ESVV2025

13th International Congress for Veterinary Virology
Including an EPIZONE Session

2-5 September, 2025
Grand Hotel Bernardin
Portorož, Slovenia

Abstract Book

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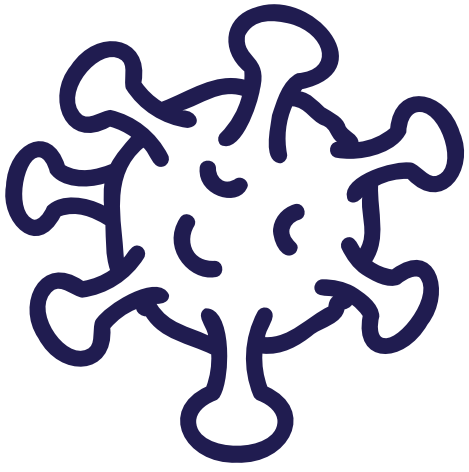

ESVV 2025 – 13th International Congress For Veterinary Virology

Portorož, Slovenia from September 2 to September 5, 2025

Abstract Book

Editor:
Sandra Blome, Enric Mateu, Ivan Toplak

Publisher:
European Society for Veterinary Virology (ESVV)

Year:
2025

E-publication:
Published on www.esvv2025.org

Kataložni zapis o publikaciji (CIP) pripravili v Narodni in univerzitetni knjižnici v Ljubljani

COBISS.SI-ID

ISBN

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Welcome to Slovenia!

On behalf of the European Society for Veterinary Virology (ESVV), it is my great pleasure to welcome you in Portorož which is located on western part of Slovenia, right by the sea, for the 13th International Congress For Veterinary Virology!

The 13th ESVV is held on September 2-5, 2025, in Grand Hotel Bernardin, Portorož, Slovenia.

The ESVV was founded in 1988 in Belgium to promote exchange of information in the field of veterinary virology. The first ESVV meeting was organized in 1989 and recent editions with more than 400 participants were held in Ghent (2022), Vienna (2018) and Montpellier (2015).

Plenary sessions will cover pathogenesis, viral immunology, epidemiology, diagnostics and vaccines, and (reverse) zoonoses. Veterinarians, virologists, academics, authorities, pharmaceutical industries as well as PhD students are encouraged to participate.

St. Bernardin Resort Portorož is located right by the sea, on the most popular Slovenian seaside promenade. It is the perfect starting point for exploring small old town Piran on one side, and Portorož's world of entertainment on the other. There are well-kept beaches all around, and the hotels offer both indoor and outdoor pools filled with sea water, as well as wellness centres. Our authentic Istrian cuisine is sure to delight you with its diversity, passion, and harmony of flavours. Characterised by Mediterranean and continental influences, it is distinguished by fresh local ingredients, Piran sea salt, authentic Istrian spices, premium olive oils, and a range of fine Istrian wines.

Sandra Blome (ESVV President)

on behalf of the ESVV Board and the ESVV2025 Scientific and Organizing Committees.

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Congress Information

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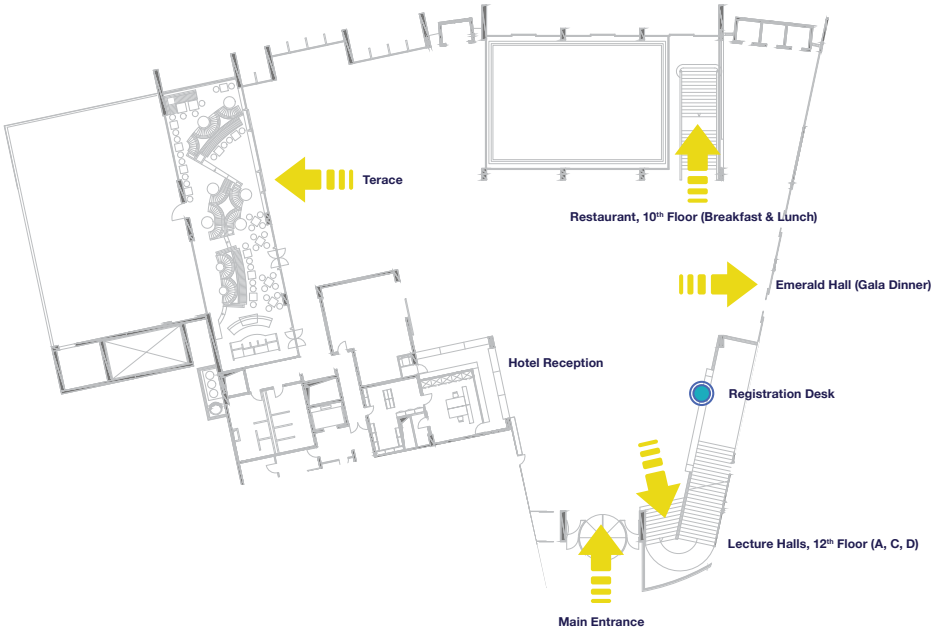
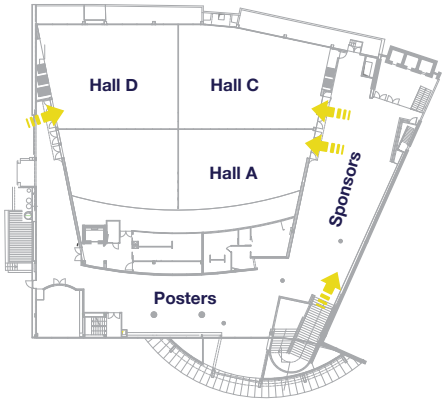
Hotel Lobby, 11th Floor



Europa Hall, 12th Floor



Congress Policies and Information

Name Badges

As a registered attendee, you will be issued an ESVV2025 name badge when you pick up your registration materials. You will be required to display your name badge for admission to all official functions. In the event of a lost badge, please visit the Registration Desk.

Liability

The Congress organizers cannot accept responsibility for personal injuries, or loss or, or damage to private property belonging to you or any accompanying people during or as a consequence of the Congress.

Programme Changes

ESVV2025 reserves the right to make any necessary changes to the program at short notice. There will be no pro rata refund of registration fees for changes to the program.

Scientific Data

Information communicated by presenters should be considered “personal communication.” Please seek permission from the presenter before quoting unpublished research results or using data as a basis for further investigations. Photographs or recording (audio or visual) of data, whether displayed on screens, in posters, or elsewhere is forbidden. Your cooperation is greatly appreciated.

Photography

By attending the Congress, you allow the congress photographer to take pictures of you. These pictures will only be made accessible to the participants of the Congress.

Other Considerations

As a courtesy to all Congress attendees, please make sure all mobile phones are turned off when sessions are in progress.

Taxes

The Value-Added Tax, or VAT, is a general, broadly based consumption tax assessed on the value added to goods and services. It applies to most goods and services that are bought and sold for use or consumption in the European Union. The standard VAT rate in Slovenia is 22%. Prices indicated are always including VAT.

Registration

GH Bernardin, Obala 2, Portorož, Slovenia.

Registration is possible at Registration desk (opposite the hotel reception) on Tuesday, September 2 from 14:00 to 19:00 and on Wednesday, September 3 from 9:00 to 12:30.

Presenter Guidelines

Presentations should be formatted for widescreen (16:9), and all presentations must be in PowerPoint file (media and special fonts must be embedded).

If you included a video, please test the playback on the site.

A good presentation has a clear objective, a well-defined structure, and uses clear and simple illustrations.

Tailor your presentation to a diverse, international audience with varying microbiological backgrounds.

Focus on key findings, implications, and scientific relevance.

Avoid heavy text—use visuals, diagrams, and graphs where possible.

Ensure all images and data are clearly labeled and cited.

Do not use abbreviations or explain them clearly at the beginning.

Rehearsal of your presentation is strongly recommended! Please note that presenting to a live audience can take a little longer than during a rehearsal.

Please be very careful that your presentation is scientific and not commercial.

It is important to adhere to the time schedule.

Session Talks

12 minutes for presentation and 3 minutes for Q&A.

Plenary Keynote Speakers

25 minutes for presentation and 5 minutes for Q&A.

Posters

The measurements of your printed poster should be in A0 Portrait (maximum 84.1 cm wide x 118.9 high – 33.1 in. wide and 46.8 in. high) .

There is one (1) poster board available per presentation; you are required to use ‘Portrait’ layout for your poster.

Include the presentation title, name of authors, institution, city and country on your poster.

Please be very careful that your presentation is scientific and not commercial.

Mounting and taking down your poste

We will provide you with the materials needed to put up your poster, which you can collect on site.

Place the highlights at eye level (i.e. the upper half of the wall element).

Posters can be mounted on Tuesday, 2 September 2025, from 15:00 onwards.

Posters can be taken down after 12:00 on Friday, 5 September.

Poster presenter tips

The dedicated poster session will take place on Wednesday, 3 September 2025, and we kindly ask you to be present at your poster during this time to address any questions from participants.

Programme

Tuesday, September 2, 2025

10:30 – 12:30	**Hall D** **Young EPIZONE/Young Scientist Meeting** (Hall D)	Sponsored by EPIZONE
12:30 – 13:30	**Coffee Break**	Sponsored by EPIZONE
13:30 – 15:30	**Hall D** **EPIZONE CF/EC (Closed Meeting)** (Hall D)	Sponsored by EPIZONE
14:00 – 19:00	**Registration Opening** Registration is possible at Registration desk on Tuesday from 14:00 to 19:00 and on Wednesday from 9:00 to 12:30	
16:00 – 18:00	**Hall A** **Congress Opening** Introduction: Sandra Blome, Ivan Toplak **Opening Speeches** (ESVV President, Local Organizer, Epizone and Rector of University of Ljubljana) **Thomas Mettenleiter:** One Health **Tamaš Petrović:** The Epizootiology Situation in Western Balkan Region: Emerging and Re-emerging Animal and Zoonotic Diseases	
20:00	**Hotel Histrion, Church of St. Bernardin** **Welcome Reception**	

Wednesday, September 3, 2025

09:00 – 10:00	**Hall A** **Plenary Session: Vector-borne Diseases and Zoonoses** Moderator: Matthijn de Boer, Enric Mateu **Melle Holwerda:** Bluetongue virus in the Netherlands, 2023-2025 **Norbert Nowotny:** Update on West Nile virus infections in Europe	Sponsored by EPIZONE
10:00 – 10:30	**Coffee Break**	Sponsored by EPIZONE

10:30 – 12:30

Hall A

Parallel Session: Pathogenesis

Moderator: Tamaš Petrović, Daniel Perez Nunez

Stephanie Clive: Visualization of PRRSV dual infections for investigation of recombination

Jonas Johansson Wensman: Vertical transmission of BTV-3: experiences from Sweden concerning congenital malformations in calves

Hans Nauwynck: Differential infection behavior of African swine fever virus (ASFV) genotype I and II in the upper respiratory tract

Jesús Urquiza Lopez: Identification of a potential entry fusion complex of African swine fever virus and its interaction with endosomal Niemman-Pick-C1 cholesterol transporter

Xuanxuan Zhang: Discovery and Rescue of Porcine Bastroviruses Associated with Polioencephalomyelitis in Domestic Pigs

Alexander Postel: The CD46 Receptor – Does this "Pathogens' Magnet" Restrict the Risk for a Pestiviral Host Switch?

Joren Portaels: Aspergillus Fumigatus Spore Proteases Alter the Respiratory Mucosa Architecture and Facilitate Equine Herpesvirus 1 Infection

Hans Nauwynck: Porcine ex-vivo intestinal mucus has age-dependent blocking activity against transmissible gastroenteritis virus

Hall C

Parallel Session: Immunity

Moderator: Laurent Gillet, Enric Mateu

Svenja Hartenberger: iRhom2 is a major determinant of cellular susceptibility to BVDV infection

Melle Holwerda: Deciphering molecular markers for Bluetongue virus virulence using a vessel-on-chip model

Samuel Kindylides: Transcriptomic profile of chicken monocyte-derived dendritic cells in response to H5N1 highly pathogenic avian influenza virus infection

Sergio Roberto Montaner Tarbes: Live attenuated vaccine-induced cytotoxic T cells correlate with protection against African swine fever virus

Hans Nauwynck: Hiding in plain sight – persistence of feline coronavirus in cats in the presence of neutralizing immunoglobulins at the intestinal mucosa

Pablo Nogales: PPRV modulates caprine dendritic cell function and induces immunosuppression through IL-10 upregulation

Marléne Pinto: Aedes albopictus produce virus-derived DNA upon Batai virus infection

Noemí Sevilla: Bluetongue Virus Evades Innate Immunity by Inhibiting the cGAS-STING Pathway

12:30 – 14:00 **Lunch**

14:00 – 15:00

Hall A

Plenary Session: Re-emerging Animal Diseases

Moderator: Hans Nauwynck, Noemi Sevilla

Guillaume Girault: Foot-and-Mouth disease is back in Europe in 2025: cases in Germany, Hungary and Slovakia

Nick de Regge: Key epidemiological aspects of lumpy skin disease virus determining control measures

15:00 – 15:30 **Coffee Break**

15:30 – 17:30 **Poster Session**

Thursday, September 4, 2025

9:00 – 9:30

Hall A

Plenary Session: Influenza

Moderator: Benoit Muylkens

Ashley Banyard: The ongoing challenges of avian influenza

9:30 – 11:00

Hall A

Parallel Session: Epidemiology

Moderator: Drago Nedic, Nick de Regge

Bryony Armson: Seneca Valley Virus: The Cause of Vesicular Disease in Pigs in England in 2022

Pierre Bessière: Seroprevalence and genetic diversity of feline immunodeficiency virus in outdoor cats in France

Dragan Brnić: Epidemiological Significance of Nasal and Oral Secretions in Rotavirus A Infections in Cattle

Armin Elbers: Lack of biosecurity compliance of poultry farms with a recent history of HPAIv infection determined by video camera monitoring

Ivona Ćorić: Protoparvovirus carnivoran 1 in golden jackals in Croatia

Davide Lelli: Emergence of a Novel Tick-Borne Flavivirus in Domestic and Wild Ungulates in the Italian Alps

Hall C

Parallel Session: Diagnosis

Moderator: Alexander Postel, Benoit Muylkens

Jovita Fernández Pinero: Targeted Metagenomics NGS for the Detection and Characterization of Porcine Viruses

Irene Comtet: Full set of new solutions for a rapid and reliable Foot and Mouth Virus detection : Double Antibody Sandwich ELISA, freeze dried RT-qPCR and Lateral flow device for point-of-care diagnosis

Elmar Erwin Ebner: Honeybee pathogen prevalence in Vienna and on hive validation of the FASTest® BEE 3T: A rapid, applicable and accurate diagnostic tool for honeybee viruses in a citizen science approach and controlled conditions

Jean-Luc Guérin: Genomic surveillance of emerging avian coronaviruses: a field assessment of Oxford nanopore clinical metagenomics

Sarah McGowan: Hyper-mutation of the Equine Infectious Anaemia Virus (EIAV) capsid retains serological detection but reduces infectivity

Semaha Gül Yılmaz: Assessing SRLV Load Dynamics and Genetic Diversity in a Swedish Dairy Goat Herd Around Parturition

11:00 – 11:30 **Coffee Break**

11:30 – 13:00

Hall A

Parallel Session: Epidemiology

Moderator: Laurent Gillet, Matthijn de Boer

Ylva Lindgren: Fowl adenovirus (FAdV) infection in young commercial broiler breeder pullets suggests mitigation of early vertical spread

Clara Montagnin: An update of the epidemiological situation of African Swine Fever in Italy

Ana Moreno: Influenza A virus inactivation in raw milk Grana type cheeses

Van Herzele: New insights into the honeybee virome by an epidemiological follow-up of three honeybee apiaries, using third-generation nanopore sequencing

Gaia Muroni: First detection of Lumpy Skin Disease in Italy: first evidence and response strategies

Hans Nauwynck: The outcome of porcine circovirus 2 infections in porcine lymphoblasts is the result of an intriguing interplay between viral and host genetics

Hall C

Parallel Session: Vaccines

Moderator: Ivan Toplak, Sandra Blome

Liani Coronado: FlagT4G vaccine protects pregnant sows from classical swine fever virus transplacental transmission two weeks After Single vaccine dose

Ilse De Leeuw: Limited field efficacy of a single-dose Syvazul BTV-3 vaccination during the 2024 Bluetongue outbreak in Belgium

Virginia Friedrichs: Safety assessment of the African swine fever vaccine ASFV-G-ΔI177L in mature breeding boars

Enric Mateu: Experimental evaluation of combined vaccination protocols using MLV and experimental inactivated vaccines against a highly virulent PRRSV-1 challenge

Daniel Perez-Nunez: Insight into the molecular mechanisms leading to reversion to virulence of live attenuated vaccines against ASFV

Sara Puente-Marin: Cyclic di-AMP incorporated into the oral formulation enhances the Protective Efficacy of E2-CD154 Chimeric protein Against Classical Swine Fever in Pigs

13:00 – 14:00 **Lunch**

14:00 – 16:00	**Hall A** **ESVV General Assembly and Vote**
20:00	**Emerald Hall** **Gala Dinner with Poster Awards**

Friday, September 5, 2025

9:00 – 10:00	**Hall A** **Plenary Session: Viral Infections in Swine** Moderator: Hans Nauwynck, Carmina Gallardo **Enrique Mateu:** The tale of highly virulent PRRS viruses **Yolanda Revilla:** ASFV: Molecular basis studies in view of Vaccine development
10:00 – 10:30	**Coffee Break**
10:30 – 12:15	**Hall A** **Plenary Session: One Health – Wildlife Diseases, Zoonoses** Moderator: Noemi Sevilla, Daniel Perez Nunez **Ursula Höfle:** Wildlife Diseases **Pierre Bessiere:** Outdoor cats and H5Nx avian influenza: Sentinel role, exposure risk, and broader implications for cross-species transmission **Luca Bordes:** Viral fitness of highly pathogenic avian influenza H5N1 viruses in mammals **Camille Johnston:** Phylogenetic insights into the first Usutu virus outbreak in Denmark, 2024 **Gorana Miletić:** First Serological Evidence of Crimean-Congo Hemorrhagic Fever Virus Infection in Croatian Sheep Populations **Violeta Santrač:** Establishing integrated surveillance for CCHF/CCHFV in Bosnia and Herzegovina: a neglected and unresolved challenge
12:15 – 12:45	**Hall A** **Oral Presentation Award and Closing Remarks**
12:45 – 14:00	**Lunch and Departure**

Keynote Lectures

Opening Lectures

One Health

Thomas C. Mettenleiter

President (retired), Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswsald-Insel Riems, Germany

The ‘One Health’ approach considers the intrinsic interdependence between human, animal and environmental (ecosystem) health in a holistic way. It is based on transsectoral, interdisciplinary cooperation, communication, coordination and capacity building. In particular during the COVID-19 pandemic, this approach gained significant visibility, although it is rooted in the much older concept of ‘One medicine’ which dates back to Rudolf Virchow in the 1870s. Actually, veterinarians have been (and still are) in the lead in the implementation of One Health approaches. This is particularly evident in the context of zoonotic infections. Avian Flu, Swine Flu, MERS, SARS, Ebola, COVID-19, mPox: zoonotic infections are in the limelight more than ever before. More than 60% of human infections are estimated to originate from animals and more than 75% of newly emerging human infections are zoonoses. The human population consisting of 8 billion individuals which are highly mobile, capable of spanning huge geographic distances in a very short time, and aggregate in urban centers, form an ideal host population. The increasing contact between humans and animal reservoirs favors spill-overs of infectious agents, which can lead to epidemics with possible pandemic potential. Thus, significant efforts to establish systems-based One Health in integrated health approaches have been undertaken. On a global level, the One Health High-Level Expert Panel (OHHLEP) was established to scientifically advise the Quadripartite of WHO, FAO, WOAH and UNEP in One Health issues. OHHLEP’s One Health definition is now generally accepted and forms the basis for many One Health activities including the political level. This culminated in the inclusion of One Health in the Agreement on Pandemic Prevention, Preparedness and Response that has recently been adopted by the World Health Assembly.

The Epizootiology Situation in Western Balkan Region: Emerging and Re-emerging Animal and Zoonotic Diseases

Tamaš Petrović[1]*, Budimir Plavšić[2]

[1] Scientific Veterinary Institute „Novi Sad“, Novi Sad, Serbia
[2] World Organisation for Animal Health (WOAH), Regional Representative for Europe, Head of Regional Representation in Moscow

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Diseases of animals, especially those that are highly contagious or transmissible, can cause huge consequences, i.e. threaten the global food supply, reduce the availability of non-food-related animal products, or influence human health, production or quality of life. During the past decades animal diseases with or without zoonotic dimension are spreading at an ever-increasing speed to regions hitherto uninfected, mainly caused by overall globalization, highly increased intensity of international travel, movement of animals including the trade of animals and animal products all over the globe, and by the effects of climate change. The Western Balkan countries constitute a critical epizootiological interface, acting as a primary land bridge for the incursion of transboundary animal diseases (TADs) and zoonoses from endemic reservoirs in the Middle East and Africa into the European region. This strategic sub-region is characterized by a deep-rooted agricultural sector dominated by traditional, low-biosecurity backyard farming, representing a unique landscape for animal health governance.

This presentation aims to give a short overview of epizootiological situation of some of the most important TADs or other emerging or re-emerging diseases i.e. pathogens introduced / endemically present / currently circulating or eradicated from the area of Western Bakan region in the last 10 to 20 years. It is based on a synthesis of epizootiological data from the WOAH information system (WAHIS), strategic documents and reports from regional bodies such as WOAH - Europe and regional GF-TADs, data from regional and international expert and scientific meetings, and an extensive review of peer-reviewed scientific literature.

Due to the economic importance and animal and public health influence, the epizootiological situation for following diseases will be presented and discussed in more detail: African (ASF) and classical swine fever (CSF), highly pathogenic avian influenza (HPAI), bluetongue (BT), lumpy skin disease (LSD), peste des petits ruminants (PPR), sheep pox & goat pox (SPP/GPP), as well as rabies and West Nile fever (WNF). Out of listed diseases, some of them were successfully eradicated (like CSF and LSD) or almost eradicated (rabies), some are present and circulating for a couple of years, or are even endemic for some parts and currently causing a significant economic and/or health issues (ASF, WNF), some present the continuous threat with sporadic occurrence each year (like HPAI), or currently emerging (PPR, SPPV/GPPV), or are of sporadic emergence, with an ongoing introduction in this moment (like BTV).

Whatever the single most important reason for the introduction and spread of any of discussed animal diseases, it is obvious that the underlying causal process is anthropogenic. Anthropogenic factors, such as the prevalence of high-risk, low-biosecurity farming systems and the illicit animal trade, are exacerbated by significant effects of climate change on vector ecology. Strengthening national veterinary services capacities, cross-border collaboration and the involvement in international networks, enhancing farming biosecurity and development of modern digital disease early-warning systems with protocols for rapid, effective response, and the institutionalization of a comprehensive One Health approach, should be priorities to contain, control or eliminate existing, or emerging and reemerging animal diseases.

Key words: transboundary, emerging and re-emerging animal diseases; overview of epizootiological situation; Western Balkan region

Acknowledgments: The study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract number 451-03-136/2025-03/20031).

Plenary Session: Vector-borne Diseases and Zoonoses

Bluetongue virus in the Netherlands, 2023-2025

Melle Holwerda [1]*

[1] Wageningen Bioveterinary Research (WBVR), The Netherlands

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Bluetongue virus (BTV) is an arthropod borne virus belonging to the Orbivirus family and is transmitted by blood meals of vector competent *Culicoides*-midges. The virus can cause Bluetongue (BT) in susceptible ruminants, where primarily sheep are vulnerable for infection. The virus is classified based on serotypes where more then 30 have been described, however, only serotype 1-24 are notifiable according to the World Organization of Animal Health (WOAH). In 2006, BTV serotype 8 (BTV-8) emerged in Northwestern Europe which became a major outbreak until emergency vaccination became available in 2008. The Netherlands achieved the BTV-free status again in 2012 after a 3 year period of absence of virus in animals. In September 2023, clinical signs of BTV in sheep like hypersalivation, fever and facial edema were observed on multiple locations in the middle of the Netherlands and notified to the authorities. Real-time PCR confirmed the presence of BTV and the entire genomic sequence was determined within two days using whole genome sequencing. The generated sequences were compared to other sequences from publicly available databases, but no other virus isolates with high nucleotide homology could be found and therefore it's geographical origin and route of introduction remains undetermined. In the following months, the virus continued to spread within the Netherlands and surrounding countries. During the spring of 2024, emergency vaccinations became available, but the virus still continued to spread throughout the Netherlands. In September 2024, a new clinical suspicion of BTV was made in a ram lamb that was fully vaccinated against BTV-3 and molecular analysis showed the presence of BTV-12. Retrospective analysis showed that the virus was only recently introduced and finally the virus was only diagnosed on 12 locations in 2024. For the 2025 BTV-season, a new monitoring program was developed to investigate the presence of BTV in the Netherlands, yet no active infections have been observed. This indicates that BTV-3 and BTV-12 might not be circulating anymore in the Netherlands, but caution should be raised for the introductions of new BTV serotypes.

Update on West Nile virus infections in Europe

Norbert Nowotny[1,2]* and colleagues

[1] Centre of Pathobiology, Department of Biological Sciences and Pathobiology, University of Veterinary Medicine Vienna, Vienna, Austria; and
[2] College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai Health, Dubai, United Arab Emirates.

* *Norbert.Nowotny@vetmeduni.ac.at*

West Nile virus (WNV) received its name due to its first isolation from a human patient in the West Nile district of Uganda in 1937. It is a mosquito-borne flavivirus that is maintained in an enzootic transmission cycle between avian hosts and mosquito vectors. Incidentally, the virus can be transmitted to humans and other mammals through the bite of infected mosquitoes. Infection in humans is frequently asymptomatic, but in 20% of cases it presents as West Nile fever, and in less than 1% of cases it results in neuroinvasive disease. However, asymptomatic blood donors may transmit the virus through their blood donation. Up to 9 genetic lineages have been described, however only lineages 1 and 2 have a significant veterinary and public health impact. Initially only lineage 1 WNV was present in Europe, which did not pose a major animal and public health risk with a few exceptions such as a large outbreak among humans in Bucharest and a few smaller outbreaks in horses in the Mediterranean area. This changed with the introduction of a lineage 2 WNV to Hungary in 2004 (Bakonyi et al., Emerg Infect Dis. 2006 Apr;12(4):618-23). Following an adaptation period of about 4 years, this virus started to spread, first to Austria, where it was detected in 2008, and soon thereafter to the Balkan region in the south, where it caused a significant outbreak among humans in Greece in 2010. This central/southern European clade of WNV lineage 2 continued to expand its geographic range also towards the west and the north, and it is now present essentially all over Europe. Besides humans, this virus is pathogenic for certain species of birds and horses. The “indicator” bird species, which usually succumbs to the infection, is the Northern goshawk, in which the infection is generally neuroinvasive. Slightly after the introduction of this virus to Hungary in 2004, another lineage 2 WNV emerged in the Volgograd region of Russia (eastern European clade of WNV lineage 2). This strain spread within Russia and also to Romania. Most likely migratory birds brought the virus to Europe, where the transmission-competent mosquito vector, i.e. Culex species, were already present. Besides these two newly introduced lineage 2 WNVs, in certain regions of Europe still WNV lineage 1 is circulating. Depending on various, mainly environmental and climatic factors, larger outbreaks may occur, such as in 2018 and 2024. This year a larger outbreak was observed in central Italy. In my talk, I will share the latest epidemiological data and trends.

Plenary Session: Re-emerging Animal Diseases

Foot-and-Mouth disease is back in Europe in 2025: cases in Germany, Hungary and Slovakia

Guillaume Girault[1]*, Martin Tinak[2], Peter Malik[3], Aurore Romey[1], Zuzana Dirbakova[2], Anthony Relmy[1], Anne-Laure Salomez[1], Cindy Bernelin-Cottet[1], Stephan Zientara[1], Michael Eschbaumer[4], Sandra Blaise-Boisseau[1], Labib Bakkali Kassimi[1]

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[4] Friedrich-Loeffler-Institut, NRL for Foot-and-Mouth Disease, Riems Island, Germany

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Foot-and-Mouth disease (FMD) is one of the most contagious animal diseases, and is caused by the Foot-and-mouth disease virus (FMDV). The disease is essentially present in seven geographical pools, and some countries are endemic while others are free with or without vaccination. Even if the disease mortality can be considered as low, the morbidity can reach 100% according to species, and the consequences in FMD-free countries can be dramatic, with important economic losses.

The European Reference Laboratory (EURL) for FMD has a role into the preparedness of EU laboratories, essentially for quick and reliable detection and identification of any FMD case within the EU. The EURL also has a role of assistance to EU member states to confirm FMD suspicion, by performing viral isolation, molecular and serological analyses, so as sequencing methods.

Since 2011, the European Union was free from FMD, but on January 2025, the German National Reference Laboratory (NRL) identified FMDV from samples taken from a dead Asian buffalo showing clinical signs characteristic of FMD. This outbreak was related to lineage O/ME-SA/SA-2018, phylogenetically close to strains reported in Iran and Türkiye in 2023 and 2024 respectively.

At the beginning of March, the Hungarian NRL for FMD also confirmed FMD in a farm at the border with Slovakia. The virus identified during this outbreak was belonging to sublineage O/ME-SA/PanAsia-2^{ANT-10}, thus different from the virus that had emerged in Germany two months earlier. This virus was phylogenetically close to strains identified in Pakistan in 2017 and Türkiye in 2024. Slovakia subsequently reported outbreaks of FMD on the Hungarian border. By the end of April 2025, six outbreaks had been reported in Slovakia and five in Hungary, all of which led to massive depopulation measures.

Even if EU was free from FMD for 14 years, two different incursions of FMDV occurred in three months. The outbreaks have been contained, thanks to a global effort from the countries and different stakeholders, including the NRLs and EURL. These outbreaks remind us that FMD still represent a threat to FMD-free countries and that preparedness is essential.

Key epidemiological aspects of lumpy skin disease virus determining control measures

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Lumpy skin disease virus (LSDV) is the causative agent of lumpy skin disease, a nodular dermatitis observed in cattle and water buffalo. LSDV is listed as a notifiable disease by the World Organisation for Animal Health (WOAH) and as a category A disease in the European Animal Health Law.

In 2024, northern African countries like Algeria, Tunisia and Libya reported for the first time LSDV circulation, thereby posing an increasing risk for a reintroduction of the disease in Europe. By the end of June 2025, this anticipated threat became reality with the notification of LSDV cases in Sardinia, mainland Italy (Lombardia) and France (Savoie and Haute-Savoie). This presentation will provide a short update on the epidemiological situation and summarize available information on LSDV transmission and vaccination as these are determinants of the control measures that are being implemented to eradicate the disease from the European territory.

LSDV is considered to be a vector-borne disease, and different vectors like stable flies, mosquitoes and ticks have been implicated as mechanical vectors for clade 1 strains. Also clade 2 strains are transmitted by vectors, but indications emerge that mosquitoes could also act as biological vectors for these strains. Furthermore, there is a growing body of evidence that clade 2 strains can also spread efficiently via direct and indirect contact, without the involvement of vectors. These findings could impact the epidemiology and control of the disease.

Vaccination is considered the most important control measure to prevent LSDV transmission and to control outbreaks. Both heterologous (based on sheeppox or goatpox virus strains) and homologous (based on LSDV strains) vaccines have been tested and used. Based on available experimental and field data, live attenuated homologous vaccines based on the Neethling strain are recommended to stop LSDV spread based on their good efficacy, long duration of immunity and limited side effects.

The current outbreaks in Europe indicate that an early detection and rapid implementation of vaccination, together with other control measures as stamping out and movement restrictions, can lead to a fast eradication of the disease upon introduction.

The ongoing challenges of avian influenza

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Avian influenza viruses constitute a diverse group of viral pathogens that affect a broad range of avian species globally. On top of their impact on avian species, these viruses pose risks to other species, and many subtypes are considered to also be a significant zoonotic risk. Of note, the clade 2.3.4.4b H5N1 high pathogenicity avian influenza virus (HPAIV) has emerged globally to cause a panzootic unlike anything seen before. The virus has reached areas previously untouched by HPAIV threatening fragile ecosystems in remote locations as well as decimating the poultry sector.

The H5N1 panzootic has not only impacted upon avian populations but has also had a profound impact upon both wild and captive mammalian species. The most unexpected twist in the story of H5N1 was seen following the emergence of infection of dairy cattle with H5N1 in the United State of America during early 2024. Following emergence, the virus has continued to circulate in cattle, with sporadic detections also reported in other ruminant species. The detection of high viral loads in milk from infected cattle, has also resulted in several human infections, once again underscoring the zoonotic potential of these viruses. In response, several countries have intensified surveillance in non-avian species to evaluate the potential for undetected viral circulation in captive mammals. In Great Britain, bulk milk tank testing of cattle and targeted surveillance of captive mammalian species on an infected premises is undertaken in accordance with the outcome of a rapid risk assessment and has resulted in the detection of infection in a single sheep. Such findings are being replicated in other areas where facilities and resources are available to look for evidence of infection in atypical species.

From a poultry perspective, the profound impact of these viruses on the global poultry sector has driven increased pressure to vaccinate flocks in countries that have never previously considered vaccination. Historically, enhanced biosecurity has been the frontline defense against these viruses in many countries with culling of infected flocks being undertaken where incursions of disease were seen. However, the global intensity of outbreaks has focused thoughts on vaccination as a tool to protect poultry from HPAIV.

Plenary Session: Viral Infections in Swine

The tale of highly virulent PRRS viruses

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The emergence of the *Porcine reproductive and respiratory syndrome virus* (PRRSV) marked a turning point in pig farming worldwide. The economic and sanitary impact of the virus has fuelled the development of new pig management strategies, such as three-site production and all-in/all-out procedures, which have become standard in many countries. It also served to accelerate the implementation of stricter and more comprehensive biosecurity measures, as well as the genetic editing of pigs as a means of controlling infectious diseases. Unfortunately, more than 30 years after its emergence, victory is still far from certain. One factor contributing to the persistence of PRRSV is the constant emergence and selection of new viral variants, some of which exhibit enhanced virulence traits. For many years, PRRSV1 (the so-called European type) was considered less virulent than PRRSV2 (the North American type), and the European perception of the disease was less dramatic than that of the Americans. The emergence of highly virulent PRRSV2 strains such as JXA1 in China and MN184 in the USA has reinforced this idea. The discovery of PRRSV1 strains with enhanced virulence, such as the Lena strain in Belarus, raised some concerns. However, as Lena belonged to subtype 3, which was restricted to the easternmost parts of Europe, many people thought that this problem was only of local interest. In 2014, Italian researchers reported the emergence of the PRRSV1.1 strain PR40 in Italy, which is the most virulent strain of this species to date. Once again, this did not attract the necessary attention. In subsequent years, other strains of enhanced virulence emerged in Europe, but none compared to the emergence of the so-called 'Rosalía' strain in Spain, the largest pig producer in Europe. The impact of the disease has been huge, contributing to a pig deficit of millions. Interestingly, Rosalía and the highly virulent PRRSV2 L1C share many features in terms of their origin, pathogenesis and evolution.

ASFV: Molecular basis studies in view of Vaccine development

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African swine fever virus (ASFV) is the causative agent of the highly contagious disease African swine fever, which has an upsetting impact in pigs farming. Beyond the high mortality taxes (up to 100% in infected pigs by virulent strains), as soon as an infected animal is detected in a farm in EU all the other animals must be sacrificed. The coast-to-coast spread of ASF occurred on at least three separate occasions, most significantly in Georgia, in 2007, where it spread from the Black Sea into Eastern Europe. In the following decade, the disease became epizootic in Russia, and by 2018 it had spread as far west as Belgium, Poland, Romania, Hungary and east to China, quickly taking over most of Southeast Asia and Oceania. According to the World Organization for Animal Health (WOAH), from January 2020 to January 2022, ASF outbreaks were reported in 35 countries or regions around the world.

The complexity of the ASFV genome and the viral mechanisms of interference with the host response, have probably been the main factors hindering fully safe vaccine development. In addition, issues such as genetic and antigenic diversity, as well as poor cross-protective immunity among genotypes, further complicate the vaccine production. Challenges to achieve protection against ASFV using classical techniques such as DNA vaccines or recombinant subunit vaccines, have so far failed. Above all these strategies, live attenuated vaccines (LAVs) are the current more promising strategy in ASF vaccine, and so, efforts of our group have been focused on unravel the molecular basis of the virulence and the development of LAVs by manipulating genes involved in virulence, most of them related to the control of type I interferon (IFN-I). Whereas some LAVs displayed high efficacy data, safety concerns and lack of cross reactivity between different strains/genotypes still remain. Furthermore, vaccine virulence may change due to duplications, mutations, or deletions of certain sequences in the ASFV genome occurring in the vaccinated animals. Because of that, defective and/or non-fully replicative forms for ASF next generation vaccines development are also presently approaching in our group.

Plenary Session: One Health – Wildlife Diseases, Zoonoses

Wildlife Diseases - Understanding the interplay between wildlife ecology and diseases emergence

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Viral disease emergence in wildlife has frequently considerable impacts on human and domestic animal health but generally also causes profound impacts in wildlife. Such impacts, especially large scale local or regional mortalities, have often difficult to foresee cascading ecological consequences and effects on biodiversity and landscape immunity which in turn can impact human and livestock health. The ecology and, not always evident, interactions of different species and of these with their habitat are important, albeit often overlooked drivers. An evident example is the current panzootic of H5N1 clade 2.3.4b, and its toll on wild birds and marine mammals and terrestrial carnivores. The resulting decline of colony breeding gulls has subsequently affected endangered duck species that used gull colonies for protection against predators

Other examples include orthoavula and Flaviviruses, the former emerged in free-living columbiforms in the 1980s and has recently expanded to hitherto unaffected species. The latter may have severe regional impacts on passerine (e.g. blackbird) populations and, a less well-known feature for Flaviviruses, increasing susceptibility to secondary infections in birds of prey, partridges and other species.

In change, canine distemper epidemics in Serengeti lions are an example of interaction of the ecology of several hosts and pathogens and their environmental drivers. When these systems are disturbed by external factors such as climate extremes or land use changes, severe outbreaks may be the consequence. These and other examples that will be discussed highlight the importance of the interaction of host and pathogen ecology.

Taking the ecology of wildlife species into account is key to understanding disease emergence and impacts and thus needs to be included in One Health concept driven actions to prevent and mitigate impacts of disease emergence.

Short Lectures

Pathogenesis

Visualization of PRRSV dual infections for investigation of recombination

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Porcine reproductive and respiratory syndrome virus (PRRSV-1) is the causative agent of porcine reproductive and respiratory syndrome and is endemic in the UK. Recombination between field strains, between vaccine strains or between field and modified-live vaccine strains has been reported in the UK or Europe previously. Due to its huge economic impact, PRRSV diversity in the field is monitored in the UK, including the identification of vaccine-like strains and monitoring occurrences of recombination between these and field strains. Recombinant variants have sometimes been reported to be more pathogenic than their respective parental strains. However, recombination and its likelihood has never been studied in depth *in vitro* with strains of PRRSV-1.

As a prerequisite to recombination at least two strains of PRRSV-1 must be present simultaneously in the same cell. The aim of this work is to create a system where such dual infections can be effectively visualized to determine the percentage of dual infected cells. The effect of varying the relative timings of the infection and the different strains used in infection (which will include both vaccine and field strains), can then be evaluated. The conditions which create the most dual infections will then be taken forward to look for evidence of successful recombination using PCR. ViewRNA, a fluorescent in situ hybridization system with strain specific molecular probes, was used to visualize infections. Varying timings between infections were used, and strains allowed to infect cells for a minimum of 18 hours. Initial results have shown that levels of dual infection can be increased with optimal timings between two different strains.

Vertical transmission of BTV-3: experiences from Sweden concerning congenital malformations in calves

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Bluetongue virus serotype 3 (BTV-3) recently emerged in Europe, reaching Sweden in September 2024. BTV-3 is a midge-borne virus affecting sheep and cattle with clinical signs ranging from mild or subclinical to severe, including mortalities. After the first reported case, the virus spread rapidly in South-Western and Southern Sweden from September to November 2024. As the average daily temperatures dropped and the vector activity ceased, the number of reported cases decreased in cattle and stopped completely in sheep by the end of November. In February 2025, an unusual accumulation of malformed calves from the area in Southern Sweden that had been most affected by BTV-3 in 2024 was reported. The predominant clinical signs in these calves were weak born “dummy calves”, that were unable to nurse and had a decreased general condition. Blindness and abnormal leg conformation were also reported. Tests run confirmed that other pathogens known to cause malformations in calves were negative, and BTV-3 specific PCR were positive. This study presents data on clinical signs, pathology and virology findings of these BTV-3-induced malformations in calves in Sweden. Until the end of May 2025, 42 calves presenting with malformations of the central nervous system and being positive for BTV-3 by real-time RT-PCR (tissue samples from spleen) have been necropsied. For seven of these calves, tissue samples from the central nervous system were available and analyzed by pan-BTV real-time RT-PCR and BTV-3 specific PCR. All seven calves were positive in the pan-BTV assay and six were positive for BTV-3. The majority of the included calves (n=42) were beef cattle (79%), while 14% were dairy cattle and 5% crossbreeds. The primary diagnosis from necroscopy were hydrocephalus and hydranencephaly. Currently, virus isolation attempts are ongoing. In conclusion, BTV-3 induced congenital malformations in calves during the first season of BTV-3 in Sweden, most likely due to vertical transmission during the 4th to 5th gestation month, when the central nervous system was being developed. Whether calves born PCR positive are also virus positive and thus can be a source for virus transmission warrants further studies, including studies of early vector activity.

Differential infection behavior of African swine fever virus (ASFV) genotype I and II in the upper respiratory tract

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African swine fever virus (ASFV) is a substantial threat to pig populations worldwide, contributing to economic disruption and food security challenges. Its spread is attributed to the oronasal transmission route, particularly in animals with acute ASF. Our study addressed the understudied role of nasal mucosa in ASFV infection, using a nasal explant model. The explants remained viable and revealed a discernible ASFV infection in nasal septum and turbinates post-inoculation. Interestingly, more infected cells were found in the turbinates despite its thinner structure. Further analyses showed (i) a higher replication of genotype II strain BEL18 than genotype I strain E70 in the epithelial cell layer, (ii) a preference of ASFV infection for the lamina propria and a tropism of ASFV for various susceptible cell types in different areas in the nasal mucosa, including epithelial cells, macrophages, and endothelial cells. Using porcine respiratory epithelial cells (PoRECs), isolated from nasal tissue, we found a difference in infection mechanism between the two genotypes, with genotype I favoring the basolateral surface and genotype II preferring the apical surface. Moreover, disruption of intercellular junctions enhanced infection for genotype I. This study demonstrated that ASFV may use the respiratory mucosa for entry using different cell types for replication with a genotype difference in their infection of respiratory epithelial cells.

Identification of a potential entry fusion complex of African swine fever virus and its interaction with endosomal Niemman-Pick-C1 cholesterol transporter

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African swine fever virus (ASFV) belongs to the family Asfarviridae, part of the group of Nucleocytoplasmic Large DNA Viruses (NCLDV). Little is known about the internalization of ASFV in the host cell and the membrane fusion events that take place at early stages of the infection. Poxviruses, also members of the NCLDV and represented by vaccinia virus (VACV), are large, enveloped, double-stranded DNA viruses. Poxviruses were considered unique in having an elaborate entry-fusion complex (EFC) composed of 11 highly conserved proteins integrated into the membrane of mature virions. Recent advances in methodological techniques have again revealed several connections between VACV EFC proteins.

In this study, we explored the possibility of an analogous ASFV EFC by identifying ten candidate proteins exhibiting structural similarities with VACV EFC proteins. Furthermore, we investigated the potential interactions of this putative fusion complex with key endosomal proteins involved in the infection process and membrane fusion at the late endosomal stage.

This study sheds light on the mechanisms governing ASFV entry and membrane fusion, highlighting conserved strategies among NCLDV members and potential targets for antiviral strategies.

Discovery and Rescue of Porcine Bastroviruses Associated with Polioencephalomyelitis in Domestic Pigs

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Bastroviruses (BastV) are non-enveloped single-stranded positive-sense RNA viruses that have been discovered recently in faeces samples of different animals and humans. The non-structural proteins of these viruses show similarities to those of Hepevirsues, and the structural proteins exhibit similarities to those of Astroviruses. However, the association of bastrovirus infection and clinical disease manifestations has not yet been established.

In this study, we report the identification of porcine bastrovirus (PoBastV) in brain samples of domestic pigs that presented fatal neurological disease in two unrelated disease outbreak scenarios in Australia and Switzerland. Viral metatranscriptomics identified genomic sequences of two genetically closely related PoBastV strains (PoBastV AUS/2015 and CHE/2022). Genomic RNA of both strains was readily detected by in situ RNA hybridisation in neurons and glia cells of brain tissues presenting histopathological lesions, thus supporting a plausible causal relationship between neurotropism and disease. We also generated a molecular cDNA clone of PoBastV CHE/2022 and rescued infectious virus by reverse genetics in swine kidney cells (SK-6) and further virus passage in intestinal porcine enterocytes (IPEC-J2). To investigate viral replication and protein expression, we performed replication kinetics analysis and antisera-based immunofluorescence detection. Early findings demonstrated efficient PoBastV replication in IPEC-J2 cells, reaching a plateau at 48 hours post-infection (h.p.i.). Moreover, Ruxolitinib, a JAK1/JAK2 inhibitor, influenced viral replication, offering insights into the host innate immune response.

These findings pave way towards PoBastV being a potential etiological agent of neurological disease outbreaks in pigs and lay the groundwork for further studies into the molecular biology and pathogenesis of emerging bastrovirus infections.

The CD46 Receptor – Does this "Pathogens' Magnet" Restrict the Risk for a Pestiviral Host Switch?

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The complement regulatory protein CD46 is a multifunctional membrane protein with orthologues in many mammalian species. It is exploited by bacterial and viral pathogens including viruses of different species within the genus *Pestivirus*.

In the presented study, genetically engineered knockout cells revealed strict dependency on the ovine CD46 receptor of different small ruminant pestiviruses including border disease virus (BDV), Tunisian sheep virus (TSV), Aydin pestivirus (Aydin PeV) and ovine Italy pestivirus (ovIt PeV). We further investigate how CD46 contributes to host cell specificity and its possible role in a host cell switch. Even the pestiviruses that originate from ovine hosts, but show highest similarity to the pig-specific classical swine fever virus (CSFV), proved to be highly adapted to ovine cells. Although the viruses showed flexibility to use different isoforms as well as genetically modified variants of ovine CD46, they were not able to utilize the porcine orthologue. Porcine cells expressing ovine CD46 showed increased permissivity, indicating that CD46 is a restriction factor preventing a host switch.

Although CD46 is used by different pathogens as receptor, it is at the same time a molecular determinant contributing to host cell specificity of pestiviruses. Overcoming this bottleneck at the cell entry level could result in efficient infection of porcine hosts with severe consequences for pig production, established CSF diagnosis and control.

This project was funded by the Deutsche Forschungsgemeinschaft, project number 427829762.

Aspergillus Fumigatus Spore Proteases Alter the Respiratory Mucosa Architecture and Facilitate Equine Herpesvirus 1 Infection

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Numerous Aspergillus fumigatus (Af) airborne spores are inhaled daily by humans and animals due to their ubiquitous presence. The interaction between the spores and the respiratory epithelium, as well as its impact on the epithelial barrier function, remains largely unknown. The epithelial barrier protects the respiratory epithelium against viral infections. However, it can be compromised by environmental contaminants such as pollen, thereby increasing susceptibility to respiratory viral infections, including alphaherpesvirus equine herpesvirus type 1 (EHV-1). To determine whether Af spores disrupt the epithelial integrity and enhance susceptibility to viral infections, equine respiratory mucosal ex vivo explants were pretreated with Af spore diffusate, followed by EHV-1 inoculation. Spore proteases were characterized by zymography and identified using mass spectrometry-based proteomics. Proteases of the serine protease, metalloprotease, and aspartic protease groups were identified. Morphological analysis of hematoxylin-eosin (HE)-stained sections of the explants revealed that Af spores induced the desquamation of epithelial cells and a significant increase in intercellular space at high and low concentrations, respectively. The increase in intercellular space in the epithelium caused by Af spore proteases correlated with an increase in EHV-1 infection. Together, our findings demonstrate that Af spore proteases disrupt epithelial integrity, potentially leading to increased viral infection of the respiratory epithelium.

Porcine ex-vivo intestinal mucus has age-dependent blocking activity against transmissible gastroenteritis virus

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Transmissible gastroenteritis virus (TGEV) causes high mortality in young piglets (< 3 days of age). With aging, the susceptibility/morbidity/mortality rates drop. We previously hypothesized that the age-related changes in the intestinal mucus could be responsible for this resistance. Hence, this study investigated the effect of porcine intestinal mucus from 3-day and 3-week-old pigs on the free mobility of the virulent TGEV Miller strain, and on the infection in swine testicle (ST) cells. Single particle tracking (SPT) revealed that TGEV had significantly higher diffusion coefficients in 3-day mucus compared to 3-week mucus. TGEV and charged and uncharged control nanoparticles diffused freely in 3-day mucus but were hindered by 3-week mucus in the diffusion model; TGEV mimicked the diffusion behavior of negatively charged carboxylated particles. Inoculation of ST cells with TGEV in the presence of 3-week mucus resulted in a significantly lower average number of infected cells (30.9 ± 11.9/5 fields) compared with 3-day mucus (84.6 ± 16.4/5 fields). These results show that 3-week mucus has a significant TGEV-blocking activity compared to 3-day mucus in free diffusion and infection of the underlying susceptible cells. Additionally, a label-free proteomics analysis revealed an increased expression of mucin 13, known for negatively regulating the tight junctions in intestinal epithelium, in 3-day-old pigs. In 3-week-old pigs, a higher expression of mucin 2, a type of secreted mucin which is known for inhibiting coronavirus infection, was observed. Concludingly, this study demonstrated a protective effect of 3-week mucus against viral infections.

Immunity

iRhom2 is a major determinant of cellular susceptibility to BVDV infection

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Host cell invasion of Bovine viral diarrhea virus (BVDV, a *Pestivirus* from the *Flaviviridae*), is mediated by several cellular factors for virus binding and internalization. As cellular receptors several candidates have been put forward including CD46, LDL receptor, high affinity laminin receptor and ADAM17. According to current data cells lacking ADAM17 are completely resistant to BVDV infection, the other candidates result in reduction of permissivity. ADAM17 is broadly expressed and a key factor on inflammation, for instance ectodomain shedding of TNFα. For this reason, biosynthesis and proteolytic activity of ADAM17 are tightly regulated. To further elucidate the exact role of ADAM17 in BVDV entry CRISPR/Cas9 knock out (KO) screens targeting 30 different genes related to the ADAM17 interactome were performed in MDBK cells. For this purpose, MDBK cells inducibly expressing Cas9 were transduced with lentiviral vectors encoding for one sgRNA and two fluorophores. 5 sgRNAs were applied for each target gene. Survival of sgRNA-expressing cells in comparison to non-sgRNA-expressing cells after infection with two different cytopathic BVDV isolates were assessed by flow cytometry. The knock outs were confirmed by sequencing. Using Nanoblade technology defined KO cell lines were generated with larger deletions in the target genes. iRhom2 turned out as an essential cellular factor within the ADAM17 interactome whose absence rendered cells resistant to deadly BVDV infection. Transcomplementation of iRhom2 in iRhom2$^{-/-}$ cell lines rescued the susceptibility to BVDV infection. iRhom2 and its homologue iRhom1 are members of the rhomboid protease family. Both are known to facilitate trafficking of the ADAM17 pro form from the ER to the cell surface via Golgi apparatus, where ADAM17 is cleaved by Furin protease into its mature form. Surprisingly, iRhom1$^{-/-}$ knock out cells remained susceptible to BVDV. Since iRhom1 is functionally equivalent to iRhom2 in ADAM17 trafficking, we postulate that iRhom2 has more functions in BVDV entry than just the transport of ADAM17 to the cell surface.

This work was funded by the Austrian Research fund (FWF, P35674)

Deciphering molecular markers for Bluetongue virus virulence using a vessel-on-chip model

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Since September 2023, The Netherlands has been facing an outbreak of bluetongue virus serotype 3 (BTV-3), primarily affecting sheep and cattle. BTV is transmitted through the bloodmeal of an infected *Culicoides*-midge, whereafter the virus targets the endothelium causing hemorrhages and oedema. BTV-3 is responsible for great losses among sheep and cattle and has proven more virulent than the BTV-8 serotype causing the previous outbreak in 2006-2008. Additionally, BTV-12 has been detected in September 2024 whereof the virulence remains undetermined. The molecular mechanism behind the difference in virulence of BTV serotypes is unknown. Normally, animal experiments are performed to analyze the virulence of serotypes of BTV, however, these experiments come with ethical issues, are expensive and labor intensive. These problems shows the urge for alternatives for animal experiments that are still biological relevant like the vessel-on-chip technology. Therefore, this study aimed to explore for molecular markers that are indicative for the virulence of BTV in a biologically relevant *in vitro* model. First, we successfully cultured primary sheep umbilical vein endothelial cells (ShUVECs) under flow conditions in a vessel-on-chip (VoC) model, resulting in 3D vessel structures. Subsequent, infection with BTV-3, BTV-6, BTV-8 and BTV-12 showed that all viruses efficiently infect and replicate within these artificial vessels. Next, using quantitative PCR, we showed significant innate immune gene activation like CXCL10, Mx1, ISG15 and IFIT3 at 24 hours post-infection. Additionally, damage of the 3D vessels was observed after staining for the tight-junction marker Zonula Occludens-1 (ZO-1) and actin for BTV-3 and BTV-12 after 24 hours of infection. The next step will be to investigate for differentially expressed genes in endothelial cells during infection with the viruses to decipher for molecular markers indicative of *in vivo* virulence. These findings will give more insight into the molecular mechanism of BTV virulence and can contribute to the development of novel intervention strategies. Additionally, the developed 3D VoC model contributes to the 3Rs of animal research and can serve as a tool to quickly assess the virulence of (newly emerging) BTV variants.

Transcriptomic profile of chicken monocyte-derived dendritic cells in response to H5N1 highly pathogenic avian influenza virus infection

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Since 2021, clade 2.3.4.4b H5N1 highly pathogenic avian influenza viruses (HPAIVs) have become the most predominant worldwide. These viruses are associated with high mortality rates in poultry and wild birds, leading to sporadic spill-over infections in humans and other mammals. Notably, aquatic wild birds, such as Anseriformes and Charadriiformes, act as the natural reservoir for avian influenza A viruses (AIVs) and were historically asymptomatic upon AIV infection. However, current clade 2.3.4.4b viruses induce severe symptoms and high mortality rates in infected waterfowl. Within the framework of the WiLiMan-ID project, our main goal is to gain insight into the mechanisms that shape host-pathogen interactions and contribute to a higher HPAIV virulence. To this end, an *ex vivo* HPAIV infection model was developed using chicken dendritic cells, key antigen-presenting cells that play a crucial role in triggering both innate and adaptive immune responses. More specifically, the transcriptomic profile of monocyte-derived dendritic cells (MoDCs) was investigated 6 hours post-infection with two H5N1 HPAIV strains: a 2023 isolate belonging to clade 2.3.4.4b (genotype AB), and a 2013 strain from clade 2.3.2.1c. This second virus historically showed a lower virulence in waterfowls and also exhibited a significantly reduced replication in our model. Transcriptomic profiling of the infected MoDCs revealed over 17,000 differentially expressed genes when compared to mock-infected controls, including many involved in immune pathways. Additionally, significant differences were observed in the expression profiles of immune-related genes induced by these two HPAIV strains when evaluated by RT-qPCR. These results represent an important first step towards identifying host cellular factors that could be responsible for the increased virulence of recent H5N1 HPAIVs.

Live attenuated vaccine-induced cytotoxic T cells correlate with protection against African swine fever virus

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African swine fever (ASF) is a panzootic disease causing severe economic losses to the porcine industry. The lack of prophylactic treatments hampers its control, and the insufficient knowledge regarding the immunological mechanisms underlying protection hinders rational vaccine design. To overcome this limitation, live attenuated vaccines (LAV) have become useful tools to analyse ASF virus (ASFV)-specific immune responses. While the role of humoral responses needs to be further explored, there is increasing evidence that cytotoxic cellular responses are involved in ASF immunity. However, there is controversy regarding their contribution in the protection or pathogenesis during ASF infection. Previously we demonstrated the presence of various cytotoxic cell subsets during *in vitro* recall response in pigs immunized with the LAV prototype BA71ΔCD2. Here, we aimed to characterise ASFV-induced cytotoxic responses in ASF immunity. First, we demonstrate that ASFV-infected pigs with clear clinical signs and lesions showed high percentage of perforin-producing NK and both αβ T- and γδ T-cells. Interestingly, the levels of γδT-cells were associated with viremia levels, indicating their contribution to the ASFV-induced systemic uncontrolled immune response. Second, we show the presence of perforin-producing ASFV-specific CD8β+CD45RA- CCR7- effector memory T-cells upon *in vitro* stimulation in PBMC from immune pigs inoculated with BA71ΔCD2. Importantly, high levels of these cells significantly correlated with lack of severe clinical signs, as demonstrated when comparing variable clinical courses in challenged pigs after a suboptimal vaccination regimen. Moreover, such correlation was not observed with either ASFV-specific antibodies or IFNγ-producing cells, indicating that cytotoxic T-cells are a critical arm of the adaptive cellular response against ASFV. This study further demonstrates the central role of cytotoxic cells during ASFV infection, highlighting the importance of their early appearance before a systemic infection is established, thus being an important immune mechanism to be targeted in the rational development of effective ASF vaccines.

Hiding in plain sight - persistence of feline coronavirus in cats in the presence of neutralizing immunoglobulins at the intestinal mucosa

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Feline coronaviruses (FCoVs) are widespread and highly prevalent in multicat-households, sustained by a short-lived immunity and the presence of persistent virus shedders. A longitudinal survey monitoring virus shedding and fecal immunoglobulin levels was conducted in a closed breeding colony that was naturally infected with FCoV. A total of 418 fecal samples from 21 cats were analyzed by real-time reverse transcriptase polymerase chain reaction (RT-qPCR), identifying 7 transient, 3 chronic intermittent, and 4 chronic persistent FCoV shedders. Fecal FCoV-specific immunoglobulin levels were semi-quantitatively measured by an IPMA. Secretory anti-FCoV IgA and IgM levels were significantly higher in cats actively shedding viral genomic RNA, though, were still detectable long after virus clearance and could not discriminate transient and persistent shedders. Following primary FCoV infection, anti-FCoV IgA was detected in feces from day 14 onwards and IgA fractions targeting the S, M, and N structural proteins appeared sequentially. Functional neutralization assays demonstrated that fecal immunoglobulins retained the capacity to inhibit FCoV infection in vitro (feline intestinal epithelial cells), with 61/152 extracts neutralizing the homologous FECV clade A strain 03.01.15 and 17/152 neutralizing the heterologous UU2 strain. Total and FCoV-specific fecal IgA levels were strongly correlated, though neither distinguished neutralizing from non-neutralizing samples. An impeccable success rate of raising FCoV-naïve offspring by isolation from FCoV-positive cats attributes clinical protection to maternally derived, lactogenic FCoV-specific IgA which were demonstrated in pooled fecal samples from litters born to resident queens regardless the FCoV-status of their mother. This field study provides insights in the complexity of FCoV mucosal immunity and suggests that while secretory antibodies exhibit neutralizing potential, they may coexist with the virus in the intestines. The protective efficacy of mucosal antibodies against FCoV reinfection and their role in long-term viral clearance are questionable. How FCoV evades from immunity in the intestines will be examined in the future.

PPRV modulates caprine dendritic cell function and induces immunosuppression through IL-10 upregulation

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PPRV infection induces immunosuppression, which can cause animal death due to opportunistic infections. In general, the disease is more severe in goats than in sheep, but the mechanisms behind this species-specific severity remain unclear. Dendritic cells (DC), which are central to mounting adequate immune responses to pathogens, can be targeted by PPRV in sheep. In the present work, we assessed the effects of PPRV on goat immune cells, specifically in CD14+ monocytes and monocyte-derived dendritic cells (MoDC). Our results show that PPRV infects caprine monocytes without impairing their differentiation into DC. Infected MoDC upregulated maturation markers and exhibit reduce phagocytic capacity, indicating a shift toward a mature phenotype. However, mixed lymphocyte reaction assays revelead that PPRV-infected MoDC has a diminished capacity to stimulate CD4+ and CD8+ T cell proliferation. This impair function was associated with an increase of IL-10 production and reduced immunological synapse between DCs and T cells. Overall, PPRV infection induces an atypical maturation stage in goat MoDCs, characterized by partial activation but impaired antigen presentation. These findings demonstrate that PPRV-driven modulation of DC function contributes to the immunosuppression observed during PPRV infection in goats.

Aedes albopictus produce virus-derived DNA upon Batai virus infection

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Batai virus (BATV) is a tri-segmented negative-sense single-stranded vector-borne RNA virus belonging to the *Orthobunyavirus* genus. BATV infection is of veterinary concern and transmitted by mosquitoes. Novel strategies to control mosquito-borne viruses are needed; one way is breaking the immune balance between mosquitoes and viruses. Viral-derived DNA (vDNA) has been shown to play an essential role for this balance. However, the role vDNA could play for vector control strategies remain poorly understood. Therefore, this study aims to improve our understanding of the mosquito immune response, in particular involvement of vDNA response, in defense against orthobunyavirus infections.

For the *in vitro* experiment, U4.4 *Aedes albopictus* cells were infected with BATV (MOI 0.1). At 24h, 48h, 72h and 96h post infection (pi), the vDNA was detected by PCR, targeting regions across S, M and L viral genome segments. The viral replication was confirmed by a real-time PCR. For the *in vivo* pilot experiment, adult *Ae. albopictus* (n=7) were microinjected with BATV (144.9 PFU). vDNA production and viral replication/ dissemination was assessed 7- and 14-days pi. The analyses were performed as described for the *in vitro* experiment.

The BATV infected U4.4 cells produced, at all three time points, vDNA from the S, M and L segments. For each segment, vDNA was produced from different regions. The real-time PCR confirmed viral replication. The mock-infected cells did not produce vDNA and no virus was detected. The in vivo data show that mosquitoes also produced vDNA upon BATV infection. We detected vDNA from the S segment (3 and 1 regions at 7- and 14-days pi, respectively) and the M segment (3 and 2 regions at 7- and 14- days pi, respectively). No vDNA produced from the L segment was observed at any of the time point.

We showed that the *Ae. albopictus* produce vDNA in response to viral infection, both *in vitro* and *in vivo*. The *in vivo* preliminary results indicate that the vDNA produced is unequally distributed across segments, suggesting that some segments are more targeted than others. The findings add knowledge about vDNA dynamics, improving our understanding of immune balance between viruses and mosquitoes.

Bluetongue Virus Evades Innate Immunity by Inhibiting the cGAS-STING Pathway

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Cyclic GMP-AMP synthase (cGAS) is a cytosolic DNA sensor that triggers type I interferon (IFN-I) responses upon detection of pathogenic or host-derived DNA, but it can also be implicated in restricting RNA virus replication. Several viruses have evolved strategies to inhibit cGAS signaling. Bluetongue virus (BTV) is a double-stranded RNA virus of the *Orbivirus* genus, which infection in ruminants produces a notifiable disease that has a severe economic impact on the livestock industry. Here, we investigate the interaction between BTV and the cGAS pathway. BTV infection induces mitochondrial damage and cytosolic DNA accumulation, which would typically activate IFN-I responses. However, BTV suppresses DNA-induced IFN-I transcription by promoting the degradation of both cGAS and STING. Functional assays reveal that inhibition of cGAS enhances BTV replication, underscoring cGAS role in antiviral defense during BTV infections. We identify BTV non-structural protein NS3 as the viral antagonist responsible for this immune evasion, demonstrating that NS3 interacts with cGAS and induces its degradation via an autophagy-dependent mechanism. These findings uncover a novel strategy by which a dsRNA virus subverts DNA sensing to evade innate immunity.

Epidemiology

Seneca Valley Virus: The Cause of Vesicular Disease in Pigs in England in 2022

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Background

We describe vesicular disease in pigs due to Seneca Valley virus (SVV) infection on five pig-breeding units in England during 2022: the first reported cases of SVV in Europe. Evidence of SVV infection has been described in other countries such as Brazil, Canada, China, Chile, Colombia, India, Thailand, Vietnam and USA. SVV is not notifiable or reportable in the UK, nor is it listed by the World Organisation of Animal Health (WOAH); however, it may cause clinical signs indistinguishable from notifiable vesicular diseases such as foot-and-mouth disease, swine vesicular disease and vesicular stomatitis.

Materials and Methods

Samples collected from affected pigs were tested for SVV at The Pirbright Institute after official investigation and testing ruled out the presence of notifiable vesicular diseases. Real-time RT-PCR was used to detect SVV RNA, and viral isolates were recovered which were characterised using VP1 and Next-Generation sequencing. Other sample types were tested for subsequent epidemiological analysis including environmental swabs, tonsils, semen and feed with the aim of identifying possible transmission routes. Serology was undertaken using a virus neutralisation test and a competitive ab-ELISA kit (BioVet, Canada).

Results

Clinical presentation mainly involved lameness in sows associated with lesions at the coronary bands of the hooves and interdigital spaces that then healed. Evidence of SVV infection without clinical signs was also found in breeding and growing pigs on affected and linked premises. Full genome sequences for SVV isolates recovered from the affected herds share a common ancestor with a virus from the United States: SVV/USA/TN/NADC6/2020. SVV RNA was detected in tonsils and environmental swabs, but not in historic batches of feed, nor semen from boars supplying affected premises.

Conclusions

No further clinical cases of vesicular disease associated with SVV infection have been detected since September 2022. These SVV cases reinforce the value of passive surveillance for notifiable vesicular disease and the legal requirement for pig keepers and vets to report vesicular lesions promptly. The timing and means by which SVV was introduced into pigs in England has not yet been determined. Investigations to identify the source of the virus and current geographical distribution are ongoing.

Seroprevalence and genetic diversity of feline immunodeficiency virus in outdoor cats in France

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Feline immunodeficiency virus (FIV) is a retrovirus that causes lifelong infections in cats and can lead to immune dysfunction. Despite its significance, especially for cat welfare and population health, FIV remains understudied in Europe, and data are particularly scarce. This study aimed to estimate the seroprevalence of FIV in outdoor cats across France and to characterize the genetic diversity of circulating strains. A total of 728 cats with outdoor access (642 domestic and 86 stray) were sampled throughout France between December 2023 and January 2025. Serological screening was performed using a commercial ELISA. PCR was conducted on ELISA-positive sera, and select samples underwent Sanger sequencing for phylogenetic analysis. One sample was also subjected to metagenomic shotgun sequencing using Oxford Nanopore technology. The national seroprevalence of FIV, estimated using a Bayesian hierarchical model accounting for ELISA test uncertainty, was 16% (95% Credible Interval [CrI]: 9%–21%). When stratified by ownership status, prevalence was 14% (CrI: 8%–20%) among owned cats and 24% (CrI: 13%–36%) among strays. Outdoor exposure was a strong predictor of seropositivity: cats with moderate (1–12 hours/day) and high (>12 hours/day) outdoor access had 2–3 and 3–5 times higher odds of infection, respectively, compared to cats with minimal outdoor time (<1 hour/day). Stray cats exhibited the highest risk, with 5–6 times greater odds of infection than owned cats. Among strays, each additional year of age significantly increased the odds of seropositivity (OR: 1.52–2.86, $p < 0.001$). By age six, the likelihood of seropositivity exceeded 75%. All sequenced FIV strains belonged to subtype A. Phylogenetic analysis revealed genetic heterogeneity suggestive of at least two independent introductions into France. Although all sequences clustered with European strains, some had distinct common ancestors. These findings underscore the limited surveillance and genetic data available for FIV, hindering comprehensive understanding of its international spread. The metagenomic dataset yielded approximately 100,000 FIV reads among 2 million total reads, confirming the feasibility of this approach for complete viral genome recovery. Taken together, these results could help inform veterinary practitioners and support the development of FIV prevention and surveillance strategies in Europe.

Epidemiological Significance of Nasal and Oral Secretions in Rotavirus A Infections in Cattle

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Rotaviruses, particularly Rotavirus A (RVA), continue to pose a significant burden on public health and animal production worldwide. Their constant presence in cattle herds presents a considerable challenge for intensive farming. While the fecal-oral route has long been considered the main way of transmission, recent experimental studies suggest that saliva and nasal secretions may also play an important role. This study aimed to assess the importance of nasal and oral secretions in RVA spread in cattle under field conditions.

In late winter 2024, 120 calves (median age: four weeks) from 20 farms in two counties of continental Croatia were sampled. Diarrhea was observed in 30 calves, while the remaining 90 showed no visible gastrointestinal symptoms. From each calf, nasal and oral swabs, as well as a fecal sample, were collected, totaling 360 samples. RVA detection and quantification were performed using VP2 RT-qPCR. For genotyping, a subset of samples was tested with conventional RT-PCR targeting the VP7 and VP4 segments, followed by Sanger sequencing and phylogenetic analysis.

All nasal and oral swabs tested positive for RVA, while only 62.5% of fecal samples were positive. The median genome copy number was highest in nasal swabs, followed by oral swabs and feces. In 90% of calves, the nasal or oral swab had the highest RVA genome copy number. Among fecal-positive calves, 75.8% had the lowest RVA genome copy number in feces compared to the other two sample types. Detected RVA genotypes were typical for cattle: G6, G10 and G8 (VP7) and P[11], P[5], P[14] and P[1] (VP4), listed in decreasing order of frequency. In total, five different G and P combinations were found. Genotype P[14] is rarely detected in cattle, but has importance in sporadic zoonotic transmission.

This is the first field-based study showing that nasal and oral secretions can be highly relevant in RVA transmission among calves, having potentially higher significance than the fecal-oral route. These findings underscore the complexity of controlling RVA infections in intensive cattle farming and suggest that aerosolized secretions may facilitate interspecies transmission.

Lack of biosecurity compliance of poultry farms with a recent history of HPAIv infection determined by video camera monitoring

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Many poultry farms become infected with highly pathogenic avian influenza virus (HPAIv) without conclusive evidence about the route of entry. Several putative introduction pathways have been proposed but it is virtually impossible to proof how the virus was introduced to susceptible poultry. Poultry farmers tend to blame HPAIv-introduction to factors they do not have influence on (e.g. airborne-transmission), but (incidental) breaches of biosecurity measures by farmer or visitors is a fair possibility. Biosecurity compliance on poultry farms, with a recent history of HPAIv infection, was monitored using 24/7 video monitoring (recording period 4 to 6 weeks) at entry and exit zones of farm buildings by farmers and visitors. Biosecurity compliance definitions used were based on internationally acknowledged norms published in poultry biosecurity reference guides and handbooks. Farms of four different production types (two broiler, two layer, two breeder broiler farms and one duck farm) were selected from a list of approximately 70 eligible poultry farms. Observations of entry to and exit from the anteroom (room separating the outside environment from the unit in which the poultry is housed and which is equipped with a hygiene lock) revealed low compliance in six poultry farms and good compliance in one, in strictly maintaining the separation between clean and potentially contaminated areas in the anteroom. Handwashing with soap and water and/or using disinfectant gel was rarely observed at entry of the anteroom by the farmer and was almost absent at exit. Egg transporters did not disinfect fork-lift wheels when entering the egg-storage room, nor changed or properly disinfect footwear. The egg-storage room was not cleaned and disinfected after egg transport by the farmer, enabling introduction of contaminated material into the poultry unit by the farmer. Similarly, footwear and trolley wheels were not disinfected when introducing young broilers or ducklings. Substantial biosecurity compliance gaps were observed when introducing bedding material in the duck farm. In contrast, veterinary practitioners demonstrated excellent biosecurity practices. This study shows a need for an engaging awareness and training campaign for poultry farmers and their co-workers as well as for transporters, to promote good practices in biosecurity compliance.

Protoparvovirus carnivoran 1 in golden jackals in Croatia

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Parvoviruses are significant animal pathogens that can cause various health issues, from asymptomatic and mild infections to severe and often fatal diseases in both domestic and wild animals. The golden jackal (*Canis aureus*), a highly adaptable carnivore with a growing presence across Europe, has been identified as potential reservoir of numerous pathogens, including zoonotic ones. As this species expands into rural and peri-urban areas, the likelihood of pathogen transmission between wild and domestic animals increases. Such spillover events may contribute to the emergence of novel parvoviral variants and pose challenges for disease surveillance and control. Based on all mentioned, it is clear that golden jackals play an essential role in viral ecology, emphasising the need to improve our understanding of their role in cross-species transmission dynamics.

Samples of small intestine from 55 golden jackals, collected during the 2024/2025 hunting season as part of routine execution of game management plan, were analysed for the presence of Protoparvovirus carnivoran 1 using real-time PCR. Of these, 22 individuals (40.0%) tested positive. The carcasses originated from the continental part of Croatia, specifically from the following counties: Zagrebačka, Sisačko-Moslavačka, and Osječko-Baranjska. The highest prevalence was observed in the central part of Croatia, particularly in Sisačko-Moslavačka County, where 7 out of 11 animals tested positive (63.6%). Partial VP2 gene sequences were successfully obtained by Sanger sequencing from eight positive samples (36.4%) and subjected to BLAST analysis, revealing the highest nucleotide similarity to Protoparvovirus carnivoran 1, genotype Feline panleukopenia virus. This study offers a rare perspective on parvoviruses among the golden jackal population globally and marks the first documented presence of parvovirus in Croatia's golden jackals. Further phylogenetic analyses will be conducted to determine the genetic relationships among the detected strains in golden jackals and other wild and domestic animals.

Emergence of a Novel Tick-Borne Flavivirus in Domestic and Wild Ungulates in the Italian Alps

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A novel tick-borne flavivirus (TBFV), tentatively named Alpine Chamois Encephalitis Virus (ACEV), has recently been identified in the Alps. This emergence raises concerns regarding the health of both domestic and wild ruminants and highlights the importance of surveillance at the wildlife livestock interface. To investigate its circulation, pathogenic potential, and possible vectors, targeted surveillance was carried out in the Lombardy region between 2023 and 2024, involving domestic goats and wild ungulates.

Neurological signs were initially observed in goats grazing in the northeastern Orobic Alps. A young Orobic goat died, and post-mortem examination revealed non-suppurative meningoencephalitis with significant *Ixodes ricinus* infestation. TBFV was detected in the brain using real-time RT-PCR and confirmed by immunohistochemistry. Of 31 ticks collected from the carcass, 5 (16.1%) tested positive. Within the same herd, 11 of 67 goats were seropositive for anti-TBFV antibodies, and 3 were RT-PCR–positive on whole blood. Further neurological cases were confirmed on a nearby farm, including a deceased kid and a febrile adult goat. Another seropositive individual was identified in a transhumant flock grazing in the same alpine area, suggesting ongoing virus circulation.

TBFV infection was also confirmed in an Alpine chamois presenting neurological signs. Sequencing of viral amplicons obtained from affected animals and ticks showed 98–99% identity with ACEV, supporting the hypothesis of a shared viral strain circulating between wild and domestic hosts. Serological screening of 1,489 wild ruminants revealed 19 seropositive individuals (1.3%), primarily red deer and Alpine chamois, indicating a low-prevalence exposure in wildlife.

These results confirm the localized circulation of ACEV within a confined Alpine ecosystem, involving both wild and domestic ruminants and likely transmitted by *I. ricinus*. The emergence of this novel virus underlines the need for integrated One Health surveillance strategies to monitor and respond to emerging tick-borne flaviviruses that may have ecological, veterinary, and potential zoonotic significance. Continued monitoring and molecular characterization are essential to assess its epidemiological dynamics, host range, and potential public health implications.

Fowl adenovirus (FAdV) infection in young commercial broiler breeder pullets suggests mitigation of early vertical spread

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This paper describes the investigation of the epidemiology of fowl adenoviruses (FAdVs) among fast-growing meat-type parent chickens in Sweden, based on serology, PCR and partial genome sequencing of samples collected during rearing and egg production. Blood samples (N=1,910) from parent flocks at 16 (79 flocks), 24 and 36 (52 flocks) weeks of age were analysed for FAdV-antibodies. Ninety-four percent of the flocks had seroconverted at 16 weeks of age, and at 36 weeks of age all flocks were seropositive. From these flocks, dead-in-shell chicks (DIS, N=949) from eggs laid at 26–27 and 36–37 weeks of age were sampled, and pooled liver and caecal tonsils were analysed by PCR. Nucleic acid from FAdV was not detected. Negative PCR results were confirmed by testing tissues from 62 unrelated seropositive broiler parents (from four flocks) and 80 DIS, collected from the same parent flocks at age 24–26 weeks. From six other parent flocks, liver and caecal tonsils were sampled from one bird (when available) per day during rearing from 1–112 days of age. From six weeks of age, in four out of six flocks, FAdV species A and/or D were detected by PCR and partial hexon gene sequencing. In conclusion, FAdVs were detected during rearing in parent birds but not in DIS, indicating that vertical transmission of FAdV was prevented by immunity acquired early in life. However, vertical transmission could still occur at a later age when immunity wanes or if the broiler parents become infected with another FAdV species/serotype.

An update of the epidemiological situation of African Swine Fever in Italy

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Introduction
Italy experienced African Swine Fever (ASF) genotype I disease from 1978 to 2024, that involved domestic pigs, illegal-free range pigs and wild boars in Sardinia. In 2022, Italy notified the ASF genotype II disease. Currently ASF involves 3 clusters in 8 regions and 22 provinces. Infection is present in wild boar population in some of these areas and is seasonally notified in domestic pig farms.

Material and Methods
In Italy, a national passive surveillance plan is in force since 2020, for early detection in wild boar population and domestic pig farms in ASF free area. In the affected territories, the eradication plans require enhanced passive surveillance, improvement of biosecurity and management of wild boar population. A national information system (VETINFO) enables portal tracking of all surveillance and eradication ASF data at the central/regional/local level.

Results and Discussion
Currently, the largest and most worrisome cluster is the northwestern one, due to its contiguity with the most relevant areas for national pig production and its tendency to become larger: the spread of infection in the wild boar population is ongoing to the southeast (Tuscany, Emilia Romagna) and to the north (Ticino Park). Two epidemic waves occurred in domestic pigs in the summer in 2023 (Lombardy) and 2024 (Piedmont, Lombardy, Emilia Romagna). In Campania, the infection is still active in the wild boar population, but it remains confined. In Calabria, there is epidemiological silence; in the last period, enhanced passive surveillance activities have been increased. ASF eradication in Latium was ratified in January 2025, thanks to an over-time implementation of measures (active search for carcasses; physical containment of infection within the affected area; wild boar depopulation by low-impact techniques). Finally, in Sardinia, ASF-free status was achieved in September 2024, applying a strong eradication strategy based on increased passive surveillance.

Conclusions
The Italian epidemiological scenario for ASFV genotype II appears definitively challenging; nevertheless, ASF eradication in Latium was based on the small size of the affected territory and on the effectiveness of measures. ASF eradication in Sardinia is a goal achieved after some decades of endemic.

Influenza A virus inactivation in raw milk Grana type cheeses

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Recent reports of H5N1 HPAIV infections in lactating cattle in the United States, with high titer virus elimination in milk, are raising major concerns about the ability of the virus to infect humans through consumption of milk and dairy products made from raw milk. So far, some studies have been conducted on virus inactivation in pasteurized milk, but almost nothing on cheeses produced from raw milk. In this study, we evaluated the dynamics of the behavior of two subtypes (H1N1 and HPAI H5N1) of avian influenza virus (AIV) in finished cheeses obtained by simulating all stages of the cheese making process of Grana-type cheeses made from raw cow's milk.

Viral stocks were prepared by inoculation into SPF embryonated chicken eggs (ECE) and the viral titer (EID50/ml) was determined by Reed and Muench method. Viral growth in contaminated cheeses was evaluated in three 1-gram inner portions of each cheese by inoculation onto SPF-ECE with two blind passages, HA test and Mab-based-virological-ELISA on allantoic fluid, before confirming negativity. In the first study, three batches of 1l raw cow's milk were used; two contaminated with 107.75EID50/mL of A/duck/Italy/281904-2/06/H1N1 each and the third as negative control. In the second study, two batches of 1l each, one contaminated with 106.75EID50/mL of A/duck/Italy/326224-2/22/H5N1HPAI (clade 2.3.4.4b) virus and the other as negative control were used. Cheeses were produced following all the process steps of the Parmigiano-Reggiano production specification, and the produced cheeses were aged for 1 month at 5-6 °C.

Inoculation tests on SPF-ECE revealed the absence of viral growth after two blind passages, MAb-based-virological-ELISA and HA tests for cheeses produced with all contaminated batches.

This work provides evidence that the traditional production process of Grana-type hard cheeses is effective in inactivating both LPAIV and HPAIV present into raw milk, even when viral loads significantly higher than those reported during natural outbreaks were used. Inactivation appears to result from the combined effect of technological steps as well as pH and aw values in the cheese. In conclusion, processing of raw-milk hard cheeses and cooked curds can eliminate the biological risks posed by raw milk contaminated by AIV.

New insights into the honeybee virome by an epidemiological follow-up of three honeybee apiaries, using third-generation nanopore sequencing

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Honeybees are threatened by a disease complex presumably caused by a combination of parasites, viruses, pesticides and nutritional deficiencies. Alarmingly, these factors also threaten other insects, further endangering our environmental health and agriculture industry. Historically, viral prevalence has been determined via PCR at one timepoint. Because PCR can only detect predetermined viruses, some important viruses can be missed. Furthermore, genetic junk can stay in bees for a long time after primary infection. By only sampling at one timepoint, viral kinetics and their importance in the complex cannot be determined. These limitations result in an incomplete understanding of viral circulation in honeybee colonies and, consequently, a lack of comprehension regarding the role of viruses in honeybee mortality. We addressed this gap, utilizing third-generation nanopore metagenomic sequencing of honeybee hemolymph to identify all replicating viruses over time without contamination from the gut microbiome. Four hives on three apiaries were followed-up from March till February, 2023-2024. We sampled honeybees of different ages once to twice a month. Twelve different honeybee viruses were detected, including viruses which are not routinely screened for and have never been identified in Belgium/Europe before. This innovative study provides valuable insights into the dynamics of viral loads in honeybee colonies over time. However, many questions remain, such as the pathogenicity, symptoms, or genome structure of the identified viruses. Understanding what viruses are circulating is essential for investigating their role in honeybee mortality. Just as a puzzle cannot be solved without all the pieces, this research represents a crucial step towards completing the whole puzzle of honeybee health. The findings from this study will serve as a foundation for developing novel strategies to protect honeybees, ultimately contributing to improved colony health.

First detection of Lumpy Skin Disease in Italy: first evidence and response strategies.

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Lumpy skin disease (LSD) is a viral disease caused by *Capripoxvirus* that affects cattle, transmitted by blood-feeding insects (flies and mosquitoes, or ticks), included in the list of notifiable diseases according to the Animal Health Law. The main symptoms are fever and skin nodules, with death occurring rarely. On 20th June 2025, in a farm located in Orani, province of Nuoro, skin nodules were observed in 16 out of 131 animals. Suspicion of LSD prompted the immediate submission of samples to the National Reference Laboratory for Exotic Diseases, where the infection was confirmed and genotyping was performed to trace the source of the outbreak. Since then, other outbreaks have been reported. This study describes the first confirmed case of LSD in Sardinia and in Italy. An immediate notification was sent to the Ministry of Health, which in turn informed the European Commission and the WOAH. In the restriction zones, the movement of farm animals was suspended, infected animals were isolated, an urgent epidemiological investigation was initiated along with clinical and laboratory exams focusing on farms where there was a high risk of infection. Traceability activities were carried out, leading to the identification of a case in the Mantova area of Lombardy, involving animals imported from Sardinia at the beginning of June.

The disease, widespread in many African countries, spread in 2012 from the Middle East to southeastern Europe, affecting EU Member States such as Greece and Bulgaria, as well as several Balkan countries. A vaccination program subsequently halted the epidemic in the affected territories. In Sardinia, the control strategy will include stamping out, vaccination of the cattle population present in the region, high levels of biosecurity, epidemiological investigation, disinfection, public awareness activities and the suspension of movements of susceptible species.

Due to the limited information available in the literature, further studies are underway to better understand the situation and this disease.

The Sardinian experience highlights the importance of continuous surveillance, targeted vaccination strategies, and strong cooperation between local and international authorities.

The outcome of porcine circovirus 2 infections in porcine lymphoblasts is the result of an intriguing interplay between viral and host genetics

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Porcine circovirus type 2 is a very small DNA virus that has been circulating in pigs for a very long time. As the virus does not have its own DNA dependent DNA polymerase, it hijacks it from mitotic target cells, such as lymphoblasts and fetal cells. During an EU project, we demonstrated that, nowadays, pigs are infected with a high number of respiratory/intestinal/general viruses/bacteria during their first weeks of life. As every infection is leading to a blastogenesis stage, which boosts the replication of PCV2, these co-infections may lead to PCV2-associated diseases. A logical consequence of this increased PCV2 replication is a high mutation rate and a rapid evolution during the last decades. We demonstrated that, following the law of the fittest, the virus is adapting to a more efficient replication in lymphoblasts. One notable example is the improvement of the viral binding to its receptor, chondroitin sulphate, due to the concentration of positively charged amino acids on the surface of the capsomer, forming a three-winged windmill on the surface of the PCV2 trimers. In addition to the effect of viral genetics, there is a genetic difference in susceptibility of pigs to PCV2-infection and its associated diseases. We demonstrated that the disassembly of PCV2 upon internalization by serine proteases only occurred at pH7 in Piétrain pigs whereas it happened at a high and low pH for Landrace pigs. This may explain the different clinical outcomes of PCV2-infections in different breeds. Currently, we are testing the susceptibility of lymphoblasts of a high number of Piétrain, Landrace, Large White and Duroc boars in order to find genetic differences within and between these breeds.

In conclusion, modern swine husbandry and animal breeding have given PCV2 the opportunity to become an important pathogen in swine and will shape the new PCV2 variants for the future. The final impact of this evolution on pathology and disease cannot be predicted and should be followed over time.

Diagnostics

Targeted Metagenomics NGS for the Detection and Characterization of Porcine Viruses

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In the absence of a clearly suspected viral pathogen, metagenomics could be a useful diagnostic tool, mainly in emergency situations. However, a first enrichment step is needed to get a reliable viral identification in a test sample. The aim of this study was to design and evaluate a capture probe panel for the detection and characterization of porcine viruses using targeted metagenomics NGS techniques.

An enrichment panel (*KAPA Hyper Explore, Roche Sequencing Solutions*) was custom-designed targeting a wide range of swine viruses, comprising 32 viral species from 14 families (DNA and RNA). These include all notifiable pathogens plus other relevant viruses to pig production. Most viral genomes were fully covered in the hybridization probe library designed (*VIRSWINECap1*). Over 20 samples containing different viral DNA or RNA pathogens were selected for panel evaluation. Samples were pooled from 4 to 8 before the hybridization step to optimize enrichment cost. NGS runs were done with the basic Illumina® benchtop device (iSeq-100), and reads were analyzed using a series of bioinformatics tools. The main steps included quality filtering (fastqc and fastp), taxonomic classification (Kraken2), reference-based mapping (bwa-mem2), and de novo assembly (Unicycler).

All expected viruses were detected in the samples, with some reaching 100% genome coverage and more than 1,000x of mean depth (such as PTV or SVD). Additionally, co-infecting viruses, included in the probe panel were also identified in some samples. Field samples previously diagnosed with PCVII showed the lowest horizontal coverage, highlighting the limitations of this technique for sequencing ssDNA genomes. Besides, the percentage of porcine reads ranged from 0.03% to 39.78% (median 12.1%). No viral pathogens were identified in either the undiagnosed field samples or the negative control.

Results demonstrate that this panel for targeted metagenomics NGS is effective for identifying and characterizing porcine viruses in clinical samples. It enables recovery of complete viral genomes even using Illumina®'s smaller-scale devices like the iSeq-100 (up to 4 million reads), while generating a low percentage of host genome reads. Achieving such results would be nearly impossible with shotgun metagenomics, even on high-performance platforms, which also entail significantly higher costs.

Full set of new solutions for a rapid and reliable Foot and Mouth Virus detection: Double Antibody Sandwich ELISA, freeze dried RT-qPCR and Lateral flow device for point-of-care diagnosis

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Introduction

Foot and mouth disease (FMD) is one of the most contagious viral diseases in ruminants, causing epizootic diseases in a few weeks and with devastating economic consequences. Rapid and specific identification of the agent is of utmost importance. Innovative Diagnostics has developed a new set of diagnostic solutions to detect all known serotypes of FMDV: a lateral flow device, the ID Rapid® FMD Antigen, providing result in the field in under 15 minutes ; a freeze-dried RT-qPCR, the ID Gene LyoTM FMDV Triplex, which includes an ultra-rapid amplification program giving results in 40 minutes ; a double antibody sandwich (DAS) ELISA, the ID Screen® FMDV pan-serotype Antigen Capture, which enable a first economic screening so that downstream serotyping is only performed on positive samples. This study presents validation of these new solutions.

Methods

Inclusivity, as well as diagnostic sensitivity of the 3 tests was evaluated on a panel of 10 inactivated strains from all 7 serotypes and panels provided by different Reference Laboratories. Diagnostic specificity were assessed for the LFD and the DAS-ELISA on 60 negative bovine tongue epitheliums from a non-infected area (France) and for the RT-qPCR on: 160 bovine samples, 50 swine whole blood samples and 100 goat milk from the same area. Exclusivity of each test with respect to closely related viruses (Swine Vesicular Disease Disease (SVV) and vesicular stomatitis (SVV) viruses) was tested.

Results

The 3 kits correctly detected all FMDV tested strains, including SAT strains, showing 100% inclusivity. The LFD's, RT-qPCR's and DAS-ELISA's measured sensitivities were 100% (95%CI [97.3-100], n=113) ; 100% (95%CI [51-100], n=3) ; 100% (95%CI [97.3-100], n=113). For all kits, all tested negative samples gave negative results and the measured specificity was 100%. SVV and VSV were not detected.

Conclusions

The ID Gene LYO™ FMDV Triplex, the ID Screen® FMDV pan-serotype Antigen Capture and the ID Rapid® FMD Antigen exclusively detects all FMDV known serotypes. While the ID rapid® kit provides results in the field in under 15 minutes, the DAS-ELISA and the RT-qPCR's performance enable a reliable confirmation of cases or an efficient first line screening of susceptible animals.

Keywords: FMDV, RT-qPCR, DAS-ELISA, LFD

Honeybee pathogen prevalence in Vienna and on hive validation of the FASTest® BEE 3T: A rapid, applicable and accurate diagnostic tool for honeybee viruses in a citizen science approach and controlled conditions

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Varroa destructor combined with viral infections are major drivers of honeybee colony losses and weakening in Europe. Viruses like Deformed Wing Virus (DWV), Acute Bee Paralysis Virus (ABPV), Sacbrood Virus (SBV) and Chronic Bee Paralysis Virus (CBPV) are widespread in the honey bee population often without clinical signs. Morbidity is linked to a high virus load within the bee colony. Therefore quantitative diagnostics are mandatory for detecting colonies at risk at an early stage. qRT-PCR tests are standard in science but not applicable in field for multiple reasons. A lateral flow immunoassay for on hive detection of clinical relevant amounts of DWV, ABPV and SBV was developed.

This study aimed at validating the FASTest® BEE 3T under field conditions by comparing its diagnostic performance to RT-qPCR assays. A second objective was to explore correlations between viral load, Varroa infestation levels, and winter colony losses.

126 bee colonies from 22 beekeepers were monitored from April 2024 to April 2025. The majority of samples were provided by a citizen science project in the region of Vienna. Consigned beekeepers tested their bees at three seasonal timepoints, submitting both samples and colony health data, including Varroa mite counts. A second dataset originated from a controlled longitudinal field trial conducted within the EU BeeGuards project in Graz, focusing on Varroa monitoring.

The FASTest® BEE 3T was simple to use and showed high sensitivity and specificity for detecting clinically relevant DWV infections when benchmarked against RT-qPCR. Detection rates for ABPV and SBV were low, consistent with the low prevalence for high virus loads. Subclinical infections, often identified by qRT-PCR, were not captured by the lateral flow assay, underscoring its utility as a rapid field-screening tool for overt infections.

Colonies exhibiting high virus loads together with elevated Varroa infestation have a clearly increased risk of winter losses. However it is more complex as other factors like CBPV and Nosema sp. which were frequently observed in our qRT-PCR analyses contribute to the morbidity of the bee colonies. Conclusion: Adding on-site virus diagnostics like the FASTest® BEE 3T test to the standard Varroa monitoring offers quick and precision in assessing bee colonies' health risks.

Genomic surveillance of emerging avian coronaviruses: a field assessment of Oxford nanopore clinical metagenomics

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Clinical metagenomics presents transformative opportunities for managing viral outbreaks and enhancing the monitoring of transmission dynamics and viral evolution. A clinical metagenomics pipeline utilizing Oxford Nanopore Technologies (ONT) has been developed and evaluated to generate complete coronavirus genomes directly from clinical samples.

Avian coronaviruses (AvCov) are responsible for infectious bronchitis (IB) in chickens and enteritis in various avian species, including turkeys and guinea fowl. The genomes of AvCov exhibit significant genetic diversity, particularly in the spike gene, driven by recombination events. Whole-genome sequencing is crucial for identifying new viral lineages and distinguishing wild-type strains from vaccine-derived IB viruses.

ONT sequencing technology operates by reading DNA or RNA strands as they pass through a nanopore, with theoretically no read length limitations. This capability enables comprehensive viral genome sequencing with a limited number of reads and can be performed in near real-time under field conditions.

Tracheal swabs were collected from chicken flocks suspected of IB and/or vaccinated with live vaccines. Similarly, intestinal contents or cloacal swabs were obtained from guinea fowl and red-legged partridges suspected of acute viral enteritis. All samples were tested via RT-PCR for AvCov; positive samples were processed using various ONT pipelines, including RNA depletion-based enrichment.

A Sequence-Independent, Single-Primer Amplification (SISPA) protocol was implemented. Following quality control with TapeStation and library preparation, ONT sequencing was conducted using either the Mk1C or PromethION 2 Solo devices. The entire sample-to-result protocol was completed within a single working day.

Utilizing these pipelines, over 20 complete AvCov genomes were assembled and analyzed. Phylogenetic analysis revealed striking diversity in the spike gene, even among viruses infecting the same bird species. This information, generated within a few days post-sampling, was instrumental in elucidating transmission patterns. Whole-genome analysis of IBV facilitated the differentiation between wild-type and vaccine strains.

Clinical metagenomics will intensify genomic surveillance in veterinary virology and contribute to a deeper understanding of the genomic evolution of emerging viruses.

Hyper-mutation of the Equine Infectious Anaemia Virus (EIAV) capsid retains serological detection but reduces infectivity

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Equine Infectious Anaemia (EIA) is a notifiable disease of equids with global distribution, caused by the EIA virus (EIAV). Due to a varied clinical presentation, fluctuations in viraemia, and significant genetic variability among EIAV strains, antibody tests remain the preferred choice for diagnostics. Serology, including the agar gel immunodiffusion test (AGIDT), ELISA and Western blots (WBs), mostly target the capsid (p26). Serologically undetectable EIAV positive horses have been reported, however the underlining cause is unknown, with antigenic diversity suggested as a potential factor. Therefore, this study aimed to assess how p26 mutations affect serological detection and test if a significantly altered EIAV would retain viability.

A hyper-mutated p26 sequence based on the Wyoming reference strain containing all reported amino acid mutations in a single sequence, and two p26 sequences from diverse field strains (Newmarket1975 and Miyazaki2011-A) were synthesized and expressed in *E. coli* to create recombinant proteins (rp26). EIAV anti-p26 sera were produced by inoculating horses with the rp26 and resulting sera were tested on AGIDT, commercial ELISAs and two WBs (rp26 and whole antigen). Full-length molecular clones of EIAV were created using synthetic fragments and golden gate cloning. Canine D17 cells were transfected and recovered virus was used to infect equine monocyte-derived macrophages (eMDMs).

Equine anti-$p26_{NEWMARKET1975}$ and anti-$p26_{MIYAZAKI2011-A}$ sera were detected by all tests. Two anti-$p26_{HYPER-MUTATED}$ sera (with high and low titers) were negative by AGIDT but positive by WB. All ELISAs detected p26 antibodies in the high titer anti-$p26_{HYPER-MUTATED}$ serum but only one detected antibodies in the low titer anti-$p26_{HYPER-MUTATED}$ serum. EIAV RNA was detected in the transfected D17 cells and supernatant, however unlike the non-mutated EIAV clone, the $EIAV_{HYPER-MUTATED\_p26}$ clone was unable to productively infect eMDMs.

The serological detection of both diverse field strains and a hyper-mutated protein provides assurance for the detection of diverse viral strains. Accordingly, it seems unlikely that viable p26 mutations would not be identified serologically. Conversely, the theoretical hyper-mutated p26 in this study was not infectious. As expected, the AGIDT had the lowest sensitivity but was still able to detect antibodies against the most diverse p26 field strains.

Assessing SRLV Load Dynamics and Genetic Diversity in a Swedish Dairy Goat Herd Around Parturition

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Introduction
Small ruminant lentiviruses (SRLVs) are globally distributed retroviruses affecting goats and sheep, often leading to chronic infection, productivity loss, animal suffering and trade restrictions. Accurate detection remains challenging due to the slow replication of the virus and host immune variability, making the combined use of serological and molecular methods essential.

Aims
This study aimed to evaluate changes in SRLV nucleic acid levels across late pregnancy and lactation stages using paired blood and milk samples, and determine the optimal sampling time for molecular diagnostics. A secondary objective was to characterize the circulating SRLV genotype and explore intra-herd viral diversity.

Methods
Samples were collected from 16 pregnant goats in a seropositive Swedish herd at two time points: around kidding and one month postpartum. SRLV nucleic acid was quantified via nested qPCR. Genetic characterization was performed using partial *pol* gene sequences.

Results
At approximately one month postpartum, 14 of 16 goats exhibited decreased Ct values—indicating elevated proviral loads—and/or converted to qPCR positive status compared to the time around parturition. A statistically significant increase in proviral load was observed between the two sampling points ($p = 0.0042$, two-tailed binomial test). Five goats transitioned from qPCR negative to positive, and among 10 goats that had not yet given birth at the time of the first sampling, nine showed increased proviral load or turned to qPCR positive ($p = 0.0215$) in the second sampling, indicating a post-kidding rise in viral activity. Blood and milk qPCR results showed complete agreement at the second time point (Cohen's $\kappa = 1.00$). Phylogenetic analysis placed both isolates within genotype C. Pairwise comparisons revealed 16% divergence from the reference genotype C and 5% intra-herd variability, suggesting potential subtype-level diversity, albeit with low genetic heterogeneity among the goats in the herd.

Conclusion
These findings indicate that proviral load is higher one month after, compared to around parturition, highlighting this period as a more suitable time point for molecular detection. The genetic data suggest a relatively homogenous SRLV population, with signs of subtype variation requiring further investigation to clarify classification. The study is ongoing and expanding to other lactation stages.

Vaccines

FlagT4G vaccine protects pregnant sows from classical swine fever virus transplacental transmission two weeks After Single vaccine dose

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Classical swine fever (CSF) remains a significant threat to swine health worldwide, particularly due to its capacity for transplacental transmission, which can result in severe reproductive losses. The availability of safe and effective vaccines capable of preventing congenital and postnatal persistent infections in pregnant sows is crucial for controlling CSF in endemic regions. In this study, we demonstrate the high efficacy of FlagT4G vaccine in conferring robust protection at 14 days post single vaccination against transplacental transmission of a highly virulent CSFV strain. Vaccinated pregnant sows remained clinically healthy throughout the trial, with no signs of disease. Importantly, no macroscopic lesions compatible with CSFV were observed in their fetuses, and viral RNA from the challenge strain was undetectable in fetal tissues and sera. Remarkably, this protective effect was achieved even in the absence of high antibody titers in the sows at the time of challenge, suggesting early innate immune mechanisms may be involved. These findings add to the growing body of evidence supporting the use of DIVA-compatible vaccines as FlagT4G in CSF eradication programs and provide new insights into their potential to reduce reproductive losses and viral persistence in swine populations.

Limited field efficacy of a single-dose Syvazul BTV-3 vaccination during the 2024 Bluetongue outbreak in Belgium

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Bluetongue virus serotype 3 (BTV-3) emerged in the Netherlands and Belgium in 2023. During the 2024 vector season, the virus spread rapidly in Belgium, causing widespread clinical disease in sheep, including mortality and production losses. Inactivated BTV-3 vaccines became available in spring 2024 as a preventive measure, although solid experimental safety and efficacy data were missing. Despite the limitations associated with some uncontrollable parameters in the field, two complementary field studies were performed to obtain insights into the efficacy of the first available BTV-3 vaccine on the Belgian market, Syvazul BTV-3, marketed as a single-dose vaccine for sheep.

In preparation for an experimental infection study, we screened sheep on a commercial farm which had been vaccinated end May 2024 with a single dose of Syvazul BTV-3. At first sampling on August 1st, two of 18 animals were PCR-positive and ELISA-negative. The remaining 16 sheep were PCR-negative and 6 showed weak or borderline ELISA reactivity. The 16 PCR-negative animals were subsequently transported to a vector-free stable for further monitoring. Within the first week in the stable, 7 became PCR-positive, confirming that BTV infection occurred around the time of first sampling and relocation. The PCR-positive animals displayed typical BTV clinical signs, and virus isolation confirmed active infection. All PCR-positive animals developed strong post-infection immune responses. In three euthanized animals, all tested organs were PCR-positive, with highest viral loads in the spleen. Among PCR-negative animals, some showed increasing antibody levels in ELISA, indicative of a controlled infection, while others remained seronegative.

On another farm, 22 sheep vaccinated with Syvazul BTV-3 were sampled two months post-vaccination, prior to local virus circulation. Only 68% and 18% of animals were ELISA-positive and VNT-positive, respectively, with low neutralizing antibody titers. Booster vaccination markedly improved antibody levels as all animals became ELISA and VNT positive with increasing titers 3 to 4 weeks later, though natural infection could not be excluded.

These findings show that a single dose of the Syvazul BTV-3 vaccine does not provide adequate protection under field conditions and highlight the importance of thorough and independent evaluation of vaccine efficacy before large-scale deployment.

Safety assessment of the African swine fever vaccine ASFV-G-ΔI177L in mature breeding boars

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The ongoing global spread of African swine fever virus (ASFV) remains a threat for local pig populations and associated economies, due to severe disease manifestation followed by often high fatality rates in Eurasian suids. A vaccine against ASFV would offer protection from lethal disease and ultimately hamper the spread of ASFV in wild populations. However, although recent research advancements were made to define a safe alternative vaccine, only live attenuated vaccines (LAVs) have proven to be efficient and effective. While the vaccine candidate ASFV-G- ΔI177L is already in use in a few countries, a prerequisite for licensing on the European market is the execution of numerous safety studies. In this study, we assessed the effects of vaccination with ASFV-G- ΔI177L on the reproductive performance of mature breeding boars. Therefore, eight adult breeding boars were intramuscularly inoculated with 1 ml 103.5 HAD50/ml of industry-grade ASFV-G- ΔI177L. However, boars started to show clinical signs 6 days post vaccination (dpv), accompanied by onset of fever 8 dpv, which lasted up to six days and reached up to 41.5°C. By 14 dpv, 4/8 boars had to be euthanized upon reaching a moderate humane endpoint. Additionally, vaccine virus caused stable viraemia in all eight boars, with most boars positive in blood at 4 dpv and oral fluids at 7 dpv. Vaccine virus was also detected in semen samples from 7 dpv until the end of the trial at 28 dpv. This evidence points towards a high probability of transmission to naïve recipients Furthermore, onset of clinical signs caused by vaccination ultimately affected the semen quality – reduction in viability of spermatozoa, as well as increase of abnormal spermatozoa and infiltrated leukocytes could be observed. Vaccine virus ASFV-G- ΔI177L was detected in the majority of organs tested upon necropsy. All boars seroconverted, but antibody titers were significantly lower compared to titers in survivors of field virus infection. In conclusion, this vaccine has proven to be not safe for use in mature breeding boars.

Experimental evaluation of combined vaccination protocols using MLV and experimental inactivated vaccines against a highly virulent PRRSV-1 challenge

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The emergence of highly virulent strains of PRRSV-1 has become a serious problem in Spain. In the present study we aimed at assessing the efficacy of combined protocols involving modified live (ML) and inactivated PRRSV vaccines against the highly virulent strain R1. Seventy 4-week-old PRRSV-naïve piglets were randomly allocated to 7 groups (G1-G7, n=10). G1 was vaccinated once IM with a commercial MLV PRRSV (PYRSVAC-183); G2 initially received the MLV and 3 weeks later animals were boostered with an experimental inactivated vaccine containing the same strain (ALL-183); G3 was vaccinated as G2 but booster was done with an R1 inactivated vaccine; G4 received two doses of the inactivated ALL-183 vaccine and G5 received 2x inactivated R1. G6 and G7 were kept as unvaccinated controls. Four weeks after the booster, all groups except G7 were IN challenged with R1 strain at $10^{5.5}$ $TCID_{50}$/ml. Pigs were followed for the next 4 weeks. Vaccination induced the development of antibodies before the challenge except for some pigs receiving 2x R1. On the challenge day most animals showed significant frequencies of interferon-γ secreting cells but G5 animals did not show a significant booster of the interferon responses after challenge. After inoculation, unvaccinated animals developed high fever, lethargy and severe growth retardation (on average 10.7 kg less than unchallenged controls). Clinical scores and weight gains were better for the MLV-receiving groups (+2.8 and +4.3 Kg compared to unvaccinated controls). In contrast, groups receiving only the inactivated vaccines, showed increased clinical scores and reduced weight gains (between -0.8 and -4.1 Kg compared to unvaccinated controls). Overall, at 7 days post-challenge, pigs showed extensive pneumonic lesions (7.5%-86% of the lung) although lesions <20% were only observed in vaccinated animals. Interestingly, groups receiving two doses of the inactivated vaccines showed a trend for having more severe lesions at 28 days post-challenge. Regarding viremia and nasal shedding, control of the infection was limited in all groups. Taken together the present results support the notion that MLV vaccines helped reducing clinical scores and weight losses. Protocols based solely on inactivated vaccines can result in enhancement of the disease after challenge.

Insight into the molecular mechanisms leading to reversion to virulence of live attenuated vaccines against ASFV

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African swine fever virus (ASFV) infects domestic pigs and wild boar and is currently the biggest animal pandemic affecting more than 40 countries in four continents and the greatest threat to the global swine industry. Currently, the most realistic vaccination strategy is live attenuated vaccines (LAV), where attenuated prototypes with high protection rates are usually generated by manipulating genes involved in virulence. However, safety issues associated with these prototypes remain a challenge to be addressed. Recently our group has generated a prototype from the virulent Arm/07/CBM/c2 strain in which both the A238L and EP402R ASFV genes were deleted, generating a fully attenuated prototype in pigs¡, which further induces 100% protection against a circulating virulent strain. However, regulatory-required back-passage tests, consisting in inoculating virus from blood/tissues from the vaccinated animals (8-10 days after vaccination) into naïve pigs, eventually resulted in the death of the newly blood-inoculated animals, indicating that the prototype had somehow reverted to virulence. Due to the importance of these results in terms of safety, and in order to analyze the causes, viruses were isolated from different tissues of the animals and viral DNA subjected to next generation sequencing (NGS). By doing so, we have identified mutations on two genes probably involved in the viral the phenotype change. The molecular mechanisms of this reversion to virulence, currently under study, would help to answer important issues that commit the safety of LAVs vaccines and could help to develop new, safer LAVs in the near future.

Cyclic di-AMP incorporated into the oral formulation enhances the Protective Efficacy of E2-CD154 Chimeric protein Against Classical Swine Fever in Pigs

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The development of mucosal vaccines has gained significant attention in recent years. The mucosal route of vaccination offers distinct advantages over parenteral delivery, including the ability to elicit both systemic and local immune responses at the primary entry points of many pathogens. Adjuvants play a crucial role in mucosal vaccine formulations by influencing the strength, quality, and duration of the immune response. Recently, the cyclic di-adenosine monophosphate (c-di-AMP), a stimulator of the STING pathway, has emerged as a promising mucosal vaccine adjuvant capable of enhancing host immune responses. In this study, classical swine fever virus (CSFV) was used as a model to evaluate the immunogenicity and protective efficacy of a mucosal subunit vaccine prototype composed of the E2-CD154 chimeric protein adjuvanted with c-di-AMP in domestic pigs.

The co-administration of E2-CD154 and c-di-AMP via sublingual conferred strong clinical protection following CSFV challenge, comparable to those induce by the licensed Porvac® intramuscular vaccine. This combination reduced clinical signs, hindered viral replication, and a strong humoral response. Notably, the addition of c-di-AMP significantly enhanced the systemic IgG and IgA responses and promoted the development of neutralizing antibodies post-challenge. In contrast, E2-CD154 alone, failed to induce a robust humoral response. These findings underscore the critical role of c-di-AMP as a mucosal adjuvant and support its further evaluation for parenteral and mucosal immunization strategies. This work represents a foundational step in the development of a non-replicating CSFV vaccine for sublingual delivery and highlights its potential applicability in broader veterinary and human vaccine platforms.

One Health - Wildlife Diseases, Zoonoses

Outdoor cats and H5Nx avian influenza: Sentinel role, exposure risk, and broader implications for cross-species transmission

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The emergence and sustained circulation of clade 2.3.4.4b high pathogenicity avian influenza viruses (HPAIV) H5Nx in wild birds and poultry across Europe has raised significant concerns about cross-species transmission and pandemic risk. Domestic cats, which frequently interact with wild birds and live in close proximity to humans, are known to be susceptible and permissive to both avian and human influenza viruses, making them potential intermediate hosts or sentinels for avian influenza exposure. This study aimed to assess whether domestic and stray outdoor cats in France had been exposed to H5Nx viruses during the ongoing HPAIV epizootics and to identify risk factors associated with seropositivity. Between December 2023 and January 2025, serum samples from 728 outdoor cats (642 domestic and 86 stray) were collected through 44 veterinary practices across 30 French departments. All cats had regular outdoor access and were not from densely urban areas. A commercial anti-H5 ELISA was used for initial screening, and positive results were further tested by virus-serum neutralization assays against both a HPAIV H5N1 clade 2.3.4.4b strain and a low-pathogenic avian H5N3 virus. Overall, 2.6% of cats tested positive, with a majority exhibiting higher titers against the HPAI virus, indicating a stronger exposure to circulating clade 2.3.4.4b strains than to low pathogenicity strains endemic in France. Seropositivity was significantly associated with stray status (Spearman's $\rho = 0.074$; $p = 0.047$). Owned cats that did not hunt were 94% less likely to be seropositive compared to strays (OR = 0.06; 95% CI: 0–0.53; p = 0.01). These findings demonstrate that outdoor cats in France can be naturally exposed to H5Nx viruses, particularly those with hunting habits, likely through ingestion of infected prey. Given the demonstrated ability of HPAIV to infect mammals and occasionally cause severe disease or onward transmission, cats may play a more active role than previously recognized in the ecology of influenza viruses. In addition to national data, international case observations and studies, and future research perspectives—including experimental and epidemiological studies—will be discussed to contextualize the role of felids in influenza emergence.

Viral fitness of highly pathogenic avian influenza H5N1 viruses in mammals

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Highly pathogenic avian influenza (HPAI) H5N1 clade 2.3.4.4b viruses have caused recurring outbreaks in poultry and mass mortalities in wild birds. Since the re-emergence of the H5N1 subtype in 2020, the virus has rapidly expanded its host range to mammals, including red foxes, polecats, otters, badgers, cats, dogs, and minks. The virus has continued to spread via the transatlantic flyway to North-America, where numerous outbreaks have been reported in poultry, wild birds, and mammals. Ruminants were not considered susceptible to HPAI viruses, but in March 2024 HPAI H5N1 was detected in goats and cattle in the United States. Transmission via milk resulted in spread to over 1000 dairy herds. The continued circulation of HPAI H5N1 in ruminants creates opportunities for mutations that increase the zoonotic potential of the virus. Incidental transmissions from cattle to humans raise concerns about its potential to evolve into a pandemic threat.

We investigated the host range of emerging influenza viruses using both traditional animal models and novel *in vitro* models. We examined the tissue tropism of HPAI H5N1 infections in wild red foxes by immunohistochemistry and investigated the replication dynamics in human, dog and chicken cell lines. Additionally, we investigated replication, pathology and direct transmission of three HPAI H5N1 viruses in ferrets as a model for human infections. *In vitro* models such as well-differentiated airway epithelial cells offer an interesting alternative to the traditional methods to determine the host range of a virus. As a case study we assessed the potential for European HPAI H5N1 viruses to cross the species barrier to ruminants using bovine well-differentiated airway epithelial cells. While additional viral mutations are required for efficient mammal-to-mammal transmission, these studies underscore the urgent need for enhanced surveillance in both wildlife and domestic animals and a timely response to new outbreaks in mammalian species.

Phylogenetic insights into the first Usutu virus outbreak in Denmark, 2024

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Usutu virus (USUV) is a neurotropic, mosquito-borne, virus belonging to the Japanese encephalitis serocomplex within the genus *Orthoflavivirus* of the *Flaviviridae* family. USUV has been classified into 8 lineages: Africa (AF) 1, 2, and 3, and Europe (EU) 1, 2, 3, 4, and 5. After the first identification of USUV in Europe in Austria in 2001, it has continued to circulate causing outbreaks in several countries. USUV caused a significant die-off of birds, predominantly Eurasian blackbirds (*Turdus merula*) across the European continent in 2018. In late summer of 2024, we detected the first cases of USUV in blackbirds in Denmark.

Brain samples from diseased blackbirds (euthanized or found dead), that scored positive in RT-qPCRs, were selected for sequencing by Illumina MiSeq, employing either a shotgun or tiled-PCR approach. Reads were *de novo* assembled using both MEGAHIT and metaSPAdes, and resultant contigs were queried to a local blast nt_viruses database to identify the closest matching reference. Draft genomes were reconstructed using these references as scaffolds, when possible, allowing for contig mapping and extraction of polished consensus sequences. Various gap-filling strategies were applied as needed. Additionally, a reference-based consensus sequence was generated by mapping the trimmed reads directly to the selected reference sequence. The consensus sequences were aligned and manually inspected to obtain the final draft genomes. Final consensus sequences were manually curated after alignment and inspection, and subsequently aligned to a custom USUV database using MAFFT. A maximum-likelihood phylogenetic tree was inferred with IQ-TREE.

Phylogenetic analysis revealed that most samples belonged to the USUV EU2 lineage, with additional representation from AF3 and EU3. Notably, the sequences from the outbreak in Denmark, from the same lineages did not consistently cluster together, but grouped into different clades alongside strains from various regions of Europe.

The phylogenetic analysis indicates that there likely have been several introductions of USUV into Denmark.

First Serological Evidence of Crimean-Congo Hemorrhagic Fever Virus Infection in Croatian Sheep Populations

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Crimean-Congo hemorrhagic fever virus (CCHFV) is one of the most widely distributed tick-borne zoonotic viruses. While it causes asymptomatic infections in animals, it can result in severe and fatal hemorrhagic disease in humans. The confirmation of viral circulation in neighboring Bosnia and Herzegovina in 2022 highlighted the urgency of investigating the presence of CCHFV in Croatia.

In a study assessing the role of sheep as sentinels for vector-borne pathogens, Barbić et al. (2025) did not detect CCHFV-specific antibodies in sheep sampled from inland Croatia, specifically Vukovar-Srijem County, during 2022. To expand the surveillance area, we analysed 152 archived sheep serum samples collected in 2023 from Dubrovnik County and 184 samples collected in 2024 from Zadar County. Both regions have been reported as endemic for *Hyalomma* spp. ticks, the primary vector of CCHFV.

All samples were stored at –20°C until testing. Detection of antibodies against CCHFV nucleoprotein was performed using the commercially available ID Screen® CCHF Double Antigen Multi-species ELISA kit (ID-vet, Grabels, France). Samples testing positive were further assessed for active viremia using the Crimean-Congo Hemorrhagic Fever Virus One-Step RT-qPCR Kit (NZYtech, Lisbon, Portugal). Results were analysed using R software version 4.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

Seroconversion was confirmed in 95 out of 184 sheep serum samples from Zadar County. No positive results were detected among samples from Dubrovnik County. This corresponds to an overall seroprevalence of 28.3% in the tested Croatian sheep and 51.6% within Zadar County. Samples originated from 14 farms; eight had no seropositive animals, while six had at least one. Seroprevalence on positive farms ranged from 63.6% to 85.7%, suggesting high intra-farm transmission or shared risk exposure. All seropositive samples tested negative by RT-qPCR. Statistical analysis showed significantly higher seropositivity in adult sheep compared to lambs (OR = 23.4; p = 0.003) and juveniles (OR = 11.7; p = 0.001), with no significant influence of gender.

This study confirmed, for the first time, CCHFV antibodies in Zadar County sheep, suggesting possible past local circulation of virus. For better assessment of CCHFV activity, continued monitoring of sentinel animals in tick-endemic areas is warranted.

Establishing integrated surveillance for CCHF/CCHFV in Bosnia and Herzegovina: a neglected and unresolved challenge

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The Crimean-Congo haemorrhagic fever (CCHF) is a severe tick-borne viral disease in humans, caused by the Crimean-Congo haemorrhagic fever virus (CCHFV), a member of the genus *Orthonairovirus*. The virus is transmitted by bites from infected ticks, mainly of the *Hyalomma* genus or by direct contact with blood or tissues of infected ticks, viremic patients or viremic livestock as well as indirectly by fomites. According to information presented on MediLabSecure regional meeting 2023, integrated surveillance system for CCHF in Bosnia and Herzegovina was not in place. Human cases were not reported but prioritization of CCHF is necessary step and strong recommendation under https://www.onehealthsecure.com/ One Health and MediLabSecure project action.

Retrospective and recent scientific data point out the first report on antibodies against Crimean-CCHFV in sheep in Bosnia and Herzegovina in 2018 (Satrovic et al., 2022). CCHFV specific antibodies were detected in 9.66% of tested animals (17/176) using the ELISA test. Seroprevalence of CCHFV in the cattle population was estimated at 14.97% (72/481). The PCR based investigation of vectors (n=760 ticks) and bovine blood samples (n=206) were performed in the period 2019 – 2021. Results showed that one pool of three male *Hyalomma marginatum* ticks tested positive for viral RNA (0.39%). In the same period no viral RNA was detected in 206 bovine blood samples (Goletic et al., 2022).

Complexity of CCHF epidemiology (appear suddenly, without warning, rapid spreading, difficulties in identifing the source of infection), lack of: equipment, appropriate biosafety levels and expertise, intra- and intersectorial cooperation and multidisciplinary approach, legislation and socio-economic factors (funds, trust), are chellenging issues that must be overcome as crucial precondition for nationally and regionaly needed and coordinated actions. Published data clearly confirm the presence and ongoing circulation of CCHFV in Bosnia and Herzegovina.

Despite previous recommendations, by 2025 no significant integrated surveillance progress has been made. We continue to comunicate the urgent need for coordinated action between the veterinary services, human health sector, and governmental authorities. The active engagement of the scientific community at this conference is expected to provide momentum toward mobilizing the broader sectoral participation required for sustainable progress.

Poster Section

Pathogenesis

Different replication behavior of a recent porcine hemagglutinating encephalomyelitis virus strain (P412) compared with the old neurotropic VW572 reference strain

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Porcine hemagglutinating encephalomyelitis virus (PHEV), first identified in 1957, was originally associated with neurological disorders (e.g. vomiting and wasting disease). However, more recent strains have mainly been associated with subclinical infections and respiratory problems. In the present study, it was the purpose to find out if there are differences in replication of old and new PHEV strains in relevant tissues that may explain the clinical outcome. This study compared the replication kinetics of an old neurotropic PHEV strain, isolated in Belgium in 1972 (VW572), with that of a recent 2020 strain (P412), using RPD (swine kidney) cells, nasal and ethmoidal mucosa explants, and primary porcine respiratory epithelial cells (PoRECs). EGTA was used to disrupt intracellular junctions to facilitate the access of the virus to basolateral receptors. VW572 strain replicated more rapidly and reached its peak earlier while P412 strain exhibited slower, more progressive infection dynamics. Results showed no significant difference in the replication kinetics of both strains in nasal explants, regardless of EGTA treatment. In olfactory mucosa explants, VW572 infected more sustentacular cells than P412, especially upon EGTA treatment, suggesting a stronger reliance on basolateral receptors of sustentacular cells. In PoRECs, the replication of both strains was very similar. Only with VW572, there was again a positive effect by EGTA. Genomic analysis identified two spike amino acid changes at the receptor binding site that may be responsible for the different behavior of the two PHEV strains. In conclusion, these findings highlight strain-specific variations of PHEV in its tropism for cells in the upper respiratory tract. Further in vivo studies will be performed to confirm the observed *ex vivo/in vitro* differences.

Structure and promoter-specificity of the early transcription factor AETF in African Swine Fever Virus

Gwenny Cackett, Christopher Dulson, Simona Pilotto, Michal Sýkora, Christos Spanos, Jerome Gouge, Anthony Roberts, Finn Werner

African Swine Fever Virus (ASFV) causes a lethal haemorrhagic fever in pigs, due to the absence of approved antivirals and vaccines, it presents a severe threat to global food security with a large socio-economic damage. ASFV belongs to the Nucleocytoplasmic Large DNA Viruses (NCLDV) or *Nucleocytoviricota*, it inhabits the cytoplasm of the infected cell, and thus depends on its own DNA-dependent transcription- and mRNA processing machinery, demonstrating a remarkable independence from the host for viral gene expression.

We study ASFV using a two-pronged approach, (i) characterising viral transcriptomes *in vivo* including bioinformatics analyses of promoter motifs (J Virol (2020) 94:10.1128/jvi.00119-20, J Virol (2022) 96:e01939-21), combined with (ii) biochemical and structural analyses *in vitro* using a recombinant ASFV RNA polymerase (RNAP) system. We have previously solved cryo-EM structures of the eight-subunit ASFV RNAP that reveal both fascinating evolutionary conserved- and virus-specific features (Nature Comm (2024) 15:1606). This further demonstrated the power of insect cell expression to produce fully functional ASFV protein complexes recombinantly in low biosafety labs.

Here, we focus on the early transcription factor complex comprising subunits G1340L and D1133L, henceforth called AETF (ASFV Early Transcription Factor). This heterodimer is an essential bottleneck during ASFV infection, being packaged alongside the RNAP in viral particles. We have produced recombinant AETF in insect cells, solved it's structure via cryo-EM, and shown that it specifically recognises the cEPM (core Early Promoter Motif) of ASFV promoters, which direct transcription during early infection. AETF is ~280 KDa and comprised of 11 domains, some of which are structurally- and functionally related to general transcription factors of eukaryotic Pol II, including an extended TBP-like domain in AETF1, and a TFIIH-like helicase/translocase residing in AETF2. Moreso than the viral RNAP, AETF features large ASFV-specific domain insertions in addition to elements that are conserved with other viral and host factors. Interestingly, despite Poxviruses being the best studied NCLDVs, the AETF appears to be a better representation of early transcription factors in other NCDLVs. Key additional features in ASFV are widely conserved in NCLDVs, in contrast to Poxviruses whose ETF appears more 'condensed'. This highlights the importance of studying each of these viruses in their own right, with many NCLDVs being causative agents in animal disease.

Feline Enteric Coronavirus Utilizes Serine Protease-Mediated Redundant Cleavage for Intestinal Adaptation

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Feline enteric coronavirus (FECV), a common feline pathogen, replicates in the intestinal tract and establishes infection through mechanisms that remain incompletely understood. Like other coronaviruses, FECV requires proteolytic activation of the spike (S) protein to mediate receptor binding and membrane fusion. In this study, we investigated the role of host proteases in FECV entry. We screened a panel of proteases and found that serine proteases are essential for FECV infection. Specifically, three pancreatic serine proteases—trypsin, chymotrypsin, and elastase—significantly enhanced viral infection and promoted syncytium formation in vitro, despite their divergent substrate specificities, suggesting that FECV employs a flexible activation strategy. In addition, we observed that the membrane-bound serine proteases TMPRSS2 and TMPRSS11D also facilitated infection in a strain-dependent manner.

The regions on the S protein where serine proteases cleave are referred to as cleavage sites. To elucidate the mechanism of serine protease-mediated spike activation, we analyzed potential cleavage sites in the S protein. Sequence alignment with related coronaviruses revealed two putative cleavage motifs: the S1/S2 and S2' sites. Using recombinant spike constructs and western blot analysis, we experimentally confirmed these two cleavage sites and identified additional processing regions within the S protein. Importantly, mutational studies revealed that even when the primary S2' cleavage motif was disrupted, spike activation was preserved due to compensatory cleavage near the S2' site. This finding underscores the critical role of the S2' site in spike activation and highlights a functionally redundant cleavage mechanism.

Collectively, our findings reveal that FECV has evolved to exploit the protease-rich intestinal niche by adopting a redundant and flexible activation mechanism. These insights advance our understanding of enteric coronavirus biology and suggest that targeting host protease-mediated activation may provide a viable antiviral strategy.

RBSN and HSPA13 are essential for BVDV infection of MDBK cells

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The invasion of BVDV in its host cell has been studied extensively with a focus on the cell surface receptors such as heparin sulphate, CD46, LDLR, LamR and recently ADAM17. Following receptor binding, clathrine mediated endocytosis (CME) and pH dependent fusion have been determined as route of entry. The aim of the study is to identify the essential factors for BVDV invasion after the interaction with the cellular receptors. A targeted CRISPR Cas9 knockout screen with MDBK cells was designed to identify the key factors of endocytosis and intracellular vesicle trafficking during BVDV infection. The screen included 41 genes encoding Rab GTPases, various kinases and subunits of the CORVET/HOPS-ESCRT complex. The readout of the CRISPR/Cas9 knockout screening was the loss of susceptibility towards two cytopathogenic BVDV strains. Inactivation of RBSN and HSPA13 genes resulted in a complete loss of susceptibility whilst 25 additional candidates, including CORVET/HOPS complex subunits, showed increased survival upon virus infection. Experiments to confirm strong candidates by transcomplementation of inactivated target genes and to scrutinize their function in BVDV entry are underway.

Funded by the Austrian Research fund (FWF P35674)

African swine fever virus infection of porcine monocyte-derived macrophages induces the formation of tunneling nanotube-connected large vesicle-like cell segments: a potential mechanism for intercellular ASFV trafficking

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African Swine Fever (ASF) is a highly fatal viral disease in pigs, with mortality rates that can reach 100%. The causative agent, African swine fever virus (ASFV), primarily targets cells of the mononuclear phagocytic system (MPS), particularly monocyte-derived macrophages (MDMs). Despite the severity of the disease, there are currently no effective antiviral treatments available in Europe. A significant barrier to therapeutic development is the limited understanding of how ASFV interacts with its primary target cells. A deeper understanding of the morphological changes induced by ASFV in infected cells is crucial to this effort.

To address this knowledge gap, we used conventional and confocal immunofluorescence microscopy, as well as transmission electron microscopy to investigate ASFV-infected primary MDMs.

Our analysis revealed that ASFV infection leads to the formation of large cellular protrusions, which are characterized by vesicle-shaped cellular segments (CS) at their tips. These protrusions contain all major cytoskeletal components, showing characteristics similar to tunneling nanotubes (TNTs). In 84.93% of the cases, the nucleus remained in the cell body (CB) near the viral factory. In the remaining cases, the nucleus was found within these CS, while the viral factory was present in the CB. Additionally, 57.6% of the cells showed contact between the CS and distant cells, suggesting a potential mechanism for ASFV transmission.

These findings suggest that ASFV induces cellular segmentation linked by TNT-like structures. Further research is needed to better understand the biogenesis and functional significance of these segmented cells, which could inform future strategies for combating ASFV.

Using Stem Cell Derived Macrophages to Investigate Differences in Responses to African Swine Fever Virus from Different Suids.

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African swine fever virus (ASFV) is a lethal haemorrhagic disease of domestic pigs and wild boar with up to 100% case fatality. There is currently no commercially available vaccine or anti-viral treatment, and control relies on early diagnostics, increased biosecurity, and slaughter. The virus targets macrophages/monocytes where replication occurs in the cytoplasm of the infected cell. The virus encodes for many genes which allows the virus to evade the host's immune defences including proteins that target interferon induction, interferon signalling, the NF-κB pathway and apoptosis.

In Africa ASFV transmission routes include the sylvatic cycle between warthogs and the soft ticks which infest their burrows which contributes to the persistence of the virus in the field. In African wild pigs such as warthogs, red river hogs and bushpigs ASFV can replicate but does not cause disease in these animals. However, the mechanisms of this effective host resistance to ASFV are poorly understood. Using primary macrophages from these species to study these mechanisms is not feasible and so we have developed pluripotent stem cell derived macrophages from pigs, wild boar, red river hogs and warthogs to try to gain some insights into the differences between the species. We have found that ASFV is able to replicate to comparable levels in these species and so there does not appear to be an intrinsic barrier to replication. We are now starting to look at host responses to the virus to see if there are any indications which could explain the differences seen in the animals.

Interspecies Transmission of Border Disease Virus from a Persistently Infected Calf to Pigs and Sheep

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Border Disease Virus (BDV) is a pestivirus endemic in small ruminants in the UK and globally. Morbidity in sheep and goats is predominantly seen in pregnant animals, causing stillbirth, abortion, and infertility. BDV occasionally infects swine where antigenic similarity can complicate classical swine fever (CSF) diagnosis, as well as cattle, where disease is similar to bovine viral diarrhoea (BVD). Persistently infected (PI) animals are major contributors to sustaining pestivirus infections within cattle. BDV has been shown to create PI-cattle and is known to spread from there to other cattle but transmission of BDV from cattle to other species had not been documented.

This study investigated BDV transmission from a PI calf via indirect exposure to sheep and pigs in adjacent pens. Cross-species contact was limited to husbandry practices and fomites, simulating a mixed-species farm. Animals were monitored for clinical signs, and blood samples were collected for detection of viraemia and seroconversion by RT-qPCR and p80 BDV antibody ELISA for 85 days post exposure.

Four of ten sheep developed transient fever ≥40°C, which correlated with a detectable viraemia. One sheep, which had a prolonged viraemia (53 days), had recurrent fever on days 10, 35, and 56. None of the animals showed other overt clinical signs of infection, such as changes in liveliness, eyes, respirations, appetite, diarrhoea, posture, or gait. By day 85, all nine pigs and four sheep had seroconverted. Three sheep were within the ELISA suspect range and three were seronegative. A calf co-housed with the PI calf started to seroconvert at day 20 and was seropositive by day 45. The co-housed calf developed facial papillomas, possibly linked to BDV-related immunosuppression, however, no other clinical signs were observed.

Environmental swabs taken weekly from the pig and sheep pens were PCR negative. Within the cattle pen, BDV was consistently detected on the walls, water, and food troughs.

These findings indicate BDV transmission occurred from cattle to pigs and sheep, likely via fomites or human-mediated transfer. Our results highlight the risk of BDV transmission on mixed-species farms and the potential of BDV infections in confounding BVD and CSF diagnostic and control efforts.

Molecular analysis of bovine leukaemia virus isolates emerging in Poland in the years 2021-2024

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Objectives

Bovine leukemia virus (BLV) belongs to the Retroviridae family and is the etiological agent of enzootic bovine leukosis (EBL). Poland has EBL-free status since May 2017, however, in the last few years a certain number of new infections were registered, mostly in Warmia and Mazury and less in Greater Poland and Mazovia regions. The reasons for emergence of these infections are unknown; perhaps they can be associated with the presence of BLV variants with specific genetic features and a high copy number of proviral DNA.

Materials & methods

Full-length genome of 30 BLV isolates were amplified. NGS libraries were prepared and subjected to sequencing. The data were quality checked, trimmed and mapped against reference sequence. Phylogenetic analysis and genetic variability was assessed by the MEGA6.

Results

In our first step we focused on the most genetically variable region of BLV- the fragment of env gene, encoding the viral surface protein gp51. Phylogenetic analysis revealed that majority of isolates 28/30 (93%) belonged to genotype 4 (G4) while remaining one was affiliated to G7 and another one to G8. Pairwise comparison of these sequences showed that the sequence identity varied between 96.1-100%. Amino acid sequence analysis revealed a total number of 24 aa substitutions. Four substitutions (S7P, P11S, L25F and Q27P) were localized at signal peptide, three (S56F and S58A or S58A) were found in epitope H and five (N50H, P73A, R74K, F82L, H121R) in epitope G. Substitution L80S was localized at CD8+ T-cell epitope, V106L in the ND1 domain and two (A133T and I144T) were seen in the ND2 region. The remaining 4 substitutions were localized in linear epitopes, I231V and S235G in B-B' epitope, R267K in DD' epitope and A291V in epitope A. Three substitutions T48A, Q61R, and L62I were found outside the specific domains of the gp51 protein.

Conclusion

Preliminary results of phylogenetic analysis show consistency with our previous results obtained from samples collected before 2017 and comparable variability rates. In order to fully characterize emerging isolates, we will trace the genetic variability of their entire genomes and BLV DNA copy number.

Porcine Hemagglutinating Encephalomyelitis Virus VW5.72 (not Gent/PS412 and Labadie) uses the CD81 receptor and MVB-derived exosomal pathway for efficient entry and spread in neuronal cells

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Porcine Hemagglutinating Encephalomyelitis Virus (PHEV) is considered a neurotropic coronavirus that invades the peripheral (PNS) and central (CNS) nervous system of the pig and causes acute encephalomyelitis, also known as 'vomiting and wasting disease'. Recently, PHEV has been proposed as a potential surrogate virus model to further elucidate the neuropathogenesis of other betacoronaviruses. In this study, we compared key steps in the replication cycle of 3 distinct PHEV isolates (VW5.72, Gent/PS412, and Labadie) in mouse PNS (N2a) cells. We found that PHEV-VW5.72 replicates more efficiently in these cells compared to the other 2 isolates. Interestingly, PHEV-VW5.72 showed high intracellular virus titers without efficient extracellular release. Further investigation revealed that PHEV-VW5.72, but not PHEV-Gent/PS412, utilizes multivesicular body (MVB)-derived exosomes for viral egress. Transmission electron microscopy confirmed the presence of complete PHEV-VW5.72 virions within intracellular vesicles and the release of fused PHEV-exosome structures near the plasma membrane. Finally, we showed that PHEV binding is restricted for all isolates. Still, we demonstrated that only PHEV-VW5.72 entry into cells is mediated by the tetraspanin CD81 receptor. Overall, these results suggest that PHEV-VW5.72 uses MVB-derived exosomal pathway as a unique strategy to promote efficient infection and overcome the early restriction in neuronal cells. In addition, these findings highlight isolate-specific differences in PHEV neurotropism.

Keywords: PHEV; neuropathogenesis; CD81 receptor, MVB-derived exosomal pathway

Immunity

Immunity Interrupted: How Virulent ASFV Strains Undermine Antigen Presentation

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The African swine fever virus (ASFV) is a large, double-stranded DNA virus that belongs to the *Asfarviridae* family. It causes a severe hemorrhagic disease in domestic pigs and wild boars. Highly virulent strains, such as “Armenia2008” (ASFV-A), result in fatal outcomes ranging from peracute to acute, while moderately virulent isolates, such as “Estonia2014” (ASFV-E), are associated with lower lethality and prolonged survival. Monocytes are the main target cells of ASFV and play a central role in initiating adaptive immunity via antigen presentation.

To analyze viral-mediated mechanisms of immune modulation, we compared the impact of ASFV-A and ASFV-E on antigen presentation *in vivo* and in primary porcine monocytes ex vivo. ASFV-A-infected monocytes exhibited significantly reduced surface expression of swine leukocyte antigen class I (SLA-I), despite unaltered transcript and total protein levels. This reduction was linked to a marked decrease in SLA-I maturation and transport to the cell surface. The intracellular maturation block of SLA-I was accompanied by a loss of functional rough endoplasmic reticulum (ER) structures and the formation of pronounced ER-associated aggresomes. This leads to cellular stress, which coincides with the inhibition of host protein translation, mitochondrial depolarization, and the activation of caspase-3-mediated apoptosis. In contrast, ASFV-E-infected monocytes retained normal SLA-I surface expression and showed no signs of ER stress or translational shutdown.

Our findings suggest that, in domestic pigs infected with the highly virulent ASFV-A strain, subversion events occur sequentially in infected monocytes, likely leading to compromised T cell activation and impaired responses against ASFV downstream.

Transcriptomic Profiling of Host Innate Immune Responses to Batai Virus Infection Using Nanopore Sequencing

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Bataivirus (BATV), a member of the *Peribunyaviridae* family, is a globally distributed mosquito-borne virus infecting e.g., humans, livestock and birds. Serological surveillance in Europe has shown a BATV seroprevalence of up to 46% in ruminants. Although infections in ruminants and humans are typically mild or asymptomatic, neurological disorders have been reported in infected seals. However, the molecular mechanisms underlying host-virus interactions in ruminants remain largely unexplored. Therefore, in this study, Madin-Darby bovine kidney (MDBK) cells were infected with BATV (MOI = 1), and RNA was extracted from both infected and mock-infected cells at 24h, 48h, and 72 hours post-infection. Nanopore cDNA sequencing coupled with bioinformatics analysis was used to characterize transcriptomic changes.

Preliminary results revealed a strong innate immune response as early as 24 hours post-infection, with 960 genes differentially expressed (DE) (FDR ≤ 0.05; fold change ≥ 2). Up- and down-regulated genes were evenly distributed; however, functional analysis revealed marked differences in biological processes. Up-regulated genes were predominantly associated with antiviral responses, particularly the type I interferon pathway, and included Interferon Induced Protein 44 (IFI44), Interferon Stimulated Gene 15 (ISG15), Interferon-Induced Protein with Tetratricopeptide Repeats 1 (IFIT1), and 2'-5'-Oligoadenylate Synthase 1Z (OAS1Z), all of which showed high fold changes. In contrast, down-regulated genes were mostly involved in metabolic pathways. However, the most downregulated gene was YPEL3 (FC = 19), a p53-regulated gene known to induce cellular senescence, and its downregulation can lead to reduced apoptosis. Several additional genes associated with apoptosis was also identified among the downregulated genes including BCL2 Interacting Protein 3 (BNIPL3), TNFRSF1A Associated Via Death Domain (TRADD) and PRA1 family protein 2 (PRAF2) suggesting a potential viral strategy to dampen host apoptotic responses. Together these findings provide new insights into the viral-host dynamics of BATV infection in its main host (*Bos taurus*). Understanding the functions of the DE genes can provide insights into BATV pathogenesis and host defense mechanisms. Further functional studies are needed to elucidate their specific roles during infection.

A novel in vitro system to analyse African swine fever virus replication dynamics at both population and single-cell levels

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African Swine Fever Virus (ASFV) is a large, enveloped, dsDNA virus from the *Asfarviridae* family. Since its discovery in Kenia in 1927, multiple viral strains have been described alongside the expansion of the virus around the world. The degree of virulence of these strains varies, from highly to moderately pathogenic viruses, depending on the disease severity. On the other hand, during the last years new recombinant ASFV strains have been developed through the deletion of genes related to virulence. Importantly, a few of these strains have shown potential as live attenuated vaccine (LAV) candidates. However, the mechanisms underlying these differences in virulence are poorly understood. Importantly, macrophages are the main target cell of ASFV, and their infection is a critical feature of the disease pathogenesis. Thus, the characterization of virus-host interactions is important to decipher mechanisms associated to the varying infection outcomes. Here, we developed an *in vitro* system using time-lapse microscopy to monitor the infection of porcine alveolar macrophages (PAMs) over time and at single-cell level. As a test case, we used three ASFV strains with different degrees of *in vivo* virulence, namely the virulent Ba71 and Georgia2007/1 strains, and the LAV candidate Ba71ΔCD2. All three virus strains were modified to express a fluorescent tag fused to the structural p54 ASFV protein to visualize infection. We found a high variability among individual cells regarding the different parameters linked to the virus replication cycle, such as the duration of the infection and the time of death. Interestingly, there were no significant differences in the replication kinetics of these three virus strains at population level, despite the different infection outcomes observed *in vivo*. Nevertheless, we did detect differences between the virus strains at the single cell level. For example, we observed a shift towards higher viral load in individual cells in Ba71ΔCD2v compared to the virulent ASFV strains. In summary, we present here a new analytical tool that provides novel dynamic parameters derived from ASFV replication *in vitro*. Further studies will be required to evaluate the potential of this technology to decipher critical viral and cellular factors associated with ASF pathogenesis.

Meta-analysis of duck (*Anas platyrhynchos*) lung transcriptome studies after highly pathogenic H5N1 infection

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Ducks and other Anseriformes serve as the natural reservoir for avian influenza. While earlier, high pathogenic H5N1 emerged sporadically in Galliformes, the outbreak in wild waterfowl in 2005 at Qinghai Lake represented a turning point towards persistence and spread of HPAIV in Anseriformes. The virus acquired mutations that altered its virulence, transmissibility, and replication dynamics in these hosts. The high variability in H5N1's impact and host responses among Anseriformes has driven considerable research interest.. RNA-sequencing has emerged as a powerful tool for studying host responses upon infection but studies are often limited by sample size. This study set out to remedy this by using a meta-analysis approach. Four relevant raw RNA-sequencing datasets of *Anas platyrhynchos* lungs were systematically collected from public databases. They include both control and HPAI H5N1-infected animals sampled at one, two or three days post-infection. These were processed together and a weighted Fisher p-value combination method was used to combine the differential expression results. After jackknife sensitivity analysis, an overrepresentation analysis was performed to find relevant Gene ontology and KEGG terms. Results showed that despite the high inter-study variability portrayed in the principal component analysis, there is a considerable subset of differentially expressed genes that is common for all experiments. This study also pinpointed a small number of differentially expressed genes that could not be identified using single studies, highlighting the advantages of this method. The overrepresentation analyses revealed both innate immune pathways—such as RIG-I–like and toll-like receptor signaling—as well as cell damage processes. Our findings demonstrate the importance of public sharing of raw RNA-sequencing datasets and the value of meta-analyses as a viable method to look for novel insights.

Single-Cell RNA-Seq Analysis of Major PBMCs Exposed to Virulent African Swine Fever Virus

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African swine fever virus (ASFV) is the causative agent of a viral hemorrhagic disease of domestic pigs and European wild boar. ASFV mainly replicates in monocytes/dendritic cells. The pathogenesis of the disease is complex and has not been fully understood. In this study, peripheral blood mononuclear cells (PBMCs) were isolated from pigs infected with the virulent strain Arm/07/CBM/c2, and subject to single-cell RNA-sequencing (scRNA-Seq) using Chromium single cell technology. Gene set enrichment analysis (GSEA) was performed on the PBMCs exposed to but not infected with ASFV, using hallmark gene sets and the gene sets derived from the Gene Ontology (GO) biological process ontology. Based on the expression profile of marker genes, 22 cell types were determined, including monocytes, B cells, gamma/delta T cells, naïve and cytotoxic CD8 T cells, natural killer (NK) cells, natural killer T (NKT) cells, and CD4 T cells, etc. A total of 3504 differentially expressed genes (DEGs) were identified in PBMCs other than monocytes. Of the up-regulated DEGs, the ten most common DEGs are part of the innate immune response, specifically the type I interferon-stimulated genes and antiviral defense mechanisms, such as IFI6 and ISG15. The findings were consistent with GSEA results, which showed significant enrichment of pathways related to innate antiviral and interferon responses. Cell type-specific enrichment was evident. For example, B cells' ability to bridge innate and adaptive immunity was disrupted whereas NK cell cytotoxicity is impaired by suboptimal activation signaling and mitochondrial stress during persistent interferon response. These results provide useful insights into the pathogenesis of African swine fever.

Characterization of Japanese Encephalitis Virus Infection Dynamics and Target Cells in Porcine PBMCs

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Japanese encephalitis virus (JEV) is a zoonotic mosquito-borne flavivirus endemic in Asia which poses a threat to Europe. Pigs serve as important amplifying hosts for this virus, and are thus of high epidemiological importance. While it is established that JEV interaction with immune cells in humans and mice is important for virus spread within the host and modulation of the immune response, such information is largely lacking in pigs.

Therefore, this study aimed to characterize JEV infection kinetics in porcine PBMCs and identify target cells. PBMCs from 3 pigs were infected with JEV at a multiplicity of infection (MOI) of 0.1, 1 and 10 and studied at 8, 24 and 48 hours post-infection by virus isolation, qRT-PCR and flow cytometry.

Lymphocytes showed a limited susceptibility for JEV, with an infection rate below 1%. CD172+ antigen-presenting cells (APC) appeared to be the most susceptible immune cell subset to JEV. An increasing percentage of JEV-infected APCs was observed with increasing MOI and infection time. At 48 hpi, up to 15.23% of APCs were JEV-NS3 positive. An in-depth flow cytometric phenotypic analysis showed that within the infected APCs, plasmacytoid dendritic cells (CD172a+CD14-CD4+ cells) and monocytes (CD172a+CD14+ cells) accounted for 51.9% and 33.63% of infected cells, respectively, representing 44.67% and 10.9 % of those populations to be JEV infected. Interestingly, indications were found that also CD3-CD8a+CD16+ cells became infected by JEV, what could reflect JEV infection of NK cells. Follow-up experiments are ongoing to confirm this unexpected finding. Also, virus isolations and qRT-PCRs are being finalized to study JEV replication and release kinetics in PBMCs.

The results indicate that JEV has a tropism for porcine plasmacytoid dendritic cells and monocytes, suggesting that these cells could play an important role in virus dissemination within the host, consistent with knowledge from humans and other flavivirus models. The JEV infection of porcine NK cells is being explored in more detail as this property has not been reported for other flaviviruses. Our findings support the usefulness of this model to investigate JEV pathogenesis and interaction with and evasion of the immune response.

Setting up an ELISpot-ifnγ Assay to Assess the Cellular Immune Response to MYXV Vaccine in Rabbits: A Preliminary Study

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Myxoma virus (MYXV) is the etiological agent of myxomatosis, a highly contagious and lethal viral disease affecting the European rabbit (*Oryctolagus cuniculus*). MYXV replicates in the cytoplasm of infected cells; cytotoxic T lymphocytes and other immune effectors play a central role in infection control. Conversely, circulating antibodies are less indicative of protection.

This study aims to evaluate the activation of the cellular immune response in rabbits vaccinated against MYXV.

Whole blood was collected before vaccination, 35- and 90-days post-vaccination (dpv), from ten 90-day-old female breeding rabbits, vaccinated once with *Nobivac Myxo-RHD PLUS* (MSD Animal Health). Additionally, 5-month-old breeding females vaccinated once with *Cunivax Mixoma* (Fatro) were sampled at 76 dpv. PBMCs were purified using Histopaque®-1077 (Merck). ELISpot assay was performed with the commercial ELISpot Flex Rabbit IFN-γ kit (Mabtech), using a lab-produced antigen in combination for *in vitro* recall. Data were collected using the CTL-Immunospot S6 Universal Analyzer and analyzed with GraphPad Prism. Humoral response was evaluated by a competitive ELISA developed at the WOAH Reference Laboratory for Myxomatosis (Chapter 3.7.1), and by Virus Neutralization test, carried out using RK13 cells and 100 $TCID_{50}$ of Cunivax Mixoma.

PBMC yields averaged approximately $2x10^6$ PBMCs/mL of whole blood, with a minimum of $5x10^5$ PBMCs/mL. To obtain a sufficient number of cells, 2–6 mL of blood was needed per animal. The cellular immune response to a specific stimulus was observed in PBMCs from vaccinated rabbits with *Nobivac Myxo-RHD PLUS* at both 35 and 90 dpv. Specifically, at 35 dpv, there were two non-responders, four low responders (consistent with low humoral immune response), and two high responders. Two animals died during the study. The response was stronger at 35 dpv and diminished by 90 dpv, possibly indicating the natural decline of effector T-cells in circulation. The PBMCs from rabbits vaccinated with *Cunivax Mixoma* appeared to exhibit a stronger immune response compared to those from rabbits given *Myxo-RHD PLUS*; however, this observation is preliminary and requires further exploration. Only one rabbit developed neutralizing antibodies.

In conclusion, these results provide a starting point to deeper insights into rabbit cell-mediated immune response against MYXV.

Evaluation of Mucosal antibody detection as a support DIVA tests for H5 highly pathogenic Avian Influenza surveillance in H5 vaccinated flocks

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In the context of the recent highly pathogenic avian influenza (HPAI) epidemics, the implementation of H5 vaccination as a complementary strategy to current control measures at the European level is being evaluated through vaccination/infection experiments in controlled and field conditions. HPAI vaccination presents significant challenges, particularly regarding surveillance. To ensure effective monitoring and control of HPAIV in vaccinated poultry flocks, the development of novel diagnostic tools is essential. This study aimed at developing innovative serological DIVA tests using minimally invasive sampling such as mucosal swabs to complement the range of diagnostic tools already available. Specific pathogen-free chickens were challenged at 42 and 114 days of age with two low pathogenic avian influenza (LPAI) strains via oculonasal inoculation. Serological kinetics were monitored over a period of 100 days. During this time, tracheal (TRS), oropharyngeal (OPS), cloacal (CLS), eye, and blood samples were collected. The results indicate a significant detection of anti-nucleoprotein (NP) antibodies in blood, TRS, OPS, and eye samples, whereas CLS samples showed very low antibody levels. Furthermore, the development of an indirect ELISA specific for anti-NP IgA confirmed the production of this isotype in the same types of samples. These findings confirmed the feasibility of detecting mucosal NP-specific antibodies as an alternative to their serum detection and allowed the characterisation of mucosal IgA kinetics following AIV infection.

Duration of immunity following infection with moderately virulent ASFV

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African swine fever virus (ASFV) represents a significant global threat to both domestic pig production and wild suid populations. This study evaluated the durability of immunity and immunological responses in animals recovered from inoculation with the moderately virulent ASFV '*Estonia14*' strain, employing a methodological framework aligned with duration-of-immunity assessments for live attenuated vaccines. Pigs inoculated with ASFV '*Estonia14*' primarily exhibited mild clinical signs and transient viremia. Six months post-inoculation, after full recovery, all animals were challenged with the highly virulent ASFV '*Armenia08*' strain. Only one previously exposed pig displayed mild clinical signs, while all naïve control animals developed characteristic severe symptoms and ultimately fatal ASF. Post-challenge testing showed ASFV genomes in only a subset of inoculated pigs, with virus isolation confirming low titers of infectious virus in tissues 28 days after challenge. Humoral immune responses, monitored through IgM and IgG profiles, demonstrated sustained IgG levels throughout the study period, with a slight increase following challenge. Plasma analysis demonstrated elevated levels of complement component C3a in recovered pigs after inoculation and challenge, correlating with detectable virus. In contrast, both C3a and C5a levels increased in control animals. Further *in vitro* studies indicated that complement activation appeared to be mediated by the lectin pathway, likely involving direct interaction of mannose-binding lectins with ASFV virions. These findings suggest that protective immunity persists for at least six months post-recovery, with no evidence of persistent or chronic disease in convalescent animals. The outcomes have important implications for vaccine development, efficacy evaluation, and epidemiological control measures, including surveillance strategies.

Epidemiology

Exploring the Enteric Virome of Calves from Central Ethiopia using Metagenomics – Detection of a Putative Novel Virus

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Over the years, viruses and the virome composition have received increased attention in the context of calf diarrhea, and various viruses have been associated with the disease. With the advancement of high-throughput sequencing (HTS) the detection and discovery of viruses has greatly improved, and multiple novel viruses have been detected in cattle and other livestock animals in recent years. In Ethiopia, the cattle industry is heavily impacted by diarrheic disease, but at the same time studies on the infectious agents in calves, primarily viruses, are lacking in the country. Therefore, we decided to apply viral metagenomics to investigate the virome composition in both diarrheic and non-diarrheic calves from Ethiopia.

Fecal samples were collected from diarrheic as well as healthy calves from dairy farms surrounding the capital, Addis Ababa, in January and February of 2023. RNA was extracted from the fecal samples, pooled, and sequenced using Illumina. Sequencing revealed a large variety of viruses, including known diarrheic viruses such as rotavirus and bovine coronavirus. Furthermore, several other bovine enteric viruses were detected for the first time in Ethiopia, for example astrovirus, norovirus, and torovirus. Interestingly, in one of the sequencing pools, we identified a highly divergent, complete viral genome of a virus that we gave the working name suluvirus. Investigation of the polyprotein sequence and genome layout together with BLAST searches revealed the closest matches to be within the *Picornaviridae* family. This was further supported by phylogenetic analysis where suluvirus clustered together with crohiviruses, a viral genus which has been identified in African bats and shrews. We propose that suluvirus is either a new genus or species within the *Picornaviridae* family. Lastly, qPCR-screening found 6/47 calves to be positive for suluvirus, indicating that suluvirus is a bovine enteric virus and that the detection using metagenomics was not an isolated occurrence.

To conclude, this is the first characterization of the enteric virome in cattle in Ethiopia, revealing a large number of viruses circulating among calves. Furthermore, the detection of suluvirus demonstrates that HTS is a powerful tool in viral discovery as well as characterization.

Wind*í* App, an R Shiny Tool to Rapidly Assess the Risk of Culicoides Wind Dispersal.

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Bluetongue and Epizootic Hemorrhagic Disease are non-contagious hemorrhagic diseases that are currently causing considerable damage to domestic ruminant livestock in Europe. A small flying midge, *Culicoides* spp, transmits them. The insect can easily be dispersed by the wind over great distances, thereby resulting in new disease introductions. Here, we present the Wind*í* App, an R shiny web-based tool designed to rapidly assess the risk of *Culicoides* wind dispersal across Europe thanks to a wind connectivity matrix built from Hysplit atmospheric simulations. This application generates maps of both forward and backward dispersal probabilities, which assist users in identifying potential high-risk destinations from a known source or, conversely, a potential high-risk source area from where a new outbreak could have been originated. This tool, leveraging customizable and easy-to-use maps, is designed to advise stakeholders of the risk of *Culicoides*-borne disease introduction, thereby supporting them in their decisions regarding disease surveillance and control.

Survival at +4°C, +20°C and +37°C of Italian genotype I and II African Swine Fever strains

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ASF is a viral disease affecting domestic and wild pigs with high lethality representing a major threat to the swine industry worldwide. The environmental contamination during an outbreak is one of the critical issues in limiting the disease spread. ASF persistence and spread is a function of the virus survival to physical and chemical factors. Data on ASFV resistance in swine tissues and inanimate materials have recently increased, however baseline data on residual virus infectivity at different temperatures are scarce. This study generated data on survival of 2 strains detected in Italy, at 3 temperatures (+4°C, +20°C, +37°C) for 15 days: a genotype I virus isolated in Sardinia in 2008, a genotype II strain isolated in Genova in 2022, and the BA71/V genotype I as a reference strain. Virus persistence was monitored also on the spleen of an experimentally infected pig with the Armenia/2007 strain, exposed for 180 days at +4°C and +20°C. At 0, 7, 15 days, 3 independent virus aliquots were exposed and tested for residual infectivity by virus titration on cell cultures. Difference in virus titres according to the strain, temperature and time point were statistically analysed. All tested strains were still infectious at day 15 at +4°C and +20°C. A difference was observed at +37°C with genotype I being inactivated at day 12. Comparing survival curves of each ASFV, a statistically significant difference was observed among temperatures and time points, indicating higher survival rates at +4°C and +20°C than at +37°C. The decrease in virus titres at +37°C was meaningfully statistically dependent on the strain and the time: differences were detected at each time point for genotype I, between days 7 and 15 for genotype II, and between days 0 and 7 for BA71/V. The, Armenia/07 infected spleen was completely inactivated following 60 days at +20°C, while ASFV remained viable at +4°C for 6 months. This study showed a different survival behaviour between genotype I and II at 37°C and provided benchmark data on ASFV resistance at temperatures, necessary to properly assess survival rates associated to biological matrices and inanimate materials.

Monitoring of viral pathogens in pooled fecal samples from captive parrots in northwestern Croatia

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In the context of avian health surveillance, early detection of subclinical infections is crucial for the management of captive bird populations. This study aimed to screen for the presence of viral pathogens in pooled fecal samples collected from parrots housed by nine breeders (designated 1–9) in Northwestern Croatia. A total of 71 fecal samples were collected and pooled separately for each of the nine parrot breeders, representing a combined population of 399 parrots across 43 different species. Samples were collected separately by aviary and species (in range: 7–26 birds per breeder). All birds appeared clinically healthy at the time of sampling. Fresh fecal material was collected from clean, non-contaminated surfaces using sterile containers, avoiding contact with urine, feathers, or bedding. Pooled fecal samples per breeder were homogenized using physiological solution, and 0,5 ml was mixed with PathoSense (Ghent, Belgium) kit for metagenomic analysis. Clearly labeled samples were stored at 4–8 °C prior to shipment. Samples were packed in leak-proof, insulated containers with ice packs and sent to the closest collaborative PathoSense lab, PoulPharm (Budapest, Hungary). A completed submission form including sample identifiers and relevant metadata was provided. The laboratory was informed in advance, and samples were shipped early in the week to prevent delays.

Results revealed the presence of a complex bacterial community in all pooled samples. Additionally, three viral pathogens were identified: avian orthoreovirus was detected at a moderate level in samples from breeder 9, avian bornavirus at a low level in breeder 8, and avian hepatitis E virus in breeder 7. Avian orthoreovirus is associated with enteric disease, arthritis, and tenosynovitis in poultry; avian bornavirus has been linked to proventricular dilatation disease; and avian hepatitis E virus can cause hepatitis-splenomegaly syndrome, typically detected in liver or fecal samples.

These findings highlight the potential for subclinical viral circulation in captive parrot populations and underline the importance of routine screening as a preventive measure. The use of pooled sampling from clinically healthy individuals offers a cost-effective approach for broad surveillance and early detection of pathogens with potential zoonotic or avian health implications.

Keywords: parrots, fecal samples, viral pathogens, avian orthoreovirus, avian bornavirus, hepatitis E virus, surveillance

Mapping and managing Lagovirus impact: A data-driven strategy for wild rabbit and hare recovery in Sardinia

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In Sardinia, the wild rabbit (*Oryctolagus cuniculus huxleyi*) and the Sardinian hare (*Lepus capensis mediterraneus*) are endemic subspecies. Despite various attempts to monitor Rabbit Haemorrhagic Disease Virus (RHDV), data on its actual presence remains limited, based primarily on a small number of samples submitted to the Experimental Zooprophylactic Institute of Sardinia. In recent years, the rapid spread of RHDV2 appears to be a major cause of disease and mortality in both wild and domestic rabbits, and potentially also in hares. A multidisciplinary approach is essential to assess the actual presence of lagovirus-related diseases in areas inhabited by the target species. Investigations were focused on carcass collection for specific RHD diagnostics (ELISA and/or RT-PCR) and viral detection in fresh faecal samples (via RT-PCR). The activities have been developed throughout the regional territory, a protected zone where hunting is prohibited and where restocking can therefore be carried out. The data obtained from passive surveillance activities included coordinates useful for geolocation. Out of a total of 185 samples collected, the majority (145) were faecal samples. Twelve organs resulted to be positive for RHDV2. Following the usual checks regarding the completeness and consistency of the data, a specific dashboard was created using business intelligence software (Power BI). The created data warehouse centralises and organises data in an optimised format for analysis and support of business and laboratory decisions. The data undergoes extraction, transformation, and loading processes to ensure consistency and quality. Within the platform, data is structured to enable fast and complex analyses, with tables designed specifically for analytical purposes. Aggregated statistics provide both an overview and the ability to focus on historical trends and timing. This procedure will allow, through continuous updating, targeted interventions in identified areas of interest in the future. A thorough investigation into the actual presence of lagovirus-related diseases in the area would enable the development of a strategy for the recovery of wild rabbit populations in Sardinia. This strategy would involve establishing high-density rabbit groups in suitable habitats to create new dispersion areas and facilitate the colonisation of individuals from these groups.

Business intelligence software as a tool to support antimicrobial resistance monitoring

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Antimicrobial resistance (AMR) is a growing global threat affecting both humans and animals. In veterinary medicine, the prudent use of antibiotics is essential to limit the spread of resistant strains, in line with the One Health approach, which recognizes the interconnection between human, animal, and environmental health. The surveillance of AMR development is a key point in the recommended strategies to fight it with integrated actions (EU, WHO, FAO, WOAH) through the collection and the analysis of human and veterinary medicine data. In addition, the European One Health Action Plan against Antimicrobial Resistance recommends that Member States to monitor AMR trends in zoonotic and non-zoonotic microorganisms that may pose a threat to public health. In Italy the National Plan for Antimicrobial Resistance Control outlines surveillance protocols, promotes responsible antibiotic use, and supports research and training to safeguard public health. Once the usual data completeness and consistency checks were completed, a tailored dashboard was built using Power BI. The Data Warehouse is targeted to implementing and strengthening AMR surveillance in veterinary medicine as a stable and representative activity, with the aim of monitoring the epidemiological trend of resistant bacteria, estimating the circulation and impact of these pathogens on animal and human health in order to study, plan and implement control measures. It is powered with the results of standardized antimicrobial sensitivity tests performed on bacteria of veterinary origin isolated at the laboratories of the Experimental Zooprophylactic Institute of Sardinia, as part of passive surveillance. It is a tool structured to facilitate complex and fast analysis, with tables designed specifically for analytical purposes. This information can be used for epidemiological studies of risk assessment, for surveillance plans and as an instrument for a rational use of antibiotics in empirical therapies (i.e. administered in an emergency) by the clinician. This activity could represent an initial step towards the integration between surveillance in human and veterinary medicine, which can also be consolidated worldwide by promoting integration with the analysis of existing data to produce a shared reporting with a standardize One-Health approach.

Investigation of pseudorabies virus (PRV) infection in wildlife in Slovenia with the first detection of PRV in the golden jackal

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Pseudorabies virus (PRV) is an alphaherpesvirus that infects a variety of animal species, including the pig as a natural host and sole latent carrier (Sun Y et al., 2016). Mammals other than Suidae are considered as dead-end hosts in which PRV infection leads to severe acute and fatal disease characterised by insatiable pruritus. In this study, a golden jackal with neurological clinical signs was examined for infection with PRV. Cytopathic effect in cell culture and positive qPCR results confirmed the presence of PRV in the golden jackal. In addition, we investigated PRV infections in wild animals found dead. A total of 375 samples from different animal species (stone marten, badger, nutria, wolf, wild boar, jackal, otter, skunk, brown bear and red fox) were analysed for the presence of PRV in lung tissue samples using qPCR. The only confirmed PRV case was in a golden jackal with neurological clinical signs, while all other samples from wild animals found dead were negative. The complete genome of the golden jackal PRV strain was sequenced. Phylogenetic analysis of the gC gene revealed a close relationship with PRV strains isolated from hunting dogs in Slovenia. This study provides the first evidence of PRV infection in the golden jackal, with phylogenetic analysis of the virus strain suggesting that similar PRV strains are circulating in the Slovenian wild boar population.

Keywords: Pseudorabies virus; golden jackal; phylogenetic analysis; complete genome; Slovenia

Emergence of H1C2.4NX Clade in Swine Influenza A Virus: Surveillance in Northern Italy (2020–2025)

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Influenza A viruses (IAVs) are among the most widespread agents of respiratory infections in humans and swine. The subtypes of swine IAVs (IAV-S) currently circulating in Europe are H1N1, H3N2, H1N2, and H1N-1pdm09. Considering the genetic diversity within the hemagglutinin (H) gene, particularly in the H1, a deep classification into the genetic clades H1A, H1B, and H1C is used.

This study monitored IAV viral circulation in swine farms in the Emilia-Romagna and Lombardy regions (Northern Italy) between January 2020 and May 2025.

Samples collected during Porcine Respiratory Disease (PRD) outbreaks were screened for IAV using real-time RT-PCR. Positive samples were molecularly subtyped via RT-PCR, and viral isolation on suitable cell lines was attempted. Then, a selection of viral isolates was fully sequenced.

In total, 1081/9424 samples tested positives (11.47%) and 465/543 viral isolates underwent full genome sequencing. Among those, 441 isolates belonged to H1 clades (H1av–1C, H1hu–1B, and H1pdm–1A) and were classified into nine subclades, with H1C2.4 being the most prevalent (129 isolates). Moreover, 51 and 44 strains were isolated in 2023 and 2024, respectively.

The genotypes H1C2.4NX were firstly identified in Northern Italy in October 2020 and rapidly spread among pig farms (5% in 2020 – 56% in 2023 – 42% in 2024). Moreover, the H1C2.4 clade is not effectively detected by hyperimmune sera against genotypes historically identified in Italy. Thus, the use of outdated diagnostic strains in HIA tests may limit the accurate identification of these viruses in serological studies.

Due to their susceptibility to both avian and human influenza viruses, pigs serve as mixing vessels for genetic reassortment, a process that can result in the emergence of novel influenza strains with pandemic potential. This, along with the difficulty of keeping diagnostic tools continuously updated, underscores the importance of implementing surveillance programs to monitor IAV circulation in the swine reservoir, given the threat it poses to both human and animal health.

ASFree M.e.a.t. (meet export agreement on trading): safeguarding the Italian cured pig products from African Swine Fever

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Processed meat exports account for approximately 56% of Italy's total pork meat exports. Since 2019, the Italian swine sector has been significantly affected by African Swine Fever Virus (ASFV) spread in Europe and North America. Major repercussions on international trading of Italian cured pig products were reported since the first ASF notification in peninsular Italy in 2022. Available data on presence and persistence of ASFV in pork products dated back between 1980s–1990s using the no longer circulating genotype I ASFV and less sensitive detection methods than those available today. This highlighted the need for generation of updated data and additional strategies beyond curing to ensure importing countries of the absence of ASFV in Italian cured products. The national funded ASFree M.e.a.t. project aims to investigate the presence of ASFV in the most exported cured meat products (i.e. salami and ham) and to assess the efficacy of High Pressure Processing (HPP) in achieving complete virus inactivation. The ASFree M.e.a.t. consortium will produce cured pork products through both artificial contamination and in vivo experimental infection of pigs. Six types of Italian salami, representative of the main exported products, were selected for the project. Three batches of Milano, Spianata and Cacciatore salami were experimentally contaminated, according to the ISO and FSIS guidelines, with 1% w/v with a BA71/V virus suspension and cured for two months in BSL3 facilities. At 1/3, 2/3 and the end of aging 3 salami were sampled for ASFV detection by qPCR and virus isolation weight loss, water activity, pH and mesophilic lactic acid bacteria. At the end of curing all contaminated ASFV salami were treated by HPP. Virus isolation assays were conducted on all batches of Milano and Spianata salami. All artificially contaminated salami resulted PCR positive but virus isolation negative at the end of curing and post HPP. This preliminary data indicated that, at experimental conditions, ASFV is completely inactivated in tested salami. Such data will be further validated by feeding naïve pigs with contaminated salami and by in vivo infections to produce pig products from experimentally infected pigs.

Prevalence and Genetic Characterization of Porcine Rotavirus B in Hungarian Pig Farms

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Rotaviruses (RVs) are small RNA viruses belonging to the *Sedoreoviridae* family and are among the most significant viral pathogens causing acute gastroenteritis in both animals and humans. The *Rotavirus* genus comprises nine species (A–I), distinguished by the antigenic properties of VP6. The RV genome consists of 11 segments of double-stranded RNA enclosed in a triple layered particle (TLP), encoding six structural (VP) and five or six non-structural (NSP) proteins. RVs are considered endemic in swine populations worldwide. RVB (*Rotavirus betagastroenteritidis*) was first detected in pigs with diarrhea and later in cattle, lambs, rats and horses. RVB has been detected in Brazil, the US and Japan in pigs with enteric signs, and in a few countries in Europe, usually in older pigs. To assess RVB prevalence in Hungary, 228 oral fluid samples were collected from 27 asymptomatic pig farms and tested by RT-qPCR. Viral RNA was detected on approximately 35% of the farms, indicating widespread subclinical circulation. For genetic characterization, 97 fecal swabs were collected from 16 herds experiencing diarrhea outbreaks. Samples with high viral load were genotyped using nanopore next-generation sequencing method. VP7 genotyping identified G20 and G12 types, while VP4 analysis revealed P[4] genotype. In one case a full RVB genome was successfully obtained, displaying the following constellation: G20-P[4]-I11-R4-C4-M4-A7-N5-T4-E4-H4. RVB was frequently detected in co-infections with other viral and bacterial enteric pathogens, suggesting a multifactorial etiology in diarrheic piglets. These findings enhance our understanding of RVB epidemiology and evolution in Hungarian pig populations.

Detection of novel coronaviruses in European wild mustelids

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Members of the family *Mustelidae* (mustelids) are recognised as natural hosts for a variety of species-specific coronaviruses (CoVs). In addition, spillback of SARS-CoV-2 has been observed in both wild and domestic mustelids. A novel genus of coronaviruses, provisionally named *Epsiloncoronavirus* (ε-CoV), has recently been identified in European badgers (*Meles meles*) and pine martens (*Martes martes*). However, genomic data are currently limited to three partial sequences, with no single sequence exceeding 56% genome coverage. As ε-CoV could represent a fifth recognised CoV genus, further investigation into its ecology, evolution and host tropism is crucial. The aims of this study were (i) to determine the frequency of ε-CoV in badgers and martens from northern Italy, (ii) to investigate its tissue tropism, and (iii) to assess viral diversity. Lung samples were collected from 259 mustelids (badgers, pine martens and stone martens), exploiting passive rabies surveillance samples (January–August 2024). RT-PCR revealed 22 positive individuals (8.49% overall): 4/148 badgers (2.7%) and 18/106 stone martens (17%). Additional tissues (lung, spleen, intestine, central nervous system, trachea, and oronasal and rectal swabs) were collected from badgers and martens during a second collection period (February–May 2025) to assess tissue distribution. Of the 65 badgers tested, seven (10.8%) were positive in the lungs, with some also showing positivity in the spleen or tracheal swabs. Of the 30 martens tested, eleven (36.6%) were positive for ε-CoV, which was also detected in the spleen, tracheal swabs, and oronasal swabs of some individuals. Genetic analysis of partial RdRp sequences revealed two host-specific subgroups, *MartesCoV* and *MelesCoV*, with intra-group nucleotide identities of 88.1–100% and 89.1–100%, respectively, and inter-group divergence of 45.9–64%. This supports the idea that they belong to separate species. The tree topology also indicated geographic clustering. Next-generation sequencing (NGS) of representative *MartesCoV* and *MelesCoV* was performed to provide further genetic insights, despite whole genomes not yet being available. The respiratory tropism, coupled with a sporadic systemic detection, warrants further investigation into the pathogenesis and transmission dynamics of the virus. NGS is essential to characterise this divergent lineage, highlighting the importance of targeted wildlife surveillance within a One Health framework.

Epizootic Coinfection of West-Nile Virus and Avian Poxvirus in a Red-footed falcon (Falco vespertinus) colony

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Highly endangered Red-footed falcon (*Falco vespertinus*) fledglings were found debilitated and dead at a nesting colony site in Eastern Hungary during the last week of July 2023. Ten birds showing symptoms of weakness, conjunctivitis and proliferative blepharitis were transferred to a rehabilitation facility and 3 carcasses were also recovered. Clinical and pathological examinations established the preliminary diagnosis of avian pox in all individuals, based on typical proliferative and squamous lesions on the birds' eyelids and legs. Total nucleic acids were extracted from pharyngeal and cloacal swabs taken into DNA/RNA Shield (Zymo Research) of clinical cases, and from carcass organ samples and lesions. Avipoxvirus and WNV infections were confirmed in all cases by PCR and qPCR. BG-Sentinel CO2 mosquito traps (Biogents USA) were deployed at the outbreak site and at an unaffected colony within the region to confirm local flavivirus circulation. Captured mosquitoes were pooled by species and tested for WNV and Usutu virus. At the outbreak site we established a WNV infection rate in *Culex pipiens* mosquitoes of 7.63 (2.03-20.79) and Usutu virus infection rate of 4.96 (0.90-16.34) Corrected MLE per 1000 mosquitoes while all control site mosquitoes tested negative. The avian poxvirus strain characterized by partial 4b Core Protein and DNA polymerase gene sequencing was assigned to Clade A4 (falconpox) of the Avipoxvirus genus while the amplicon based whole genome sequencing of WNV isolates corroborated the qPCR diagnosis of lineage 2 WNV infection. Our diagnostic results confirmed the diagnostic utility of clinical swab sampling for WNV diagnosis and characterization. Unfortunately, none of the ten clinical cases subjected to palliative treatment survived the infection. The outbreak caused a significant loss of fledglings in the breeding population of 80 Red-footed falcon pairs, underscoring the potential conservation threat of such concurrent epizootics. Although birds of prey are among the most susceptible species to WNV infection, they are rarely affected by significant mass mortality events in the Old World and usually only sporadic avian cases would signal active WNV circulation. The same is true for Avipoxvirus impact on European wild birds. This study was supported by grants SA-99/2021, TKP-2021-EGA-01, RRF-2.3.1-21-2022-00006.

Foot-and-Mouth disease is back in Europe in 2025: cases in Germany, Hungary and Slovakia

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Foot-and-Mouth disease (FMD) is one of the most contagious animal diseases, and is caused by the Foot-and-mouth disease virus (FMDV). The disease is essentially present in seven geographical pools, and some countries are endemic while others are free with or without vaccination. Even if the disease mortality can be considered as low, the morbidity can reach 100% according to species, and the consequences in FMD-free countries can be dramatic, with important economic losses.

The European Reference Laboratory (EURL) for FMD has a role into the preparedness of EU laboratories, essentially for quick and reliable detection and identification of any FMD case within the EU. The EURL also has a role of assistance to EU member states to confirm FMD suspicion, by performing viral isolation, molecular and serological analyses, so as sequencing methods.

Since 2011, the European Union was free from FMD, but on January 2025, the German National Reference Laboratory (NRL) identified FMDV from samples taken from a dead Asian buffalo showing clinical signs characteristic of FMD. This outbreak was related to lineage O/ME-SA/SA-2018, phylogenetically close to strains reported in Iran and Türkiye in 2023 and 2024 respectively.

At the beginning of March, the Hungarian NRL for FMD also confirmed FMD in a farm at the border with Slovakia. The virus identified during this outbreak was belonging to sublineage O/ME-SA/PanAsia-2^{ANT-10}, thus different from the virus that had emerged in Germany two months earlier. This virus was phylogenetically close to strains identified in Pakistan in 2017 and Türkiye in 2024. Slovakia subsequently reported outbreaks of FMD on the Hungarian border. By the end of April 2025, six outbreaks had been reported in Slovakia and five in Hungary, all of which led to massive depopulation measures.

Even if EU was free from FMD for 14 years, two different incursions of FMDV occurred in three months. The outbreaks have been contained, thanks to a global effort from the countries and different stakeholders, including the NRLs and EURL. These outbreaks remind us that FMD still represent a threat to FMD-free countries and that preparedness is essential.

Spatiotemporal dynamics of ASF in Serbia's wild boar population

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African swine fever (ASF) poses severe economic challenges globally due to its highly fatal nature in domestic pigs and wild boar. The goal of this study was to evaluate patterns of ASF spread and determine factors influencing its prevalence among wild boar in Serbia from 2020 through 2024. Surveillance data encompassing 480 confirmed ASF cases from Serbia's National Reference Laboratory were analysed. Infection confirmation was done through real-time PCR assays targeting the ASF virus p72 gene. Spatiotemporal clustering was assessed using the SaTScan™ space-time permutation model, while associations with environmental features including roads, water bodies, and elevation were investigated using Quantum Geographic Information System (QGIS). Chi-square tests were utilised to establish statistical significance ($p < 0.01$). The prevalence of ASF in wild boar gradually increased from 0.66% in 2020 up to 1.47% by 2023. Clear seasonal variation emerged, with case numbers significantly peaking during winter (68%) and spring (24%) months ($p < 0.00001$). The analysis identified five significant clusters, three located close to international borders (North Macedonia and Bulgaria), indicating possible cross-border virus transmission, while two were centrally situated, pointing to domestic spread. Furthermore, proximity analysis demonstrated a statistically significant correlation ($p < 0.01$) between ASF cases and closeness to major roads, with 65% of cases occurring within 5 km of such infrastructure. In contrast, water proximity had no significant effect on the disease distribution. Elevation data showed that most infections (40%) were identified in areas situated between 500 and 1000 metres altitude, consistent with preferred wild boar habitats characterised by dense woodland and limited human interference. This study underscores significant weaknesses in Serbia's existing passive surveillance strategy, likely causing underreporting of ASF in remote and forested areas. Highlighting cross-border risk factors emphasises the need for collaborative regional surveillance and integrated control initiatives. Effective strategies recommended include mandatory carcass reporting, educational programmes for hunters, and the implementation of GIS-based real-time surveillance, critical to mitigating ASF transmission and economic impacts in Serbia and neighbouring regions.

Epidemiological study of early age virome in different poultry categories in Croatia

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Global food security is highly dependable on poultry meat production, broiler chickens and turkey poults. Their complex gastrointestinal microbiome is crucial for overall health, affecting metabolism and immunity, while the impact of viral infections still remain insufficiently studied. This study investigates the virome of 5 and 25 days old broilers from two farms, and 14 and 28 days and 5 and 19 days old turkey poults on one farm. PathoSense (Ghent, Belgium) diagnostic tool and platform was used to identify and quantify viral loads in small intestine swabs, using nanopore sequencing methodology. In case of broiler chickens at 5 days of age, only Astrovirus was identified on both farms. In contrast, at 25 days, on Farm 1 three viruses were identified - Astrovirus, avian Orthoreovirus and infectious bronchitis virus, while on Farm 2 five viruses were revealed - Astrovirus, chicken Calicivirus, Gallivirus, Sicinivirus and infectious bronchitis virus. On turkey poult farm at 14 days of age, the results revealed mixed infection with medium quantity of Astrovirus, and low quantity of Parvovirus, Turkey Avisivirus and Gallivirus. At 28 days of age, results revealed mixed infection with lower quantity of Parvovirus and Turkey Avisivirus. At 5 day old turkey poult flock results showed high quantity of Avastrovirus, Rotavirus G, and medium quantity of Turkey Avisivirus, while at 19 days of age medium quantity of Avisivirus, Avastrovirus, Rotavirus A and G, and lower quantity of Gallivirus and Parvovirus was detected.

These findings across all poultry categories suggest an early and intensive infection via vertical transmission and later horizontal spread and viral diversification. The pathogenicity of some viruses, like Astroviruses, in birds with compromised immunity at an early age, could facilitate viral spread and diversification with clinical manifestation and elevated losses. This study highlights the necessity for enhanced biosecurity measures and targeted interventions in poultry management, from parent flocks, hatcheries to production farms. The used simple and rapid method could be a good bioindicator for improvement of management and biosecurity practices on different levels of production and mitigation of the economic impacts of viral diseases.

Keywords: chickens, turkey poults, virome, nanopore sequencing, PathoSense, Avisivirus, Avastrovirus, Rotavirus, Gallivirus, Parvovirus, Calicivirus, Sicinivirus, infectious bronchitis virus

The epidemiology of bluetongue in Europe and BTV confirmed cases in Slovenia

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Bluetongue is a non-contagious, insect-transmitted, viral disease. Bluetongue virus (BTV), an *Orbivirus*; can infect any ruminant species, domestic and wild and does not pose any health risk to humans. The infection is caused by more than 30 possible serotypes that have been described in the serologically very heterogeneous group of the *Orbivirus* genus. Bluetongue virus is spread by biting midges, small hematophagous insects of the genus *Culicoides*. Infection can occur in a wide range and describes a constellation of several disease entities. Most of the serotypes do not cause a clinically detectable form of the disease. The bluetongue, the clinical sign after which the disease was named, is sporadically observed only in severe clinical cases. Following recovery, animals may also exhibit a decreased yield of milk and meat, wool break in sheep and temporary infertility. BT infection was first described in Cape Province of South Africa in the late eighteenth century following the Merino sheep infection. In the first half of the twentieth century Cyprus, Israel and USA in1952 first reported the BTV infection. In early 70-ies the BTV was confirmed in Asia, India, Australia. The first outbreak in Europe owing to BTV-10 was reported from Spain and Portugal between 1956 and 1960 with the mortality range up to 180.000 animals. Greek islands were also affected by BTV, first reported in 1979. Following years of no massive BTV outbreaks, Italy has experienced multiple incursions of different serotypes of Bluetongue. In August 2006 the first ever case of the BTV (BTV-8) infection was confirmed in Western Europe, in the Netherlands and in 2014 major outbreak in Italy (BTV-4). Slovenia was no exception to the disease, first confirmed in 2015. For 2 positive samples, RT-qPCR as confirmatory method was used and successful determination of BTV-4 nucleotide sequences was done. Since vaccination is one of the most effective ways to prevent the disease, Slovenia entered the vaccination program. For several years different serotypes of BTV have shot up in many European countries. After a decade we confirmed the BTV-4 case in Slovenia again in January 2025.

Prevalence Estimation and Phylogenetic Analysis of Porcine Rotavirus C in Hungarian Pig Herds

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Rotaviruses (RVs) are small RNA viruses belonging to the *Sedoreoviridae* family and are among the most significant viral pathogens causing acute gastroenteritis in both animals and humans. The *Rotavirus* genus comprises nine species (A–I), classified based on the antigenic properties of the VP6 protein. The RV genome consists of 11 segments of double-stranded RNA, encoding six structural (VP) and five non-structural (NSP) proteins. RVs are considered endemic in swine populations worldwide. RVC (*Rotavirus tritogastroenteritidis*) was first identified in pigs with diarrhea in 1980 and has since been detected in several species, including cattle, ferrets, dogs, and humans. In pigs, it is mainly associated with diarrhea outbreaks in suckling and weaned piglets, but it also circulates among post-weaning animals with potential pathogenic relevance. To assess RVC prevalence in Hungary, 228 oral fluid samples were collected from 27 asymptomatic pig farms and tested by RT-qPCR. Viral RNA was detected on approximately 75% of the farms, indicating widespread subclinical circulation. For genetic characterization, 97 fecal swabs were collected from 16 herds experiencing diarrhea outbreaks. Samples with high viral load were genotyped using nanopore next-generation sequencing method. VP7 genotyping identified G1 and G5–7 types, while VP4 analysis revealed P[1], P[4], P[6] and an unclassified P[X] genotype. In one case a full RVC genome was successfully obtained, displaying the following Cowden-like constellation: G1-P[6]-I1-R1-C1-M1-A7-N9-T6-E1-H1. RVC was frequently detected in co-infections with other viral and bacterial enteric pathogens, suggesting a multifactorial etiology in diarrheic piglets. These findings enhance our understanding of RVC epidemiology and evolution in Hungarian pig populations.

African Swine Fever in Croatia – Lessons Learnt

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African Swine Fever (ASF) emerged in Croatia on June 26th 2023 and spread vastly, presenting a critical threat to pig farming and a significant challenge for the veterinary sector as well as the agriculture economy. In total, almost 1200 outbreaks in domestic pigs were recorded and more than 141.500 pigs died or were culled until the end of the year. In 2024, a decrease in the number of outbreaks was recorded (only 2), followed by a single outbreak in 2025. The majority of the outbreaks were concentrated in Vukovar-Srijem County (VSC), with limited incursions reported near the borders with Brod-Posavina and Osijek-Baranja Counties. The disease primarily affected backyard farms and highlighted some weaknesses regarding the early detection, biosecurity, control measures and public awareness.

After the confirmation of ASF in a wild boar on July 5th 2023, 12 additional positive cases were detected in 2023 (9 in VSC, 2 in Sisak-Moslavina, and 1 in each, Karlovac and Zadar), followed by 38 cases in VSC in 2024, with additional conformations until the end of May 2025, complicating eradication efforts. In total, from 2023 to mid-May 2025 the National Reference Laboratory for ASF in Croatia (NRL) tested 64.701 samples of domestic pigs and 25.587 samples of wild boars by PCR. Despite the enhancement of its capacity, the high throughput of samples in 2023 led to the opening of a Regional Official Laboratory for ASF in Vinkovci in mid-2024, that highly contributed to the testing procedures.

The virus derived from pigs and wild boars belonged to the same genotype II, subtype 19, indicating a spill-over from the pigs to the wild boar population and shown to be the cause of severe acute infections with almost 98% lethality's in both species.

According to the lessons learnt, early detection, sustained investment in surveillance, biosecurity training, and multisectoral collaboration are crucial to control ASF. Croatia's ASF outbreak is a valuable experience in early preparedness and management of transboundary animal diseases, demonstrating that effective containment is achievable through coordinated, science-based interventions and stakeholder involvement.

Whole-Genome Identification of Rotavirus C in Croatian Pig Populations

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Rotavirus C (RVC) is an enteric virus associated with gastroenteritis in various species, primarily affecting pigs. Its segmented double-stranded RNA genome enables frequent reassortment and recombination, enhancing its potential for interspecies and possible zoonotic transmission. Despite its global presence, genomic data on RVC in European swine populations remain limited. This study aimed to determine the first whole-genome constellations of RVC strains in domestic pigs in Croatia and contribute novel sequences to the global database.

Metagenomic datasets from 21 porcine rectal swab samples, generated by next-generation sequencing (NGS) during a previous Rotavirus A (RVA)-focused study, were reanalyzed for RVC detection. Samples were collected from suckling piglets and weanlings during winter and spring between 2018 and 2021, primarily in continental Croatia. Existing FASTQ data were remapped to RVC reference genomes using CLC Genomics Workbench (Qiagen, Germany), and consensus sequences were validated using BLASTn. In the absence of established RVC genotyping criteria, genotypes were assigned to genes covering >50% of the ORF based on RVA criteria, and meeting specific RVC genotype identity thresholds. Phylogenetic analysis was performed in MEGA 11 using the Maximum Likelihood method and visualized using iTOL software.

RVC was detected in seven of the 21 samples, collected during the winters of 2018 and 2019 from 3- to 6-week-old piglets in the Slavonia region. Only five samples contained sufficient dataset enabling complete or near-complete genome classification. Three strains resulted with the whole genome constellations: G3-P[6]-I1-M1-C1-R1-A1-N8-T5-E5-H1, G5-P[20]-I6-M3-C1-R1-A7-N8-T1-E1-H1, and G5-P[20]-I6-M3-C1-R1-A7-N8-T1-E1-H1, while two strains were partially characterized: G7-P[6]-I13-M1-C1-R1-A7-NX-T6-EX-HX and G1-P[4]-I4-M1-C1-R1-A8-N9-T1-EX-HX.

This study provides the first genomic evidence of RVC in Croatian pig populations. Notably, two P[20] and one I4 genotype sequences were identified, significantly enriching the genomic database where these genotypes had been scarcely represented. Although genotype variability was observed, all detected genotypes were porcine-specific, with no indications of interspecies transmission. Additionally, the co-detection of RVA from the previous study in all RVC-positive samples supports the notion of frequent mixed rotavirus infections in host populations.

Continued genomic surveillance remains essential for monitoring RVC diversity and understanding its implications for animal and public health, including vaccine development.

New orbivirus surveillance program confirms widespread presence of bluetongue virus serotype 3 in wild ruminants in Denmark

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The risk of infection for livestock in northern and western Europe with midge-borne orbiviruses e.g. bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) has increased in recent years. To address this challenge, a national program for active surveillance of BTV and EHDV in wild ruminants was initiated, as part of the veterinary contingency. Sampling was planned for the winter season 2024/2025 at organized deer hunts in different regions of Denmark with collection of samples from around 300 newly shot animals (blood and spleen). Samples from wild ruminants submitted for necropsy through the national passive wildlife disease surveillance program were also included.

By the time of the first sample collection (November 2024), Denmark had experienced its first BTV serotype 3 (BTV-3) outbreak and ongoing infection was circulating in cattle and sheep farms throughout the country.

In total, samples were collected from 339 different wild ruminants - the majority from red deer (172) and fallow deer (107), additionally from roe deer (29), mouflon (11), European bison (11) and sika deer (9). Serum was screened for antibodies against BTV and EHDV using a commercial ELISA kit from ID-Vet. Animals were seropositive for BTV in all of the sampled regions of the country except for the south-eastern part, which had also been sampled less intensively in the program. Seropositive samples were detected from red deer, fallow deer and European bison and corresponding EDTA-stabilized blood samples from the same animals were confirmed PCR positive for BTV. This is not surprising, as BTV is known to persist for months in the blood of infected animals.

Our findings indicate a seroprevalence of BTV in red deer of 28% and in fallow deer of 11%, in Denmark. For European bison, the samples originated from a single fenced group and the findings of 4 positive animals out of 11 tested, does not predict an overall seroprevalence in this introduced species in Denmark.

For EHDV, all 339 serum samples were negative, however, two samples had S/N%-values close to cut-off. EDTA samples from the same animals were tested in PCR and found negative for EHDV but positive for BTV.

Prevalence of Beak and Feather Disease Virus in Samples Received at the Department of Poultry Diseases with Clinic, FVMUZ, Croatia (2021-2025)

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Beak and feather disease virus (BFDV) is the causative agent of psittacine beak and feather disease (PBFD), which is one of the most important viral diseases in wild and captive birds. The disease has been reported all over the world, mainly because of the global trade of parrots and physicochemical properties of the virus that enable its persistence in the environment. The infected birds may contain a high number of genome variants as a result of high mutation rates, which presents a replication strategy that allows host-switching and easy transmission of the virus within a population of birds. The most common manifestation of the disease includes abnormal feather development and feather dystrophy, loss of plumage and beak deformities. Considering the increasing number of birds with PBFD symptoms in the last few years, the aim of this study was to determine the prevalence of BFDV-positive samples received at the Department of Poultry Disease with Clinic, FVMUZ, Croatia, in the period between March 2021 and June 2025. The analysis included 165 samples - feathers (n=91), individual fecal samples (n=38), pooled fecal samples (n=22), tissues (n=12) and cloacal swabs (n=2), originating from 128 birds, excluding the pooled samples. The tissue samples were frozen at -20°C until the analysis, while all other samples were immediately processed or stored at 4°C until the analysis. Total DNA was extracted using NucleoSpin DNA RapidLyse kit (Macherey-Nagel, Germany), after which PCR analysis was performed according to the protocol as described by Ypelaar et al. (1999). In total, 37/165 (22,42%) samples and 27/128 (21,09%) birds, respectively, were positive for BFDV. Forty-one out of 128 (32,03%) birds showed extensive feather and/or beak lesions, and 34,15% of the birds with clinical symptoms were positive for BFDV. As the symptoms can vary and some birds may be asymptomatic carriers, the prevalence of the disease might also be underdiagnosed and underreported. Although PBFD is not a reportable disease in Croatia, the prevalence results indicate there is a need for continuous screening; especially of the breeders and pet shops which have a high turnover of birds originating from various sources.

Keywords: circovirus, BFDV, PBFD, parrot, bird

Prevalence and molecular characterization of porcine cytomegalovirus in suckling piglets and growers of commercial herds farms in Spain

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Porcine cytomegalovirus (PCMV) is a roseolovirus causing inclusion body rhinitis, although it is also commonly found in apparently healthy animals. Like other herpesviruses, PCMV can establish latency in infected individuals. Despite its assumed wide distribution, the available epidemiological data are scarce and the presence and involvement of PCMV in clinical conditions of pigs remains almost unexplored in Spain. The aim of this study was to assess the prevalence of PCMV infections in suckling piglets and weaners and to genetically characterize the local strains circulating in commercial farms in some of the major pig-producing areas of swine of Spain.

Nasal swabs from nursery and farrowing pigs of 74 farms (2,264 samples in total) were tested for the presence of PCMV using RT-qPCR. From farms where samples with Ct<31 were available, one sample of each positive farm was selected and used to amplify the partial gB gene by PCR. PCR products were purified and sequences on both strands were obtained by SANGER sequencing. The sequences obtained (n=46) were aligned with MEGA 11 and a phylogenetic tree was constructed using the maximum likelihood method.

PCMV was detected in at least one animal in 71.6% of farms analyzed and the average prevalence was 22.6% of the tested individuals. No significant differences in prevalence were found regarding the age of the animals or their health status. Sequencing showed that PCMV isolates circulating in Spain belonged to two distinct sequence groups, A and B, as reported for other countries. Group A comprised 36 sequences and contained three different clades. Of those 36 isolates, 16 clustered with Korean and Chinese strains, eight with Japanese strains and twelve with a Spanish reference strain. Other 10 isolates of the present study were classified in PCMV group B together with some Chinese strains. All B isolates were characterized by a 3-nucleotide deletion in gB. To our knowledge this is the first report on the epidemiology of PCMV in Spanish farms.

Bluetongue Virus in Sardinia: Multiple Serotypes, One Strategy

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Bluetongue is a vector-borne viral disease of domestic and wild ruminants. Bluetongue virus (BTV) is trasmitted through the bites of certain species of *Culicoides*, does not affect humans and causes considerable damage to livestock populations. In Sardinia, the 2024 Bluetongue epidemic was characterized by intense circulation of the BTV-8 strain already present in 2023. This strain caused severe clinical signs with high mortality in sheep, along with increased circulation of the North African-origin BTV-3 strain. This strain was no longer confined to the historically affected southern area of the island but spread throughout the region, leading to a rise in mortality and the appearance of typical clinical signs of the disease. The presence of numerous co-infections among the three circulating serotypes, the earlier onset compared to recent years, and low immunological coverage led to a severe epidemic situation. This highlighted the need for intense surveillance and prevention efforts, particularly through an effective vaccination campaign planned for 2025. So far, the final scenario of the recent epidemic reports 4,066 outbreaks, totaling 207,626 cases and 71,754 deaths. The BTV-3 strain circulating in this epidemic appears genetically distinct from previous strains (2022–2023). It is a reassortant strain with a different genomic segment, likely resulting from a co-infection with the currently circulating BTV-4 strain. The complexity of the epidemiological situation necessitated a re-evaluation and update of the territorial risk analysis, considering the potential for re-emergence and resurgence. This involved focusing outputs through GIS technology to create a new risk map, which provides a list of farms and associated risk levels. This was used as a support tool for prioritizing vaccination efforts for all circulating serotypes, with the goal of launching an early vaccination campaign (March–June). This campaign, combined with other on-farm prevention measures (vector control, improved biosecurity, and animal welfare), aims to protect animals from the disease and its consequences while reducing viral circulation. This study aimed to assess the adequacy of the strategy and the challenging implementation of the relevant regulations in different territorial contexts, including costs related to the vaccine, potential compensations, and restrictions on the movement of susceptible animals.

Outbreak and control of African swine fever in the Republic of Srpska (BiH) in 2023

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African swine fever is a particularly dangerous infectious disease of domestic and wild pigs. The disease is not zoonotic, but it causes great economic damage. The causative agent of the disease is a virus from the Asfarviridae family. The disease was previously limited to Africa, but in recent decades it has spread to Europe, Asia and other parts of the world. International trade and transport contribute to the global spread of ASF. The wide distribution of domestic and wild pigs complicates control measures, as wild reservoirs can serve as a source of infection for domestic pigs. This paper analyses key aspects of the epidemiology of African swine fever, including the first detection of infection, the number of cases per municipality, the mode of transmission, the geographical spread by days and months, the number and size of farms affected, risk factors, establishment of restrictive zones, field sanitation and crisis management.

The first case was detected in the Municipality of Bijeljina, which also had the highest total number of cases during the year. Epidemiological investigation could not precisely identify the source of infection, but it is more likely that the virus was introduced from a neighboring country, by wild boars or indirectly through contaminated surfaces and food, fomites or *Ornithodoros* ticks. Risk factors include the movement and transport of live pigs and pork products, inadequate biosecurity measures on farms and the presence of vectors. In the Republic of Srpska, in the period from 22.6.2023 to 31.12.2023, the disease was detected in 24 municipalities on 1,166 farms, where 48,325 pigs were harmlessly destroyed. The highest number of outbreaks in a single day was recorded on the 41st day after the disease was first identified. On average, 41 pigs were kept on each affected farm. On all farms where ASF was confirmed, all affected pigs were euthanized and safely disposed of by deep burial on the ground, applying all necessary measures to prevent further spreading of the disease. The total direct damage is estimated at around 13 million euros. Despite the successful control of the disease, certain weaknesses have been identified that contributed to the spread of the disease, such as the inability to fence off restricted areas and prevent the movement of animals, the lack of sufficient sanitary abattoirs, insufficient involvement of local authorities in ensuring sufficient logistical equipment and materials and determining places for burying animals.

Keywords: African swine fever, epidemiology, pigs, Republic of Srpska (B&H)

Foxes Carry FPV, Dogs CPV-2c: Divergent Parvovirus Lineages in Croatia

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A partial parvovirus VP2 gene fragment was amplified from the brain of apparently healthy red foxes (*Vulpes vulpes*) collected in north-east Croatia. The nucleotide sequences were compared with 456 publicly available carnivore protoparvovirus-1 sequences (canine parvovirus, CPV; feline parvovirus, FPV; and mink enteritis virus, MEV), including all Croatian dog isolates deposited in GenBank.

The relationships between haplotypes were analysed using the median joining network algorithm (DnaSP v6 PopART), while the evolutionary history was reconstructed in BEAST v1.10 using the Yang-96 codon model, a lognormal relaxed molecular clock and a constant-size coalescent prior. Markov chain Monte Carlo analyses were performed until all parameters reached an effective sample size (ESS) > 200, and the maximum clade confidence (MCC) tree was summarised using TreeAnnotator.

Both approaches clearly assigned the Croatian fox sequence to the FPV clade and clearly separated it from the CPV-2c lineage currently prevalent in Croatian domestic dogs. The long internal branch leading to the fox haplotype suggests a longer independent evolution in wild animals, and no recent epidemiological link between the two host species could be established.

These results suggest that (i) red foxes in Croatia are more likely to be infected with FPV than with CPV variants adapted from dogs and (ii) virus exchange between wild foxes and the local dog population is currently unlikely. Continuous molecular surveillance of wild and domestic carnivores is therefore essential to identify potential future host switches and refine vaccination strategies at the wild–domestic interface.

Lentivirus antibody levels in serum and milk from goats before and after kidding

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Small ruminant lentiviruses (SRLV) are a group of retroviruses responsible for chronic diseases in sheep and goats, known as Maedi/Visna (MV) and Caprine Arthritis and Encephalitis (CAE), respectively. CAE primarily manifests as polyarthritis, encephalitis, and chronic wasting. Infected herds often exhibit a high within-herd prevalence, despite only a small number of animals showing clinical signs. Because CAE is frequently sub-clinical, reliable diagnostic tests are essential for effective disease control.

There is ongoing discussion about whether lactation status influences antibody levels in both serum and milk, which may impact the optimal timing and method of sample collection for establishing an accurate diagnosis.

To investigate how antibody levels change during lactation, both serum and milk samples were repeatedly collected from all 63 goats in a CAE-positive herd during the lactation period. Sampling was performed before kidding (0-61 days) and after kidding (1-17 and 2-53 days, respectively). All samples were analysed using the ID Screen® MVV/CAEV Indirect ELISA (Innovative Diagnostics, Grabels, France) and the ELITEST® MVV/CAEV (Hyphen BioMed, Neuville-sur-Oise, France). The results are expressed as S/P% (Sample-to-Positive percentage) values.

The agreement between serum and milk test results was 95.6% at the first sampling and 87.0% at the second. The number of animals identified as CAE-positive based on serum remained unchanged between the two sampling occasions, although the mean S/P% value in serum increased from 116 ± 69 to 146 ± 93. In milk, however, the mean S/P% value declined from 162 ± 85 to 90 ± 79, leading to a shift from positive to negative results in 0.05% (1/20) of cases. In animals sampled for serum antibodies both before and after kidding, the mean S/P% was slightly lower prior to kidding (103 ± 71) compared to post-kidding (124 ± 80).

The results indicate that S/P% in serum increase after kidding, suggesting that early postpartum may be a suitable time point for sampling. In contrast, S/P% in milk were relatively high close to kidding but declined rapidly postpartum. This pattern should be taken into account when determining the timing of milk sampling. Further knowledge about how S/P% fluctuate throughout lactation is needed to improve disease detection and control.

Comparison Between Open Reading Frame 5 and 7 of PRRSV with a Phylodynamic Approach

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The Porcine Reproductive and Respiratory Syndrome (PRRS) is caused by the *Betaarteivirus suid* virus (formerly PRRSV). It is an ssRNA+ virus characterized by a high mutation rate and potential for recombination events. Two genetically distinct genotypes exist: *Betaarterivirus suid* 1 and *Betaaerterivirus suid* 2. Both genotypes have a 15 kb long genome, organized in Open Reading Frames (ORFs). Among the 10 identified, ORF5 and ORF7 have always been used for taxonomic and phylogenetic purposes. Here, we compare PRRSV-1 strains sequenced for both ORF5 and ORF7 to assess the degree of consistency between the two genomic portions from a phylodynamic perspective.

Overall, 578 PRRSV-1 positive samples from a 4-year period were sequenced for both ORF5 and ORF7, using the Sanger method. Sequences were then aligned with the MAFFT algorithm, implemented in Lasergene (DNASTAR), and a Maximum Likelihood tree was calculated using *iqtree* with automatic selection of the best substitution model. Tree tips were then associated with the sampling date, for a preliminary inspection of the data. Phylodynamic analysis was performed using *BEAST 1.10* and related tools. The best molecular clock and tree models were selected through path sampling and stepping stone sampling implemented in *BEAST*.

Several differences emerged from the analysis, starting from the mutation rate, which was faster for ORF5 ($8{,}92\times10^{-2}$) and slower for ORF7 (1×10^{-3}). While ORF5 required a GTR+I substitution model, ORF7 required a GTR+G+I model. The Bayesian Skygrid population model was the best choice for both datasets, but the relaxed molecular clock was ideal for the ORF5 dataset, while the more complex random local clock was needed for the ORF7 dataset. Given these settings, the MCMC for ORF7 took much more run time when compared to the ORF5 one. Finally, the dynamics of the viral population inferred from the BEAST results showed very different trends.

This study quantifies the differences coming from the analysis of different genome regions of the same PRRSV strains. Changing the analyzed ORF of the virus can lead to substantial differences in delineating viral spread, therefore, a whole genome approach is preferable to properly asses the epidemiology of PRRSV.

National surveillance plan for infectious bovine rhinotracheitis (IBR) in autochthonous Italian cattle breeds: Results of five years of activity

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Infectious bovine rhinotracheitis (IBR) is a highly contagious disease caused by *Bovine alphaherpesvirus* 1 (BoAHV-1) that affects wild and domestic bovines worldwide. The disease is endemic in almost all Italian cattle herds causing significant economic losses due to decreasing in weight and milk production, reproductive disorders and trade restrictions on animals and germinal material imposed by disease-free countries. For these reasons, in 2015-2016 were approved two voluntary surveillance plans for IBR in 6 beef cattle breeds recorded in the two National Herd Books. The breeds included were Marchigiana, Romagnola, Podolica, Chianina and Maremmana races, and as of 2017, the Piemontese race has been added. The data on IBR prevalence from 2018 to 2022 were obtained from cattle over 12 months that were involved in surveillance plans and tested using an Enzyme-linked immunosorbent assay (ELISA). This test can discriminate cattle immunized with a gE-deleted marker vaccine (gE-) and from conventionally vaccinated animals or infected cattle (gE+). A farm was considered seropositive for IBR if a single animal tested positive. The overall annual herd seroprevalence rate and the seroprevalence rates for beef cattle were determined with 95% confidence intervals (CI). On average, each year, 2,891 farms and approximately 132,000 cattle were tested. From 2018 to 2022, the prevalence showed a decreasing trend, dropping from 42% (CI: 40.3%-43.7%) to 21.6% (CI: 20.1%-23.2%) for farms and from 11.2% (CI: 11%-11.3%) to 6.3% (CI: 6.2%-6.4%) for cattle. This study represents an initial phase of a National IBR Control plan, which will include restrictive measures such as “test and removal” use of marker vaccines.

A comprehensive study on early detection of WNV circulation in the environment in Vojvodina Province, Serbia

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Identifying the areas where WNV circulates before the occurrence of human infection cases is of a crucial importance for disease risk reduction and epidemic prevention. Screening field-collected mosquitoes can reveal WNV circulation two to five weeks before the first human cases occur. Timely surveillance for the early detection of WNV circulation is the primary measure and a prerequisite for the successful implementation of prevention and control strategies. To define the exact timing and the conditions that play an essential role in appearance and multiplication of WNV in vector mosquitoes and the onset of natural WNV circulation during a season, a comprehensive study on WNV presence in vector mosquitoes was conducted at 10 localities in the Vojvodina Province of Serbia during 2024. From April 20th to September 26th, 2024, eggs, larvae, and adults of *Culex pipiens*, the most competent vector of WNV in the region, were collected on a weekly basis. In addition, to each CO_2 baited mosquito trap for adult collection, a commercially available FTA card soaked with liquid honey was fixed to the outer side of the the trap in order to collect the saliva of captured adults. In the laboratory, *Cx. pipiens* egg rafts, larvae, and adult mosquitos were individually processed (mechanically disrupted) and prepared for molecular detection of WNV. More than 7.000 individual samples were tested. Testing is still ongoing, but preliminary data confirmed that the first WNV detection was in an adult mosquito in mid-May 2024, and just a week later on an FTA card. WNV was detected only in adult mosquitoes and on FTA cards; none of the tested egg or larval samples tested positive for WNV. The highest number of WNV-positive samples was recorded in August and September 2024. Valuable and practical conclusions will be drawn once all sample testing will be completed, and after the inclusion of other systematically collected environmental data during the sampling period (meteorological data, habitat type of sampling sites, etc.). The goal is to develop a prediction model expected to provide the necessary data for designing an effective monitoring system for early detection of WNV circulation in some area.

Key words: WNV, early detection, surveillance; *Culex pipiens*, mosquitoes

Acknowledgments: The study was supported by the European Commission (project 101046133 - ISIDORe - HORIZON-INFRA-2021-EMERGENCY-02: JRA ID: ISID_JRA_zj0y), and by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract numbers 451-03-136/2025-03/20031 and 451-03-137/2025-03/200117).

USUTU virus in Serbia: data on virus circulation in the Vojvodina Province in the last 15 years

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Usutu virus (USUV) is an arbovirus of the Flaviviridae family that was first isolated in South Africa in 1959. The virus is maintained in the environment through a typical enzootic cycle involving mosquitoes and birds. In Europe, USUV mainly leads to substantial avian mortalities. In humans, USUV infection is most often asymptomatic or causes mild clinical signs. Nonetheless, a few cases of encephalitis or meningoencephalitis have been reported.

In Vojvodina Province of Serbia, USUV was for the first time detected - serologically confirmed in 0.3% (1/349) horses tested during the period 2009– 2010, and 1.3% (4/318) wild boars tested during the period 2011–2012. Additionally, USUV was confirmed in 0.4% *Culex pipiens* mosquito pools in 2014, 0.93% Cx. pipiens pools in 2015, 2.75% (3/109) *Cx. pipiens* pools in 2017, and in 9,73% (18/185) *Cx. pipiens* pools in 2018. One isolate from 2014 was genotyped as USUV Europe 1 linage and two isolates from 2017 as USUV Europe 2 lineage. Moreover, USUV antibodies were detected in 5% human serum samples in South Bačka District (Vojvodina) tested during 2015.

In 2024, in total 298 pooled *Cx. Pipiens* mosquito samples were collected from 65 localities and tested by real-time RT-qPCR. The presence of USUV was detected in a total of 8 (2.68%) samples, namely in 0% (0/50) of samples collected in May, in 0% (0/62) of samples from June, in 4.07% (5/123) of samples from July and in 4.76% (3/63) of samples from August 2024, at the area of 5 out of 7 counties (Central Banat (3/43, 6.98%), West Bačka (2/46, 4.35%), North Banat (1/24, 4.17%), South Bačka (1/48, 2.08%) and North Bačka districts (1/49, 2.04%)).

Given the established constant circulation of USUV in Vojvodina Province of Serbia, as well as its pathogenicity, especially for some species of wild birds, but also as a possible cause of fever and neuroinvasive disease in humans, it is necessary to raise attention and to officially monitor the circulation of this virus, especially in relation to West Nile virus in areas where both viruses circulate together, as it is the case in Serbia.

Key words: USUV, Vojvodina Province, Serbia

Acknowledgments: The study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract numbers 451-03-136/2025-03/20031 and 451-03-137/2025-03/200117).

Comprehensive Analysis of the Honeybee Family Virome in Slovenia: Viral Diversity and Distribution Dynamics

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The honeybee virome is crucial for understanding viral threats to colony health, pollination performance and biodiversity. It provides information on pathogen surveillance, viral ecology and management strategies, especially under the pressure of the *varroa* mite, climate change and global pollinator decline. In this study, we determined the virome of 19 honeybee families, focusing on the relative abundance of viral families and the distribution of virus species, using robust bioinformatics approaches. RNA from honeybee samples was sequenced using Illumina RIP-seq. Virome analysis of honeybee samples from Slovenia revealed a diverse spectrum of virus families with varying degrees of relative abundance, reflecting the complexity and dynamic nature of the virome in this region. The Slovenian honeybee families (*Apis mellifera carnica*) virome reveals high diversity dominated by *Iflaviridae* (up to 80.93%) and *Dicistroviridae* (36.35%), alongside vast unclassified sequences (≤97.52%). *Apis mellifera filamentous virus* (AmFV) prevails unevenly across colonies, highlighting ecological and host-driven variability. Bacteriophages (e.g., *Myoviridae*, <3%) modestly influence gut microbiota interactions. *Iflaviridae* and *Dicistroviridae* include pathogens like *deformed wing virus* (DWV) and *acute bee paralysis virus* (ABPV), exacerbated by *varroa destructor* mites. Their co-occurrence underscores multi-viral pressures, with black queen cell virus (BQCV) persisting opportunistically. *Partitiviridae* (15.35%), typically plant/fungal-associated, may cross kingdoms via pollen, raising questions about bee health impacts. AmFV's patchy distribution mirrors Central European trends, suggesting immunity or transmission variations. *Lake Sinai virus* (LSV) and *chronic bee paralysis virus* (CBPV) show lower, stress-responsive abundances. *Rhabdoviruses* and *sacbrood virus* (SBV) appear sporadically, likely suppressed by management or viral competition. Unclassified sequences dominate, hinting at novel viruses or atypical bacteriophages. Their roles in colony resilience demand urgent study. The Slovenian honeybee virome reflects complex host-environment-pathogen interactions. Continued metagenomic surveillance is essential to identify unclassified viruses and assess their potential impacts on colony health and pollinator resilience.

Identification of Twenty-Two New Complete Genome Sequences of Honeybee Viruses Detected in Apis mellifera carnica Worker Bees from Slovenia

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Honeybee viruses were identified in naturally infected honeybee colonies (*Apis mellifera carnica*). From nine selected samples of clinically affected and ten samples of healthy honeybee colonies, different strains of honeybee viruses were first detected using quantitative real-time RT-PCR methods. Twenty-two nucleotide sequences of the complete genomes of honeybee viruses were identified using the Illumina Next-Generation Sequencing (NGS) method: *acute bee paralysis virus* (ABPV) (n = 4), *black queen cell virus* (BQCV) (n = 3), *chronic bee paralysis virus* (CBPV) (n = 2), *deformed wing virus* (DWV) (n = 5), *Lake Sinai virus* (LSV) (n = 4), *sacbrood bee virus* (SBV) (n = 1), *Apis rhabdovirus-1* (ARV-1) (n = 1), *bee macula-like virus* (BeeMLV) (n = 1) and *Hubei partiti-like virus* 34 (HPLV34) (n = 1). The nucleotide sequences of ABPV, BQCV, DWV and SBV are the first complete genomes of these viruses identified in Slovenia and they represent an important contribution to our understanding of the genetic diversity of honeybee viruses. ARV-1, BeeMLV and HPLV34 were detected and sequenced for the first time in Slovenia.

Circulation of Influenza A Virus in Wild Boars in the Emilia-Romagna Region, Italy (2023–2025)

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Influenza A viruses (IAVs) are single-stranded, negative-sense, segmented RNA viruses classified into subtypes based on the genetic and antigenic characteristics of their hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. IAVs are globally recognized as major respiratory pathogens in both humans and swine. The swine population plays a key role in IAV ecology, acting as a "mixing vessel" for avian and human-origin viruses, thereby contributing to the emergence of reassortant strains, as occurred in 2009. While IAV dynamics are well-documented in domestic pigs, data on viral circulation in wild boars and feral pigs remain limited. This study aimed to investigate IAV circulation in the wild boar population of Emilia-Romagna (Northern Italy) and to compare the findings with the epidemiological situation in domestic pigs from the same area, known for its intensive swine production. Virological surveillance was conducted between 2023 and 2025 (May) on wild boars either hunted or found dead. We screened 1,418 lung samples for IAV using real-time RT-PCR targeting the matrix (M) gene. Out of those, 10 samples tested positive (0.7%), all detected in 2025. All positives were subtyped with RT-PCR: six belonged to the H1N1 subtype, specifically H1pdm-09N1av, except for one H3Nx sample and three HxNx. Whole Genome Sequencing (WGS) was performed on typed samples. Genomic analysis revealed that all H1N1 strains belonged to a single genotype, not previously detected in Italian wild boars. The HA-H1 belonged to the H1A lineage, the NA-N1 was avian-derived, and the internal gene cassette (IGC) was of pdm09 origin, except for an avian-like PB1 segment. This genotype was also detected in domestic swine in Northern Italy from 2022 to 2024, suggesting a transmission event from domestic pigs to wild boars. The single H3Nx strain displayed an avian-like IGC, while its HA-H3 clustered closely with human-origin sequences. Given that human-derived H3N2 viruses have been actively circulating in Italian swine since 2021, this strain probably originated in pigs and subsequently infected wild boars. Despite the low IAV prevalence observed in wild boars, detecting genotypes already circulating among domestic pigs highlights the importance of targeted surveillance at the wildlife–livestock interface.

Detection of enteric viruses in mussels during the COVID-19 and post-COVID-19 period

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Mussels, as filter feeders, are effective bioindicators of viral contamination in marine environments, accumulating pathogens from wastewater inputs. The COVID-19 pandemic and associated non-pharmaceutical interventions have had a profound impact on human activities and environmental conditions, including water quality. A four-year study (2020-2024) utilized quantitative one-step reverse transcription PCR (RT-qPCR) to investigate the presence of enteric viruses in Mediterranean mussels (*Mytilus galloprovincialis*) collected from the Slovenian Adriatic coast. Notably, the study period encompassed two years of COVID-19 lockdown measures, providing a unique opportunity to assess the impact of reduced human activity on viral prevalence. While no SARS-CoV-2 was detected, a significant reduction in presence of noroviruses and astroviruses was observed during the lockdown periods, correlating with a decrease in wastewater discharge. We observed that both the positivity rate and the diversity of enteric viruses increased in the post-COVID period. While aichiviruses and rotaviruses were not detected during the COVID period, they were present in the post-COVID period. Additionally, a pronounced seasonal pattern emerged, with viral loads peaking in colder months, likely due to increased viral stability and higher wastewater inputs during the wet season. Mussels, beyond their ecological role, are a sustainable and environmentally friendly source of animal protein, making their monitoring essential for ensuring food safety. Our results advocate for enhanced wastewater treatment to mitigate viral contamination in coastal ecosystems and emphasize the need for continued monitoring to understand long-term trends, thereby informing public health strategies for managing foodborne viral risks.

Molecular Detection and Phylogenetic Characterization of Swine Orthopneumovirus (SOV) in Northern Italy

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Swine orthopneumovirus (SOV) is an emerging pathogen belonging to the *Pneumoviridae* family. Despite its potential relevance in the context of porcine respiratory diseases, its prevalence, pathogenicity, and epidemiology remain largely unknown. Considering the significant role of pigs as reservoirs of several respiratory viruses and the economic impact of infectious diseases in swine farming, understanding the circulation of SOV is of particular interest.

The primary objective of this study was to evaluate the circulation of SOV in pig herds from Northern Italy and to explore its genetic diversity through phylogenetic analysis of the glycoprotein (G) gene, a well-established target for molecular epidemiological studies of pneumoviruses.

A total of 612 diagnostic samples, including lungs, oral fluids, nasal swabs, and bronchoalveolar lavages, were collected from pigs exhibiting respiratory signs between January 2024 and April 2025. The samples were obtained from farms located in the regions of Lombardy, Emilia-Romagna and Veneto, three of the most important pig-producing areas in Italy. Following necropsy, samples underwent specific virological screening. Detection of SOV RNA was performed using a real-time RT-PCR assay targeting the G gene. Overall, 15 samples tested positive for SOV (2.45% prevalence): 10 in 2024 (10/343, 2.9%) and 5 in 2025 (5/269, 1.85%). Positive samples were predominantly identified in farms located in Lombardy (14/15), with a single detection from the Veneto region. Partial sequences of the G2 and/or G1 genes were successfully obtained from 7 samples through Sanger sequencing. Phylogenetic analysis of G2 sequences revealed that the Italian strains clustered with previously identified SOV strains from Asia, USA and Europe. Nucleotide homology analysis demonstrated similarities with strains from China, South Korea, USA and Sweden, suggesting potential international circulation routes for SOV.

Interestingly, SOV was frequently detected in association with other major swine respiratory pathogens, particularly porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV). Co-detection of SOV and porcine respirovirus 1 (PRV1) was observed in five samples. These findings provide new insights into the circulation and molecular characteristics of SOV in European pig populations, contributing valuable data to the limited information currently available on this emerging pathogen.

Determination of the prevalence and complete genome sequences of PCV 2 and PCV 3 in domestic pigs and wild boars in Slovenia

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Circoviruses are among the most significant pathogens affecting the pig industry. To date, four porcine circoviruses (PCV 1 to 4) have been identified, with PCV 2 being the most clinically relevant due to its association with various diseases. While circoviruses are globally distributed, data on their prevalence in Slovenian pigs remains limited. This study aimed to investigate the prevalence of PCV 2 and PCV 3 in Slovenian domestic pigs and wild boars.

Samples were collected from 131 dead animals: 88 domestic pigs (DP) and 43 wild boars (WB). A 10% tissue suspension (spleen, kidneys, lymph nodes, tonsils) was prepared, and viral DNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). Detection of PCV 2 and PCV 3 DNA was performed using real-time RT-PCR and conventional PCR (PCR SuperMix). Ten different positive samples with PCR products covering the complete genome region (five for PCV 2, five for PCV 3; 10 µl each) were submitted to external sequencing company for full-genome Sanger sequencing. Sequences were analyzed with DNASTAR software (Lasergene, USA).

PCV 2 DNA was detected in 60 (68.2%) DP and 8 (18.6%) WB, while PCV 3 DNA was found in 20 (22.7%) DP and 15 (34.9%) WB. Coinfections with both PCV 2 and PCV 3 were observed in 14 (15.9%) DP and 8 (18.6%) WB. Full genome sequences were obtained for four PCV 2 and two PCV 3 samples, showing 96–99.8% nucleotide identity for PCV 2 and 99% for PCV 3. Phylogenetic analysis classified two PCV 2 strains as genotype PCV 2b and two as PCV 2d; both PCV3 strains belonged to genotype PCV 3a.

Our findings demonstrate that PCV 2 and PCV 3 are widespread in Slovenian domestic pigs and wild boars, with prevalence rates comparable to those in neighboring and other countries. Genetic analysis revealed close similarity to international strains. This study provides the first report of PCV 3 in the Slovenian wild boar population and the first complete genome sequences of PCV 2 and PCV 3 from Slovenia.

Detection and genetic characterization of bovine viral diarrhoea virus in cattle abortions in Slovenia (2020 – 2024)

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As part of systematic annual monitoring conducted between 2020 and 2024, laboratory testing for the presence of bovine viral diarrhea virus (BVDV) nucleic acids in cattle abortions was carried out for the first time in Slovenia, using real-time RT-PCR. A total of 4089 abortions were examined, with BVDV detected in 4,98 % of the tested samples. The BVDV positive samples were genetically characterized using direct Sanger sequencing method in 5'- untranslated region of viral genome. The first genetic typing of 48 BVDV strains isolated from positive abortions revealed the presence of "indigenous" strains belonging to subgroups BVDV 1b, 1d, 1e and 1f, that have been circulating in Slovenian cattle herds for several decades. In herds where multiple BVDV positive samples were identified, identical viral strains were detected in aborted fetuses, persistently infected animals, animals with mucosal disease, and animals died from acute BVDV infection manifesting as diarrhea or pneumonia. The detection of BVDV in aborted fetuses can also serve as an early warning signal for herd owners indicating potential (re)introduction of the virus, particularly in herds previously granted BVDV free status. The detection of BVDV underscores the need for implementation of additional biosecurity measures, especially the prompt identification and removal of persistently infected animals from the herd. Phylogenetic analysis comparing the strains detected in abortion cases with previously characterized strains demonstrated that genetically identical BVDV strains were present in multiple infected herds and that the virus primarily spreads to new locations over short distances.

Sixty years of the veterinary virology laboratory in Slovenia

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The laboratory for veterinary virology in Ljubljana was established in 1964 within the Department of microbiology and parasitology at the Veterinary Institute of Slovenia (VIS). The first head of the laboratory was Prof. Zoran Železnik, who introduced virus isolation in cell cultures, the immunofluorescence test for detecting classical swine fever and rabies viruses, and the Malmquist test for detecting African swine fever virus (ASF). From the first occurrence of sylvatic rabies in 1973 until its eradication in 2016, the laboratory played a central role in the national surveillance and oral vaccination programs for foxes. In 1996, the first RT-PCR method was introduced for BVDV diagnostics, followed by the accreditation of the first real-time RT-PCR method in 2011. Sanger sequencing was first performed in 1999, and the first molecular epidemiology study was completed in 2002. Next-generation sequencing (NGS) was implemented in 2014, and complete genomes of several virus species have been published since 2015. Over the past 30 years, both the volume and complexity of the laboratory's routine and research activities have significantly increased, driven by the growing number of samples and the implementation of new methods for controlling diseases such as CSF, ASF, FMD, Aujeszky's disease, rabies, bluetongue, BVD, IBR/IPV, PRRS, equine infectious anemia, lumpy skin disease and other poxviruses, peste des petits ruminants, and various viral diseases affecting fish and bees. The laboratory has developed and introduced diagnostic methods for over 50 different viruses, employing more than 130 diagnostic protocols. Each year, it participates in several international proficiency testing schemes, applying 84 different protocols for this purpose. Seventeen diagnostic methods are accredited under ISO/IEC 17025, and the virology unit hosts eight National Reference Laboratories (NRLs). In recent years, the research team has published 68 original scientific articles in the field of virology. Today, the virology laboratory is Slovenia's leading institution for the diagnosis of viral animal diseases and serves as a key center for education and ongoing research in veterinary virology.

Progress in the voluntary programme for granting bovine viral diarrhoea virus (BVDV)-free status to cattle herds in Slovenia (2023–2025)

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Bovine viral diarrhoea (BVD) is one of the most economically significant cattle diseases. The first monitoring of BVD infections in Slovenia began in 1994. The disease is endemic across all regions, with approximately 20 % of cattle herds infected with the bovine viral diarrhoea virus (BVDV). The official process for granting, maintaining, suspending, and restoring BVDV-free status was introduced under national legislation in 2014. However, by 2022, little progress has been made, with only 16 herds achieving recognition of BVD free status. With the new EU legislation on animal health, BVD became listed disease and conditions regarding BVD status were harmonised at the EU level. National legislation was aligned accordingly. In 2023, BVD eradication was included in the Regulation on the animal welfare intervention in the frame of Common Agricultural Policy (CAP) Strategic Plan. Since then, operators who joined the BVD programme have received financial support under the CAP. Between 2023 and 2025, a total of 935 cattle herds joined the program. In 2025, 396 cattle farms had already been officially granted BVDV-free status, with up to 300 herds expected to be certified by the end of 2025. During this period, a total of 7,512 animals from 81 previously identified antibody-positive herds were tested for the presence of BVDV nucleic acid using the real-time RT-PCR method. A total of 190 BVDV-positive animals were detected during this period and immediately advised to cull or slaughter upon receiving laboratory results. This measure helps prevent the continued circulation of the virus within the herd and its transmission to other herds. However, it was unexpectedly found that not all BVDV-positive animals were removed as recommended. These findings raise concerns about whether operators (breeders) are sufficiently informed about biosecurity and whether it might be necessary to introduce mandatory marking of BVDV-positive animals and to strictly prohibit their movements to other herds. The BVD voluntary program provides a clear understanding of the current health status of each herd, facilitates disease monitoring, enables virus elimination from infected herds, and supports continuous progress tracking with the goal of implementation a nationwide compulsory BVD eradication program.

Controlling FECV in Breeding Catteries: Impact of Stepwise Protocols on Viral Shedding and Cattery Status

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Feline enteric coronavirus (FECV) is widespread in multi-cat environments and, although typically asymptomatic, can mutate into the virulent form responsible for feline infectious peritonitis (FIP), a progressive and often fatal systemic disease. Therefore, reducing FECV shedding plays a critical role in FIP prevention.

This study aimed to evaluate the effectiveness of structured protocols in reducing or eliminating FECV shedding in catteries. A total of 20 catteries were included and assigned to one of three intervention levels. Ten catteries implemented general guidelines aimed at reducing the number of shedding cats. Five catteries adopted a “FECV-low” protocol targeting less than 20% FECV shedders. The remaining five applied a “FECV-free” protocol, aiming to house no shedders. Each protocol involved a set of hygiene, management, and biosecurity measures, which were followed over time.

To assess outcomes, qPCR testing was performed on rectal swabs at three time points: one before and two after implementation. In the general guidelines group, the cat-level prevalence decreased from 84% to 59% to 49%, while the cattery-level prevalence remained 100% throughout. For the FECV-low protocol, cat-level shedding dropped from 72.7% to 33.3% to 12.9%, and cattery-level prevalence went from 100% to 100% to 60%. In the FECV-free protocol group, cat-level prevalence decreased from 55.6% to 17.1% to 2.5%, while cattery-level prevalence reduced from 100% to 60% to 20%, meaning that 4 out of 5 catteries achieved FECV-free status by the end of the study.

These results demonstrate that protocol-based management can significantly reduce FECV shedding in catteries. While full elimination remains challenging, particularly in dynamic populations, strict adherence to tailored protocols can lead to substantial improvements in breeding catteries.

Epidemiological situation of avian influenza in Serbia during 2023-2024

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Avian influenza in birds is caused by influenza A viruses, which belong to the Orthomyxoviridae family, genus Alphainfluenzavirus. Among orthomyxoviruses, only influenza A viruses are known to naturally infect birds. It is believed that aquatic birds are the primary reservoir of influenza A viruses, which are, in most cases, low pathogenic for chickens and turkeys. Highly pathogenic strains of subtypes H5 and H7 cause high mortality rates in wild birds and poultry, leading to clinical disease and significant economic damage. Some strains of avian influenza can also cause sporadic infections in humans. For all the above reasons, it is essential to carry out surveillance for this disease.

In the Republic of Serbia, the Ministry of Agriculture implements both active and passive surveillance for avian influenza.

Active surveillance was conducted over six months (October – December 2023 and March – May 2024) in 19 districts. Passive surveillance was carried out from October 2023 to November 2024 in all cases of suspected disease. During active surveillance, a total of 3,659 samples were collected, including: 2,586 samples (70.67%) from 11 types of domestic poultry, 426 samples (11.64%) from intensive production of 4 types (categories), and 125 samples (3.42%) from poultry in trade, representing 7 species. Additionally, 522 samples (14.27%) were collected from 38 species of wild birds, predominantly waterfowl.

During active surveillance, AI virus (H7) was detected in only one sample (river gull) in March 2024 in the Danube District. Due to the low quantity of the virus, it was not possible to determine the N subtype and pathotype.

During passive surveillance, 108 samples were analyzed, including 93 from 17 species of wild birds, 13 from 4 species of domestic poultry, and 2 samples from 1 species of wild mammals. Of the 80 locations with suspected cases, AI virus was confirmed in 27 outbreaks (34.0%). Positive results for AI virus (subtype H5N1) were found in 40 samples, including: 24 from swans, 15 from grey cranes, and 1 from wild geese. Some of the viruses were isolated and the whole genome was sequenced. All sequences had CS motif characteristic for Highly Pathogenic AIV and belonged to clade 2.3.3.4b.

Keywords: Avian influenza, virus, Serbia, Monitoring results

Associations of Cervid Herpesvirus 2 and Chlamydia spp. with Infectious Keratoconjunctivitis in Reindeer in Sweden

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Background
Infectious keratoconjunctivitis (IKC) is a multi-factorial, transmissible, and severe ocular disease that affects ruminants worldwide. In Sweden, the disease was first reported in semi-domesticated Eurasian reindeer (*Rangifer tarandus tarandus*) in the 1890s. Both isolated cases and outbreaks are reported annually. Previous studies indicate that cervid herpesvirus 2 (CvHV2) works as a primary causative agent. In addition, the bacterium *Chlamydia pecorum* was isolated for the first time in 2016 during an outbreak of IKC in a Swedish reindeer herd.

Objectives
The objective was to study the presence of potential pathogens with a focus on CvHV2 and *Chlamydia* spp. in eye swab samples from reindeer in Sweden.

Materials and Methods
Swedish reindeer herds (n=55) with ongoing outbreaks or cases of IKC in 2019-2025 were selected for participation in this study. Conjunctival eye swabs (eSwab, Italy) were collected from reindeer with and without clinical signs of IKC (n=306), sent to the Swedish Veterinary Agency (SVA), and analyzed for the presence of CvHV2 and *Chlamydia* spp. with real-time PCR.

Results
The preliminary results revealed a proportion of 24.3% for CvHV2 (45/185) and 33.5% for *Chlamydia* spp. (59/176). Only 4.6% (8/174) of the samples tested positive for both CvHV2 and *Chlamydia* spp. simultaneously, whereas 50.0% (87/174) of the samples tested negative for CvHV2 and *Chlamydia* spp. The most common signs observed in infected reindeer were epiphora, followed by the shedding of pus and conjunctivitis. Selected cases of IKC outbreaks where CvHV2 has been detected will be presented, focusing on herd demographics, clinical presentation, and disease progression.

Conclusions
Preliminary results support CvHV2 as a primary causative agent, detected exclusively in reindeer displaying clinical signs, which was not valid for *Chlamydia* spp. However, to conclude whether CvHV2 might precede the detection of *Chlamydia* spp. requires further studies.

Epidemiology of respiratory viruses circulating in farrowing units and nurseries in Spanish pig farms

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This study assessed the prevalence of respiratory viruses circulating in pig farms in Spain. The study was conducted in farrowing units and/or nurseries of 73 farms, which were classified as suffering respiratory disease (n=34) or not (n=39). In diseased units, nasal swabs were collected from 20 sick pigs and 20 more from apparently healthy counterparts. In farms where no respiratory disease was observed, sampling was randomly conducted on farrowing units and nurseries (20 pigs each). Nasal swabs were analysed by RT-qPCR for *swine Influenza A virus* (swIAV), *Porcine reproductive and respiratory syndrome virus* (PRRSV), *Porcine respirovirus 1* (PRV-1), *Swine orthopneumovirus* (SOV), and *Porcine cytomegalovirus* (PCMV).

Overall, at farm level, PRV-1 was the most frequently detected virus, present in 75.3% (55/73) of the farms. swIAV and PCMV were detected in 69.8% (51/73) of the farms and PRRSV in 63.0% (46/73) of the farms. Farms having respiratory disease in farrowing units or nurseries were more likely to be infected by swIAV and PRRSV (Relative risks (RR) 1.69and 1.78 for swIAV and PRRSV, respectively; $p<0.05$). When analysing individual data by age groups, PRRSV was 3.2 times more frequently detected in nursery pigs compared to suckling piglets (31.9% vs. 9.9%, $p<0.05$). Similarly, SOV and PCMV were more frequent in weaners (RR= 3.2 and 6.5, respectively; $p<0.05$). Interestingly, in farms with respiratory disease, swIAV and PRV-1 were more frequent in suckling pigs than in nurseries (RR=2.0 and 2.0, respectively; $p<0.05$), while PRRSV was associated with the disease in the nurseries (RR=2.1; $p<0.05$). Co-occurrence of different pathogens was examined using tetrachoric correlations. At the farm level, the most significant was between swIAV and PRV-1 ($\rho = 0.54$). At the individual level, in sick animals PRRSV and PCMV tend to co-coinfect animals ($\rho =0.53$), as well as PRV-1 and SOV ($\rho= 0.58$) did. Finally, a logistic regression model was built using these data. The model showed that PRRSV, swIAV, PRV-1 and SOV but not PCMV were significantly ($p<0.5$) related to the disease with odds ratio values between 3.15 (PRRSV) to 1.50 (SOV). Also, sequencing revealed the presence of both A and B clades of PRV-1.

Molecular and Phylogeographic Characterization of SRLV Genotype C and Its Evolutionary Dynamics across Sweden

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Background
Small Ruminant Lentiviruses (SRLVs) are genetically diverse retroviruses responsible for chronic infections in sheep and goats. Their diversity is categorized into distinct genotypes, some of which exhibit geographically restricted distributions. Genotype C has been identified exclusively in Sweden and Norway. Molecular analyses based on sequences obtained from different regions and time points have the potential to reveal the transmission dynamics and possible host adaptation processes of SRLVs, thereby contributing to more effective control strategies.

Objectives
This study aims to investigate the molecular characteristics, phylogenetic relationships, geographic spread, and evolutionary pressures acting on SRLV strains circulating across different geographic regions of Sweden. The main objective is to reconstruct viral transmission patterns and identify potential signatures of region-specific adaptation.

Methods
SRLV-positive samples collected from multiple Swedish regions and time periods will be subjected to genetic characterization using partial gag and env gene sequences. Phylogenetic relationships will be inferred using maximum likelihood analysis, and pairwise genetic distances will be calculated to assess sequence divergence, alongside the examination of amino acid variation in immunologically relevant motifs. Time-calibrated phylogenetic trees will be used to investigate how the virus has spread across regions over time. To evaluate selection pressures, nonsynonymous to synonymous substitution ratios (dN/dS) will be compared across geographic clusters. At the same time, amino acid signature patterns in important antigenic parts of the env gene will be examined to detect differences that may be related to how the virus spread between regions.

Expected Outcomes
This study is expected to characterize SRLV strains circulating in different geographic regions of Sweden through molecular and phylogenetic approaches. It aims to reveal region-specific genetic clustering, provide insights into how the virus has spread over time, and identify signals of positive or purifying selection. By combining dN/dS analyses, amino acid signature pattern evaluation, and phylogeographic reconstruction, the study may help clarify how genetic variation relates to regional viral spread. Findings from gag and env gene analyses may also support a better understanding of antigenic variation, contributing useful information for future surveillance and diagnostic strategies.

Serological and Molecular Surveillance for Seneca Valley virus in England

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Background
Seneca Valley virus (SVV), a picornavirus, may cause clinical signs in pigs indistinguishable from notifiable vesicular diseases. Primary viral replication occurs in tonsils, while SVV-specific antibodies are produced 3-10 days after infection. In 2022, vesicular disease due to SVV was detected on four outdoor pig farms in England with a fifth herd identified through tracing. To date, no further clinical evidence of SVV in England has been reported.

Materials and methods
Two anonymised archives were established, each with sera and tonsils collected from batches of pigs at English abattoirs in 2019 (12 abattoirs, 676 sera/tonsils) and 2023/24 (9 abattoirs, 800 sera/tonsils). SVV-antibody results were compared with two sets of sera from Switzerland (Swiss National Serumbank 2018 and 2022; 400 each) with no SVV disease reported to date. Sera were screened for SVV-specific antibodies using a blocking ELISA (BioVet, Canada), followed by in-house virus neutralization test (VNT) to confirm positive results. Selected tonsils were tested by real-time RT-PCR for SVV RNA; these were from all ELISA-positive batches and randomly selected negative batches (187 and 218 tested for 2019 and 2023-24 archives respectively).

Results
The proportion of sera with positive ELISA values was highest for the England 2023-24 archive (17.8%) compared to the England 2019 archive (15.0%) and Swiss National Serumbank (3.5% and 6.0%). Neutralizing antibodies were detected in 18.3% of ELISA-positive samples within the England 2023-24 archive (3.3% inferred for whole archive; titres ranging from 1/45 to 1/1413) and in none of the ELISA-positive sera in the other three archives. Viral RNA was detected in 2.2% of tonsils, only from batches of pigs with VNT-positive sera in the 2023-24 archive.

Conclusions
Evidence of SVV exposure or infection was found in a low proportion of the England 2023-24 archive, mainly in pigs from the east of England. It remains unclear what triggers SVV infection to manifest as vesicular disease. The ELISA was a useful screening test, however, with the prescribed kit cut-off, data indicated low specificity (40.6%) and so VNT was required to confirm seropositivity. Specificity could be increased to 81.7% if a higher cut-off (Percentage of Inhibition: 58%) was applied.

Bluetongue virus in biting midges: observations in the field and in the laboratory

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Bluetongue virus (BTV), family *Reoviridae*, is the causative agent of bluetongue disease in ruminants. With the exception of the Arctic and Antarctic, BTV is found worldwide. Between the vertebrate hosts, it is primarily transmitted by biting midges of the genus *Culicoides*. Bluetongue disease is characterized by a hemorrhagic fever with ulcerations, hyperaemia and oedemas of the facial mucosa and the coronary band, but it can also affect internal organs causing breathing difficulties and death. Several BTV serotypes emerged and caused outbreaks all over Europe. The first outbreak of bluetongue disease in central and northern Europe in 2006 was caused by BTV serotype 8 (BTV-8). In September 2023, BTV serotype 3 (BTV-3) was detected for the first time in the Netherlands. From the place of detection, BTV-3 rapidly spread to neighbouring countries, including Germany. After a limited circulation in the west of Germany in autumn 2023, BTV-3 reappeared in spring 2024 leading to the detection of BTV-3 cases in domestic ruminants all over Germany.

To investigate the prevalence of BTV-3 in the putative *Culicoides* vectors, biting midges were trapped from September to November 2023 and from April to September 2024 in the western federal states of Germany. A total of 5,510 pools from 2023 and a total of 4,937 pools from 2024, containing up to 50 culicoids per pool, were tested by BTV specific real-time RT-PCR. While only one pool (0.02%) from 2023 was positive for BTV-RNA, 365 pools (7.4%) from 2024 were positive, which corresponds to the development of the reported BTV-3 cases in ruminants in Germany. Due to the observed rapid spread of BTV-3 in 2023/2024, which was even faster than the spread of BTV-8 in 2006/2007, we compared the replication properties of the two serotypes in the *Culicoides* vector. We conducted an experimental infection study with BTV-3 and BTV-8 in a laboratory-reared colony of *Culicoides sonorensis*. Oral infection with BTV-3 resulted in a significantly higher viral load in the infected midges with demonstrated replication than BTV-8 infection, offering a possible explanation for the difference in the outbreak progressions.

Newcastle Disease Outbreaks in Backyard Poultry in Slovenia

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Newcastle disease (ND) is a devastating, highly infectious poultry disease with significant economic impact and worldwide spread despite large-scale vaccination. It is caused by virulent strains of Orthoavulavirus javaense (OAVJ), commonly known as ND virus (NDV) or avian paramyxovirus 1 (APMV-1). In Slovenia, vaccination against ND is mandatory in poultry flocks with more than 350 birds, ostrich flocks, parent flocks of pheasants and partridges, and pigeons intended for exhibitions or competitions. However, vaccination in hobby flocks is neither regulated nor controlled.

In January and February 2025, in two unrelated flocks of laying hens in northeast Slovenia, consisting of 14 and 160 birds of different ages, there was increase in mortality, 57% and 73% within 5 days, respectively, accompanied by depression, weakness, and cyanosis of the heads. Post-mortem examination revealed consistent findings, including mucoid to hemorrhagic exudate in the trachea, liver discoloration with multifocal pale foci suggestive of necrosis, fibrinous oophoritis and salpingitis, and hemorrhages in the mucosa of the gastrointestinal tract. The splenic lesions ranged from splenomegaly to splenic atrophy. Histopathological examination revealed multisystemic necrosis, hemorrhages, and lymphocytic infiltration. Bacteriological analysis revealed the presence of *Escherichia coli* and *Clostridium perfringens* in liver and spleen samples (first outbreak) and *Pasteurella multocida* and *Gallibacterium anatis* in liver and heart samples (second outbreak). Quantitative real-time RT-PCR excluded avian influenza virus and confirmed the presence of OAVJ in oropharyngeal, cloacal, and brain swabs. Phylogenetic analysis of the fusion protein gene (F) showed that the OAVJ strains from both outbreaks were identical. In addition, the identified strains were assigned to genotype VII.1.1. and showed 100% nucleotide identity with the OAVJ strains (accession no. PQ880030) detected in the ND outbreak in Poland in 2024. However, the OAVJ strains detected in backyard poultry differed from the OAVJ strain (genotype VI.2.1.1.2.1) detected in free-living pigeons in Slovenia in 2024, which showed only 85% nucleotide identity. These results highlight the ongoing threat of ND in inadequately regulated backyard flocks and the importance of biosecurity, vaccination and disease surveillance in commercial and backyard poultry flocks. These were the first confirmed cases of ND in poultry in Slovenia since 1991.

Diagnosis

A Multi-Country Evaluation of ELISA Kits for Bovine Viral Diarrhoea Virus Antibody Detection

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Bovine viral diarrhoea (BVD) remains one of the most significant cattle diseases worldwide, with substantial economic and health implications for the livestock industry. Accurate serological diagnosis is essential for BVD surveillance and control in livestock populations. Enzyme-linked immunosorbent assay (ELISA) kits are widely used for BVDV antibody detection, yet their performance varies across sample panels and epidemiological contexts. This study evaluates six commercial ELISA kits using 481 bovine serum samples from France, Sweden, the UK, and the Netherlands, with serum neutralisation test (SNT) as the gold standard reference.

Diagnostic performance indicators, including correlation, diagnostic sensitivity and specificity, accuracy, positive and negative predictive values (PPV and NPV), were assessed to determine each kit's fitness for purpose. Optimal threshold values were identified, balancing sensitivity and specificity, and compared to manufacturer-recommended cutoffs. Differential SNT assessed cross-reactivity with the related Border disease pestivirus using a subset of discordant samples with varying inter-kit outcomes.

The PrioCHECK and IDVET kits had the highest accuracy (≥97%), with sensitivity and NPV exceeding 96%. Conversely, BIOX had significantly lower sensitivity (91%) despite perfect specificity (100%). Pairwise McNemar's tests evaluated inter-kit agreement, revealing strong concordance among most tests but highlighting systematic classification deviations for BIOX and, to a lesser extent, Svanova. Strong positive associations between ELISA kits and SNT were identified, with Pearson correlation coefficients (r) between 0.8669 and 0.9549. PrioCHECK and IDVET short protocol performed by Sweden exhibited the highest correlations, with all p-values statistically significant ($p < 0.0001$), confirming robust relationships.

To assess performance variability, the dataset was stratified by geographical origin, unknown vaccination status, and history of PI animals. The vaccinal status of the samples seemed to impact the performance of the kit. PrioCHECK and IDVET maintained stable classification metrics across all panels, indicating diagnostic robustness. However, BIOX and Svanova had significantly reduced sensitivity depending on sample origin and status.

These findings highlight the need for careful ELISA kit selection based on epidemiological context. While most kits demonstrated high performance, some showed classification variability across sample populations. Further research should investigate the causes of these discrepancies, including antigen composition and potential cross-reactivity with related pestiviruses, to refine diagnostic tools for broader field applications

Two proteins, one goal: fast and accurate ELISAs for LSDV antibody detection

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Introduction

Lumpy skin disease virus (LSDV) poses a serious threat to livestock health and the economy, primarily due to its rapid transmission and expanding geographic range. Accurate serological diagnostics are essential for effective surveillance and control strategies. Recombinant protein-based ELISAs offer a scalable, safe, and standardized alternative to assays using whole-virus antigens, eliminating the need for viral culture. In this study, we report the development and validation of two serological ELISAs based on LSDV recombinant proteins p32 and ORF060 (a homolog of L1R in Vaccinia virus), designed to detect specific antibodies in both infected and vaccinated cattle.

Methods

The p32 and ORF060 genes were cloned into plasmid vectors with a six-histidine tag and expressed in E. coli BL21(DE3). Proteins expression was confirmed via SDS-PAGE and Western blotting using specific and anti-histidine monoclonal antibodies (mAbs). A trapping ELISA was developed for p32 using a capture mAb, while ORF060 was used in an indirect ELISA format by direct adsorption to ELISA plates. The assays were validated using 90 sera from 18 experimentally infected cattle (sampled weekly up to 28 days post-infection), 412 negative sera from a capripox-free country, and 658 field sera from vaccinated or naturally infected animals in Serbia and Albania. These sera were previously analysed with virus neutralisation test (VNT).

Results

The p32 protein was obtained in native form as crude lysate, while ORF060 was purified under denaturing conditions and renatured, yielding 0.5 mg/L. Both assays demonstrated approximately 91% concordance with the VNT, with the Cohen's Kappa resulting over than 0.8 for both the tests (0.81 for ORF060 and 0.85 for p32).

Discussion

These recombinant antigen-based ELISAs offer significant advantages over traditional methods, including elimination of viral culture, reproducible antigen preparation, and suitability for large-scale testing. Some samples positive in both ELISAs but negative by VNT suggest that the ELISAs may detect low-titre or early antibody responses missed by VNT. The inclusion of the novel ORF060 ELISA, performing comparably to the p32-based assay, supports a multi-antigen approach to enhance diagnostic reliability and reinforces their value as effective tools for LSDV surveillance and control.

Development of a molecular toolbox to detect and pathotype Eurasian H7 avian influenza viruses

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Rapid and sensitive diagnostic methods for avian influenza virus (AIV) are essential for the timely implementation of control measures. Low pathogenicity (LPAI) strains of the H5 and H7 subtypes are of particular concern, as they can cause highly pathogenic avian influenza (HPAI) through the insertion of polybasic amino acids at the hemagglutinin cleavage site (HA CS). Recent outbreaks in domestic birds across Africa and Europe caused by Eurasian H7 LPAI and HPAI strains, along with detections in wild birds, emphasize the need for updated molecular diagnostics. To address this, we developed and validated a toolbox of molecular assays for subtyping and pathotyping Eurasian H7 AIVs in clinical samples.

HA nucleotide sequences from AIVs indentified in Eurasian and African birds and environmental samples collected since 2020, along with zoonotic H7N9 strains from China, were retrieved from GISAID. Primers and probes were designed using Geneious Prime to target conserved HA regions. Three assays were developed: a TaqMan-based qRT-PCR for H7 subtyping (assay #1); an end-point RT-PCR combined with Sanger sequencing to identify HA CS motifs (assay #2); and an intercalant-based qRT-PCR to raplidly distinguish between H7 HPAI and LPAI strains (assay #3).

Various primer combinations and thermal profiles were tested to optimize conditions. The analytical and diagnostic performance, based on the WOAH standards, was compared to existing protocols widely used in Europe. Assays #1 and #2 outperformed former H7 detection methods, demonstrating improved sensitivity (limit of detection: 10^{0}–10^{-1} EID_{50}/100 µl) and inclusivity with respect to contemporary H7 strains, without cross-reactivity to non-H7 AIVs. Both were repeatable across a range of virus loads and were able to successfully detect and characterize H7 AIVs in clinical samples. Assay #3 showed promising results for pathotype discrimination using melting curve analysis. However, further analyses (e.g., gel electrophoresis) may be needed for viruses with A-T rich motifs within the CS sequence.

In conclusion, the new qRT-PCR and RT-PCR assays provide sensitive and broad-spectrum tools to detect and characterize contemporary Eurasian H7 viruses, supporting their integration into routine AIV surveillance. Assay #3 offers rapid pathotyping but may require supplementary verification for specific strains.

A new high sensitive indirect Enzyme-Linked Immuno Sorbent Assay for the detection of antibodies against the VP7 protein of the African Horse Sickness Virus

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African horse sickness (AHS) is an infectious, but non-contagious vector-borne disease of equids caused by the Orbivirus AHSV (family Reoviridae). The main vectors are *Culicoïdes* midges. AHS is endemic in sub-Saharan Africa, but outbreaks occurred in many countries surrounding the Mediterranean Sea (Morocco, Spain, Portugal,…), in the Middle East and Asia. AHS is listed by the WOAH; and specific guidelines must be followed to obtain official recognition of AHS-free status, particularly for international animal movements. Vaccination is prohibited in officially free countries.

The ID Screen® African Horse Sickness Indirect ELISA kit has been developed to detect antibodies against the VP7 protein, which is conserved among all 9 AHSV serotypes. The aim of the study was the evaluation of specificity and sensitivity of the ELISA.

Diagnostic specificity and sensitivity were evaluated on 1015 sera from non-infected animals (France, Brazil, Argentina, Iceland) and 26 positive sera from different reference laboratories (EU/WOAH AHS Reference Laboratory, Spain; the Pirbright Institute, UK; Friedrich-Loeffler-Institut, Germany). For exclusivity evaluation sera from ruminants infected with Bluetongue Virus or Epizootic Hemorrhagic disease Virus were tested.

The observed specificity of the ID Screen® ELISA was 100% (95%CI [99.6, 100.0]) and the observed diagnostic sensitivity was 100% (95%CI [87.13, 100.0]). No-cross reaction with BTV or EHDV positive sera was observed.

The ID Screen® ELISA is a reliable and easy-to-use test for the detection of AHSV VP7 antibodies. It shows high specificity and excellent diagnostic and analytical sensitivity. VP7 antibody detection is the prescribed WOAH method to prove freedom of infection, estimation of prevalence and surveillance of a disease-free status.

Key-words: AHSV, ELISA, diagnostic, surveillance, orbivirus

Two complementary tools for diagnosis and management of Equine Herpesvirus 1 and 4 infections: Discriminating ELISA and high performance triplex qPCR

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Introduction

Equine herpesviruses 1 and 4 (EHV-1 and EHV-4) are widespread among horses globally, causing respiratory symptoms and abortions. EHV-1 can further result in serious neurological disorders and death, making its detection and monitoring - particularly during equestrian sporting events - critical. Several international competitions have been cancelled in the past due to EHV-1 outbreaks. In contrast, EHV-4 is very common in horses and typically causes milder symptoms. Given the close genetic relationship between EHV-1 and EHV-4, reliable diagnostic tools are essential for distinguishing between them and ensuring effective disease management.

IDvet has developed an ELISA and a qPCR:

- **ID Screen® EHV-1/EHV-4 Discrimination test Indirect ELISA** kit based on specific recombinant proteins, in order to distinguish between EHV-1 and EHV-4 antibodies in equine sera
- **ID Gene LyoTM Equine Herpes virus 1 and 4 Triplex**, a freeze-dried triplex qPCR kit for EHV-1 and EHV-4 containing an exogenous internal control.

Methods

Both diagnostic tests were validated as follows:

ID Screen® ELISA: Diagnostic specificity and sensitivity were evaluated on VNT-pretested sera. Seroconversion of experimentally (EHV-1) or naturally (EHV-4) infected horses was monitored.

ID Gene LyoTM qPCR: Limit of detection of the PCR (LDPCR) and Method Detection Limit (MDL) were determined and specificity was assessed.

Results

ELISA: Observed specificity for EHV-1 was 99% (95% CI [96.6-99.7%]). For EHV-4 the VNT negative sera tested showed negative results. EHV-1 antibodies were detected between 13–17 dpi, and EHV-4 antibodies appeared 6–10 days after clinical onset. No cross-reactivity was observed between EHV-1 and EHV-4.

PCR: The LDPCR and MDL will be presented for the different sample types.

Conclusion

The ID Screen® ELISA is user-friendly and effectively distinguishes EHV-1 from EHV-4. The ID Gene Lyo™ qPCR enables rapid, reliable detection of both viruses separately thanks to the internal control included. The combined use of both tools supports accurate equine herpesvirus infection management.

Keywords: EHV-1, EHV-4, Triplex qPCR, ELISA

Strategic use of biological matrices for ASF diagnosis: experimental insights into detection across infection stages

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African swine fever (ASF) is a complex and expanding transboundary disease that threatens both domestic pigs and wild boar populations. In Europe and Asia, the widespread circulation of genotype II ASF virus (ASFV), along with growing endemicity in wild boar and human-mediated spread, complicates control efforts. The co-circulation of ASFV strains with differing virulence further challenges early detection and diagnosis. In this context, having sensitive, validated techniques for a broad range of biological matrices is essential for timely outbreak confirmation and effective surveillance. This study evaluated the diagnostic performance of various sample types under experimental conditions using well-characterized genotype II ASFV isolates.

The study: 4,179 samples were collected from 125 pigs experimentally infected with 20 ASFV isolates. Matrices included blood, serum, oropharyngeal and nasopharyngeal swabs, and 21 tissues. ASFV genome detection was performed using the WOAH real-time PCR (procedure 2), and antibody detection was assessed by ELISA and immunoperoxidase test (IPT). Virus isolation was conducted on PCR-positive samples.

Results: In blood and swabs, ASFV genome was detected with kinetics depending on strain virulence. Blood was highly effective for early detection and remains the matrix of choice in acute cases. Swabs—particularly oropharyngeal—were useful at later stages, especially with attenuated strains. Infectious virus was isolated from blood during the first month, matching the active disease phase. In tissues, ASFV genome was consistently detected by PCR during the first month, declining thereafter. However, IPT detected antibodies in 100% of tissue samples after 31 days post-infection, including bone marrow, thus extending diagnostic value when PCR turned negative. Tonsils, lymph nodes, and joint-associated tissues retained infectious virus beyond 90 days, underscoring their relevance for long-term surveillance. For antibody detection, IPT was more sensitive than ELISA, identifying antibodies earlier and more consistently. Combining PCR and IPT increased ASFV detection by over 10%, particularly between 15–36 days post-infection.

Conclusion: ASF diagnosis is a critical pillar for disease control, especially under complex epidemiological conditions with co-circulating viruses of differing virulence. The complementary use of varied sample types and validated diagnostic methods enhances detection across all disease phases and supports effective surveillance and outbreak response.

Multispecies serological tools for Influenza A surveillance: validation on bovine serum and milk.

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Introduction

Influenza viruses belong to the family Orthomyxoviridae and include four types : A, B, C, D; defined by the nature of their nucleocapsid antigen. Type A (IA-V), the most conserved genus, can be divided into subtypes based on their Hemagglutinin and Neuraminidase antigens. Some subtypes are associated with highly pathogenic forms and high mortality rate. The emergence of cases of Influenza A subtype H5N1 in dairy cattle in USA in 2023, involving more than 1,000 farms in 17 states, raises major health issues and zoonotic risks. In this context, two species-independent ELISAs kits, ID Screen® Influenza A Antibody Competition (kit A, detection of anti-nucleoprotein antibodies) and ID Screen® Influenza H5 Antibody Competition 3.0 (kit B, detection of anti-H5 antibodies) have been validated for use on bovine samples, on serum, individual and tank milk matrices.

Methods

For both kits, the diagnostic specificity was assessed on negative bovine samples : 187 serum, 180 individual milks and 178 bulk milks. The capacity to detect antibodies directed toward IA-V clade 2.3.4.4.b, those circulating in bovine herds, was evaluated in an external study2. Analytical sensitivity for both ELISA was assessed by testing serial dilutions of 3 serums and 3 milks spiked with either low or high pathogenic strains of IA-V.

Results

Measured diagnostic specificity was 100% [99.3-100] for kit A and 99.6% [98.6-99.9] for kit B. Both kits efficiently detect antibodies against the clade 2.3.4.4[2]. With kit A : 3 spiked sera and 2 spiked milks detected positive until 1:256 and 1 milk was detected positive until 1:8. With kit B : 2 sera and 2 milk were detected positive until 1:256, 1 serum until 1:8 and 1 milk until 1:16.

Conclusions

Both ID Screen® kits efficiently detect antibodies toward nucleoprotein (kit A) and H5 (kit B), including following the infection with strains from clade 2.3.4.4. In addition, ID Screen® H5 3.0 offers a 10-times superior limit of detection of antibodies directed toward H5 strains, clade 2.3.4.4b, compared to the previous version. The validation data acquired offers multi-species tools that can be used for surveillance if an outbreak occurs in cattle or other species.

Keywords: Influenza, Bovine, Milk, serum

[2] Halwe, N.J., Cool, K., Breithaupt, A. et al. H5N1 clade 2.3.4.4b dynamics in experimentally infected calves and cows. Nature (2024). https://doi.org/10.1038/s41586-024-08063-y

Production and Characterization of the Toscana Virus Nucleoprotein and Its Preliminary Use in a Multispecies Serological ELISA

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Toscana virus (TOSV) is an arthropod-borne virus belonging to the genus *Phlebovirus* within the family *Phenuiviridae*, transmitted primarily by *Phlebotomus* sandflies. Endemic to Mediterranean countries, TOSV infection in humans can range from asymptomatic presentations to severe neurological disorders, including meningitis. Serological evidence suggests that a variety of animal species—including dogs, livestock, and wildlife—may also be exposed to the virus, although their role in the transmission cycle remains unclear. In this context, the availability of a multispecies serological assay is critical for monitoring viral circulation within a One Health framework.

In this study, we describe the development of a double-antigen ELISA (DAg-ELISA) for the detection of antibodies against the TOSV nucleoprotein (NP) in both human and animal sera. The TOSV NP gene was codon-optimized for *E. coli* expression based on the sequence available under GenBank accession KU922126. The recombinant NP (rNP) was expressed under native conditions, and purified via immobilized metal ion affinity chromatography, with a yield of 3.75 mg/L. The ELISA was configured using rNP to coat the plates, serum samples diluted 1:10, and HRP-conjugated rNP as the detection reagent. The assay was evaluated using a panel of 11 TOSV-positive and 74 TOSV-negative human sera.

Additionally, the DAg-ELISA was tested on animal sera, including one TOSV-positive hyperimmune rabbit serum, 11 negative sera from various animals species, and 135 sheep sera collected from the pre-Apennine hills of Italy, a region endemic for sandflies and phleboviruses. All animal samples were also tested using a TOSV-specific serum neutralization (SN).

The DAg-ELISA successfully distinguished between TOSV-positive and -negative human sera. Results were expressed as optical density (OD), with a tentative cut-off value set at 0.2 OD. Among the 147 animal samples tested, 77% (114/147) of ELISA results were concordant with SN. Additionally, 14% (20/147) of the samples were positive only by ELISA, and 9% only by SN.

Further studies are ongoing to assess potential cross-reactivity of the TOSV nucleoprotein with other *Phlebovirus* species. However, the current findings from both human and animal sera are encouraging and support the potential of DAg-ELISA as promising tool for TOSV and broader *Phlebovirus* surveillance across species.

A Longitudinal Study of BTV-3 and BTV-4 Detection in Bull Semen Samples: Comparison of Different Extraction and Amplification Assays

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Bluetongue (BT) is an infectious, vector-borne, viral disease, listed by the WOAH as a multispecies disease with a large economic impact, affecting wild and domestic ruminants. It is caused by a segmented, double-stranded RNA (dsRNA) virus belonging to the *Orbivirus* genus (family *Sedoreoviridae*). To date, thirty-six BTV serotypes (BTV-1 to BTV-36) have been identified, among which 24 serotypes (BTV1- BTV-24) are notifiable under the Animal Health Law.

The potential economic loss for bull breeders impelled the investigation of reliable diagnostic methods for BTV-RNA detection in bovine semen. A longitudinal study aiming at determining the most valuable assay was conducted from end October 2024 to February 2025 on coupled specimens (K3EDTA and semen samples) collected weekly from five bulls, belonging to a certified Italian artificial insemination Station (AI) that had experienced a natural, acute BTV-3 and BTV-4 infections in September 2024.

All collected semen samples underwent virus investigations by means of two different pretreatments, two automated extractions and three molecular assays at the Italian NRL for BTV and at IZSVe laboratory, based in the area of the certified Italian AI Station. Blood samples were investigated with validated diagnostic tests, in accordance with WOAH guidelines. Insect KC and C6/3 and VERO cell cultures were inoculated with PCR-positive samples for isolation attempts.

Semen was pretreated as described by Hoffmann B. et al, (2014) and according to a protocol issued by VetMAX PRRSV EU & NA 3.0 Kit. Three different NS3-targeting protocols were applied to the extracted RNAs.

All molecular assays gave comparable results, and endogenous co-extracted controls were detected in all samples. In blood samples, viral RNA was detectable throughout the study, with Cycles threshold (Cts) ranging from 25 to 36, while in semen specimen Cts ranged from 27.3 to 40.4, but viral RNA was sometimes undetectable.

To conclude, all methods applied in this study are suitable for the detection of BTV viral RNA in bull semen samples. Nevertheless, virus viability and residual infectivity in semen remain unsolved issues, as does the cause of negative results observed, possibly explainable by an intermittent viral excretion or a lack of test sensitivity.

When Negative Isn't Negative: Improving the Diagnostic Specificity of BoHV-1 glycoprotein B-ELISA Screening through Characterisation, Epitope Mapping, and Statistical Approaches

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All over the world, Infectious Bovine Rhinotracheitis (IBR) causes significant concern in the livestock industry due to its substantial economic impact. IBR is caused by the Bovine herpesvirus 1 (BoHV-1) and often leads not only to respiratory disease, abortions, enteritis, mastitis, balanoposthitis, pustular vulvovaginitis, and death, but also to infertility, milk drop, and immunodepression, resulting in suboptimal production on cattle farms. Therefore IBR is considered an economical important disease and surveillance is implemented across Europe. However, many diagnostic challenges hinder smooth surveillance often hindering eradication. One such difficulty is the occurrence of false-positive glycoprotein B (gB) results in antibody-ELISA testing. Some of these false positives are due to accidental vaccinations. However, not all false-positive gB results can be explained this easily.

Therefore, we attempted to characterize existing commercial ELISAs (anti-gB or whole-virus antibodies). Diagnostic sensitivity and specificity, a kernel density estimate (to evaluate discriminatory capacity between negative and positive samples), positive and negative predictive values, and analytical sensitivity were determined. Based on these results, a ROC analysis was performed to propose new test cut-offs. Next, a testing protocol using statistical modelling was developed to reduce the number of unidentified false positive cases. A classification and regression tree (CART) analysis was conducted to build a decision tree to distinguish false positives from truly infected animals. Especially in an IBR-free context where gE-screening is no longer allowed, this approach could prove useful.

Finally, the epitopes corresponding with the commercial ELISA's monoclonal antibody were identified. Using Western blot and immunostaining, the existence of linear epitopes corresponding to the intracytoplasmic and transmembrane domain of gB was confirmed, after which an epitope-mapping experiment using the pepscan method was set up. Some identified epitopes were located in a region that is highly conserved within the varicelloviruses. Identifying the kits' epitopes, together with the CART analysis, could prove useful in establishing an IBR gB screening strategy to reduce false-positive results. Furthermore, the suggested approach could be valuable for other diseases managed through serological testing-based control programs.

A lateral flow device to detect and differentiate between EBHSV and RHDV2

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The genus *Lagovirus* (*Caliciviridae* family), which emerged around the 1980s, comprises highly pathogenic viral species, including Rabbit Haemorrhagic Disease Virus (RHDV/genotype GI.1), with rabbit-specific tropism, and European Brown Hare Syndrome Virus (EBHSV/genotype GII.1), whose host spectrum is restricted to the hare. In 2010 a new RHDV-related virus, named RHDV2 (GI.2), emerged in France, exhibiting expanded tropism for both rabbits and hare species. These viral groups cause fulminant hepatitis and high mortality in their respective host species.

RHD surveillance is primarily conducted using molecular and serological diagnostic techniques. Additionally, a lateral flow device has been developed to enable a rapid differentiation of RHDV from RHDV2. However, in geographical regions such as part of Europe and North Africa, the sympatric coexistence between the wild rabbit (*Oryctolagus cuniculus*) and the European brown hare (*Lepus europaeus*) poses additional diagnostic challenges.

Since European hares can be infected by either RHDV2 or EBHSV, it is crucial to have diagnostic tools that discriminate between these two viral infections in this species.

This study aims to develop a lateral flow device (LFD) for antigen detection capable of differentiating their presence in liver samples.

The test relies on the detection of the virus using specific Monoclonal Antibodies (MAbs), selected from those currently employed in the ELISA virological diagnostics of the WHOA reference laboratory for rabbit haemorrhagic disease. Three specific MAbs for RHDV2, two specific for EBHSV, and one cross-reactive MAb able to detect conserved epitopes of pathogenic lagoviruses were chosen. ELISA results demonstrated the ability of these antibodies to detect RHDV2 and EBHSV. The developed LFD prototype features four bands: one for EBHSV, one for RHDV2, one for both viruses, and a control line. Viral capture occurs through the immobilization of specific MAbs on the sample loading membrane, while detection is mediated by gold nanoparticle- conjugated MAbs.

Further studies are ongoing to optimize and validate the device as a unified diagnostic test. Thanks to the use of specific MAbs, the developed system holds the potential for diagnostic application not only in hare species but also in rabbits.

Acknowledgements: This research was supported by European Partnership Animal health and Welfare (HORIZON-CL6-2023-FARM2FORK-01-2) to IZSLER and by the Project CH4.0 under the MUR program "Dipartimenti di Eccellenza 2023-2027" (CUP: D13C22003520001).

Air Sampling as a tool for detection and surveillance of respiratory pathogens in pig herds

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Airborne micro-organisms pose a significant threat to animal health, with major economic implications. Porcine Respiratory Disease Complex (PRDC), caused by pathogens like *Mycoplasma hyopneumoniae*, Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), and Swine Influenza Virus (swIV), is a growing global concern. Current diagnostics often target individual pathogens, missing the multifactorial nature of PRDC. Traditional diagnostics for PRDC, such as blood or respiratory tract sampling, are invasive, costly, and time-consuming. The sector is seeking a non-invasive, efficient alternative for early outbreak surveillance and pathogen monitoring.

Three commercially available air sampling devices, with different operating mechanisms, were evaluated for their ability to detect respiratory pathogens in pig pens under field conditions. Sampling was conducted across nine compartments of nursery and fattening pigs on three farms experiencing clinical respiratory signs. The tested air samplers included the Coriolis µ (Bertin Technologies), the MD8 Airport (Sartorius), and the AeroCollect (AeroCollect). After air sampling, nucleic acids were extracted using the Indimag Pathogen Kit (Indical Bioscience) and analyzed by real-time PCR for *Mycoplasma hyopneumoniae*, PRRSV EU, PRRSV NA, and swIV. Air sampling results were compared to conventional diagnostic methods, including nasal and tracheobronchial swabs, serum, and oral fluids, to evaluate their diagnostic performance.

The first results demonstrated that all three air samplers could capture the targeted respiratory pathogens. However, the detected concentrations varied according to the sampling protocols and locations. The Coriolis µ and MD8 Airport samples yielded higher pathogen loads compared to the AeroCollect. For the Coriolis µ, the highest concentrations were observed under the ventilation outlet. In the case of the MD8, elevated pathogen levels were found when using mobile sampling between pens.

Notably, preliminary results for swIV and PRRSV indicated that several air samples showed higher pathogen loads than the corresponding conventional samples. This highlights the potential of air sampling as an alternative approach for detection of respiratory pathogens in swine herds. Further research will determine if this new approach could become a low- cost, non- invasive monitoring tool for improvement of early intervention and disease control in pig populations worldwide.

Viral metagenomics in diagnosis: an external quality assessment on e-lab skills for laboratories of the Mediterranean, Black Sea and Sahel regions

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Introduction

Metagenomics plays an important role for the detection of emerging pathogens, outbreak tracing and pandemic preparedness. The MediLabSecure/OneHealthSecure projects, funded by the European Commission, have implemented a unique training program on metagenomics for rapid viral surveillance, including wet-lab and e-lab modules. Here, the first external quality assessment (EQA) exercise organized to evaluate the acquired knowledge on metagenomics data analysis is presented.

Materials and Methods

The EQA was organized for the human and animal virology, and entomology beneficiary laboratories of the project. This exercise aimed to evaluate the laboratories´ capacities to correctly apply bioinformatics tools for NGS data analysis and to properly interpret the obtained results. The EQA panel was designed upon 4 epidemiological scenarios and included 4 raw datasets generated by sequencing real samples in MinION (Oxford Nanopore Technologies, ONT) device. The selected samples contained genetic material from 4 zoonotic viruses: SARS-CoV2, West Nile, Rift Valley Fever and Monkeypox. The participants were requested to analyze the data using 2 platforms: Epi2Me (ONT) and the TELEVIR module from INSaFLU (https://insaflu.insa.pt/); the use of additional analytical tools was also encouraged.

Results

The global results were very satisfactory. Nineteen laboratories participated in the EQA. Seventeen labs (89.5%) used Epi2Me for a first basic analysis, and 89.5% used also the INSaFLU-TELEVIR platform for final pathogen identification. Almost 60% of the participants employed additional bioinformatics tools, being Genome Detective and Chan Zuckerberg ID platforms the most frequently reported. Overall, 89.5% of the labs correctly identified the main etiological agent present in the samples and provided a correct interpretation combining the metagenomics analysis and the epidemiological scenarios.

Conclusions

The results of the EQA confirmed the interest and the proficiency of the participating laboratories in bioinformatics analysis. Most laboratories correctly identified viral pathogens from metagenomics experiments performed in real samples, but also expressed their need to reinforce their capacities. Moreover, this EQA exercise was specifically designed for laboratory professionals of different one-health sectors addressing viral diseases. Currently, the new OneHealthSecure project will strengthen capacity building by offering an updated training program on genomic surveillance and data analysis, including environmental scientists among their participants.

Restoration of the inclusivity of an APMV-1 screening qRT-PCR assay upon the first detection of a pigeon strain of genotype VI 1.2.1.2 in Italy

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Newcastle disease (ND), caused by virulent strains of avian paramyxovirus type 1 (APMV-1), is a listed disease under the EU Animal Health Law that affects domestic birds causing significant economic losses worldwide. *Columbiformes*-adapted strains are genetically and antigenically distinct from other APMV-1 and are often referred as pigeon paramyxovirus type 1 (PPMV-1). These viruses belong to genotypes VI, XX or XXI and are endemic in pigeons and doves, where the infection is generally mild in healthy birds. The majority of *Columbiformes* are synantropic animals, and as such represent a potential source of infection to poultry farms and other domestic birds. Upon natural transmission to chickens, PPMV-1 strains may indeed evolve into virulent viruses leading to major outbreaks. For this reason, surveillance in wild and feral birds is a key element to detect emerging APMV-1 variants.

In 2024, a PPMV-1 strain of genotype VI 1.2.1.2 (APMV-1/Collared dove/Italy/24VIR9980-1/2024) was reported for the first time in found-dead pigeons in Sardinia, Italy. This strain showed higher similarity with viruses previosly detected in Europe and Africa and, importantly, determined a diagnostic dropout of a widely-used screening qRT-PCR targeting the L-gene of current APMV-1 and PPMV-1 genotypes. To restore assay inclusivity, an *in silico* study was undertaken, resulting in the design of an additional MGB-probe to complement the existing ones. An extended validation of the updated assay was performed, preserving the original reaction chemistry and thermal cycling conditions.

The updated assay successfully detected the APMV-1/Collared dove/Italy/24VIR9980-1/2024 strain with high sensitivity (LoD determined in lung tissue and tracheal swabs: 10^2 EID_{50}/100 µl) and repeatability (Cq CV ≤ 3%). The new probe did not determined cross-reaction with non-target pathogens. Diagnostic sensitivity and specificity assessed on a variety of APMV-1 genotypes, species and matrices were 100%.

The new qRT-PCR proved to be fit for routine screening and allowed a broader and more sensitive detection of circulating APMV-1 in clinical specimens. This study also highlights the critical impact of the variability of APMV-1 genome on the performance of molecular diagnostic protocols, and the importance of surveillance for the maintenance of an up-to-dated status of laboratory methods.

Complete Genome Sequencing of Infectious Hematopoietic Necrosis Virus (IHNV) in Finnish Rainbow Trout Farms in 2021 and 2022

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Infectious hematopoietic necrosis virus (IHNV) is a significant viral pathogen affecting rainbow trout and other salmonids. IHNV belongs to the genus *Novirhabdovirus* within the family *Rhabdoviridae*. In Finland, the virus was first detected in 2017.

In 2021, IHNV outbreaks occurred in five rainbow trout farms in the Åland Islands. Before these outbreaks, IHNV had been identified in May 2021 in Denmark, with fish movements from infected sites to two farms in the Åland Islands. IHNV was subsequently detected on these two farms in June 2021, followed by three additional farms in June and October 2021. In June 2022, an additional rainbow trout farm in the Åland Islands tested positive for IHNV.

This study presents the complete genome sequences of IHNV isolates from Finnish fish farms in 2021 and 2022. Whole-genome sequencing is essential for tracking the virus's origin and identifying potential evolutionary mutations.

Whole-genome sequencing was conducted on two samples from a single farm in 2021 and two samples from another farm in 2022, using paired-end sequencing technology on an Illumina MiSeq platform. The genome length of IHNV was determined to be 11,148 nucleotides (nt). The two isolates from 2021 exhibited 100% sequence identity, as did the two isolates from 2022. Comparisons between the 2021 and 2022 isolates revealed sequence differences of 0.08%, corresponding to a shared similarity of 99.92%.

Phylogenetic analysis was performed using publicly available IHNV sequences from GenBank. The Finnish isolates showed the closest relationship to an IHNV strain from Italy, identified in 2005 (GenBank accession number MK829693) with nucleotide identity of 96,95–97,03%. The findings indicate that the Finnish IHNV isolates belong to genotype E.

Funding
European Union's Horizon Europe under Grant Agreement No 101136346: European Partnership Animal Health &Welfare, Joint Internal Project SOA12 BETO – Better tools for diagnosis of infectious diseases.

Genomic Dinucleotide Frequency-Based Classification of Dengue Virus Serotypes Using Machine Learning

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The classification of dengue virus (DENV) serotypes based solely on nucleotide composition represents a promising in silico approach for viral genomic surveillance and outbreak response. In this study, a robust supervised classification model was developed, capable of accurately predicting DENV serotypes (1–4) based on dinucleotide frequency patterns. A total of 49,215 complete and partial DENV genomic sequences were downloaded from NCBI's GebBank, and sequences shorter than 300 bp were excluded. The final dataset consisted of 46,562 sequences distributed across four serotypes: DENV-1 (18,581), DENV-2 (15,357), DENV-3 (8,370), and DENV-4 (4,254). To ensure fair representation across both serotype and sequence length, a stratified data splitting based on a combined variable generated from serotype and decile-based length bins. Dinucleotide frequency features were computed for each sequence and used to train multiple machine learning classifiers. Metadata including sequence headers and lengths were retained to track performance post-classification. A random forest classifier achieved the best performance and was used for final evaluation. On the held-out (test) validation set (n = 9,313), the model achieved an overall accuracy of 99.08%. The precision, recall, and F1-scores for individual serotypes were consistently high, ranging from 0.98 to 0.99, with a macro-averaged F1-score of 0.99. Serotype 4, the smallest class, showed slightly lower recall (0.98), though still within excellent performance bounds. These results demonstrate that dinucleotide composition, even without alignment or positional features, carries strong serotype-discriminatory information across a wide range of sequence lengths. This approach is scalable, alignment-free, and suitable for rapid genomic classification tasks in large viral datasets. It offers a computationally efficient alternative for serotyping and has the potential to assist genomic epidemiology efforts, particularly in low-resource or high-throughput scenarios.

First detection of Usutu virus in Denmark

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In 2024, Denmark experienced its first documented outbreak of Usutu virus (USUV) among wild birds, primarily affecting the common blackbird (*Turdus merula*). USUV is a mosquito-borne virus (genus *Orthoflavivirus*, family *Flaviviridae*) that has been circulating in Europe since its initial detection in Austria in 2001, causing die-offs of blackbirds in several European countries. Following public reports of increased blackbird mortality during the summer of 2024 in Denmark, USUV RNA was initially detected in brain tissue from three blackbirds using an USUV-specific RT-qPCR assay. During the autumn season, a total of 149 dead birds were submitted to University of Copenhagen, including 85 blackbirds. Birds were submitted from most regions of Denmark, although the geographical distribution was uneven, with the highest incidence of positive birds in the southern part of the country.

Pathological examination performed on all the birds revealed that many of the birds were emaciated, with empty stomachs and incomplete plumage. While most birds showed no macroscopic organ changes, the most frequent findings observed included enlarged spleen, intracranial bleeding and congestion.

Of the 85 examined blackbirds, USUV RNA was detected in 56 individuals. Most often they had a high level (Ct<20) of viral RNA in the brain. Both male and female blackbirds were USUV RNA positive, with a predominance of males (~62%), and almost half of the birds (~46%) were characterized as juveniles. In addition, low levels (Ct>30) of viral RNA were detected in the brains of two great spotted woodpeckers (*Dendrocopos major*) and one red kite (*Milvus milvus*). Cloacal and throat swabs were tested from 23 blackbirds positive for USUV RNA in the brain. The levels of viral RNA detected in swabs corresponded qualitatively with that observed in brain tissue. Liver and kidney samples from another five USUV RNA positive blackbirds were tested, and in these tissues, comparable levels of viral RNA were detected as the corresponding brain tissue sample.

This outbreak highlights the importance of USUV surveillance in Denmark, considering that USUV has zoonotic potential and its role as a possible early indicator for the emergence of the closely related West Nile virus.

The detection and phylogenetic analysis of equine herpesviruses 1 and 4 in Slovenia

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Equine herpesvirus (EHV), also known as equine rhinopneumonitis, is a highly contagious viral infection caused by the equid alphaherpesvirus-1 and -4 (EHV-1 and EHV-4). EHV-1 and EHV-4 are closely related and share nucleotide sequence identity more than 55% and amino acid sequence identity up to 96%. With the exception of EHV-1 in Iceland infection is endemic to horses worldwide. It represents one of the most important infectious diseases and causes economic loss in the equine breeding industry globally. EHV affects only domestic and wild equids and does not pose any health risk to humans or other animals. It occurs in several forms and describes a constellation of several disease entities. The severity of the diseases is related to age and immunological status of the infected animal. The disease manifests itself primarily as an upper respiratory infection in young horses following viraemia causing abortion and perinatal foal death in unvaccinated pregnant mares.

EHV induces dormant, long-lasting latent infection. This allows the virus to continually reside within the horse and can be reactivated following stress. Consequently, neurological signs of the EHV infection may develop.

Over the past decade and more, we have tested a large number of samples from placenta and fetal tissues from suspect cases and nasal/nasopharyngeal swabs from asymptomatic horses for the presence of the EHV virus. We have confirmed 16 positive samples using the PCR method described and published by Varrasso and co-authors. 11 samples were identified as EHV-1, and 5 samples were confirmed as EHV-4.

The detected viruses were subsequently characterised by partial nucleotide sequencing for EHV-1 glycoprotein H (gH) and EHV-4 partial glycoprotein B (gB).

Phylogenetic analysis of the ORF39 (EHV-1) and ORF33 (EHV-4) glycoprotein genome regions showed sequence similarities among all Slovene isolates. The amino acid sequence homology was above 97 % when compared to other strains from GeneBank. The evolution of the EHV strains correlates with time and their geographical site of isolation.

Virus detection using a new broad surveillance procedure

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To be well prepared for outbreaks of virus threats, early detection of new and emerging viruses are extremely important.

To deal with unknown or unexpected virus variants, we have created a Veterinary Probe Capture Set (VetCap). This set includes custom designed probes derived from a large range of viruses derived from the virus disease lists of WOAH, DISCONTOOLS, EmZoo and our WBVR institute (diagnostic) list. For each selected target virus a combination of specific probes was selected to be included in the VetCap probe set, with this probe set we were able to detect all the included viruses.

However, challenges arise when environmental, host, or bacterial DNA/RNA is abundant, leading to sequencing failures. This means the selection of the appropriate starting matrix is important and in addition, a proper virus pre-enrichment is crucial. This has been executed for different matrixes, including faeces, organ tissues, water, blood, serum, respiratory swabs, dust or air samples. Subsequently, enrichment of the DNA/RNA can be accomplished via target-specific PCRs or a random amplification (Sequence-Independent, Single-Primer Amplification (SISPA)-PCR). The sensitivity of this newly developed probe set has been tested using a variety of archived viruses from the list and with different sample batches from diverse animals from the field.

The VetCap is a comprehensive tool for capturing and identifying a broad range of viruses in veterinary surveillance, both animal and environment-derived.

Development of a Reverse Transcriptase Quantitative PCR Targeting the tat Region for the Detection of Equine Infectious Anaemia Virus

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Equine infectious anaemia virus (EIAV) is a notifiable lentivirus, which causes disease in equines and is characterised by viral persistence. Like other lentiviruses, EIAV contains three main genomic elements: *gag* (an area coding for structural and non-structural proteins); *pol* (coding for the polymerase complex) and *env* (coding for the glycoproteins). Since EIAV genomes display great diversity, serological detection remains the relied upon method for diagnostics. Attempts have been made to develop polymerase chain reactions (PCR) assays targeting different regions of the EIAV genome, including the gag gene which was identified as a conserved region. However, further investigation revealed insufficient conservation of nucleotide sequences across EIAV strains, thereby limiting their detection by PCR. This study aimed to identify an alternative conserved region within the EIAV genome and to develop a reverse transcriptase quantitative PCR (RT-qPCR) assay targeting this newly identified region. Analysis of 18 whole genome sequences and 2 near complete genomes (~7.8kb) identified the *tat* gene to be highly conserved and a pair of primers and probes were designed to target this gene. Development of the PCR components was performed manually using MegAlign identifying a sense primer, antisense primer and two TaqMan dual-labelled probes. The performance of this RT-qPCR was evaluated by testing viral RNA extracted from serum/tissue samples and viral isolates, including all available UK strains and the Wyoming strain. Synthetic DNA generated from European and South American strains was also produced for testing of any strains which showed mismatches in the primers/probe sequences (n=11). The tat-based RT-qPCR was able to detect all available EIAV strains tested, representing all known EIAV variability in this region, with results showing a detection limit of 1 copy/reaction indicating high sensitivity. The assay demonstrated high specificity, as amplification was observed only in the positive samples and no signal detected in the negative controls. Both intra-assay and inter-assay tests analysed reproducibility and precision for the detection of viral RNA, with results showing coefficient of variation values of <10%, indicating high reproducibility. These results indicate that this assay could be used as an effective tool alongside serological assays for the early detection of EIAV.

Efficient primer-design for pan RT-qPCR: A semi-automated workflow using sequence clustering and varVamp

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Pan RT-qPCR assays are essential tools for detecting genetically diverse viruses, enabling broad and sensitive identification across genotypes, lineages, or even at the genus or family level. These assays target conserved genomic regions, ensuring robustness despite rapid viral evolution – critical for identifying emerging or re-emerging viruses where strain-specific assays may fail. Pan-assays are particularly valuable as screening tools, allowing broad detection before applying more targeted approaches.

Designing truly pan-specific primers can be challenging for viruses with high genomic variability. Primers must be placed in conserved regions with minimal variability and avoid spanning insertions or deletions. Degenerate nucleotides can increase primer inclusivity, but their use must be balanced to maintain specificity. varVAMP enables automated design of degenerate primers for (RT)-qPCR, requiring only a multiple sequence alignment as input. However, varVAMP assumes that the input alignment accurately reflects viral diversity, which is often not the case due to biases in public repositories, such as the overrepresentation of human-pathogenic strains and the underrepresentation or absence of rare or recently identified variants. Careful curation and data reduction are thus essential.

We developed a semi-automated workflow that integrates varVAMP with upstream database construction and post-processing steps. Viral genomes are curated from sources such as NCBI, excluding vector, synthetic, or chimeric sequences and filtering for length, duplication, and ambiguity. Metadata in TSV-format (e.g. accession and species) is used to structure the dataset. Clustering is performed using CD-HIT and VSEARCH to define major and subclusters. A representative subset of sequences is selected from each cluster using Python's random.sample, ensuring inclusion of all subclusters.

The selected sequences are aligned and passed to varVAMP with varying consensus thresholds and ambiguity limits. If an off-target database is provided, expanded primer candidates are queried against it using BLASTn and filtered by criteria including 3′-end matching, alignment length, and mismatches. For on-target checks, similar, but stricter, filtering ensures correct primer orientation and expected amplicon size within a certain range.

We applied this workflow to two different genera: *Orthoflavivirus* and *Alphavirus*, generating pan RT-qPCR assays that were compared to existing published assays, demonstrating its utility in designing broadly reactive primers for virus detection.

First outbreak of bluetongue virus serotype 3 in Denmark – data from clinical suspicions indicate severe impact on animal health in infected sheep and cattle

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Denmark, a country with 1,440,000 cattle and 150,000 sheep, has officially been free of bluetongue virus (BTV) since 2011 after the first outbreak of BTV (serotype 8) occurred in 2007-2008. However, on the 8th of August 2024, clinical suspicions of BTV were raised on two sheep farms in the south-western part of Denmark, close to the German border. Diagnostic samples were confirmed positive for BTV serotype 3 (BTV-3) and from subsequent whole genome sequencing, the virus was found to be more than 99.8% identical to the strain circulating in the Netherlands during the outbreak in 2023. Following the index case, the number of clinical suspicions of BTV increased rapidly in both sheep and cattle, with the number of new confirmed infected farms reaching 142 within a single week in mid-September. Hereafter, the number of suspected and confirmed infected sheep farms decreased drastically and was near zero from mid-October, while there was a much slower decline for cattle farms, only reaching a low level in mid-December.

As of the end of 2024, BTV-3 had been detected in nearly all regions of Denmark with a total of 904 confirmed positive farms (70% diagnosed from cattle and 29% from sheep, respectively). In early 2025, samples from clinical suspicions were still submitted to the laboratory and new positive cattle herds continued to be identified – albeit at a lower rate – with an additional 64 farms confirmed by the end of March.

From August 2024 through March 2025, veterinarians submitting samples for laboratory diagnosis most often reported fever, lesions, limb ailments and local swelling as the basis for suspecting BTV infection. Mortality was reported as a clinical sign prompting suspicion of BTV in 8% of positive sheep farms and 2% of positive cattle farms, where information on clinical signs was available. During the winter/early spring period (December-March), clinical suspicions were most often reported on the basis of airway or ocular symptoms, abortion or disease in calves. In summary, in Denmark, as seen when BTV-3 first emerged in Europe in 2023, both sheep and cattle were affected by the disease and with reported mortality in both species.

At the forefront of Orbiviruses diagnosis: highly performant freeze-dried RT-qPCR

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Introduction

BlueTongue Virus (BTV) and Epizootic Hemorrhagic Disease Virus (EHDV) are Orbiviruses which were reported in 2023/2024/2025 across Europe. Both have huge economic impacts and similar clinical symptoms, making laboratory testing essential for diagnosis. Innovative Diagnostics offers a versatile range of RT-qPCR: **ID Gene LYO™ BTV & EHDV Advantage Triplex**, a freeze-dried solution enabling the detection and differentiation, in a single well, of both BTV & EHDV. RT-qPCR for downstream genotyping of BTV-3, BTV-1, BTV-12, BTV-8 and BTV-4 are also available. **Results** can be obtained **in 50 min** with a **rapid amplification program, compatible** with **all ID Gene™ kits**, making possible to **test** on the **same run** with **different Orbiviruses RT-qPCRs**, therefore offering maximum **flexibility & lab ressources optimization**.

Materials and methods

Diagnostic specificities and sensitivities for all kits were assessed on panels of positive and negative samples. For each kit, inclusivity was assessed on 3 reference panels (adapted to the differents kits): 13 EHDV RNAs, 36 BTV RNAs (French national reference laboratory for BTV & EHDV, Anses), 7 EHDV RNAs (The Pirbright Institute, UK) and 10 BTV RNAs (FLI, Germany).

Results

The couple [Specificity;Sensitivity] for the ID Gene Lyo™ kit and ID GeneTM BTV-3, BTV-1, BTV-12, BTV-8 and BTV-4 RT-qPCR kits were respectively : [100%;100%] (for both targets), [100%;100%] , [100%;100%], [100%;100%], [100%;100%] (for both targets).

All kits showed an 100% inclusivity, including the different strains that were detected in Europe.

Discussion

The new ID Gene Lyo™ kit enables the efficient detection and differentiation of BT and/from EHD in only one reaction. Its freeze-dried format allows for an eco-friendly shipment at room temperature. The Orbivirus range include highly performant BTV genotyping kits to further characterise samples that are positive with the ID Gene Lyo™ kit. Most of the kits are already registered in several EU countries and validated by national reference laboratories. In regions where both viruses, or several BTV genotypes, can co-circulate, this range is the most versatile for **differential diagnosis testing, disease surveillance and testing before animal movement**s.

Keywords: EHDV, BTV, RT-qPCR

Continued Identification of Lesions Associated with Pseudocowpox Virus on Dairy Farms in Slovenia

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Parapoxvirus infections in cattle are widespread globally but are rarely reported in scientific literature due to their typically mild clinical course. Although these infections generally have limited economic impact, they have veterinary significance as differential diagnosis of serious infectious diseases. The objective of this work was to identify and confirm the etiological agent responsible for oral and nasal lesions in calves housed in a separate calf-rearing facility of large commercial dairy farms.

In a herd of over 800 Holstein-Friesian cows, characteristic erosive lesions on the oral mucosa and muzzle were observed in calves in the first month of life, especially in those suffering from severe and long-lasting diarrhoea throughout the year. Swabs of erosive lesions on the oral mucosa and scrapings from nasal lesions were collected from the two most severely affected calves. The sampled material was inoculated into a bovine turbinate cell culture. The presence of the virus was confirmed in the cell culture supernatant by electron microscopy and PCR using primers targeting the ORF045 gene. The PCR amplicons were subjected to direct sequencing. The sequences were analysed and compared with parapoxvirus sequences available in GenBank.

Parapoxvirus was confirmed by virus isolation and electron microscopy. Nucleotide sequencing revealed 97 % to 99 % similarity with pseudocowpox virus (PCPV), and significantly lower similarity with other parapoxviruses.

The findings suggest that the mucosal lesions in the calves were caused by PCPV, a virus less commonly associated with such clinical manifestations. It is presumed that the infection was transmitted from cow to calf during nursing, as PCPV is more often associated with teat lesions in lactating cows. Review of the medical records of the cows revealed only a few instances of skin lesions potentially related to PCPV, which resolved without any complications and treatment. Due to the mild nature of lesions in cows, bovine papular stomatitis was initially considered the most likely diagnosis. No human cases were reported among farm workers.

Future investigations should include detailed screening of all lactating cows, sampling of teat lesions, and molecular comparison with the virus isolated from calves to better understand transmission pathways and circulation dynamics of the virus on the farm.

Novel insights into cell tropism, kinetics of virus growth and prevalence of Phocoena pestivirus in harbour porpoises

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The first marine pestivirus, Phocoena pestivirus (PhoPeV), has been described a few years ago. Phylogenetic analyses showed that PhoPeV is more closely related to the porcine pestiviruses Bungowannah pestivirus (BuPV) and Linda virus than to classical swine fever virus (CSFV). In contrast to other pestiviruses, the PhoPeV genome does not encode the N-terminal protease N^{pro}, which mediates proteasomal degradation of interferon regulatory factor 3 (IRF-3) resulting in inhibition of the interferon type 1 (IFN-1) mediated antiviral response.

To further characterize the virus, *in vitro* cell tropism, viral growth kinetics and the prevalence of PhoPeV were analyzed. While BuPV exhibits a broad cell tropism, PhoPeV infects porcine, bovine, ovine and feline cells as described for CSFV. Infections of these cell lines over a period of 96 h showed a correlation between virus titer and amount of intracellular PhoPeV-specific RNA. In accordance with the loss of N^{pro}, no inhibition of the IFN-1 signaling pathway was detected in PhoPeV infected PK-15 cells.

A total of ninety-six harbour porpoises, four dolphins, two minke whales, one sperm whale, and one humpback whale were tested for PhoPeV genome. Five samples of harbour porpoises tested genome positive and two of them allowed isolation of infectious viruses in porcine cells. As no serum samples were available from the animals found dead, virus neutralization tests were performed using liquids collected from organ samples and homogenates. Six out of eighty-seven samples were tested positive for neutralizing antibodies including four samples showing an antibody titer ≥ 320 ND_{50}.

In conclusion, PhoPeV is more closely related to BuPV than to CSFV but showed a more restricted cell tropism compared to BuPV. Unlike other pestivirus species, PhoPeV does not encode for N^{pro} and consequently, does not cause degradation of IRF-3 in porcine cells. Screening of samples collected from ninety-six harbour porpoises over the last four years resulted in a PhoPeV genome detection rate of 5.2%. Moreover, a virus neutralizing antibody test has been established in which various organ matrices from animals found dead are used instead of the standard serum sample matrix. This assay resulted in detection of PhoPeV-specific antibodies in several samples.

Development and Validation of a Multispecies Double-Antigen ELISA for the Detection of anti-H5 HPAIV (clade 2.3.4.4b) Antibodies in Human and Mammalian Sera

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Since 2021, the detection of the highly pathogenic avian influenza virus (HPAIV) H5N1 in multiple countries, and its ability to infect both avian and mammalian species has raised significant concerns about its potential to cross the species barrier and adapt to humans. In 2024, H5N1 clade 2.3.4.4b was identified in dairy cattle, followed by human infections associated with exposure to infected animals. Specific diagnostic tools are crucial for managing emerging infectious diseases and preventing outbreaks. In this study, we describe the development and validation of a double-antigen ELISA (H5-DAg-ELISA) for detecting antibodies (Abs) against H5 HPAIV in human and mammalian sera.

H5N1 HPAIV was propagated in SPF embryonated chicken eggs. The recombinant HA1 (rHA1) subunit of the H5 protein (clade 2.3.4.4b) was produced in mammalian cells and purified using a histidine tag. In the H5-DAg-ELISA, plates were coated with the rHA1, sera were diluted 1:5, incubated, and the HRP-conjugated rHA1 protein was used as the detection tracer. Results were expressed as a sample-to-positive control ratio (S/P %) using net optical densities (ODs).

A panel of 1,057 sera (94 H5 positive and 963 negative) from humans and domestic and wild mammals was used for validation. Positive sera, confirmed by HI and VNT assays, were obtained from mammals either experimentally or naturally infected with H5N1 HPAIV. Negative samples were collected from healthy humans who had been vaccinated against seasonal influenza, as well as from domestic and wild animals sampled during surveillance programs in areas where H5 AIVs were not circulating. ROC analysis was used for performance evaluation.

The ROC curve showed the excellent diagnostic performance of the H5-DAg-ELISA, with an area under the curve of 0.998 ($P<0.001$), and respective Sensitivity and Specificity of 95.8% and 100%. Notably, the assay showed no cross-reactivity with human sera collected from people vaccinated against seasonal influenza.

The H5-DAg-ELISA represents a valuable tool for the serological detection of H5N1 HPAIV infections in humans and other mammals, as well as for identifying prior infections in surveillance programs. Using a recombinant antigen not only enhances biosafety but also improves the robustness, reproducibility, reliability, and efficiency of the assay.

Assessment of the minimum dose for infectivity, transmission, and early detection of highly virulent African swine fever virus in pigs

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Surveillance strategies for African swine fever virus (ASFV), particularly early diagnostic approaches, are critical for the effective prevention and control of African swine fever (ASF). In this study, we evaluated the infectivity and transmission potential of the ASFV Georgia strain administered intranasally at three different doses: high (10^4 HAU), moderate ($10^{2.5}$ HAU), and low (10 HAU), in three groups of 20 pigs each at seven weeks of age. Infection dynamics were monitored using quantitative PCR (qPCR) across an extensive panel of samples, including clinical specimens, tissues, non-invasive matrices, and environmental swabs. Additional diagnostic techniques included loop-mediated isothermal amplification (LAMP), serological assays, and cytokine profiling.

All infected animals succumbed to the disease, confirming the high virulence of the Georgia strain. Notably, in the low-dose group, only one pig developed ASF, while the remaining animals remained uninfected despite direct exposure, suggesting a potential threshold for transmission under minimal infectious dose conditions. Blood samples consistently yielded the highest diagnostic sensitivity and were confirmed as the gold standard. However, other matrices—such as serum, spleen, tonsils, bone marrow, ear tip, and tongue—also showed potential for early ASFV detection, even after using the LAMP assay as a point-of-care tool suitable for farm use.

Environmental and non-invasive samples, such as air filters and oral fluids, proved useful for surveillance, including in animals exposed to the lowest infectious dose. ASFV-specific antibodies were generally undetectable, indicating rapid disease progression and limited seroconversion. These findings underscore the importance of integrating both molecular and serological diagnostics within ASF surveillance programs. Prompt detection, combined with stringent biosecurity measures, is essential to contain ASFV spread effectively.

Experimental evaluation of cross-protection induced by naturally attenuated ASFV genotype II strains

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Introduction: African swine fever virus (ASFV) genotype II continues to evolve, leading to the emergence of non-hemadsorbing (non-HAD) variants with distinct CD2v mutations and a wide clinical spectrum ranging from acute to subclinical infections. While molecular genotyping is essential for outbreak tracing, it is not predictive of virulence or clinical outcome. Some non-HAD genotype II strains are naturally attenuated, with low or absent viremia, strong antibody responses, and reduced transmission, allowing them to go undetected and contribute to viral persistence. However, their ability to confer cross-protection remains poorly defined.

The study: This study evaluated the cross-protection conferred by the attenuated non-HAD strain Lv19/WB/Rie29/Tukuma14 (genotype II, group 3). Five pigs were infected and, at 28 days post-infection, challenged with either genotype I strain E70 or the virulent genotype II strain 22489_4_2312/RC/2023 (group 19, Italy 2022). Whole genome identity between immunizing and challenge strains was 97% (group 19) and 95% (E70). Clinical signs, survival, viremia, and infectious virus detection were assessed.

Results: No protection was observed against genotype I: all pigs developed acute ASF and were euthanized at 10 dpi, with lesions indistinguishable from non-immunized controls. In contrast, partial protection (60%) was observed after challenge with the genotype II strain: 3 out of 5 pigs survived, showing delayed disease progression and reduced clinical scores. However, infectious virus was detected in blood for approximately 20 days in most pigs, confirming extended virus circulation despite survival.

Conclusion: These results demonstrate that cross-protection is limited and group-specific, and high genomic similarity alone does not ensure protective immunity. Naturally attenuated strains can persist silently, shedding infectious virus and potentially maintaining ASFV circulation. Control strategies must adapt to detect subclinical infections, and vaccine development should consider intra-genotype diversity and the risks posed by partial immunity under field conditions.

European Swine Influenza Network (ESFLU): Harmonising Surveillance of Swine Influenza A Virus Across Europe

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Swine influenza A virus (swIAV) persists endemically in European pig herds, where its ongoing antigenic and genetic diversification hampers control and poses a zoonotic threat. To unify fragmented national initiatives and create a One-Health early-warning platform, the COST Action “European Swine Influenza Network” (ESFLU, CA21132) was launched. Between April and October 2023, ESFLU Working Group 3 (WG3) surveys were sent to 39 institutions in 25 countries, analysing 44 complete responses that described 26 surveillance programmes. In parallel, WG1 collated and compared standard operating procedures (SOPs) from 32 laboratories and provided training on monitoring, identification and characterization of swIAV, to harmonise molecular diagnostics across Europe. Of the 26 surveillance programmes reviewed, 16 operated at national scale. However, methodologies varied widely - only 42% performed routine genetic characterisation and just 35% shared data with OFFLU.Both funding stability and sampling intensity also wereinconsistent. ESFLU therefore developed the first pan-European SOP guideline set, designed reference HI antisera panels for antigenic mapping (planned delivery in 2025), and organized a training school that equipped 12 early-career scientists from nine countries with real-time nanopore whole-genome sequencing skills, cutting data-turnaround times from weeks to 24 h. Meanwhile, WG2 expanded analytical capacity by compiling the inaugural pan-European swIAV diversity report, instituting regular sequence-data calls, and running a highly rated phylogenetic training school whose materials will become a peer-reviewed SOP; additional work is under way on antigenic cartography and a Zenodo-hosted annual-report series. Complementing these technical advances, WG4 developed the ESFLU web portal and LinkedIn presence, and is preparing video clips, e-brochures and policy-facing One-Health surveillance recommendations to ensure rapid dissemination to scientists, industry and decision-makers; WG4 has also drafted biosecurity guidelines with 12 key external and internal measures to support herd-level risk mitigation. By standardising diagnostics, upskilling the workforce and promoting timely data-sharing, ESFLU’s integrated approach is closing critical gaps in European swIAV surveillance. In 2025, a harmonised template for passive and active surveillance will be published alongside pan-European antigenic maps to guide vaccine-strain updates, thereby strengthening preparedness for outbreaks with zoonotic or reverse-zoonotic potential and underscoring the value of coordinated, transnational epidemiological networks.

Development of a novel RT-LAMP based point-of-care system (fPOC) for the detection of avian influenza virus

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Avian influenza caused by type A Influenza viruses (AIVs) is a transboundary infectious disease with pandemic potential that spreads rapidly among birds and occasionally to humans, making it a priority for the poultry industry, public health, and the ecosystems. The availability of reliable diagnostic tests is pivotal for the prompt implementation of control measures. To support central diagnostic laboratories, reliable point-of-care (POC) systems would be beneficial to accelerate the detection of AIVs in the field. To this end, we developed the fPOC system consisting of i) a magnetic bead based sample preparation protocol; ii) a fluorescence-based POC device; iii) cartridges pre-filled with gelified RT-LAMP reagents that allow testing 4 samples in approximately 1 hour.

The analytical and the diagnostic validation was carried out according to the WOAH standards. The limit of detection in tracheal and cloacal swabs was $\leq 10^{2.50}$ and $\leq 10^{2.91}$ EID50/mL for the low pathogenic strains H5N3 and H7N1, respectively.

First results on 35 AIV and 37 non-AIV strains, tested for inclusivity and exclusivity, evidence a specificity of 100%.

An implementation study aiming at exploring different usage scenarios of the fPOC (i.e. poultry movement, high pathogenicity avian influenza outbreaks, active surveillance in wild birds, passive surveillance in carnivores) yielded overall 98.4% and 100% diagnostic sensitivity and specificity, respectively. We also evaluated fPOC performance on chickens experimentally challenged with a highly pathogenic H5 AIV. Tracheal, cloacal and meat swabs were collected at 1, 2, 3 days post infection (dpi) and subjected to fPOC testing comparatively with qRT-PCR and a commercially available antigenic rapid test. For all samples but one, fPOC results were consistent with the reference method, yielding a detection rate of 98% and no false positives. In contrast, the antigenic test was poorly effective at 1 dpi being able to detect only 1 out of 20 samples, while restoring its performance at 2 and 3 dpi.

The fPOC system proved to be a versatile tool for the detection of AIV with high sensitivity and specificity, outperforming antigenic tests at the onset the infection, and it is suitable for application in facilities with basic equipment and electric supply.

Development and Diagnostic Efficacy Evaluation of a Double-Antigen ELISA for Capripoxvirus Antibody Detection

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Background
Lumpy skin disease (LSD), caused by capripoxvirus, poses a significant threat to cattle industries worldwide. Currently, only a single commercial ELISA kit (ID-VET) is available for LSD antibody detection, but its sub-optimal sensitivity limits widespread serosurveillance and vaccination monitoring. Furthermore, as the sole commercial kit is imported, its availability and supply in Korea can be unreliable. To address these gaps, we aimed to develop a more sensitive double-antigen ELISA using selected capripoxvirus antigens, with the goal of creating a kit specifically suited for Hanwoo (Korean native cattle, *Bos taurus coreanae*) and Holstein (*Bos taurus*), the main cattle breeds raised in Korea, and to ensure more efficient and stable supply through domestic production.

Methods
Four capripoxvirus proteins (P32, A4L, A12L, and A33R) were selected based on published studies. They were expressed in *E. coli* and evaluated for diagnostic potential as ELISA antigens. A33R showed the highest reactivity and specificity and was selected as the diagnostic antigen. The double-antigen ELISA protocol was optimized using standard procedures. The assay was validated with serum samples from Hanwoo and Holstein in Korea. Diagnostic performance was compared with the commercial ID-VET ELISA kit and indirect immunofluorescence assay (IFA), performed according to the WOAH Manual.

Results
The A33R-based double-antigen ELISA demonstrated sensitivity either comparable to or even superior to that of the commercial ID-VET kit, while showing similar specificity. This was particularly evident in the detection of low-titer antibody samples, where the new assay consistently identified positive cases that the commercial kit sometimes missed. Receiver operating characteristic (ROC) analysis showed a higher area under the curve (AUC) for the newly developed assay compared to the ID-vet kit. Concordance with IFA was high, with the new ELISA detecting additional positive samples not identified by the commercial kit. No cross-reactivity was observed with sera from animals infected with unrelated viruses.

Conclusion
The newly developed A33R-based double-antigen ELISA offers a promising alternative for LSD antibody detection in cattle, with improved sensitivity and domestic availability compared to the currently available commercial kit. However, further clinical validation using a larger number of field samples is required to confirm its robustness and diagnostic performance under diverse field conditions.

Molecular and Serological Analysis of Lumpy Skin Disease Outbreaks in Korea, 2023–2024

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Background
Lumpy skin disease (LSD), caused by the lumpy skin disease virus (LSDV), threatens cattle industries globally, including Korea. Since its 2019 emergence in East Asia, LSD has spread rapidly, with outbreaks in China, Mongolia, and Thailand. In October 2023, LSD was first confirmed in South Korean cattle, leading to nationwide dissemination. Emergency vaccination and reduced vector activity during winter curbed transmission; however, sporadic cases were reported in 2024 among newborn calves and pregnant cows with incomplete vaccine coverage. Comprehensive analysis of these outbreaks is essential to understand the transmission dynamics and inform future control strategies.

Methods
Samples were collected from suspected LSD cases in cattle across South Korea during 2023 and 2024. Molecular diagnosis was performed using real-time PCR targeting the LSDV genome. For selected positive cases, next-generation sequencing (NGS) was conducted to analyze the genetic characteristics of the circulating LSDV strains. Phylogenetic analysis included 9 LSDV-positive cases from South Korea in 2023, 29 cases from South Korea in 2024, and 110 representative LSDV genome sequences from NCBI, selected to cover major clusters and geographic regions for comparative analysis. In addition, serological investigation was carried out using the ID-VET Capripox Double Antigen ELISA kit, targeting both clinically affected and in-contact cattle from outbreak farms in 2024.

Results
Phylogenetic analysis revealed that all LSDV strains detected in South Korea during 2023 and 2024 belonged to lineage 2.5, indicating limited genetic diversity among circulating strains. Serological testing of both symptomatic and in-contact cattle from affected farms in 2024 showed an overall antibody positivity rate of 25.04%.

Conclusion
These findings indicate that the recent LSD outbreaks in South Korea were caused by LSDV lineage 2.5, with little evidence of genetic variation among isolates. The relatively low antibody positivity rate observed in outbreak farms highlights the need for improved vaccination strategies and ongoing serological surveillance to enhance herd immunity and prevent future outbreaks.

Results of West Nile virus surveillance in the Republic of Serbia in 2024

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West Nile disease is a vector-borne zoonosis. The causative agent is a single-stranded RNA virus belonging to the family *Flaviviridae*, genus *Orthoflavivirus*, species *Orthoflavivirus nilense*. Nine genetic lineages of WNV are proposed. The virus is widely distributed in Africa, Europe, America, the Middle East, Australia, and West Asia. Mosquitoes are primarily responsible for virus transmission, but oral routes of infection are also possible. The virus has an enzootic cycle of transmission between mosquitoes and birds, while humans, horses, and other mammals are dead-end hosts, they don't develop viremia high enough to allow further transmission. Migratory birds play a key role in the spread of the virus over long distances. They can be infected but clinically asymptomatic or can develop fatal neurological symptoms leading to death. Humans can also develop a disease with a wide range of symptoms, which depend on the patient's age and immune status.

VSI "Kraljevo" is NRL in Republic of Serbia for West Nile disease in animals. As part of WNV active surveillance in Serbia, serological tests are carried out in horses (IgM antibodies) and in young cattle (IgG antibodies). Molecular testing for virus presence in vectors and dead/shot susceptible bird or their pharyngeal swabs, is carried out. In passive surveillance, sick/dead horses are serologically (paired sera) and molecularly tested for WNV or anti-WNV Ab presence. During monitoring in 2024 serological tests revealed the presence of IgM antibodies in 0.71% horse sera (8 out of 1113) and the presence of IgG antibodies in 4.5% cattle sera (103 out of 2288). The presence of virus was detected by RT-qPCR in 1.75% of mosquito samples (12 out of 684) and in 3.54% of wild bird samples (16 out of 451). Based on Ct value, location and sampling date, 9 samples were selected for NGS. Sequencing was performed on MinION (Oxford Nanopore platform) using the amplicon-based protocol for lineage 2 directly from clinical samples, which was developed at VSI "Kraljevo". These isolates showed the greatest similarity with viruses from Hungary. Phylogenetic test results indicated that the viruses analyzed are part of the Balkan clade of the West Nile virus.

Surveillance of African swine fever in domestic pigs and wild boar population in non-infected area, during period 2014 and 2024 in Slovenia

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African swine fever (ASF) is a complex and lethal disease of swine that has a significant negative impact on regional, national and international trade.

In Slovenia, both passive and active ASF surveillance were initiated in 2014, after the first confirmed cases of ASF in the European Union. Although three neighbouring countries (Hungary, Croatia and Italy) have been affected by African swine fever virus (ASFV) for several years, all tested samples in Slovenia during this period have returned negative results, indicating the absence of ASFV infection. Between 2014 and 2024, within passive surveillance a total of 7027 samples of domestic pigs from 3478 holdings were tested using real-time PCR method.

In parallel, active surveillance of hunted wild boars involved 5020 serum samples tested by ELISA for detection ASF antibodies. Additionally, 1737 wild boars found dead or involved in road/rail traffic accidents were tested for ASFV using real-time PCR as part of passive surveillance.

In response to outbreaks of ASF in Croatia in 2023, close to the southern border of Slovenia, the ASF high risk area was established in Slovenia. It this area, enhanced passive surveillance was implemented, including the testing of 829 wild boars' samples for ASFV, out of which 271 samples were tested also for the presence of antibodies.

The ASF awareness program in Slovenia is very intensive, and clear reimbursement policies and penalties ensure strong cooperation with hunters. Hunters are well aware of their role in ASF prevention and early detection of ASF in wild boars. As a result, both passive and active surveillance were strengthened during this period.

However, the high risk of ASFV incursion into Slovenia remains high. Therefore, the implementation of an early detection system, and ongoing preventive activities, including passive and active surveillance, are essential to maintaining the country's favourable situation and ASF free status.

Mapping cross reactive and serotype specific motifs within the GH-loop of foot and mouth disease virus serotypes O and A

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Background: Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed animals caused by a virus (FMDV) with seven antigenically distinct serotypes. There is no cross-protection between serotypes, yet existing antibody-detection ELISAs have poor serotype specificity and consequently are not able to accurately define the immunological history of infected or vaccinated animals in endemic settings where multiple FMDV serotypes may be present.

Hypothesis: We hypothesise that the G-H loop, which is an important antigenic site present on the surface of the FMDV capsid, can act as a serotype specific target to develop novel immunoassays to overcome the non-specificity observed with the established commercial serological immunoassays.

Methods: This study describes the development of indirect ELISAs that use different sets of peptides that represent the G-H loops for serotype O and A FMDVs. Full length wildtype peptides (35-mer), and truncated overlapping peptides (21-mer) and alanine scanning peptides (24-mer) were tested against a panel (n=267) of monovalent sera from cattle exposed to all the FMDV serotypes to test the sensitivity and specificity of these antigens. These results were compared to those generated using virus neutralisation using the corresponding virus isolates.

Results and Discussion: Results showed native and peptides that were downstream of the RGD receptor-binding motif had similar reactivity profiles with better sensitivity of 69% and specificity of 95% observed for serotype O compared to 38% and 85% for serotype A peptides respectively. Alanine scanning peptides demonstrated that the 147DLGXL150 motif was critical amino acids for serotype A sera binding. However, some monovalent Asia 1 sera was cross-reactive with the serotype A peptides. In conclusion, the G-H loop cannot be used as a single determinant for FMDV serotype specificity in a serological ELISA, although further studies using engineered peptides may improve the specificity of the ELISAs.

Impact: The improvement of serotype-specific assays will provide tools to enhance FMD surveillance, diagnosis and post-vaccine monitoring and thus, effective control of the disease.

Vaccines

Resistance of DNAJC14 Gene Edited Pigs to Classical Pestiviruses

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Diseases caused by classical pestiviruses, such as classical swine fever virus (CSFV) or bovine viral diarrhea virus (BVDV), remain a major impediment to global livestock production. Outbreaks caused by CSFV incur enormous losses due to culling and export restrictions, and BVDV infection is a serious detriment to cattle health and productivity. The pestivirus genome encodes a single polyprotein that is co- and post-translationally cleaved by both virus and host proteases. *In vitro* studies have identified the host protein DNAJC14 as a core component involved in cleavage of the viral NS2/3 protein. Specifically, DNAJC14 acts as a cofactor of the NS2 autoprotease, which is vital for the replication of non-cytopathic pestiviruses. Where key interactions between viruses and host proteins exist, it is possible to rationally devise intervention strategies using gene editing. Using CRISPR-Cas9 we produced a cohort of pigs with edits to DNAJC14, resulting in either deletion of a region of the protein or a W576A alteration which prevents NS2/3 cleavage. No changes from normal health status were observed in gene edited animals indicating no adverse effect of the alteration. Primary cells from these animals did not support replication of either classical swine fever virus (CSFV) or bovine viral diarrhea virus (BVDV) *in vitro*. Transfection of these primary cells with a plasmid encoding unedited DNAJC14 restored viral replication. In contrast to unedited control animals, DNAJC14 edited pigs inoculated intranasally with CSFV had no clinical signs of CSF or leukopenia, no detectable viral RNA in blood or tissues and no CSFV specific antibodies by 3 weeks post inoculation confirming they were completely resistance to infection. This establishes gene editing as an additional strategy that can contribute to the control of classical pestiviruses.

Assessment of Efficacy of a Subunit Vaccine against Epizootic Hemorrhagic Disease Virus Serotype 8 (EHDV-8) by vaccination and challenge in cattle

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The efficacy of the Syvac EH VP2 subunit vaccine against epizootic disease virus (EHDV) serotype 8 (EHDV-8) was evaluated in cattle by general and local examination, serological follow up and challenge. Syvac EH VP2 is a subunit vaccine which contains the VP2 protein of the EHDV-8 as an active substance. Twenty-four 3–5-month-old calves were randomly allocated into 3 groups of 8 animals each. One group was vaccinated intramuscularly (IM) with two injections of Syvac EH VP2 containing a standard payload of antigen (T02) at a 28-day interval, the second group was vaccinated with Syvac EH VP2 containing a lower payload of antigen concentration (T03) following the same vaccine administration pattern and the third group was vaccinated with a saline solution and used as control group (T01). Forty-two days after first vaccination, all calves were subcutaneously (s/c) challenged with epizootic hemorrhagic disease virus serotype 8 (EHDV-8) in a target dose of 6.0 Log10 $TCID_{50}$/animal. Vaccinated calves were seropositive for EHDV VP2 ELISA antibodies whereas all calves remained seronegative for EHDV VP7 ELISA antibodies until the day of challenge (D42) confirming the absence of an adventitious natural EHDV infection. For both vaccinated groups a reduced number of calves with viraemia and a significant reduction in viral loads and duration of viraemia was demonstrated in comparison to the control group ($p < 0.05$). After challenge, no animal showed significant signs of disease, including the control group meaning that an evaluation of the efficacy based on lesions and symptomatology was not possible in this study. It is concluded that vaccination with Syvac EH VP2 not only resulted in a significant reduction of viraemia in all calves, but also, calves vaccinated with Syvac EH VP2 and prior to challenge only had antibodies against VP2 protein, they did not have antibodies against VP7. This leads to a perfect result for our DIVA strategy (Differentiating Infected from VAccinated individuals) that plays a key role in control of infections, ultimately leading to a better control of outbreaks of vector transmitted viruses.

Comparison of African swine fever detection by qPCR in oral, nasal and anal swabs during ASF vaccine efficacy studies

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African Swine Fever Virus (ASFV) studies often assess virus shedding using oral (OS), nasal (NS), and anal (AS) swabs. To improve animal welfare, reducing the number of swabs may be considered if research objectives allow. We performed a systematic comparison of ASF viral DNA detection via qPCR in OS, NS, and AS samples during a multicenter vaccine efficacy study conducted at Friedrich Loeffler Institute (FLI), Germany, and at Wageningen Bioveterinary Research (WBVR), Netherlands, within an EU collaboration.

Live attenuated vaccine candidates were tested via oral administration (FLI: 4 groups with n=15/group; WBVR 3 groups with n=10/group). One of the candidates was tested intramuscularly (IM, n=8, WBVR) as an efficacy control, and unvaccinated controls (n=5 at both institutes) were included. All animals were challenged oronasally with the Armenia'08 strain. Swabs were collected during the vaccination and challenge phases, and qPCR was used to detect viral DNA.

At WBVR, ASFV DNA was detected in 7/120 OS (oral vaccines) and 10/32 OS (IM vaccine) samples during vaccination phase. Only 1/32 NS (IM vaccine) and no AS samples tested positive. During the challenge phase, 34/43 OS samples were positive at 2 days post-infection (dpi), compared to 6 NS and 0 AS. NS and AS positivity increased at later time points, but overall, the numbers of positive OS was higher. At FLI, one OS and one NS (out of 237) samples were positive during the vaccination phase. After challenge, 31/64 OS, 18/64 NS, and 11/64 AS samples were positive at 4 dpi. Positive OS and NS numbers equalized later, while AS remained consistently lower.

OS were the most sensitive samples for detecting virus shedding during the vaccination phase and early post-challenge and were superior in numbers or comparable to NS and AS at later stages. These results suggest that OS sampling alone is sufficient for assessing ASFV shedding in vaccine studies, improving animal welfare by minimizing invasive procedures.

Immunological effects and safety of a COVID-19 mRNA vaccine

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During the SARS-CoV-2 pandemic, innovative mRNA vaccines were approved with unprecedented speed. The COVID-19 mRNA vaccines consist of mRNA coding for SARS-CoV-2 spike-glycoprotein, enclosed by lipid nanoparticles (LNPs). The vaccines reliably protect from severe disease and had a positive impact on the pandemic. While the risk-benefit assessment favors the vaccines, rare adverse reactions like myocarditis have occurred, of which the pathogenesis remains elusive.

Our study aims to explore how mRNA vaccines modulate the immune system, with a particular focus on understanding the mechanisms behind the increased risk of myocarditis. Potential explanations could be an aberrant expression of spike-protein in the myocardium leading to apoptosis, LNPs' direct negative impact on cardiomyocytes, or a generalized inflammatory response.

Guinea pigs were vaccinated with mRNA-vaccine or empty LNPs. At specific timepoints, animals were sacrificed to perform necropsies. Tissue samples were analysed by qRT-PCR for cytokine, chemokine and spike expression. T-cell proliferation in isolated PBMCs and spike specific antibody levels in the serum were measured. Selected tissues were analysed by histopathology, including in situ hybridization of spike mRNA and immunohistochemistry of immune cell markers.

Guinea pigs remained clinically inapparent and produced a robust antibody response. Expression analysis showed main reactivity within one week after first or booster immunization. Some animals showed increased Type 1 interferon levels in the heart and elevated chemokines (CCL3, CXCL10) in injection site, draining lymph node, spleen and liver shortly after immunization. Increases in IL-1 were also observed. Histopathology revealed mild lymphoplasmacytic infiltration at the injection site. We aim to determine, if these findings can be correlated with the presence of mRNA vaccine particles.

In view of their versatility and excellent immunogenicity mRNA vaccines have become an indispensable part of our pandemic-preparedness-armamentarium. However, any potential safety issue has to be elucidated and resolved. Our efforts will contribute to this process and thereby enhance general vaccine acceptance.

Testing vaccine candidates for oral immunization against ASFV

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Keywords: African Swine Fever Virus, Oral application, Vaccination

The African swine fever virus (ASFV) is responsible for African swine fever, a devastating disease leading to the development of hemorrhagic-like fever and high mortality in suidae species. The disease spread developed into a panzootic threat with severe consequences for wild boar populations and the pig industry. Despite extensive efforts through biosafety measures and surveillance programs, the ASFV control faces a major challenge by the transmission among wild boar populations. Vaccination could be the missing tool to contain ASFV. However, effective immunization of wild boar is only feasible through oral vaccination via bait consumption and comprehensive data on the efficiency and efficacy of oral vaccination is still missing. Within the EU Consortium ASFaVIP (African Swine Fever attenuated Vaccines In Pigs), a harmonization study was conducted between partners to align methods and readouts. The study aimed to compare efficacy and efficiency of three vaccine candidates for oral administration. After immunization, the animals were challenged with the highly virulent ASFV strain Armenia08 at d28 post vaccination. Clinical signs, serological responses and viral replication were detected. Following, a baiting study was performed to optimize oral vaccine application, exploring whether increased inoculation doses, the addition of adjuvants, or modifications of the bait matrix could improve serological responses. Although the results of the vaccine comparison study revealed only 20 % seroconverted animals, we could show that each responding animal was protected during the challenge phase. Moving on with the best performing vaccine candidate ASFV-G-ΔI177L for the optimization study, we were able to increase seroconversion ratios to 80 % by increasing the vaccine titer and up to 40 % by adapting the baiting strategy. The findings reveal the high efficacy and improvable efficiency of the tested vaccine candidates for oral vaccine strategies.

A VP2 Subunit Vaccine Confers Complete Protection from EHDV-8 Infection in Calves

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Epizootic haemorrhagic disease virus serotype 8 (EHDV-8) has recently emerged in Europe, causing widespread outbreaks among cattle and wild ruminants. The absence of commercial vaccines at the time of emergence underscored the urgent need for effective immunization tools to support disease control efforts.

In this study, we developed and evaluated the safety, immunogenicity, and efficacy of a novel subunit vaccine based on the recombinant VP2 protein (EHDV8 rVP2), produced using a baculovirus expression system, against EHDV 8. Ten Holstein-Friesian calves were enrolled and randomly assigned to vaccinated (n = 5) or control (n = 5) groups. Calves in the vaccinated group received two doses of 200 µg of purified rVP2 protein, administered 21 days apart, while control animals were sham-vaccinated. Fourteen days after the booster dose, all animals were challenged with a field strain of EHDV-8. Clinical signs were monitored daily, and blood samples were collected at regular intervals to assess viraemia and immune response.

The EHDV8-rVP2 vaccine was well tolerated, with only transient fever and mild local reactions observed. Vaccinated calves developed neutralizing antibodies by day 28 post-vaccination and, upon challenge, showed no clinical signs and no detectable viraemia, as confirmed by real-time RT-PCR. In contrast, all control calves developed viraemia and exhibited mild clinical signs.

Notably, vaccinated animals did not seroconvert in the VP7 ELISA, even after challenge, indicating complete suppression of viral replication and confirming the vaccine's DIVA (Differentiating Infected from Vaccinated Animals) compatibility.

Overall, our results demonstrate that the EHDV8-rVP2 subunit vaccine is safe, immunogenic, and provides complete protection against EHDV-8 infection under experimental conditions. Combined with its DIVA capability, rVP2 represents a promising tool for orbivirus disease control and emergency response strategies.

Adjuvant-induced macrophage activation compromises live attenuated vaccine-mediated protection against African swine fever

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The African swine fever (ASF) pandemic is causing enormous economic losses to the global swine industry, yet the development of safe and effective vaccines against the disease remains a major challenge. Subunit vaccine strategies, very appealing due to their inherent safety, still provide insufficient protection. In contrast, some live attenuated vaccines (LAVs) have shown high effectiveness. However, biosafety concerns are limiting their implementation. To address this issue, novel LAV-based vaccination strategies are needed to better balance safety and immunogenicity. A hallmark of the ASF virus (ASFV) and its corresponding LAVs is the infection of macrophages and the inhibition of subsequent innate immunity. Therefore, we hypothesised that incorporating macrophage-activating adjuvants during LAV-based vaccination could increase immunogenicity, enabling the use of lower vaccine doses and thereby improving biosafety. To evaluate this hypothesis, we tested two immunostimulants based on the bacterium *Rothia nasimurium* as adjuvants for the ASF LAV prototype BA71ΔCD2. In vitro studies demonstrated that combining the vaccine prototype with either heat-inactivated *R. nasimurium* (HI-Ro) or a bacterial wash (Frac-Ro) modulated the functional responses in alveolar macrophages. Indeed, BA71ΔCD2-infected cells expressed higher amounts of inflammatory cytokines and activation markers in the presence of these immunostimulants, indicating their potential as vaccine adjuvants. However, *in vivo* co-administration of a suboptimal dose of BA71ΔCD2 with either HI-Ro or Frac-Ro impaired vaccine-induced immunity. Both ASFV-specific humoral and cellular responses were hampered in pigs vaccinated with the two adjuvants, consequently reducing survival rates following a lethal ASFV challenge. To determine whether these results were due to reduced viral antigen levels during immunization, we next evaluated the LAV's capability to replicate in adjuvant-activated macrophages *in vitro*. Importantly, the percentage of BA71ΔCD2-infected cells was lower in the presence of HI-Ro or Frac-Ro, likely as a consequence of the antiviral innate immune responses in activated macrophages. Overall, these results highlight the complex balance between triggering productive innate immunity and ensuring adequate antigen dose levels for effective ASF LAV-based immunization, thus demonstrating that the use of adjuvants to improve vaccine biosafety will require a more targeted and rational design.

Longitudinal BTVPUR®4-8 vaccine trial highlights appropriate BTV vaccination status in cattle under field conditions.

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Bluetongue virus (BTV) is type strain of the *Orbivirus* genus and causes the infectious haemorrhagic disease, bluetongue, in domestic and wild ruminants. Over the last few decades, strains of several BTV serotypes have been in circulation across Northern Europe, including BTV-4, BTV-8 and, most recently, BTV-3. Vaccination remains the most effective control measure. Inactivated monovalent vaccines have been used in the past to control BTV-8 outbreaks in Europe and bivalent vaccines are also available where multiple BTV serotypes co-circulate. These vaccines protect ruminants only from re-infection with strains of a homologous serotype, through generation of protective neutralizing antibodies against the viral outer coat protein, VP2. With at least 28 recognized BTV serotypes, however, this poses a significant challenge to effective control, given the rapidly evolving orbivirus landscape in Europe. To enable development of effective cross-protective vaccines, we require a greater understanding of the ruminant immune response to BTV vaccination. Field reports also suggest that not all cattle have detectable anti-BTV antibodies following vaccination, requiring the need to determine “appropriate vaccination status” for effective control.

Under field conditions, sixty Holstein-Friesian cattle were vaccinated subcutaneously with two doses of the BTV-4/BTV-8 bivalent inactivated vaccine, BTVPUR®4-8 (Boehringer Ingelheim). Cattle were followed longitudinally over one-year to characterize cellular and humoral immune responses to BTV vaccination. Cattle were confirmed BTV-antibody and -RNA negative before the study. Serum was collected from each animal at 0, 1, 2, 3, 6, 9 and 12 months post-prime vaccination. Serum was screened for antibodies against BTV VP7 using the commercial diagnostic competitive ELISA and dynamics of IgM- and IgG-specific antibodies were characterized following vaccination. Serum neutralization tests were used to quantify titres of protective neutralizing antibodies. Whole blood interferon gamma release assays were developed to detect primed T cell responses to BTV-4 and BTV-8 antigens over the course of vaccination.

Here, we highlight in unprecedented detail, for the very first time, results of this longitudinal BTV-4/BTV-8 vaccine field study. We highlight “appropriate BTV vaccination status” in cattle and outline the dynamics of humoral and cellular immune responses up to one-year post-vaccination.

Evaluation of vaccine efficacy against airborne transmission in a preclinical model of SARS-CoV-2

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The primary focus of vaccines developed during the COVID-19 pandemic was to prevent severe disease. However, to effectively stop a pandemic, vaccines must also reduce transmission. Insights into the impact of vaccination on pandemic control showed that vaccines were generally more effective at reducing infectiousness (transmission from vaccinated, infected individuals) than at preventing infection of vaccinated individuals, with combined effectiveness estimates exceeding 70%. However, those insights were gained retrospectively when vaccines were already deployed.

We developed a preclinical (Syrian hamster) model for evaluation of vaccine efficacy (VE) against aerosol transmission of SARS-CoV-2. Initially, we demonstrate that the duration of aerosol exposure (20, 60, or 180 min) of unvaccinated contact (N=4 per duration) to donor hamsters (N=4) infected with an early variant of SARS-CoV-2 (D614G) did not affect transmission, as all contact hamsters became infected (virus was detected in oropharyngeal swabs).

As a follow-up, an even shorter exposure time (5 minutes) was added to previously established durations (20 and 60 minutes). Either vaccinated (with mRNA vaccine) or unvaccinated contact hamsters were exposed to vaccinated or unvaccinated donor hamsters (N=4 per vaccination status and exposure duration). The results confirmed that exposure duration did not influence transmission in our model. Transmission was analyzed by quantifying the reproduction number (R) for each donor-contact combination. For transmission between unvaccinated donors and contacts, RUNV= 6, and was used as the reference for estimating VE. Vaccination reduced susceptibility (transmission from unvaccinated donor to vaccinated contact) by 33% (RS= 4), and infectiousness (transmission from vaccinated donor to unvaccinated contact) by 53% (RI=2.8). When both donor and contact hamsters were vaccinated, overall transmission was reduced by 89% (RT = 0,67), resulting in $R < 1$ (meaning that the infection will gradually die out).

Interestingly, vaccinated donors had higher viral loads in oropharyngeal swabs than unvaccinated donors, suggesting that viral load in these samples is a poor transmission predictor.

The results of this preclinical model align with epidemiological observations in humans, indicating that it can be used as a valuable tool for assessing the potential of candidate vaccines to control epidemics and pandemics.

Adenovirus-Based Subunit Vaccines Elicit Cross-Protective Immunity Against Multiple Serotypes of Epizootic Hemorrhagic Disease Virus

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Epizootic hemorrhagic disease virus (EHDV) is an emerging arbovirus in Europe, causing substantial economic losses in the ruminant industry. Current vaccines offer limited cross-serotype protection and lack DIVA (Differentiating Infected from Vaccinated Animals) capability, hindering effective disease control. In this study, we developed replication-defective adenoviral vectors expressing EHDV structural proteins VP2 from serotype 8 (Ad-VP2-8) and VP7 from serotype 2 (Ad-VP7) to assess their immunogenicity and protective efficacy in a murine challenge model. Immunization with Ad-VP2-8 and Ad-VP7 induced cross-reactive T cell responses between EHDV-6 and EHDV-8. Immunization solely with Ad-VP2-8 elicited high neutralizing antibody titers against homologous EHDV-8 and low titers against heterologous EHDV-6, indicating limited cross-serotype antibody reactivity. Protection against EHDV-8 was achieved with Ad-VP2-8, but not against EHDV-6, as shown by viremia levels in EHDV-6 challenged mice similar to those in the control group. In contrast, Ad-VP7 vaccination conferred protection against both serotypes, despite the presence of viremia, suggesting non-sterile immunity. Importantly, combined immunization with Ad-VP2-8 and Ad-VP7 conferred protection against both EHDV-6 and EHDV-8, with minimal or no detectable viremia. These results demonstrate that a multivalent adenovirus-based subunit vaccine can induce robust and cross-protective immune responses, offering a promising DIVA-compatible strategy for EHDV control. This approach could enhance disease surveillance and reduce the burden of EHDV in livestock populations.

The First Experimental Inoculation of Cattle Against Rinderpest in 18th-Century Slovenia

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Rinderpest was a highly contagious disease of even-toed ungulates, caused by the Rinderpest virus from the *Morbilivirus* genus. Known for its high morbidity and mortality, the disease had devastating socio-economic impacts. In the 18th century, several epizootics occurred in the Lower Duchy of Styria (modern-day Slovenia), severely affecting rural communities. In response, Holy Roman Emperor Charles VI issued a decree in 1731 outlining measures to be taken during outbreaks. Around the same time, medical experts across Europe began experimenting with inoculation methods, inspired by the approach used for smallpox in humans.

Rinderpest was introduced to Styria in 1777 through infected cattle from Croatia. Outbreaks continued annually until 1780, when the disease gradually subsided. During this period, an estimated 10,000 cattle perished, giving rise to the term “Great Styrian Plague.” Paul Adami (1736–1814), a professor of veterinary medicine at the Medical Faculty of Vienna, observed the outbreak and decided to apply the inoculation technique previously attempted elsewhere in Europe.

Count Ferdinand Attems (1746–1820), a nobleman from Slovenska Bistrica and patron of science, provided twenty cattle from his estate for Adami’s experiment. Ten animals were selected—seven were inoculated with infectious material, while three served as controls. The inoculum, derived from purulent nasal discharge of infected cows, was absorbed into cotton threads and inserted into skin incisions between the ribs. The first trial failed—all ten animals succumbed to the disease.

In a second attempt, Adami purchased six cattle and inoculated two with a reduced dose using smaller incisions. Both animals became ill but fully recovered after eleven days. Concurrently, he studied the infectiousness of barns, tools, feed, and other materials, and assessed the protective effect of inoculation. His findings were published in 1781 in *Contributions to the History of Livestock Plagues in the Imperial-Royal Hereditary Lands*.

Adami’s early vaccination experiment was a pioneering achievement in veterinary science—especially significant given the widespread scepticism toward inoculation in the Habsburg monarchy. Conducted on Slovenian territory, his work marks a foundational moment in the development of empirical veterinary science. Rinderpest vaccination later proved successful, the disease is globally eradicated.

Pericarditis in vaccinated pigs that had survived African swine fever virus challenge

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Insights about the protective host responses after African swine fever virus (ASFV) infection are largely lacking. To address this question, pigs were immunized in two different studies with low doses of moderately virulent attenuated ASFV strains. A substantial number of vaccinated pigs (80%) survived ASFV challenge. However, a pericarditis was observed in all survivors at necropsy two weeks post challenge. Other organs did not show macroscopic lesions at the end of the study. In contrast, non-survivors showed clinical signs and ASFV related pathology during necropsy with occasionally acute myocardial hemorrhages, but no clear pericarditis. Heart tissue of survivors and non-survivors was compared using hematoxylin-eosin staining and immunohistochemistry staining for several immune cell markers. The pericarditis in surviving pigs was characterized by infiltrates of round cells, which were positive for immune markers such as CD3 (a marker for T-lymphocytes) and CD21 (a marker for a subset of B- lymphocytes). Transcriptional analysis of heart tissue indicated a prominent increase in transcripts associated with cytotoxic cells and B-cells. No bacterial cause was identified. These preliminary findings suggest immune-mediated pericarditis in vaccinated pigs after challenge infection with virulent ASFV. Further investigation of the pathogenesis of the pericarditis and its relevance and association with the immune status of the vaccinated animals is currently being performed.

Reprogramming viral fitness: codon pair deoptimization in FMDV serotype O

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Foot-and-mouth disease (FMD) remains one of the most economically significant viral diseases in livestock farming. Many industrialized countries have eradicated FMD using inactivated whole-virus vaccines, but these have some critical shortcomings. Inactivated vaccines are expensive and dangerous to produce and the duration of immunity is shorter than after a natural infection — issues that could potentially be overcome by live-attenuated vaccines (LAV).

One approach to developing an FMD LAV is codon pair deoptimization (CPD), which increases the frequency of suboptimal codon pairs in the viral genome without changing the amino acid sequences. The exact mechanisms leading to attenuation are not fully understood, but factors such as translation efficiency, RNA stability, protein expression, protein folding, and dinucleotide frequency likely play a role.

In this study, CPD was applied to the P1 capsid-coding region of FMDV serotype O. A significant attenuation of replication of the deoptimized virus compared to the wild type was observed in LFBK-αVβ6 cells. Pigs inoculated with 10^5 $TCID_{50}$ of CPD virus into the bulb of the heel did not show any signs of infection, but also did not seroconvert. Inoculation with a significantly higher dose (4.5×10^6 $TCID_{50}$), on the other hand, led to clinical FMD indistinguishable of wild-type infection.

These results highlight both the potential of CPD for the development of safe FMDV vaccines and the challenges of evaluating such vaccines in vitro and in vivo. Further optimization is required to ensure that a CPD FMDV LAV can achieve adequate protection in vivo while maintaining sufficient attenuation.

One Health - Wildlife Diseases, Zoonoses

Zoonotic Risk Assessment of Mammalian Orthoreovirus (MRV) and Rotavirus A (RVA) in Pet Dogs and Cats: A One Health Study

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Mammalian Orthoreovirus (MRV) and Rotavirus A (RVA) are segmented, double-stranded RNA viruses belonging to the *Spinareoviridae* and *Sedoreoviridae* families, respectively. Both are capable of infecting mammals, including humans. The segmented genomes of MRV and RVA facilitate reassortment events, driving viral evolution and the emergence of new variants, which raises significant concerns about their zoonotic potential. Reports of human MRV infections describe identified strains as reassortants resulting from spillover events. In humans, these viruses can cause severe enteritis, acute respiratory infections, and encephalitis. However, limited information is available regarding pets and the role they may play in transmitting these viruses. Given the frequent contact between humans and pets, it is important to enhance surveillance of MRV and RVA in companion animals.

Since April 2023, in collaboration with several veterinary clinics across north-eastern Italy, faecal samples were collected from 248 dogs and 265 cats, both asymptomatic and exhibiting gastrointestinal symptoms, and tested for MRV and RVA using a customised multiplex real-time RT-PCR (rRT-PCR) assay. Samples were also subjected to a basic differential diagnostic panel including Parvovirus (rPCR), Coronavirus (rRT-PCR), and qualitative coprological examination. For each enrolled animal, epidemiological data were collected by veterinarians.

Sampling is still ongoing; so far, two dogs and two cats have tested positive for MRV, while four dogs and five cats have tested positive for RVA. Viral isolation in VERO cells was successful for three out of four MRV-positive samples, and full genome sequencing is currently underway. Attempts to isolate RVA from positive samples are ongoing.

All sequenced samples will undergo phylogenetic analysis to characterise the viruses and compare them with human MRV strains, in order to assess the zoonotic risk.

Considering the increasing presence of pets in households and the growing interest in Animal-Assisted Interventions for children and vulnerable people, the early detection of potential interspecies transmission and viral reassortment is crucial within a One Health framework.

Funding: Ministero della Salute, Ricerca Finalizzata 2021 (SG-2021-12374649).

Surveillance of tick-borne pathogens on selected localities in Slovakia

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The prevalence of pathogens in vector populations is considered an important part of assessment the risk of infection in host species. The aim of this work was to perform surveillance for *Borrelia* spp. and TBEV in tick populations in selected localities in Slovakia. *Borrelia* spp., the causative agent of Lyme disease, circulates in complex natural cycles involving vertebrates (reservoir and host species) and vectors (ticks). Currently, there are approximately 50 known endemic areas of tick-borne encephalitis in Slovakia; the number of TBE cases in humans has been on an upward trend since 2000.

From 26 localities throughout the Slovak Republic, 1 485 ticks (1 438 *Ixodes ricinus*, 23 *Dermacentor reticulatus*, 9 *Dermacentor marginatus*, 11 *Heamaphysalis concinna*) were collected by flagging and examined in the laboratory by PCR and RT-qPCR. Molecular genetic methods confirmed 505 ticks (34.0%) infected with *Borrelia* spp. The highest prevalence of *Borrelia* spp. (52.1%) was found in an urban park (Nitra); of 71 ticks collected in that location, positivity was confirmed in 37 ticks; the presence of TBEV was not detected in any of the examined samples, supporting the concept of active circulation of TBEV in microfocuses.

Acknowledgment: Co-funded by the European Union under the project 101132974 - OH SURVector. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or European Health and Digital Executive Agency (granting authority). Neither the European Union nor the granting authority can be held responsible for them.

OH4Surveillance – Setting Up a Coordinated Surveillance Under the One Health Approach in a Belgian Context

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OH4Surveillance (One Health for Surveillance) is a EU4Health-funded, multidisciplinary initiative running from 2024 to 2026, aimed at strenghtening zoonotic disease surveillance under a One Health approach. By fostering cross-sector and cross-border collaboration, OH4Surveillance aims to improve surveillance systems, early warning capabilities, and support coordinated responses to emerging zoonotic threats across Europe. Belgium is part of a consortium of 11 participating countries, coordinated by Denmark.

In Belgium, the project targets three zoonotic diseases of significant public health relevance: Highly Pathogenic Avian Influenza (HPAI), West Nile Fever (WNF), and Q fever. For HPAI, a multidisciplinary surveillance strategy builds on existing monitoring of wild birds and mammals (rodents and carnivores) and has been expanded to include environmental sampling to further track virus circulation and potential interspecies transmission. Surveillance for WNF includes both active and passive monitoring of wild bird populations across Belgium's regions to detect potential virus introduction and support early warning efforts. Regarding Q fever, the project focuses on innovative surveillance strategies, including airborne pathogen detection through air sampling and the investigation of rodent reservoirs. These efforts aim to address gaps in current surveillance and improve understanding of disease transmission.

During the first year of the project, surveillance activities were initiated for HPAI and WNF, while Q fever efforts focused on capacity building, protocol development, and stakeholder engagement. HPAI sampling showed no active infections in wild birds, though serological testing indicated past exposure. All rodents tested negative, while some foxes showed seropositivity, suggesting previous exposure. Environmental samples were negative. For WNF, all samples obtained through active and passive surveillance of wild birds tested negative in PCR. Nevertheless, recent WNV circulation in neighbouring countries underscores the importance of maintaining early warning systems. For Q fever, capacity building included evaluating sampling strategies based on randomly collected rodents throughout the Flemish region, which revealed a very low prevalence. As a result, future surveillance efforts will focus on targeted rodent collection on farms.

The initial phase of the OH4Surveillance project strengthened the basis for integrated zoonotic surveillance in Belgium. Ongoing collaboration and methodological refinement will be essential to improve preparedness and define fit-for-purpose approaches.

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