (3D and IRES) and the endogen cellular gene (β-actin) were performed in duplex.

Results: After treatment of “FMDV collector strips” in a 0.2% citric acid bath during 15 minutes, FMDV was found to be inactivated. Indeed no CPE was observed after two passages in cell cultures. Viral RNA was however detected by 3D and IRES real-time RT-PCR

Conclusion: After live FMDV collection onto penside strip and adequate chemical treatment, FMDV is inactivated but viral RNA is still detectable by real time RT-PCR. To determine if live virus can be rescued from treated FMDV collector strips, viral RNA transfection assays will be performed. We will finally evaluate and validate this process on field samples.

Topic: Host/virus interactions & viral immunity

Experimental infection of Houbara Bustard with avipoxvirus

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Objectives: Avipoxviruses (APV) are a serious threat for the success of reinforcement programs of endangered bird species. Avian pox, due to a wide diversity of APV, is regularly observed in conservation projects of Houbara Bustard (*Chlamydotis*spp.), despite a very strict implementation of both vaccination and biosecurity. The aim of this study is to develop an experimental model of avian pox in Houbara Bustard to study time course of infection and its impact on physiological and haematological parameters.

Methods: An APV from a cutaneous lesion of a captive-bred Houbara Bustard, previously isolated on cell cultures and characterized by P4b PCR as belonging to canarypox-like virus, subclade B2, was used as inoculum. The excised skin lesions were ground using sterile mortars and pestles in Dulbecco's Modified Eagle Medium (DMEM) containing 400 U/mL penicillin, 400 µg/mL streptomycin and 250 µg/mL amphotericin B. The supernatant was collected after centrifugation and quantified by quantitative PCR. A ten-fold dilution was used as inoculum. Twelve 2 months old captive-bred Houbara Bustard were divided in 2 groups: control (n=3) and infected (n=9). Birds were challenged by 10 needle pricks in the proximo-lateral part of the tarsometatarsus of both legs with the inoculum (infected birds) or with DMEM alone (control birds). For 3 months, birds were controlled on daily basis to assess development of lesions. Weight and food consumption were monitored and blood was collected once a week. Sample lesions were histologically analyzed and their viral DNA titre was assessed by quantitative PCR. White blood cell counts and plasmatic protein electrophoresis were performed from collected blood samples. Significances of data were calculated using a linear mixed model.

Results: Seven out nine infected birds developed at least one lesion and none control bird displayed lesions suggestive of avian pox. The 12 lesions were all localized in the inoculation site: 10 didn't reach more than the stage of papule but 2 evolved until the classical stage of scabby mass. Histological analysis of 6 collected lesions showed massive secondary infection but few cytopathic lesions and characteristic intracytoplasmic inclusions were seen. However, viral DNA was detected in all collected lesions. Mean incubation time was 39 days, which is much longer than most of previously incubation times reported. Statistical analyses showed that APV infection had a significant negative effect on weight and food consumption whereas, among haematological parameters, only basophils and alpha-2 globulins were significantly influenced by APV infections (positively and negatively, respectively).

Conclusion: An experimental model of avian pox in Houbara Bustard was developed and offers further opportunities of research on avipoxviruses. We showed that incubation times are surprising long and should be considered for epidemiological studies. Despite the observation of mainly small lesions, physiological and haematological changes shown that even sub-clinical infections can be deleterious for Houbara Bustard.

The invasion mechanisms of infectious laryngotracheitis virus (ILTV) in the respiratory and conjunctival mucosa

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Objective: To evaluate quantitatively the replication characteristics of infectious laryngotracheitis virus (ILTV) in *in vitro* models of chicken tracheal and conjunctival mucosa explants (organ) and to analyze cell viability of infected mucosal explants (organ).

Methods:

Ciliary beating and viability analysis by TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) assay

The tracheal and conjunctival mucosa explants (organ) were placed on fine-meshed gauze and maintained for 96h at an air-liquid interface. A viability analysis was performed using light microscopy (cilial beating) and fluorescence microscopy (TUNEL-positive cells).

Inoculation of ILTV and evaluation of mucosal spread

The mucosal explants were inoculated with ILTV. ILTV replication kinetics was quantitatively evaluated by measuring plaque number, plaque latitude and invasion depth underneath the BM in tracheal and conjunctival mucosa explants (organ).

Cell viability in ILTV infected tracheal and conjunctival mucosa

The TUNEL assay was performed to detect apoptotic cells in infected tracheal and conjunctival mucosa explants (organ).

Results:

Ciliary beating and Viability of tracheal and conjunctival mucosa explants

The ciliary beating of the tracheal explants was normal up till the end of the study (96h of cultivation). The viability of the tracheal and conjunctival mucosa explants (organ) was assessed based on the percentage of TUNEL-positive cells. We did not observe a significant increase in the number of TUNEL-positive cells at 0, 24, 48, 72 and 96h of *in vitro* cultivation for both the epithelium and the lamina propria.

ILTV interactions with tracheal and conjunctival mucosa, and evaluation of mucosal spread

Inoculation of chicken tracheal and conjunctival mucosa explants (organ) with ILTV led to the formation of viral antigen positive plaques (group of closely connected cells) in the mucosa. At 0h, no infection was observed. At 24, 48 and 72h pi clear ILTV infected plaques were found in the tracheal and conjunctival mucosa. Dissemination kinetics of plaques in the mucosa was evaluated by measuring the plaque number, maximal plaque latitude and invasion depth underneath the BM at 0, 24, 48 and 72h pi. The number of plaques slightly increased in time. The plaque latitude increased steadily over time from 0 to 72h pi. In the tracheal mucosa the latitude increased from 29.4 ± 9.0 µm (24h pi), to 55.4 ± 5.6 µm (48h pi) and 70.4 ± 12.9 µm (72h pi). In the conjunctiva increased from 17.2 ± 3.4 µm (24h pi), to 59.8 ± 5.3 µm (48h pi) and 97.8 ± 9.5 µm (72h pi).

Cell viability in ILTV infected tracheal and conjunctival mucosa

After inoculation with ILTV, a large number of cells were ILTV infected. A small number of TUNEL-positive cells were observed in both regions of ILTV-infected and non-infected cells. In ILTV-infected regions, TUNEL-positive cells were usually observed in the vicinity of ILTV-infected cells. ILTV-infected cells were mostly not TUNEL-positive. In tracheal and conjunctival epithelium, the percentage of ILTV-positive cells that were TUNEL-positive ranged from 0.4 % at 24h to 1.4% at 72h pi.

Conclusion: Even though *in vivo* laboratory animals are the best system for studying host-pathogen interactions, physiological inter-individual differences and different environmental conditions are important drawbacks. The use of an *in vitro* culture offers the opportunity to study host-pathogen interactions under more controlled conditions. In cell cultures, cell-cell and cell-extracellular matrix interactions are reduced due to the lack of a three-dimensional architecture of the culture. *In vitro* explant (organ) cultures are excellent alternative models that mimic natural conditions. The *in vitro* explant (organ) cultures are in line with the three Rs principle, i.e. Reduction (reduction of number of animals), Replacement (no use of living animals) and Refinement (minimizing the pain). A major advantage of the *in vitro* explant (organ) model is that explants of the same animals can be used to compare different viral strains.

To our knowledge, there are no reports on the viability of cells in the epithelial layers, lamina propria and underlying connective tissue of chicken tracheal and conjunctival explants. Hence, in this study the viability of epithe-

lial cells and cells in the lamina propria of tracheal and conjunctival explant culture were evaluated by quantifying TUNEL-positive cells at 0, 24, 48, 72 and 96h of *in vitro* cultivation. The TUNEL-positive cells remained below 1.5% up to 96h of cultivation in both epithelium and lamina propria of tracheal and conjunctival explants. Thus, we can state that tracheal and conjunctival explants were successfully maintained for at least 96h in culture at an air-liquid interface without significant changes in tissue viability.

The chicken tracheal and conjunctival explants were susceptible to an ILTV infection. The purpose of the present study was to analyze the behavior of ILTV in the mucosa by immunofluorescence and mathematical quantitative analysis using confocal microscopy and software Image J. By doing this, a complete three-dimensional picture of the horizontal and vertical spread of ILTV in the mucosa was obtained. This novel approach is an ideal tool for studying cellular and molecular aspects of the invasion mechanisms of pathogens. It allows a thorough comparison of different alphaherpesvirus replication kinetics in their respective species. The ILTV induced plaques were present in the epithelial layer starting from 24h pi. The plaque latitude increased in time. The ILTV infected plaques started to cross the basement membrane from 48h pi.

When comparing replication kinetics of ILTV in trachea and conjunctiva, we observed some interesting findings. At 72h pi, the plaque latitude in the conjunctival mucosa was significantly larger compared to that in the tracheal mucosa. The percentage of the plaques that penetrated through the BM was larger in conjunctiva (48h pi: 43% and 72h pi: 74%) compared to the trachea (48h pi: 31% and 72h pi: 56%). Plaque latitude and invasion depth kinetics indicated that at later time points (48 and 72h pi) ILTV showed a more extensive replication in conjunctival mucosa compared to the tracheal mucosa. The differences in the replication kinetics of ILTV are likely due to variation in its tropism for trachea and conjunctiva.

In BHV1, SHV1 and HSV1 infection, the lateral mucosal spread and vertical invasion depth evolved similarly with increasing time pi. In the case of ILTV, the invasion through the BM was very restricted. At 48h pi 30.9% of the plaques in trachea and 43.3% of the plaques in conjunctiva were crossing the BM. This finding is in contrast with plaques induced by SHV1, HSV1 and BHV1. With SHV1 and HSV1, 100% of the plaques crossed the BM at 24h pi. With BHV1, 90% of the plaques went through the BM at 48h pi. ILTV may not have developed enough tools to breach quickly through the BM like BHV1, SHV1 and HSV1. The restricted ILTV invasion through the BM agrees with the *in vivo* pathogenesis of ILTV, where no clear evidence exists for viremia. Otherwise, invasion of ILTV through the BM of trachea and conjunctiva is evidently corroborated. Indeed, it was recently reported that ILTV in most of the internal organs of infected chickens.

The effect of ILTV on the cell viability of tracheal and conjunctival mucosa was analyzed by a TUNEL assay. A large number of epithelial cells were ILTV-positive. But, only a few ILTV-infected cells were TUNEL-positive, even at 72h pi. Our studies are consistent with previous reports demonstrating that low levels of apoptosis were observed in cells infected with other alphaherpesviruses HSV2-, SHV1- and HSV1. Further, TUNEL-positive cells were predominantly restricted to the vicinity of ILTV-infected cells. These TUNEL-positive cells might be local leukocytes that respond to the productive ILTV infection, as seen in SHV1 and HSV2 or epithelial bystander cells that are resistant to infection but die due to the changes in their cell contacts with the infected cell. Absence of apoptosis in infected cells may be an ILTV strategy to sustain infection in the mucosa and to allow the virus to breach the BM and to reach the lamina propria. By doing this, the virus may reach sensory nerves of the trigeminal ganglion, the site of latency. All of these mechanisms help ILTV to sustain life-long infection.

Topic: Influenza

Assessment of the immunosuppressive effect of H9N2 virus infection in SPF chicken

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Objective: H9N2 Avian influenza virus (AIV) is of great interest for the poultry industry in which the virus infection is a major cause of economic losses. This virus is massively circulating in the poultry population of the whole Asia and it affects poultry also in Northern Africa, and, more sporadically, in Europe. Therefore further study to

explain the virus pathogenesis and to investigate immune responses is necessary. In this study, pathogenicity, tissue dissemination of H9N2 A/chicken/Israel/1163/2011 in various organs, shedding and the early immune response in H9N2 inoculated specific pathogen free (SPF) chickens were examined. Furthermore, to investigate whether if infection with H9N2 virus might have immunosuppressive effect i.e. if it could suppress the secretion of IFNγ, a set of ex vivo stimulation experiments was done using splenocytes and peripheral blood mononuclear cells and different mitogens.

Methods:

Avian influenza virus and chicken infection

A/chicken/Israel/1163/2011 H9N2 was kindly supplied by Dr. Irit Davidson, Kimron Veterinary Institute. Eighty eight 4-week-old SPF chickens were divided equally and randomly into two groups: forty four chickens in experimentally infected group and forty four chickens in negative control group. In experimentally infected group, chickens were inoculated intranasally- intraocularly with the virus at the dose of 10^6 EID_{50}. Samples of organs (spleen, kidney, lung, duodenum) and cloacal and trachea swab were aseptically collected at 2, 3, 4, 5 and 10 day post infection (DPI) for virus dissemination. Samples of spleen and blood were also collected at 2, 3, 4 and 5 dpi for the early immune response investigation. H9N2 inoculated chickens were monitored daily for morbidity and mortality.

Detection of viral genomic RNA by RRT-PCR

A reverse transcriptase polymerase chain reaction (RT-PCR) test was performed for virus detection in organs and swabs. RNA extraction was done by using MagMax Total RNA Isolation kit as described by the manufacture. RT-PCR was performed with the AgPath –ID One-Step RT-PCR kit and Lightcycler 480 (Roche).

Seroconversion

Sera were collected at 10 dpi and tested by HI test according to OIE standard procedures (OIE, terrestrial manual), using 4 HA units of homologous virus per well. The titer of a respective serum sample is defined by the highest dilution conferring complete inhibition of hemagglutination. Titers <4 log2 were considered negative, and titers ≥4 log2 were considered positive. The geometric mean titers were expressed as reciprocal log2.

Measurement of AIV specific cell-mediated immunity by AIV recall on splenocytes and peripheral blood monocyte cells (PBMC)

Splenocyte preparation with Pasteur pipette.

Spleens were aseptically removed just after sacrifice and squeezed with a 5-ml syringe plunger to extrude cells. Cell suspensions were filtered through 70 µm nylon (Falcon BD Bioscience, Belgium) before being washed three times in RPMI 1640 medium (Invitrogen, Belgium). Splenocytes were adjusted to 10^7 cells/ml and 100 µl of this suspension were transferred into flat-bottomed 96-well plates (Nunc).

PBMC preparation with Pasteur pipette

Peripheral blood of infected and non-infected chickens were collected into Falcon tubes containing heparin with the final concentration of 1000U/ml and mixed well. The peripheral blood was then incubated for 4 hours at 39°C in vertical position. PBMCs were collected by Pasteur pipette before being washed three times with RPMI 1640 medium. Finally, the cells were adjusted to 10^7 cells/ml and 100 µl of this suspension were transferred into round-bottomed 96-well plates (Nunc).

Contact with mitogens

Different mitogens (PMW, ConA, PHA and IL-2), previously determined as optimal concentration – 10 µg/ml, were prepared in medium containing inactivated foetal calf serum (iFCS). These mitogens were added, in duplicate, with 10^6 splenocytes or PBMC from experimentally infected chickens or negative control chickens. Non-activated lymphocyte control received 100 µl RPMI 1640 medium instead of mitogens. After 36 h or 72 h of incubation at 39°C, cell supernatant was removed from each well and frozen at -20°C until the time of assay.

ELISA ChIFNγ

MaxiSorp Nunc-Immuno F96 microwell plates were coated with anti-Mouse Ig ChIFNγ 1E12 2µg/ml in 1hour at 37°C. Then the plates were washed three times with PBS supplemented with 0.1% of Tween 80. Plates were blocked for 30 min at 37°C with PBS containing 2.5% casein and then incubated with samples for 1h at room temperature. Biotin-labeled mouse antibody directed against chicken IFN gamma (Mouse Ig anti- ChIFNγ 1D12 – BIOT) at dilution 1:10000 was then added for 1 hour at room temperature. Plates were incubated with Streptavidin – Horseradish peroxidase conjugate for 1 hour at room temperature. After six washings, peroxidase activity was revealed by adding 100µl of TMB/H2O2 for 15 min in darkness before stopping reaction with 1M H3PO4 buffer. Optical density was determined at 450nm-620nm with an ELISA reader.

Results:

Virus virulence and replication in chicken

There was no death during the experimental course.

The clinical signs observed in the inoculated chickens were red eyes and excessive lacrimation. These signs were visible from day 2dpi with 9/9 chickens showed red eyes and 2/9 chickens showed excessive lacrimation. The most remarkable clinical signs were observed at 4 dpi and remained at 10 dpi.

The lesions such as hemorrhage in liver and swelling spleens were observed in inoculated group. There was only one case of liver hemorrhage at 2 dpi. The swelling spleens were detected at 5 dpi with 6/9 chickens and at 10 dpi with 5/9 chickens.

The presence of virus was checked by RRT-PCR in all samples obtained from inoculated chickens at different dpi.

The predominant infection in respiratory tract was observed between 2 dpi (trachea swabs)/3 dpi (lungs) and 5 dpi. In the urinary tract, predominant infection was also observed between 2 and 5 dpi. In digestive tract, the virus was detected from day 2 pi and became predominant between 3 dpi and 5 dpi. The H9N2 virus shows its pneumotropism, intestinotropism and nephrotropism following intranasal inoculation through the virus detection from trachea swabs, lungs, duodenum and kidneys. The presence of viral RNA in spleens could reveal virus tropism for immune system. On 5dpi the number of infected chickens and also their organs with positive virus detection were more than the other experimental day

This study showed that H9N2 virus has multi-organic tropism: the respiratory system (lung and trachea swab), immune system (spleen), urinary system (kidney) and digestive system (duodenum).

Seroconversion

All 8 remaining H9N2 inoculated chickens seroconverted with a mean titre of 5.9 with homologous antigen.

Measurement of AIV specific cell-mediated immunity by AIV recall on splenocytes and peripheral blood monocyte cells (PBMC)

PMW, ConA, PHA and IL-2 were used to activate slenocytes and PBMC from H9N2-infected chickens and negative chickens group at 2, 3, 4 and 5 dpi and ChIFNγ production was measure by ELISA. At 2 and 3 dpi, cells from the negative group chickens were not activated by the mitogen so that the results were excluded from further antigen-activation analysis.

At 4 and 5 dpi, ChIFNγ was produced by splenocyte and PBMC from each chicken after mitogenic activation (O.D $\geq$ 0.1; S.I $\geq$ 2), validating the splenocyte activability. There was only weak proliferative response measured by the level of ChIFNγ in the infected chickens while high proliferation of mitogen-activated splenocytes and PBMC was observed in the negative control chickens. This result suggested the immunosuppressive effect of H9N2.

Presence of NDV in H9N2 viral stock

Incidentally the initial H9N2 virus stock was found to be contaminated with Newcastle disease virus (NDV).

The replicates of 10-fold of NDV were prepared to build up a standard curve to quantify the amount of NDV present in the initial infection stock. Based on the standard curve, the contamination was determined as 1.35 x $10^{0.5}$/infection dose for each chicken.

Six animals out of all experimentally infected chickens showed excretion via cloacal route at different dpi (one at 2dpi, three at 3 dpi and 2 at 10 dpi). However, no seroconversion was detected on the experimentally infected chickens.

Conclusion:

The clinical and virological data from this study confirmed the mild virulence of the H9N2 A/A/chicken/Israel/1163/2011/ virus for SPF chickens and the multi-organic tropism of the virus with detection of viral RNA at the level of the spleens and kidneys in all inoculated chickens. We developed a model to assess immunosuppressive effect of H9N2 infection in chickens. Immunosuppressive ability of virus strain was recorded for splenocytes at 4, 5 dpi and PBMC at 5 dpi. This result will be confirmed with complementary analyses in vitro, infecting splenocytes and PBMC cultures with the H9N2 virus and monitoring the immune response.

Establishment of a veterinary Biobank at Istituto Zooprofilattico Sperimentale delle Venezie

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Objective: A biobank is a type of biorepository that stores biological samples for their use in research. Biobanks traditionally store samples of human origin. In the years such infrastructures have become a significant resource for medical research as the availability of biological samples have supported many types of studies in important research fields such as genomics. The scope of biobanks is to provide the scientific community access to scientific material and data. The added value of storing samples in a biobank is that data derived from those samples can often be used by multiple researchers for multiple purposes. This significantly facilitates the sharing of data and resources.

The World Health Organization (WHO) states that, over the last decade, approximately 75% of the new diseases in humans were caused by pathogens from animals or animal products. Additionally globalisation has led to an extensive and rapid distribution of animals and animal products contributing to the spread of pathogens. This highlights the importance of investigating the role of the animal reservoir in maintaining and spreading such pathogens. In this view, a Veterinary biobank may enable the storage of samples useful for cross-species disease surveillance, to investigate cross-species disease transmission through comparative medicine and environmental research.

The Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) is part of a national network that consists of nine other Italian public veterinary Institutes (IZS).Each institute has gained experience in diagnostic testing for specific diseases that led to the recognition of National reference laboratories.

The IZSVe has 7 national reference laboratories (Avian influenza and Newcastle disease; Rabies; Salmonellosis; Beekeeping; Fish, crustacean and mollusc pathology; Diseases at the animal-human interface; Animal Assisted Interventions). The IZSVe has also 7 international reference laboratories (OIE reference laboratory for avian influenza and Newcastle disease; FAO reference centre for animal influenza and Newcastle disease; OIE reference laboratory for viral encephalopathy and retinopathy of marine fish; OIE reference laboratory for salmonella; FAO reference centre for rabies; OIE collaborating centre for epidemiology, training and control of emerging avian diseases; OIE collaborating centre for diseases at the animal-human interface). Surveillance activities, scientific research and provision of diagnostic testing facilities at national and international levels have resulted in collection and storage of invaluable biological materials. Storage of biological materials is one of the mandates of a reference laboratory and is the baseline to develop reference material in accordance with OIE requirements and to distribute to national laboratories biological reference products and any other reagents used in the diagnosis and control of the designated pathogen.

The organized collection of biological materials of animal origin has therefore multiple implications nowadays in the research and diagnostic fields. For these reasons the establishment of a network of veterinary biobanks was promoted at a national level by the OIE Collaborating Centre for cell cultures at IZS of Lombardia and Emilia Romagna involving 4 other IZS including IZSVe. Each IZS was involved in the network making available biological materials collected and stored by reference laboratories.

Since 1997 several outbreaks of Avian Influenza (AI) have occurred in the North East part of Italy affecting both industrial and rural poultry farms. The diagnosis of AI outbreaks carried out by the AI national and international reference laboratory allowed the collection of relevant samples throughout the years in a longitudinal manner. This led to an intensive research activity and resulted in analysis, publishing and dissemination of relevant epizoological data to the international scientific community. Part of invaluable samples, collected during AI outbreaks, is now part of IZSVe Biobank.

The aim of the IZSVe Veterinary Biologicals Biobank is to collect information from the OIE Reference Centres on what reference materials they collect, produce and supply. The ultimate aim is to create a biobank of correctly identified, quantified and stored materials.

Methods: The national and international reference laboratories for Avian Influenza and Newcastle disease were selected as reference laboratories present at IZSVe for the establishment of the veterinary biobank. The first step was the identification of an organizational structure to define and direct the allocation, coordination and supervision of tasks for the achievement of the goal. The organizational structure was based on the establishment of a workgroup for the entire project. The workgroup was composed by a veterinarian and skilled laboratory technicians, all with a virology background. The workgroup had the aim to coordinate the identification, collection and storage of biological materials to be included in the biobank with staff of reference laboratories. The veterinarian was assigned the role of project coordinator. Her main duties were: identify short-term goals, coordinate directly with head of reference laboratories involved and allocate tasks to members of the workgroup. Additionally she was responsible for the management and maintenance of activities defined by the national network.

Viruses included in the Biobank were selected according to the following criteria: strains involved in relevant outbreaks, presence of epidemiological information and sequence data deposited in public databases. Viruses undergone quality control analysis to ensure the absence of the most common viral pathogens included in differential diagnosis. Additionally all viruses were tested for eubacteria and mycoplasma spp. For each strain 3 different batches were generated: one master, one working and one back up batch. The master batch is used to produce the working batch and the latter is used to grant requests from clients. The backup batch, stored in a different location from the master and working batches, is maintained as a substituted batch in reserve. Each vial is identified univocally and correctly traced along the process using a barcode system. A sample is never discarded from the biobank and its liability and validity is routinely controlled. An *ad hoc* software was developed for the management of the biobank and a dedicated website was created to manage requests of biological materials.

Results: The IZSVe biobank is a member of a national network of veterinary biobanks. Such network is composed by 5 IZS each sharing biological materials of respective National and International reference laboratories. The Italian network of veterinary biobank was recognized as the OIE Collaborating Centre for Veterinary Biologicals Biobank in May 2014 during the 82th OIE General Session.

The storage of biological materials is a routine activity for Reference laboratories, and therefore they have always had the goal of routinely sharing their specimens with other laboratories. However, the establishment of veterinary biobank at IZSVe included in a national network with other veterinary biobanks and recognized by OIE has promoted and facilitated the adoption of standard operating procedures for collection, storage and quality assurance of biological materials.

Conclusion: The availability of a biobank represents a change in the nature of IZSVe research. The aim of IZSVe biobank is to progress beyond single-center research centers to a qualitatively different next-generation research infrastructure. The existence of a veterinary biobank renders important information available to the scientific community.

The veterinary biobank would assist OIE to expand its standardisation programme to evaluate and adopt more widely the reference materials that OIE Laboratories are mandated to develop.

Additionally the development of a quality management system according to ISO 9001:2008 is in progress to ensure the adequacy of services provided by the biobank.

The H5N1 HPAI crisis has demonstrated how relevant is to approach correctly public health threats at the interface between humans and animals in different ecosystems. The availability of veterinary biobanks will certainly aid to address relevant animal and public health issues globally. Additionally, the establishment of a veterinary biobank could promote intersectional collaborations and networking in the scientific community and eventually may contribute to render operative One health in policy and practice.

The Italian veterinary biobank network with its activities translates this approach as a new and fundamental paradigm at national level. In this view veterinary biobanks considered as a global public health good need support nationally and internationally.

Stronger interference of Avian Influenza than Newcastle Disease Virus specific maternal derived antibodies with a recombinant NDV-H5 vaccine

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Objective: Maternal derived antibodies (MDA) are known to provide early protection from disease but also interfere with vaccination efficacy of young chicks. This interference phenomenon is well described in the literature for viral diseases like Infectious bursal disease (IBD), Newcastle disease (ND) and avian influenza (AI). The goal of this work was to investigate the impact of H5- and/or Newcastle disease virus (NDV)-MDA on the vaccine efficacy of a recombinant NDV-H5-vectored vaccine (rNDV-H5) against two antigenically divergent highly pathogenic avian influenza (HPAI) H5N1 challenges. Beforehand the expression of the H5 protein at the surface

of the recombinant vaccine has been confirmed with immunogold electron microscopy and ELISA. A SPF model where passive immunity had been artificially induced by inoculations of H5 and NDV hyperimmunized polysera, respectively, was used to distinguish between the respective effects of the H5- and NDV-MDA.

Methods:

Vaccine and HPAI H5N1 challenge strains

The experimental rNDV-H5 vaccine was provided by Lohmann Animal Health GmbH and was developed by the research group of Professor P. Palese at the Mount Sinai School of Medicine. The rNDV-H5 vaccine is a NDV La Sota vector expressing a modified H5 ectodomain from an HPAI A/Vietnam/1203/2004 H5N1 clade 1 strain.

Two different HPAI H5N1 challenge strains were used: A/crested-eagle/Belgium/01/2004 (clade 1) H5N1 (Crested Eagle 2004) virus and the A/chicken/Egypt/1709-6/2008 (Egypt 2008) H5N1 viruses (clade 2.2.1). The Egyptian strain was kindly provided by M.M. Aly of the Animal Health Research Institute, Giza, Egypt.

Immunoelectron microscopy

Virions purified by differential centrifugation were brought on pioloform- and carbon-coated, 400 mesh copper grids by the grid-on-drop method. The grids were pretreated with a 1% Alcian blue 8G solution to introduce positive charges on the grid surface. Five grids were incubated with purified rNDV-H5, washed and incubated with PBS containing 2% goat serum to prevent non-specific bindings. To perform immunogold labeling, each grid was incubated with one of the following primary antibodies: 10F2 (anti-F monoclonal antibody (mAb)), 7D4 (anti-HN mAb), 8C5 (anti-H5 mAb), 5A1 (anti-H5 mAb) and an anti-IFNγ mAb as negative control. After washing with PBS and incubation in PBS containing 2% goat serum, grids were incubated with goat anti-mouse IgG conjugated with 10 nm gold beads as secondary antibody. After a wash with PBS and a final wash with distilled water, immunogold labeled virions were imaged in bright-field TEM mode using a Tecnai G^2 Spirit electron microscope with Biotwin lens configuration operating at 120 kV.

Homologous clade 1 challenge

Six groups (G1 to 6) were included in this study. G1 and 2 were composed of SPF chickens. The G3 and 4 were composed of NDV-MDA- chickens and G5 and 6 composed of NDV-H5-MDA chickens. Groups 1, 3 and 5 received 10^6EID_{50}/inoculum (inoc) of the rNDV-H5 vaccine by a combination of oculonasal (ON) and drinking water/oral (DW) route, at day old (D1) and at 2 weeks of age (D14). G2, G4 and G6 were left unvaccinated as challenge controls. All groups were ON challenged with 10^6EID_{50}/inoc of the clade 1 Crested eagle 2004 HPAI H5N1 strain one week after the second vaccination (1wp2v). After challenge, birds were monitored for clinical signs, mortality and viral excretion.

Heterologous clade 2.2.1 challenge

Eight groups (G1 to G8) were included in this study: two groups of SPF birds (G1 and 2), two groups of natural NDV-MDA (NDV-nMDA) birds (G 3 and 4), two groups of artificially induced NDV-MDA (NDV-aMDA) birds (G5 and 6) and two groups of artificially induced H5-MDA (H5-aMDA) birds (G7 and 8). Groups 1, 3, 5 and 7 were ON-vaccinated at day 2 (D2) and D14 with 10^6EID_{50}/inoc of the rNDV-H5 vaccine. Groups 2, 4, 6 and 8 were left unvaccinated as challenge controls. All groups were ON-challenged at 1wp2v with 10^6EID_{50}/inoc of the clade 2.2.1 Egypt 2008 HPAI H5N1 strain. After challenge, birds were monitored for clinical signs, mortality and viral excretion.

Results:

Glycoprotein expression and their distribution at the surface of the rNDV-H5

Expression of the chimeric H5 glycoproteins (gp) at the surface of the rNDV-H5 vaccine was confirmed by immunogold labelling. rNDV-H5 purified virions were specifically labelled with the anti-H5 mAb (8C5 and 5A1), confirming the expression of the H5 gp at their surface. Anti-F and anti-HN mAb indicate that the virions also expressed the NDV gps at their surface. These results were not the consequence of any aspecific binding of the mAb, since the recombinant virions incubated with ant-IFNγ mAb did not shown gold particles at their surface.

Effect of NDV and NDV-H5-MDA on rNDV-H5 vaccine efficacy following a homologous HPAI AsH5N1 challenge

After a homologous HPAI challenge performed at 3 weeks of age, all unvaccinated SPF and NDV-MDA control birds died at 2 day post infection (dpi) while a delayed mortality, starting at 5 dpi, and complete at 15 dpi was observed in the unvaccinated NDV-H5-MDA control group. The rNDV-H5 vaccination conferred 68% protection

in SPF birds, an unexpected full protection in NDV-MDA birds and only 25% protection in NDV-H5-MDA group. The clinical signs were higher in vaccinated NDV-H5-MDA birds compared to the vaccinated SPF group and were inexistent in the vaccinated NDV-MDA birds. Results of the excretion follow-up strengthened the observed clinical sign and mortality datas.

Effect of natural and artificial NDV-MDA and artificial H5-MDA on rNDV-H5 vaccine efficacy against a heterologous HPAI AsH5N1 challenge

Chicks with an artificial-induced passive immunity after specific polyserum inoculation were used to evaluate the distinct impact of the NDV- and H5-MDA on the rNDV-H5 vaccine efficiency. In order to confirm the reliability of this artificial MDA (aMDA) model, vaccination/challenge experiment was realized in parallel in commercial chickens presenting natural NDV-MDA.

After a heterologous HPAI challenge performed at 3 weeks of age, all unvaccinated SPF, NDV-nMDA and NDV-aMDA control birds died at 3 dpi and at 4 dpi for the unvaccinated H5-aMDA control group. In SPF chickens vaccination induced no protection but only delayed full mortality at 7dpi while all vaccinated H5-aMDA birds died at 4 dpi like their unvaccinated control group. In contrast, the rNDV-H5 vaccination conferred a significant 50% protection in the NDV-nMDA group and 25% among NDV-aMDA birds. Results of the excretion follow-up strengthened the observed clinical sign and mortality datas.

Conclusion: For the first time it was shown that depending of the types of MDA, their effect on an rNDV-H5 vaccination can be completely different. The H5-MDA seems able to neutralize rNDV-H5 vaccines and block any protective capacity while NDV-MDA increase its protective potential. In the light of these results, in countries performing routine vaccination against NDV and prohibiting AIV vaccination, the rNDV-H5 vaccine combined with the high prevalence of NDV-MDA in progeny would be a valuable vaccine candidate in case of emergency vaccination. However, in countries with high H5 seroprevalence, delivery at young age should be performed after the waning of passive H5 antibodies.

The adaptation of avian influenza viruses to the respiratory epithelium of pigs

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Objectives: Pigs are important hosts for influenza A viruses and may play a crucial role in the interspecies transmission. Primary target cells for influenza viruses are cells of the respiratory epithelium. In *vitro* studies with differentiated respiratory epithelial cells are possible, for example by using air-liquid interface cultures or explant cultures. Recently we have established precision-cut lung slices from the porcine lung as a culture system for differentiated respiratory epithelial cells. In precision-cut lung slices, the differentiated epithelial cells are maintained in their original setting. Avian influenza viruses of the H9N2 subtype have been circulating worldwide in multiple avian species and have repeatedly infected mammalians to cause disease. The continued avian-to-mammalian interspecies transmission of H9N2 viruses raises concerns about the possibility of viral adaption with increased virulence for humans and poses a potential health risk to the public. Here we used precision-cut lung slices as a culture system to compare the infection of respiratory epithelial cells by avian influenza A viruses and recombinant viruses. As differentiated repiratory epithelial cells are the primary target cells for influenza virus infections, precision-cut lung slices provide an interesting system to analyze the adaptation of avian influenza viruses to the respiratory epithelium of pigs.

Methods: Avian influenza viruses of the H9N2 subtype were subjected to several passages in precision-cut lung slices. The changes in the viral properties that are associated with the adaptation process were characterized by analyzing: (1) duration of the growth cycle; (2) amount of infectious virus released into the supernatant; (3) extent of the ciliostatic effect. Sequence analysis revealed which amino acid changes occurred during the different virus passages.

Results: Adaptation of the avian viruses to growth in porcine cells was evident in a shortening of the growth

cycle. Sequence analysis revealed that few amino acid changes occurred during the different virus passages. The importance of the individual mutations has been analyzed by generating recombinant viruses that contain the respective mutated proteins. The functional importance of individual mutations will be reported.

Conclusion: Our study helps to understand the processes involved in the adaptation of H9N2 influenza viruses to new hosts.

Whole genome sequencing of influenza A viruses in pigs using Next Generation Sequencing

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Objectives: Pigs are susceptible not only to swine influenza (SIV) but also to human and avian influenza viruses. An ongoing monitoring study aims to identify SIV subtypes circulating in the Swedish pig population and to detect SIV transmissions between pigs and humans, such as those living on pig farms, farm workers and veterinarians, in order to determine new variants as early as possible. The objective of this part of the study was to evaluate the possibility to use a Next Generation Sequencing strategy in combination with an RT-PCR which amplifies all eight genomic RNA segments, to rapidly sequence the complete genome and to detect new variants.

Methods: Ten participating farms were visited by field veterinarians every second or third week during human influenza season in Sweden. Nasal swab samples were collected from 15 pigs from each farm at visits. Viral RNA was extracted from the swabs and screened for influenza A virus by a real-time reverse transcription PCR (rRT-PCR) selective for the matrix gene. Samples shown to be positive by rRT-PCR were further amplified with a multisegment reverse transcription PCR that simultaneously amplifies all eight genomic RNA segments of any influenza A subtype. PCR products were analyzed by gel electrophoresis and purified before concentrations were measured. Libraries for sequencing were prepared using the Nextera XT Preparation Kit. Quantity and quality of the libraries was analyzed by the Agilent Bioanalyzer before pooling and sequencing on Illumina MiSeq.

Data analysis was performed with CLC Genomics Workbench. FASTQ files were trimmed using quality criteria and mapped to reference genes downloaded from GenBank.

Results: Active surveillance From December to April participating farms were visited six times by field veterinarians. No clinical sign of disease were observed in the pigs during this period. From a total number of 1475 swabs collected, 90 (6%) samples were positive for influenza A viruses with rRT-PCR. Eighty-five (93%) of these influenza A positive samples were found to be pandemic influenza pH1.

Out of ten participating farms, five farms had at least one positive result during this period and four farms were tested positive in at least two occasions. The molecular characterization and phylogenetic analysis of the newly isolated viruses revealed that they possess pandemic H1N1 lineage HA and internal genes. However the NA gene was closely related to H1N2 SIV strains, previously isolated in Swedish pig population.

Conclusion: The results of this study support the application of Next Generation Sequencing for epidemiological studies of swine influenza viruses. We could successfully amplify viral RNA from clinical samples and through deep sequencing extract the sequence of the complete genome with good genome coverage and sequence quality sufficient for detection of clinically significant viruses and furthermore trace new variants.

In the last five years two new influenza A viruses were detected in the Swedish swine population. Both of these viruses were the result of multiple reassessments between avian or/and human and swine influenza A viruses. In the present study the absence of any clinical signs in examined pig farms with pigs harboring influenza A virus highlights the importance of active surveillance to reduce the risk for zoonotic transmission to humans in close contact with pig herds.

Topic: Respiratory viruses

Distribution of equine herpesvirus type 1 (EHV-1) in the tissues of aborted fetuses

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Objective: Equine herpesvirus type 1 (EHV-1) is one of the most common pathogen in horses, causing both sporadic and epidemic abortions. The purpose of this study was to present a practical application of a real - time PCR for rapid detection and quantitation of EHV-1 DNA load in different tissue samples of aborted fetuses.

Methods: Sixty nine tissue samples (lung, liver, spleen, heart, kidney, thymus, placenta) from 23 aborted fetuses, suspected to EHV-1 infection submitted to the Department of Virology of the National Veterinary Research Institute in Pulawy were tested. A real-time Taq Man PCR targeting the conserved region (glycoprotein B gene) was applied. For the evaluation of the detection limit of quantitative PCR – eight 10-fold dilutions of the synthesized fragments of EHV-1 gB gene DNA was used in triplicates to match the standard curve.

Results: The detection limit of the real-time PCR assay was 6.0 x 10^0copies of the standard DNA, corresponding to the average Ct value 37.0 (SD 0.84) and obtained standard curve exhibited a linear range from 10^0 to 10^7 molecules. Sixteen out of twenty three aborted fetuses (69.5%) were positive for EHV-1 in real-time PCR. The lowest mean Ct values which suggested the highest DNA load were obtained for liver (15.7) and lung (18.2) samples, whereas the lowest concentration of EHV-1 was detected in thymus (29.6) and placenta (28.4).

Conclusion: Tissues of aborted fetuses originated from lung, liver and spleen should be considered the best choice for EHV-1 infection diagnostic in aborted fetus regarding the high frequency of positive real-time PCR results and high mean Ct value suggesting high viral DNA loads in those tissues.

Earlier and easier diagnostic tools for PRRSV herd management: Comparison of sampling and prevalence under field conditions

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Objective: Real time RT-PCR (rtRT-PCR) and ELISA tests are often used to assess the PRRSV infection status of pig herds. rtRT-PCR tests on blood/serum and tissue samples are the most used technique to detect earlier PRRSV. Recently, the detection of many pig pathogens in orals fluids was reported as an alternative technique being more economical, easier and less invasive. The main goal of several studies was to validate oral fluid against blood/serum and tissue and also establish a sampling recommendation for oral fluids under field conditions for an earlier diagnostic of PRRSV with an easier sampling method.

Methods: Thermo Fisher Scientific requested several laboratories and research institutes throughout the world to evaluate the rtRT-PCR tools on over 800 field samples from different genotypes. A field study in Spain allowed evaluation of the performance of the kit on oral fluids samples. Results per pen are compared to individual results (blood/serum). Based on these results, a biostatistical/epidemiological study was launched to calculate the probability of genotype 1 and 2 virus detection in a pen using oral fluid samples taking into account the prevalence of PRRSV in serum as an independent variable. In all these studies, sample extraction was carried out with the MagMAX™ Pathogen RNA/DNA Kit (5X) or the MagVet™ Universal Isolation Kit. Purified RNAs were analyzed by rtRT-PCR with the LSI VetMAX™ PRRSV EU/NA kit or VetMAX™ NA and EU PRRSV Reagents.

Results: The LSI VetMAX™ PRRSV EU/NA kit has shown a sensitivity of 98.2% on more than 400 positive field samples and a specificity of 100% on more than 400 negative field samples. In the second study, the kit showed an excellent correlation at pen level (oral fluid sample) compared to animal level (blood/serum sample) with a

difference of +/-1C_T. The PRRSV RNA was identified in early infectious stages compared to antibody detection: from 7 days after infection up to 7 weeks on oral fluids and 8 weeks on blood/serum compared to 28 days after infection with ELISA test. The number of oral fluids samples that need to be sampled on herd level in order to find at least 1 positive PRRSV oral fluid sample was for example 3 for a serum prevalence of 50%.

Conclusion: Ready-to-use rtRT-PCR tools allow detecting the virus in early stage of infectious compared to antibody detection. rtRT-PCR results obtained with blood/serum and oral fluids demonstrate an excellent correlation. Based on epidemiology and prevalence of the virus in the herd, oral fluids sample are able to provide the same if not better information due to ease of use compared to randomly taken blood samples on herd level. Oral fluids allow the swine industry to generate a more accurate and reliable monitoring system where early detection can result in faster response time. It is an opportunity to increase the number of pigs tested while decreasing the cost of analysis and to estimate the circulation of pathogens (PRRSV, PCV2 and SIV) in swine population for effective herd health monitoring.

Equine herpesvirus 2 and 5 in actively racing Standardbred trotters: studies on viral load and genetic variation of gamma herpesvirus in nasal secretion

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Objectives: The equine gamma herpesviruses 2 and 5 (EHV-2 and -5) have commonly been observed in the equine population and until recently presumed low to nonpathogenic. However, recent reports linking presence of equine gamma herpesviruses with clinical signs of mild to severe lung disease, suggest that the role of these viruses in respiratory disease and poor performance is still unclear. Moreover, baseline data regarding the temporal pattern of shedding of EHV-2 and EHV-5 within stables and within actively racing individual horses have been lacking.

The objective for the first part of this study was to monitor the viral excretion from the upper respiratory tract of EHV-2 and EHV-5 in elite racing Standardbred trotters to identify if the viral load varied within individual horses and/or over season, and/or if it was associated with poor performance or clinical respiratory disease. To accomplish this we developed and validated two single qPCR assays specific for EHV-2 respective EHV-5.

The objective for the second part was to determine the genetic variation of gamma herpesvirus in nasal secretion within individual horses and over time.

Methods: Nasal swabs (NS) from 66 elite racing Standardbred trotters were collected monthly during one year. Health status and performance was registered at each sampling occasion. Viral DNA was extracted from the nasal swabs and quantified using two newly developed and validated qPCR assays targeting the DNA polymerase gene of EHV-2 and EHV-5 respectively. In order to separate the two closely related viruses we used UPL probes with Locked Nucleic Acids (LNA) incorporated into the sequence, to increases the binding strengths and thus enable the possibility to use very short probe sequences. Viral loads were calculated before comparison between individual horses. Statistical analyses were performed on Stata 13.0 software.

To study the genetic variation primers were designed to amplify a highly variable region of the glycoprotein B (gB). PCR products were purified, indexed and pooled for Next Generation Sequencing (NGS) on Illumina MiSeq. Data analysis was performed with CLC Genomics Workbench.

Results: A total of 663 samples were analyzed and 197 (30%) were positive for EHV-2 and 492 (74%) positive for EHV-5. Furthermore, 176 (27%) of the samples were positive for both EHV-2 and EHV-5 simultaneously. In 100% of the horses EHV-5 were detected at least once and 86% of the horses were EHV-2 positive at least once.

EHV-2 was found in 44% of the samples during winter compared to 17% of the samples taken during summer. The season with highest proportion of positive samples of EHV-5 was the spring (80%) and that with the lowest proportion was summer (67%).

The pattern of shed viral particles of EHV-5 clearly differed from that of EHV-2 and exhibited on average ten times higher levels of shedding.

Neither the presence nor the peak in viral load was associated with clinical respiratory signs, markers of systemic inflammation, pharyngeal or tracheal inflammation or poor performance.

For the studies on genetic variations amplicon sequencing of the gB-gene using NGS was successful. Data analysis is currently in progress and the final results will be presented at the congress.

Conclusion: To merely detect or quantify viral loads of EHV-2/5 in NS from horses may not be sufficient to elucidate disease causality. Rather the viral genomic variability in EHV-2 and/or EHV-5 might provide more insight to the potential role of these gamma herpesviruses as pathogens, and thus further work on identifying differences in gene sequences is ongoing.

Herd management strategies to control PRRSV infections using the PrioCHECK® PRRSV VIA ELISA

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Objective: PRRSV outbreaks are feared in the swine industries because of the devastating economic impact. The complex nature of the PRRSV disease indicates that single diagnostic tests may not be enough to successfully manage PRRS virus but rather comprehensive solution strategies are needed for the effective control of the disease. Here we present data for PRRSV disease management using the ELISA PrioCHECK® PRRSV VIA. The PrioCHECK® PRRSV VIA is an indirect ELISA being able to discriminate Type I (EU) from Type II (US) PRRSV infection by measuring the presence of neutralizing antibodies in serum of pigs.

Methods and Results: In total 182 serum samples relating to 19 different pig herds were investigated with the PrioCHECK® PRRSV VIA. On herd level, a sensitivity of 89.4 % was achieved, corresponding to 17 correctly identified herds. The specificity was calculated on individual samples derived from pigs with confirmed negative PRRSV status (240 samples) and resulted in 99.4% for Type I coated plates and 98% for Type II (US) coated plates.

In one particular case study pigs showed clinical signs for PRRSV despite regular vaccination with a Type I (EU) vaccines. PRRSV Screening ELISA data revealed that all samples were positive. PrioCHECK® PRRSV VIA results detected very high antibody titers to Type II (US) in more than 90% of the animals. In contrast low antibody titers to Type I (EU) were seen in 2 of the 17 animals. These results indicated that protection to heterologous PRRSV strain failed. This finding could be confirmed by positive Type I and Type II PRRSV PCR. As a consequence the veterinarian decided to change the vaccine in this farm.

In a second investigation serum samples of 10 boars known to be continuously infected with Type I (EU) PRRSV were tested with the PrioCHECK® PRRSV VIA. All samples were negative to Type I (EU) PRRSV indicating that no neutralizing antibodies against Type I were present. In contrast 50% of the samples revealed high antibody titers to Type II (US) PRRSV. The same samples were also positive in the screening ELISA, PrioCHECK® PRRSV Ab porcine. Type II (US) positive results could be confirmed by PCR indicating that the herd was affected by a fresh infection of Type II (US).

Conclusion: In summary we show that the PrioCHECK® PRRSV VIA is a reliable tool to discriminate Type I (EU) from Type II (US) PRRSV infections on herd level, detects single or mixed infections of different PRRSV types on herd level and supports decisions for the optimal type and schedule of PRRSV vaccination.

Topic: Vaccines & antivirals

Enrofloxacin is a potent antiviral against African swine fever virus in vitro but lacks detectable antiviral activity in vivo

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African swine fever (ASF) is a severe, multi-systemic disease of pigs. So far, vaccines are lacking, and control is solely based on strict hygiene measures and culling of infected herds. However, intensive research targets vaccine development and alternative control options, including antivirals.

The causative agent, ASF virus (ASFV), is a large double-stranded DNA virus and belongs to the genus *Asfivirus* in the *Asfarviridae* family. Among the approximately 200 genes is one that encodes for a topoisomerase type II enzyme (ASFV-Topo II) which is unique for a mammalian infecting virus (Baylis et al., 1992). This enzyme is more similar to bacterial topoisomerase IV and DNA gyrases than mammalian type II topoisomerases. Fluorochinolones act as gyrase inhibitors and thereby are efficacious antibiotics. It was recently demonstrated that six out of 30 fluorochinolones (Enrofloxacin, Grepafloxacin, Balofloxacin, Tosufloxacin, Gatifloxacin and Garenoxacin) showed antiviral activity against ASFV *in vitro*. Among the tested fluorochinolones were licensed antibiotics for human and/or veterinary medicine (Mottola et al., 2013).

To further investigate the antiviral properties of relevant fluorchinolones (Enrofloxacin, Danofloxacin, Ofloxacin, Marbofloxacin), inhibition tests were carried out on a permanent wild boar lung cell line (WSL). In contrast to the Vero cells that were used in the abovementioned study, this cell line originates from an ASFV host species and could be more indicative for the situation *in vivo*. In the WSL-cell system, Enrofloxacin was found to be the most effective. For this reason, it was used in a proof-of-concept *in vivo* trial with intramuscular ASFV "Lithuania 2014" (genotype II) infection.

In a group of five young fattening pigs, two randomly chosen animals were treated with Enrofloxacin (Baytril, Bayer Animal Health) at a daily dose of app. 25 mg per kg body weight and day (10-fold of the antibacterial therapeutic dose) starting one day before infection. Samples for serological and virological assays as well as blood cell counts were collected prior to infection, at day 4 post infection and at the day of death or euthanasia of the respective animals. Upon ASFV infection, all animals developed an acute-lethal disease course and no indications for a beneficial effect of Enrofloxacin on clinical disease or viral load were observed. On the contrary, the treated animals succumbed on day 4 post infection while the untreated animals were euthanized on day 6 post infection. The serum samples collected at the day of infection were tested for their antiviral effects *in vitro*. No antiviral effect could be observed.

Thus, the promising *in vitro* antiviral effect of Enrofloxacin could not be confirmed *in vivo*.

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Identification and molecular characterization of RHDV serotype 2 virus isolated in Italy

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Objective: Rabbit Haemorrhagic Disease (RHD) is an acute disease affecting European rabbits *Oryctolagus cuniculus*. Acute forms of RHD are characterized by high morbidity and mortality rate that usually ranges between 70% and 90%; the incubation period varies from 1 to 3 days and rabbits usually succumb within 12h to 36h after the onset of fever. The liver, lung and spleen are the primary target tissues of the infection; acute infections are accompanied by anorexia, apathy, neurologic symptoms, haemorrhages and congestions.

The aetiological agent of the disease, the Rabbit Haemorrhagic Disease Virus (RHDV), is a small non- enveloped single-stranded RNA virus belonging to the genus Lagovirus, family Caliciviridae. RHDV is characterized by low genetic variability and until 2010, only one serotype was known, with the two subtypes RHDV (wild type) and RHDVa (variant). In 2010, in France, a new serotype was identified. The new serotype, actually known also as RHDV2, differs from RHDV in terms of pathogenicity, phylogenetic position and antigenic profile.

To perform a genetic characterization of an RHDV2 strain isolated in Italy, complete sequence of the capsid protein VP60 gene was obtained and data were aligned and compared with all publicly available RHDV sequences.

Methods: RHDV strain IZO/13 was isolated in 2013 in a rabbitries in the North-western Italy during an outbreak of the disease. Virus was isolated from liver of infect animals. Viral RNA was extracted from approximately 100mg of infected tissue using QIAzol lysis buffer protocol (Qiagen). RNA target sequence from VP60 capsid protein gene was retrotranscribed and amplified by PCR. The PCR product was used for direct sequencing and

obtained data were analyzed in order to perform a phylogenetic analysis.

To investigate on its immunological properties, rabbits were vaccinated using an inactivated viral suspension and inducted antibody serological titre was analyzed 14 days post-vaccination using ELISA-test.

Results: The BLAST analysis of sequences revealed that RHDV strain IZO/13 is homologous (94-97%) of the corresponding fragment of the VP60 gene of several RHDV2 isolated in Italy and in Southern Europe since 2011. In particular, RHDV strain IZO/13 show the highest homology (97% of nucleotide identity and 96% of amino acidic identity) with an RHDV strain isolated in Southern Italy. Interestingly, the homology between IZO/13 VP60 nucleotide sequence and the corresponding fragment of the Italian isolate Ud11, known as the first case in Italy of an RHDV2 outbreak, is 94% (94% at amino acid level). Moreover, classical RHDV Italian isolates showed an homology lower than 80% with RHDV2 IZO/13. In particular, RHDV strain BS/89 and PV-97 show an homology, respectively, of 79% and 78%.

To perform phylogenetic analysis (Neighbor-Joining method, bootstrap resampling performed on 1,000 replicates), the nucleotide sequence from IZO/13 was aligned with the VP60 gene sequences available in GenBank using ClastalW. Obtained data show that the RHDV2 IZO/13 is close to a branch formed from the previously RHDV2 strains isolated in Italy and in Southern Europe but separated by the branch formed from classical RHDV strains.

Rabbits vaccinated with the inactivated viral suspension produced using RHDV IZO/13 as vaccine strain showed 14 days post-vaccination an average serological titre of 1/80 in ELISA-test. A preliminary challenge study reveals that vaccinated rabbits are protected from infection showing no disease manifestation.

Similar data were obtained using a trivalent inactivated vaccine in oil-emulsion against classical RHDV strains and RHDV2 IZO/13: treated animals showed no adverse reactions and serological titres able to confer protection against RHDV and RHDV2 infections.

Conclusion: RHDV2 IZO/13, originally isolated in Northern Italy during an outbreak of the disease, shows an homology with several RHDV2 strains isolated in Italy and in Southern Europe. A phylogenetic analysis showed that IZO/13 belongs to a branch formed from other RHDV2 Italian isolates and distinct from other branches formed from classical RHDV strains.

In order to investigate immunological properties of the RHDV2 IZO/13, rabbits were vaccinated with an inactivated viral suspension in oil emulsion both as monovalent and trivalent formulation using RHDV classical strains used in a commercial Italian vaccine (IZOVAC MEVAX2 from IZO srl). After 14 days post-vaccination, all rabbits showed a serological titres for each valency able to confer protection against infection, confirmed by a challenge test against classical RHDV strains and RHDV2.

A complete efficacy, safety and challenge studies will be carried out in order to register a trivalent inactivated vaccine in oil emulsion able to confer protection against RHDV and RHDV2 strains.

Pestivirus contamination of vaccines - possible source of new BVDV-2 and atypical bovine pestivirus infections in Polish cattle?

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Objective: Until recently only Bovine Viral Diarrhoea Virus type 1 (BVDV-1) strains have been detected in Polish cattle. The introduction of BVDV-2 to a Polish herd where severe respiratory problems, bloody diarrhea and mortality in calves were observed was confirmed for the first time in 2012 (Polak et al., 2014). More recently an atypical bovine pestivirus has been detected in two Polish bulls (Kuta et al., submitted). The epidemiological investigation failed to determine the sources of infections, however contamination of a vaccine or other bioproduct used in the herds has been speculated. In order to study the risk of pestivirus transmission by this route, wide selection of vaccines dedicated mostly for use in domestic ruminants available in Poland were tested for the contamination.

Methods: A total of 174 vaccine samples containing 69 different vaccines of different batches has been tested for the presence of pestivirus antigen (IDEXX BVDV Ag/Serum Plus Test) and RNA. RT- PCR using pan-pestivirus primers and type-specific real-time RT-PCR with primers and probes specific for BVDV-1, BVDV-2 and atypical bovine pestivirus (Hobi-like) within the 5'-UTR were used. Selected vaccine contaminated with pestivirus was used to challenge guinea pigs. Virus isolation in bovine turbinate cells was attempted for BVDV antigen and RNA positive vaccine batches.

Results: Except for BVDV vaccines, pestivirus was detected by classical RT-PCR in 5 (2.9%) vaccines (4 used in ruminants and one in pigs), however no BVDV antigen was detected in those samples. Only one (a batch of live IBR vaccine) of those 5 vaccines was confirmed by real-time RT-PCR with BVDV-1 specific primers and probe. Additionally, another RT-PCR negative IBR vaccine was positive in BVDV antigen ELISA with high O.D. value 3.3. The immunogenisity of the antigen was confirmed by seroconversion in one out of 3 guinea pigs inoculated with the BVDV antigen vaccine. No live viru was isolated from any of the vaccines contaminated either with pestivirus antigen or RNA.

Conclusion: No BVDV-2 or atypical bovine pestivirus RNA was detected in any of the tested vaccines, however BVDV-1 contamination was confirmed in one live IBR vaccine. Although no live virus was isolated, pestivirus antigen contaminating a vaccine can lead to change of immunological status of the herd where the vaccine is used. The study was conducted as part of the project No 198893 finansed by National Science Centre (NCN).

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Postvaccinal immune response in pigs treated with enrofloxacin at the time of vaccination against Aujeszky's disease

Malgorzata Pomorska-Mól, Ewelina Czyzewska-Dors, Krzysztof Kwit, Andrzej Lipowski

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Objective: Enrofloxacin is an anti-bacterial agent which belongs to fluoroquinolones. The fluoroquinolones have become an increasingly popular class of antibiotics for use in a variety of infections in humans and animals. They are also known to have direct effects on the immune system. The immunomodulatory effects of fluoroquinolones are probably due to their effects on intracellular cyclic AMP and phophodiesterases, on transcription factors such as NF-kappa B and activator protein 1. Enrofloxacin is metabolized into pharmacologically active metabolite, ciprofloxacin, which is also known to have modulatory effect on the immune system. Ciprofloxacin has been shown to modulate phagocytic and killing capacity of neutrophil and macrophages as well as affects the expression of toll-like receptors in monocytes. Moreover, ciprofloxacin decreased and/or delayed the synthesis of IL-1, IL-6, IL-12, TNF-α.

These data suggest that enrofloxacin (and its pharmacologically active metabolite- ciprofloxacin) may affect cellular and humoral immunity by influence on the cytokine production and secretion. In view of the potential immunomodulatory properties of enrofloxacin we evaluated the effect of therapeutic doses of enrofloxacin on selected immune parameters, with special emphasis on postvaccinal immune response.

Methods: Forty pigs were used in this study. Animals were sourced from herd with high health status, seronegative to Aujeszky's disease virus (ADV). Only pigs that not received any of antibiotics were involved in the experiment. Pigs were randomly divided into three groups: control not vaccinated (C, n=10), control vaccinated (CV, n=15), and vaccinated during enrofloxacin administration (ENRO, n=15). During the experiment, pigs were housed in isolated units, one for the each group. Food and water were offered *ad libitum*. Animal use and handling protocols were approved by Local Ethical Commission (University of Life Sciences in Lublin, Poland).

From day -1 to day 3 animals from ENRO group received enrofloxacin intramuscularly, at the recommended dose (1ml/10 kg of body weight per day). Pigs from ENRO and CV groups were vaccinated intramuscularly at 10 and 12 weeks of age with 2 ml of vaccine (0 and 14 days of study). Piglets from C group were not vaccinated and did not receive any antibiotics.

Drug and vaccine

The commercially available product containing enrofloxacin was used (Enrobioflox 5% Injectio, 50mg/mL, Vetoquinol Biowet, Poland). For vaccination the live-attenuated gE deleted vaccine against ADV (Akipor 6.3, Merial, France) was used.

Humoral response against ADV

Specific antibodies to the glycoprotein B (gB) and glycoprotein E (gE) antigen were determined using a blocking ELISA tests (IDEXX PRV/ADV gB and IDEXX PRV/ADV gI, IDEXX Laboratories, USA), as directed by the manufacturer.

Lymphocyte proliferation assay

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by centrifugation onto Histopaque 1.077 and were seeded in vials at a density of 1 x 10^6 viable cells/ml medium. PBMCs were restimulated with live ADV, strain NIA-3 (titer $10^{7.0}$ $TCID_{50}$). In control vials the PBMCs were cultivated without ADV (mock-control) or with 5µg/ml of concanavalin A (ConA) (viability control). After 72 hours of incubation at 37ºC in 5% CO2 atmosphere, the cultures were pulsed with 0.5µCi [3H]-thymidine (PerkinElmer, USA) and were harvested after next 18 hours. The incorporated radioactivity was measured in liquid scintillation counter (Quantulus, PerkinElmer, USA). Proliferation was expressed as a stimulation index (SI) calculated as the number of counts per minute (cpm) of ADV stimulated PBMCs divided by the number of cpm of the mock-stimulated cells (in each cases taking mean of triplicate vials). Based on the SI values (mean plus 3 x standard deviation) observed at day -1 (before vaccination) and in non-vaccinated animals, a SI $\geq$ 2.20 was considered positive for ADV-specific proliferation.

In vitro secretion of interleukin (IL)-4, IL-6, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α).

For analysis the ability of PBMC to secrete cytokines, the concentrations of IL-4, IL-6, IL-10, IFN–γ, and TNF-α after stimulation with live ADV ($10^{7.0}$ $TCID_{50}$) and ConA (5µg/ml) were determined (ELISA kits specific for porcine IL-4, IL-6, IL-10, IFN–γ, and TNF-α; Invitrogen Corporation, Camarillo, USA; Abcam, Cambridge, UK). Untreated cells served as control (mock control). PBMCs were isolated and cultured under the same conditions as described above.

Statistical analysis. A nonparametric Kruskal-Wallis test with post hoc multiple comparisons for comparison of all pairs was used (STATISTICA 8.0; StatSoft). For all analyses $p \leq 0.05$ was considered significant.

Results:

Antigen-specific humoral and cellular response

Before vaccination all experimental animals had no antibodies against both gB and gE. None of the pigs had antibodies to gE at the end of the study indicating that no infection had occurred during the study. No specific antibodies against gB were found in pigs from C group. Taking into consideration the S/N ELISA ratio, 9 days after the first vaccination all of animals from CV group developed specific humoral response at the level considered positive. In contrast, in ENRO group only 5 out of 15 animals were positive 9 days after the first dose of vaccine. Significantly lower ELISA S/N ratio was observed also at day 14 of study in pigs from ENRO group as compared to CV group ($p<0.05$). Starting from day 21 of study no significant differences were found between ELISA S/N ratio in pigs from CV and ENRO groups.

Six days after the first vaccination, in 9 out of 15 pigs from CV group the ADV-specific proliferation was observed, while in ENRO group only 6 out of 15 pigs revealed antigen-specific proliferation. Nine days after first vaccination 100% animals from both vaccinated groups developed a ADV-specific proliferation. The mean SI value did not differ significantly between both vaccinated groups at that time. Starting from three weeks after booster dose, the SI values were significantly higher in pigs from CV group as compared with ENRO group ($p<0.05$). In animals from C group the mean SI values ranged from 0.97 to 1.31 during period of study.

IL-4, IL-6, IL-10, IFN-γ and TNF-α secretion following in vitro stimulation of PBMCs

In vitro stimulation with live ADV did not induce detectable production of IL-4 by PBMC in the case of vaccinated and non-vaccinated pigs. In supernatants from ADV-stimulated and unstimulated cultures the level of IL-4 was undetectable (<15.6 pg/ml). The detectable level of IL-4 was found only after stimulation with ConA but no significant differences between groups were noted ($p>0.05$).

The mean constitutive production of IL-6 in vaccinated and non-vaccinated animals ranged from undetectable level to 44.4 pg/ml. From day 9 to 63 of study concentration of IL-6 after ADV stimulation in both vaccinated groups was significantly higher ($p<0.05$) as compared with non-vaccinated animals from group C. No significant differences were found between CV and ENRO groups.

No significant differences were found in the production of IL-10 between all experimental groups after ConA. In

contrast, starting from day 6 after the first vaccination significantly higher production of IL-10 were observed in vaccinated groups after ADV stimulation compared to non-vaccinated controls ($p<0.05$). Moreover, from 21 to 35 day of study secretion of IL-10 was significantly higher in pigs from CV group comparing to ENRO group ($p<0.05$).

In mock-stimulated vials no IFN-γ was detected in vaccinated and non-vaccinated animals. After stimulation of PBMC with ConA the mean level of IFN-γ did not differ significantly between groups ($p>0.05$) After in vitro exposure to live ADV naive PBMC (isolated from blood of pigs from C group) never secreted IFN-γ at level higher than detection limit (2.0 pg/ml). In contrast, in vitro ADV restimulation of PBMC from vaccinated animals resulted in high amounts of IFN-γ in the culture supernatants. In pigs from CV group the significantly higher production of IFN-γ as compared with pigs from ENRO group was noted between 21 and 49 day of study ($p<0.05$).

As regards TNF-α production, no statistically significant differences between all groups were found after mock and ConA stimulation. However from day 6 to day 14 of study the secretion of TNF-α in ENRO group seemed to be slightly lower than in CV and C groups after ConA stimulation. Significant increase in TNF-α production was observed in both vaccinated groups after ADV-restimulation ($p<0.05$). In addition, in CV group the secretion of this cytokine was significantly higher from day 21 to 35 of study compared with ENRO group ($p<0.05$).

Conclusion: The results of the present study indicate that enrofloxacin, in addition to its antimicrobial properties, possess significant immunomodulatory effects in vivo and may alter the immune response to vaccines if it is co-administered during vaccination of pigs. The exact mechanisms leading to modulatory effects of enrofloxacin are not identified however, the influence on the secretion of various cytokines may be considered as a possible cause.

Zulvac® SBV, the first vaccine against Schmallenberg virus in cattle and sheep authorized at a European level

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In November 2011, a new virus, the Schmallenberg virus (SBV) belonging to the genus *Orthobunyavirus* was identified in Germany. SBV is transmitted mainly by biting midges from the *Culicoides* genus and can infect cattle, sheep and goats. SBV rapidly spread throughout the whole Europe causing mild transient disease in adults (i.e. drop in milk production, fever, diarrhea). Main losses are associated with SBV infection during pregnancy that can lead to congenital infection, causing stillbirth, abortion and fetal abnormalities due to SBV ability to cross placental barrier.

In February 2015, the European Commission adopted a Commission Decision granting the marketing authorization for the Zoetis vaccine Zulvac® SBV. This veterinary medicine approval is valid throughout the European Union (EU).

The vaccine Zulvac® SBV is a suspension for injection intended for active immunization of cattle and sheep against SBV. The active substance is the inactivated SBV strain BH80/11-4 adjuvanted with aluminium hydroxide and saponin as adjuvants. The proposed route of administration is subcutaneous in sheep and intramuscular in cattle. The recommended dose is 1 ml in sheep and 2 ml in cattle.

The benefits of Zulvac® SBV are its prophylactic immunization:

- To reduce viraemia associated with infection by SBV in cattle of 3.5 months of age
- To prevent viraemia associated with infection by SBV in sheep of 3.5 months of age
- To reduce viraemia and transplacental infection associated with infection by SBV during the first trimester of pregnancy in breeding sheep vaccinated before pregnancy.

Zulvac® SBV has been shown to reduce or prevent viraemia associated with SBV infection, thus, reducing the presence of the virus in the blood stream and hence the likelihood of transmission of the virus from an infected animal to the vector and, consequently, the transmission to other susceptible animals.

The laboratory studies in lambs and calves of the minimum age (3.5 months) and pregnant ewes (at 2.5-5 months of pregnancy) show that the safety profile of Zulvac® SBV when used as recommended is satisfactory; no relevant safety risks have been identified neither.

Zulvac® SBV provides a new treatment possibility against SBV. Although Zulvac® SBV and some other Schmallenberg vaccines have been granted provisional forms of authorization at a national level, this is the first vaccine against SBV in cattle and sheep centrally authorized in the EU.

Topic: Virus evolution

Characterization and phylogenetic analysis of two Finnish EBLV-2 isolates

Tiina Nokireki [1], Anita Huovilainen [1], Tarja Sironen [2], Olli Vapalahti [3], Liisa Sihvonen [1,3], Miia Jakava-Viljanen [1]

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Objective: Finland has been free of rabies in terrestrial mammals since 1991. Due to the natural circulation of European bat lyssavirus type 2 (EBLV-2), rabies can remain a residual risk to public health. In 1985, a Swiss bat researcher died in Finland of rabies encephalitis caused by EBLV-2 and in 2009, an EBLV-2-positive Daubenton's bat was detected in the city of Turku. The objective of this study was to increase the understanding of epidemiology and evolution of EBLV-2. Previously only three full genome EBLV-2 sequences were known and we added to this two fuller genome sequences, thus providing critical additional data on the evolution of EBLV-2. Using the two Finnish isolates as a calibrator, we have calculated for the first time the molecular clock.

Methods: These two EBLV-2 isolates 24 years apart were fully sequenced, genetically characterized and the substitution rate of EBLV-2 and time of divergence from the phylogenetically closest lyssavirus species was estimated.

Results: The complete lengths of the FI-85 and FI-09 genomes were 11 928 and 11 927 nucleotides, respectively. The general genome organization was typical for lyssaviruses, consisting of five structural genes, N, P, M, G and L, and non-coding regions between them and at both ends of the genome. The long untranslated region between the G and L genes was the only area that varied in length between the two Finnish EBLV-2 strains. The analysis revealed that all five fully sequenced EBLV-2 strains form a monophyletic group separate from other bat-type lyssaviruses with significant support. EBLV-2 shared the most recent common ancestry with Bokeloh bat lyssavirus (BBLV) and Khujan virus (KHUV). More distant ancestry was shared with ARAV, RABV and ABLV (albeit with low bootstrap support). A second major cluster of lyssaviruses consists of IRKV, DUVV and EBLV-1. The clearly most divergent group is formed by SHIV, LBV and WCBV. In this phylogenetic analysis EBLV-2 sequences shared high nucleotide and amino acid identities and high similarity to fully sequenced EBLV-2 strains originating from the UK and the Netherlands. There were four amino acid differences between the two Finnish strains. In a phylogenetic tree of partial N gene sequences, the Finnish EBLV-2 strains clustered with a strain from Central Europe.

Conclusion: EBLV-2 showed limited diversity compared to RABV and appeared to be well adapted to its host bat species. The slow tempo of viral evolution was evident in the estimations of divergence times for EBLV-2.

Molecular characterization and phylogenetic analysis of small ruminant lentiviruses (SRLV) circulating in Germany

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Objective: Small ruminant lentiviruses (SRLV), whose prototypes are caprine arthritis-encephalitis virus (CAEV) and Maedi-Visna virus (MVV), are the causative agents of slow progressive degenerative diseases of goat and sheep, responsible for significant economic losses. These viruses cause persistent infections with long period of incubation and induce inflammatory and degenerative lesions. As no vaccine is available, most often employed schemes to prevent spread of SRLV are based on segregation and/or culling of positive animals associated with management practices, especially concerning the offspring. The high heterogeneity of nucleotide and amino acid sequences in SRLV may determine their antigenicity, virulence and growth and may affect their persistence and escape from the immune system. It has been shown in sheep that genetic susceptibility to MVV varied between virus subgroups. According to the current nomenclature based on 1.8 kb *gag-pol* and 1.2 kb *pol* sequences, the SRLVs can be subdivided into genotypes A-E, with subtypes present in A, B, and E. Genotype A can be further divided into at least 15 established subtypes named A1- A15, genotype B originally referred to CAEV type, comprises 4 distinct subtypes, B1- B4, whereas genotypes C and D are so far represented by only few isolates from goat and sheep respectively, showing high divergence from other groups. Genotype E, first described in Italian goat herds, is characterized as low pathogenic caprine viruses. The data from previous studies based on phylogenetic analysis helped to revise the classification of SRLV according to the host species. Indeed, it was demonstrated that these viruses are constantly and easily transgressing the species barrier between goats and sheep and several reports indicate that natural cross-species infection may occur.

The first documented occurrence of Maedi-Visna infection in Germany dated 1969. In various German states programs, based on serological survey, were started to eradicate SRLV infection. Pursuing this program led to a number of sero-negative flocks, however seroconversions continue to occur, causing important economic losses to the breeders.

To better understand the naturally circulating viral strains and to investigate the heterogeneity of SRLV in Germany, a phylogenetic analysis was carried out on virus isolates from sheep flocks. The aim of this work was to carry out a preliminary investigation regarding SRLV phylogeny as a basis for future molecular epidemiological studies on lentiviral infection in Germany.

Methods: For the purpose of this study, EDTA blood samples were collected in the year 2014 from ewes aged 4 years or older in 9 North German and 1 Southern German sheep flocks with clinical signs for maedi-visna disease. After serological testing with IDEXX MVV/CAEV ELISA, a total of 122 samples were selected for extraction of genomic DNA from whole blood using high salt method. DNA concentration of all samples was standardized to 50 ng/µl. A nested PCR using the forward primers *gag-F1* (5'-TGGTGARKCTAGMTAGAGACATGG-3') and *gag-F2* (5'-CAAACWGTRGCAATGCAGCATGG-3') and the reverse primers *pol-R1* (5'- CATAGGRGGHGCGGACGGCASCA-3') and *polR2* (5'-GCGGACGGCASCACACG-3') flanking a 900 bp fragment within the *gag-pol* region, was used. Bands of correct sizes were excised and purified by means of a QIAquick Gel Extraction Kit (QUIAGEN) and used as templates in sequencing reactions with Big Dye version 3.1 (Applied Biosystems). Precipitated products were run on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Both the sense and antisense strands were sequenced performing three independent reactions for each isolate. Sequence data were analyzed using the program SeqMan II from the DNASTAR package and subsequently with Clustal X version 2.00, in respect of the amino acidic coding frame and were aligned with SRLV homologous sequences available on GenBank. Editing was performed by BioEdit Sequence alignment Editor. The phylogenetic tree was inferred with the MEGA6using the Neighbour-Joining algorithm. Nodal support was evaluated by 10,000 bootstrap replications. The presence of recombinant strains was investigated through Simplot and Splits Tree programs.

Results: PCR products of the expected size were obtained for 39 samples after amplification with the primer pairs designed for the *gag-pol* region. The topology of the tree indicated that they belonged to the genetic subtypes A1 (n=2), A2 (n=5), A4 (n=1), A12 (n=2) and to one potentially new subtype (n=29) which differed significantly from all the subtypes previously described; this includes isolates with a sequence identity ranging from 81.9 %to 98.5 %,grouped in a separate cluster within the genotype A, tentatively named A16. The percentage of divergence of the proposed novel cluster compared to the other subtypes within genotype A, was 15.0 % to 27.0%.Interestingly, the new subtype was identified in 7 out of 9 positive flocks; co-circulation of multiple subtypes was detected in 4 flocks (A1+A16; A2+A16; A12+A16).Studies on recombination events did not show any evidence of recombinant signals.

Conclusion: In this study the diversity of 39 SRLVs was investigated by viral DNA amplification in the *gag-pol* region, direct sequencing and phylogenetic analysis. According to the taxonomic classification proposed by Shah *et al.* (2004), all the German isolates analyzed in this study belonged to the genotype A. Interestingly, most of them could not be assigned into existing clusters. Therefore, it is proposed to classify these viruses in a new genetic subtype, tentatively defined A16.

SRLVs belonging to this subtype were from North German flocks which were not epidemiologically related by common trade practices between sheep farmers. We cannot exclude that an ancestral virus circulated in the area prior to our study as well as the possibility that this viral subtype is endemic.

In conclusion, our results showed a low SRLV genetic heterogeneity in Germany compared to other European countries. The present study has not the claim to give a complete picture of lentiviral infection in Germany, however it provides for the first time findings of molecular characterization of German SRLVs as well as further evidence of SRLV genetic variability.

The role of quasispecies in chronical infections with classical swine fever virus

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Objective: Classical swine fever is a severe multi-systemic disease of domestic and feral pigs that is caused by classical swine fever virus (CSFV) and has a high socio-economic impact world-wide. Infections with CSFV can be divided into three different outcomes of the disease, the acute, the chronic and the prenatal form. The latter two are accompanied by long-term shedding of CSFV and are thus of importance for disease maintenance in pig populations. While chronic infections have a low impact in modern industrialized pig production due to culling policies, it may play an important role in wild boar populations or under backyard settings. However, due to the rare occurrence, little is known about the virus-host interaction in chronic infections. In this study we examined the virus populations of chronically infected animals compared to acutely infected ones and the inoculum of three different animal trials to shed further light on this special form of CSFV-infection.

Methods: Samples of chronically and acutely infected animals as well as the inoculum were sequenced with either a 454 Genome Sequencer FLX or an Illumina MiSeq. Both *de novo* assembled consensus sequences and variant profiles were compared. Besides variant calling, viral populations were compared by calculation of “Manhattan distances”, taking all variants into account. Mutational “hot spots” were identified and compared with regard to differences in the disease outcome.

Results: At the consensus sequence level, no differences were detected between the inocula and viruses from acutely or chronically infected animals within two out of three trials. Only virus from one acute-lethally infected animal within the third trial showed four substitutions at the consensus level. In addition, low frequency variants were detected by the population analysis. Due to these variants, the distance between the inoculum and the virus populations from the acute-lethally infected animal was greater than the distances between the populations of similarly long replicating viruses from chronically infected animals. Additionally, differences in the population diversity were observed over time which was expected due to the high virus replication rates. Population analysis of viruses from an animal trial in which both domestic pigs and wild boar were infected revealed no remarkable impact of host sub-species or disease outcome on the population diversity. Furthermore, no mutational “hot spots” could be detected which were related to inoculum, host sub-species, or outcome of the disease.

Conclusion: Our results imply that the outcome of disease following CSFV-infection, i.e. acute or chronic infection, is not related to changes in the viral genome. Rather, the host seems to play the major role in the development of the different disease outcomes. In addition, it could be demonstrated that the viral population diversifies over time with regard to minor variants, but is constant regarding high frequency variants and the consensus level. Since no changes at the consensus sequence level were observed between viruses from domestic pigs and wild boar, it can be also concluded that the host sub-species has most likely no marked impact on viral quasi species or the disease outcome. Future work about the chronic forms of CSF should therefore mainly focus on the role of host factors.

Time scale evolution of avipoxviruses

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Objectives: Based on basic genomic analyses (restriction fragment length polymorphism), avipoxviruses are currently divided into 10 virus species but more recent phylogenetic studies identified 3 major clades: canarypox-like viruses, fowlpox-like viruses, and psittacinepox-like viruses. The age of divergence between the 3 avipoxviruses main clades has so far not been estimated. The aim of the present study was to assess substitution rate and time to most recent common ancestor of avipoxviruses, to compare them with data available for other poxviruses and to reach hypotheses on their spread and circulation.

Methods: All available DNA sequences of avipoxviruses P4b, cnpv186 and DNA polymerase loci were retrieved from GenBank/EMBL databases. The sampling date was collected for each sequence and only the oldest sequence was kept when identical sequences were available. ClustalX 2.1 and Bioedit 7.2.5 were used for sequence alignments and editing, respectively. We analyzed 81 P4b gene sequences (partial open reading frame, ORF) from specimens collected between 1865 and 2014; 31 cnpv186 genes (complete ORF) from specimens collected between 1965 and 2014; and 50 DNA polymerase genes (partial ORF) from specimens collected between 1980 and 2014.Phylogenies taking sampling time into account were estimated using the Bayesian Markov chain Monte Carlo (MCMC) inference methods available in the BEAST package. The analyses were run using the Tamura and Nei 1993 model with gamma-distributed rate. Different combinations of demographic models and clock models were compared: constant size or Extended Bayesian Skyline Plot models, strict or relaxed (lognormal or exponential) clocks. At least 200 million MCMC iterations were run for each gene and each demographic models / clock models combination. The estimation of parameters and divergence time were carried out using Tracer and the best fitting model (lowest AICM) was selected.

Results: Phylogenetic trees based on 3 avipoxvirus genes (P4b, cnpv186, and DNA polymerase) DNA sequences confirmed the common classification in the genus: canarypox-like viruses, fowlpox-like viruses, and psittacinepox-like viruses. The lowest AICM values were obtained with a relaxed exponential clock and the Extended Bayesian Skyline Plot model for both cnpv186 and the DNA polymerase genes. For the P4b gene, the AICM value was slightly lower with a strict clock and the Extended Bayesian Skyline Plot model than with a relaxed exponential clock and the Extended Bayesian Skyline Plot model but we decided to use the same combination for our 3 genes for consistency: a relaxed exponential clock and the Extended Bayesian Skyline Plot model. Our molecular clock analyses showed that avipoxviruses diverged from a common ancestor approximately 10 to 30 thousand years ago: 8,682 (95% Highest Posterior Density (HPD): 950-60,718), 10,891 (95% HPD: 96393, 707), and 29,259 (95% HPD: 1,871-

197,714) years ago for DNA polymerase, cnpv186, and P4b, respectively, and that avipoxviruses evolved at a rate of 2 to 8 x 10^{-5} substitution/site/year. Looking at avipoxviruses collected the same year in the same location such as Chlamydotisundulata/MA/2009/LK021680, Chlamydotisundulata/MA/2009/LN795880, and Chlamydotisundula - ta/MA/2009/LN795881, all 3 isolated on houbara bustards in Morocco in 2009, the cnpv186 phylogeny and molecular clock analysis enabled to conclude to clear independent introductions of viruses and rule out the hypothesis of virus spread and evolution within the site.

Conclusion: In conclusion, the present study showed that avipoxviruses evolve at a rate of 2 to 8 x 10^{-5} substitution/site/year and diverged from a common ancestor approximately 10 to 30 thousand years ago. These new pieces of information should facilitate future epidemiological investigations on virus spread, origin and circulation.

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