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SURVEILLANCE OF ZOONOTIC AND NON-ZOONOTIC BOVINE VIRUSES CIRCULATING AMONG CATTLE IN CATALONIA AND CHARACTERIZATION OF THEIR EXCRETED VIROME

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Diana de Oliveira Ribeiro

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Training placement report presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by
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July 2024

RESUMO

Vírus zoonóticos e não zoonóticos de interesse veterinário podem chegar ao ambiente por diversas vias, dependendo do seu mecanismo de excreção, sendo capazes de infetar outros animais e humanos continuamente. Segundo o conceito da “One-health”, é importante melhorar a preparação e alerta precoce de possíveis surtos, com potencial para prejudicarem vidas animais e humanas, mantendo a sustentabilidade ambiental. O objetivo deste trabalho era estudar o potencial do uso de amostras ambientais coletivas para a deteção de vírus de interesse veterinário e/ou com potencial zoonótico em circulação entre gado, reduzindo a necessidade da coleta de amostras clínicas que requerem o uso de métodos invasivos nos animais. Adicionalmente, novos protocolos operacionais standardizados para n(RT)PCR e (RT)qPCR foram implementados no laboratório. Entre maio e junho de 2023, amostras individuais de saliva e fezes em conjunto com amostras coletivas de água potável e palha foram coletadas em 4 quintas de gado para comparar a deteção por (RT)qPCR de vírus selecionados. Estas amostras foram analisadas para otimizar os métodos usados ao longo do trabalho. Uma campanha de amostragem de amostras coletivas foi implementada: 16 lixiviados e 16 aerossóis de 2 quintas de gado foram coletados desde setembro de 2023 até abril de 2024 e 8 amostras de água residual de 2 matadouros de gado foram coletadas entre fevereiro e abril de 2024. Para a deteção viral, todas as amostras coletivas foram submetidas a um procedimento de concentração viral seguido da extração de ácidos nucleicos. As amostras foram testadas para vários vírus bovinos por (RT)qPCR: BPyV, RoV-A, BCoV, BRSV, CCHFV, EHDV, BTV e AIV. O BPyV pareceu ser um bom indicador viral bovino em amostras de lixiviados (13/15) mas não em amostras clínicas (2/99) nem em amostras de água residual de matadouros (5/8, com baixas concentrações). No geral, o RoV-A foi detetado em 31,25% dos lixiviados das quintas e em 75% das amostras de águas residuais dos matadouros e o BCoV foi detetado em amostras de lixiviados e de aerossóis e estava presente em 50% das amostras do matadouro de Sabadell, provando a circulação do vírus em gado na Catalunha. Os vírus AIV, CCHFV, BRSV, EHDV e BTV não foram detetados em nenhuma das amostras individuais e coletivas testadas por (RT)qPCR. Um n(RT)PCR para a deteção do EHDV (com alvo principal nos serotipos 6 e 8) foi desenvolvido no laboratório e mostrou ser eficiente para concentrações tão baixas como 250 GC/rx em amostras biológicas. Para além disso, NGS foi realizado nas amostras ambientais coletivas usando uma plataforma Illumina NextSeq 550 acoplada com enriquecimento do alvo para a caracterização do viroma de gado. O NGS permitiu a deteção de 16 genes virais relacionados com infeções bovinas. Outros vírus não bovinos foram também detetados nas amostras coletivas, incluindo vírus não detetados por (RT)qPCR, como BRSV e BPyV em aerossóis. Contudo, o método mostrou algumas dificuldades em capturar genomas de cadeia dupla de RNA, como é o caso do RoV-A, detetado em grandes quantidades por (RT)qPCR. Neste trabalho, o uso de amostras ambientais coletivas revelou-se apropriado para a vigilância de vírus em circulação na indústria de gado, sugerindo o seu potencial uso como uma ferramenta de deteção precoce de surtos virais de interesse veterinário na população de vacas, ou para monitorizar a circulação de vírus potencialmente zoonóticos.

Palavras-chave: Vírus bovinos; Vigilância viral; Amostras ambientais coletivas; (RT)qPCR; Viroma.

ABSTRACT

Zoonotic and non-zoonotic viruses with veterinarian interest can enter the environment through different routes, depending on its shedding mechanism, being able to infect other animals and humans continuously. Following the concept of One-health, it's important to enhance preparedness and early warning of possible viral outbreaks, with potential to jeopardize the lives of animals and humans while maintaining the environment sustainability. The goal of this work was to study the potential of using collective environmental samples to detect viruses of veterinarian interest and/or with zoonotic potential, circulating among cattle, reducing the need for the collection of individual clinical samples that require the use of invasive methods in the animals. In addition, new SOPs for n(RT)PCRs and (RT)qPCRs to detect these viruses were implemented in the laboratory. Between May and June of 2023, individual saliva and faeces samples together with collective samples such as drinking water and bed straw were collected in 4 cattle farms to compare the detection by (RT)qPCR of selected viruses. These samples were analysed to optimize the methods used in this study. A sampling campaign of collective samples was implemented: 16 lixiviates and 16 aerosol samples from 2 cattle farms were collected from September to April 2023-2024 and 8 wastewater samples from 2 cattle slaughterhouses were collected between February and April 2024. For viral detection, all the collective samples were submitted to a viral concentration procedure followed by nucleic acid extraction. Samples were tested for several bovine viruses by (RT)qPCR: BPyV, RoV-A, BCoV, BRSV, CCHFV, EHDV, BTV and AIV. BPyV seemed to be a good bovine viral marker in lixiviate samples (13 /15) but not in clinical samples (2/99) nor in wastewater samples from slaughterhouses (5/8 with very low concentrations). Overall, RoV-A was detected in 31,25% of the lixiviates from the cow farms and in 75% of the wastewater samples from slaughterhouses and BCoV was detected in both lixiviates and aerosol samples and it was present in 50% of the samples of Sabadell slaughterhouse, proving the circulation of the virus among cattle in Catalonia. The viruses AIV, CCHFV, BRSV, EHDV and BTV weren't detected in any of the individual and collective samples tested by (RT)qPCR. A nested (RT)PCR for the detection of EHDV (targeting mainly the serotypes 6 and 8) was developed "in house" and it showed to be efficient for concentrations as low as 250GC/rx in biological samples. Furthermore, NGS was performed on the collective environmental samples using an Illumina NextSeq 550 platform coupled with a target enrichment to characterize the virome. The NGS enabled the detection of 16 viral genus related to bovine infection. Other non-bovine viruses were also detected in collective samples including viruses not detected by (RT)qPCR techniques, such as BRSV and BPyV in aerosols. However, the method showed difficulties in capturing dsRNA genomes, as the case of RoV-A, detected in high amounts by (RT)qPCR. In this work, the use of collective environmental samples revealed to be suitable for surveillance of viruses circulating in cattle industry, suggesting it's potential use as an early detection tool of viral outbreaks of veterinary interest in the cows' population, or to monitor the circulation of viruses with zoonotic potential.

Keywords: Bovine viruses; Viral surveillance; Environmental collective samples; (RT)qPCR; Virome.

AKNOWLEDGMENTS

This incredible journey comes to an end, and I want to thank everyone that made part of that. Doing my master thesis in Erasmus in the University of Barcelona was the best decision in my academic and social life. I was really lucky with all the people I met during this experience and for the incredible group who welcomed me so well in the lab.

Firstly, to my supervisors, **Sílvia** for accepting me so easily in the project even though I didn't speak Catalan (now it's better!) and for creating a very comfortable and easy-to-learn environment, and **Xavi** for teaching me so much, guiding me so well in my work and for the all the trust placed in me. I never thought I could have such a close relationship with my supervisors as I had with you both, so thank you for that! Also, to **Marta R.**, who helped me a lot during the first days in the lab and took me to the first samplings in the farms (remembered by the bird's surprise!). To **Rosina**, the first person I contacted and who open the doors for me to the group. From ESB-UCP, to **Célia Manaia**, my tutor from Portugal always available for my doubts and for advising me to do the entire thesis abroad.

Now, to VIRCONT, the people who made me feel at home when I was so far from that and the best lab group I could have ever asked. **Cris** and **Marta I.**, our PhDs, thank you for all the patience to our questions, for teaching me so many things in the lab and to take us out of the lab to do a coffee. **Sandra**, I'm so glad you came on time so I could meet you and learn with you, you were incredible with me, always available to help and with a positivity and confidence that I needed for the most stressful times. Finally, the Xiquets, the VIRALERT team, the lab 8 force. **Júlia** and **Ignasi**, the inseparable team who made me feel part of their group, you've made the workdays so much better with your joy and craziness that I loved, you are incredible persons, and I won't forget the "no es pot treballar tant!". To **Ona**, who came to our team later but turned out to be the best person I met in my Erasmus, I'll miss the "enough English Diana, enough", the long days of working together in the lab, our road trips to farms with long talks and all the support you always gave to me. I've gained a lot of affection for everyone and whenever I return to Barcelona I'll visit you. Thank you for everything!

On the other hand, to the people I had the opportunity to meet in Erasmus, **Carol, Pedro, Catarina, Bea, Peigi, Finn, Nader, Sarah, Brent, Gabs** and **Emma**, who became a group of friends, with whom I lived incredible moments in Barcelona. Also, to all my friends from home that are always an important support, even far away, specially Inês and Elza who had the time to visit me. To **Andreia**, one of my best friends, for the dedication to our friendship, for always knowing the answers to my questions, for always receiving me so well in Múrcia and turn me into a basketball fan.

Finally, to the people that are always by my side no matter what. To all my **family**, specially **avó Fátima** for all the support for my stay in Barcelona and **avó Ana** for the words of encouragement. To **my parents**, thank you for doing everything for me, for supporting me in all my dreams, for giving me what I need to experience so many things and follow all the opportunities, for constantly worrying about my comfort and for loving me as you always do. To **Gonçalo**, my biggest support, thank you for never letting me give up, for always being there even at 1000 km away, for putting me first and for believing in me more than I believe, I can't thank you enough for everything you do for me.

In the end, the most important are the people!

CONTENTS

ABREVIATIONS AND SYMBOLS

AIV – Avian influenza virus

BCoV – Bovine coronavirus

BLAST – Basic local alignment search tool

BPyV – Bovine polyomavirus

BRoV – Bovine rotavirus

BRSV – Bovine respiratory syncytial virus

BTV – Bluetongue virus

CCHFV – Crimean-Congo haemorrhagic disease virus

cDNA – complementary DNA

ddNTPs – Dideoxy nucleoside triphosphate

EHDV – Epizootic haemorrhagic disease virus

GC – Genomic copies

HPAIV – Highly pathogenic avian influenza virus

LPAIV – Low pathogenic avian influenza virus

ml - mililiter

MST – Microbial source tracking

NGS – Next generation sequencing

n(RT)PCR – nested reverse transcription polymerase chain reaction

NTC – Negative template control

PAdV – Porcine adenovirus

PBS – Phosphate buffered saline

qPCR – quantitative polymerase chain reaction

RoV – Rotavirus

RoV-A – Rotavirus A

r.t – room temperature

(RT)qPCR – Reverse transcription quantitative polymerase chain reaction

rx - reaction

SISPA – Sequence-independent single-primer amplification

SOP – Standard operational protocol

TES – Target enrichment sequencing

WBE – Wastewater based epidemiology

WHO – World health organization

WW – Wastewater

µl - microliter

1. INTRODUCTION

1.1. Zoonotic viruses and viruses of veterinarian interest

Viral zoonoses are infectious diseases caused by zoonotic viruses, which are viruses of animal origin that can be transmitted directly or indirectly from vertebrate animals to humans, significantly impacting the public health and the economy of the world. Viruses that are only transmissible between animals are referred to viruses of veterinarian concern. According to the WHO, about 75% of the infectious agents that emerge and remerge in humans have a primary source in the animals or their products [3]. In fact, the biggest epidemic events in the last decades were caused by viruses transmitted from animals to humans, being the most remarkable cases the 2009 H1N1 pandemic caused by Influenza virus generated by the reassortment of human, swine and avian influenza viral genes, and the 2020 Covid-19 pandemic caused by SARS-CoV-2 virus . These zoonotic viruses may also end up being transmitted from humans to animals (reverse-zoonosis), significantly increasing the viral reservoir and consequently the appearance of new variants, as some studies already showed [4]. Moreover, the continuous increase of the world population will cause more and more anthropogenic pressure on wildlife and the environment, which will contribute to a higher probability of pathogen spillover from animals to humans [5].

Considering only the zoonotic diseases reported between 1990 and 2010, approximately 94% were caused by RNA viruses. This can be explained by their greater variability in relation with DNA viruses, mainly due to the lack of proofreading capability of their RNA dependent RNA polymerase that leads to more errors in the RNA synthesis, followed by a natural selection process that acts on these variable population in favour of the most resistant viruses [6, 7].

Zoonotic viruses can be divided in two major groups related with the primary source of transmission to humans: the vertebrate-borne viruses that are transmitted directly from vertebrate animals like rodents, foxes, bats, birds, pigs, cows and others, and vector-borne viruses that are primarily transmitted by arthropods like mosquitoes and ticks [8]. Some of the most significant zoonotic viruses that have emerged or re-emerged in the last decades in different continents, including Europe, are Influenza A, Ebola virus, Zika virus, MERS-Coronavirus (MERS-CoV) and most recently SARS-Coronavirus 2 (SARS-CoV2) [8-12].

Beyond zoonotic viruses that cause harm in the population, there are other non-zoonotic viruses of veterinarian concern with a big economic impact in the livestock industry due to the mortality and morbidity caused among animals, which includes reduced feed efficiency, lower growth rates, less production of milk and meat, and abortions [7]. For example, Bovine coronavirus (BCoV) is responsible for a respiratory or/and gastrointestinal disease in animals and is one of the main problems in beef cattle farming, even in vaccinated cows, by reoccurrence of the virus, having already showed high positive rates in several countries around the world, including an epidemic trend after 2010 [13, 14]. The Epizootic haemorrhagic disease virus (EHDV) and the Bluetongue virus (BTV) are vector -borne viruses with no human cases reported but that are able to cause severe symptoms in cattle such as several

haemorrhages and respiratory complications, with great economic losses for farmers due to the high morbidity provoked [15].

The increasing likelihood of emerging disease outbreaks shows the critical importance of early detection of unusual illness or circulation of pathogens based on the 'One health' approach: surveillance at the human, animal, and environmental interfaces (**Figure 1**).

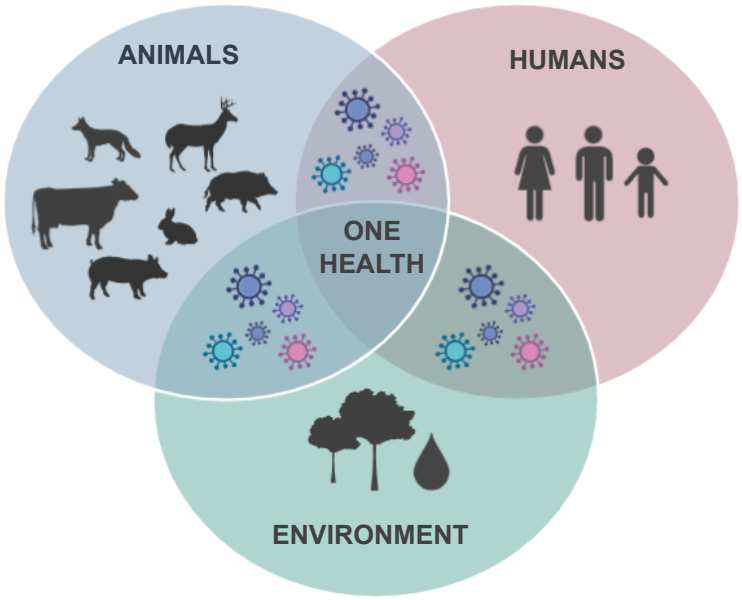


Figure 1 – The One health approach. Created with BioRender.

At the moment, there are already many organizations working to control or even eradicate infectious diseases around the world, including zoonoses, and the use of animals as a surveillance tool is widely reported by organizations such as the Centre for disease control and prevention (CDC), the Centre for One Health research (COHR), the World organization for animal health (WOAH) and the World health organisation (WHO) among others.

Knowing that most of the human health hazards have a zoonotic origin, the impact of epidemics can be mitigated or even eradicated if animals are properly used for pathogen surveillance [5].

1.1.1. Bovine viruses of veterinarian interest

1.1.1.1. Bovine coronavirus (BCoV)

BCoV is a respiratory and enteric virus due to its tissue tropism for both respiratory and intestinal tract, which may result in serious damages to both organs. The infection of the intestine can cause diarrhoea in cattle at any time of the year, although is more predominant during cold seasons. It's known for causing syndromes like calf diarrhoea and winter dysentery, with haemorrhagic diarrhoea in adult cows, because of the destruction of the epithelial cells and villous atrophy in the gut [13, 16]. The infection of the respiratory tract causes desquamation and necrosis of the epithelium of the lower respiratory tract and the respiratory symptoms are more common in adult cattle. It has been suggested that the tissue tropism of the virus is dependent on the variation of the Spike(S) protein gene [13]. The

virus is responsible for great economic losses due to the high mortality of infected calves, reduction in growth rate and decrease of milk production.

BCoV is an enveloped virus (lipidic envelope) with a single-stranded positive-sense RNA genome that encodes for 5 major structural proteins. The nucleocapsid (N) protein is very conserved among strains so it's often used as the target for detection by molecular assays. The S glycoprotein, on the outer surface of the virus, is composed by the dominant neutralizing epitopes and helps in the viral membrane fusion (**Figure 2**) [16]. The virus belongs to the order *Nidovirales*, family *Coronaviridae*, subfamily *Orthocoronavirinae* and genus *Betacoronavirus.* This genus also includes the MERS-CoV and the SARS-CoV-2, with great importance for humans [13]. This proximity may pose a danger of spillover of the virus to human through easy adaptation to a new host.

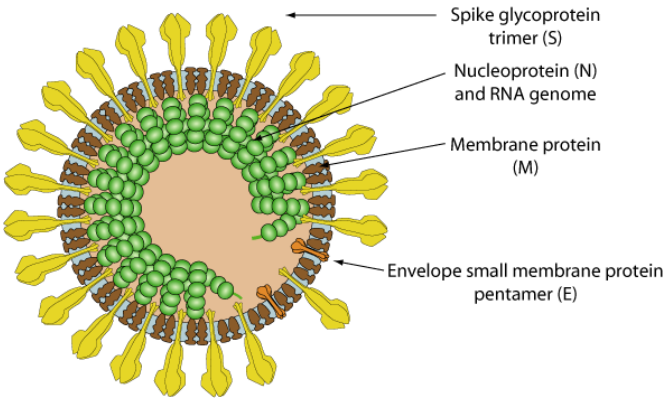


Figure 2 – *Betacoronavirus* genus: structure and genome. Font: Viral Zone, https://viralzone.expasy.org

1.1.1.2. Bovine respiratory syncytial virus (BRSV)

BRSV is a significant pathogen in cattle, causing widespread winter outbreaks of respiratory disease. The virus is prevalent globally, with isolates found in Europe, America, and Asia. Although cattle are the natural host, other species like sheep, goats, bison, chamois, and camelids might also be involved in the virus epidemiology. The disease is primarily spread through direct contact or aerosol transmission, with an incubation period of 2-5 days. The incidence of BRSV infections correlates with cattle population density and animal age and it's known that clinical signs are predominantly seen in calves but can also appear in adults, especially in herds with naïve animals. The infection can be asymptomatic and, when symptomatic it can affect only the upper airways or both upper and lower respiratory tracts. Mild cases feature cough and seromucous nasal and ocular discharge, while severe cases involve depression, anorexia, decreased milk production, fever, rapid breathing, and severe respiratory distress. In severe cases, broncho-interstitial pneumonia can be observed. Infection by BRSV presents a morbidity rate of 60-80% and mortality can reach up to 20% in some outbreaks [17].

BRSV is an enveloped virus with a single-stranded negative sense RNA that encodes for 11 proteins (**Figure 3**). G, F and SH are the transmembrane surface glycoproteins that are involved in attachment to the host cell and membrane fusion. The nucleoprotein (N) is the most conserved among different strains and the protein L corresponds to the RNA dependent RNA polymerase. The virus belongs to the genus *Orthopneumovirus* within the *Pneumoviridae* family included in the order *Mononegavirales*.

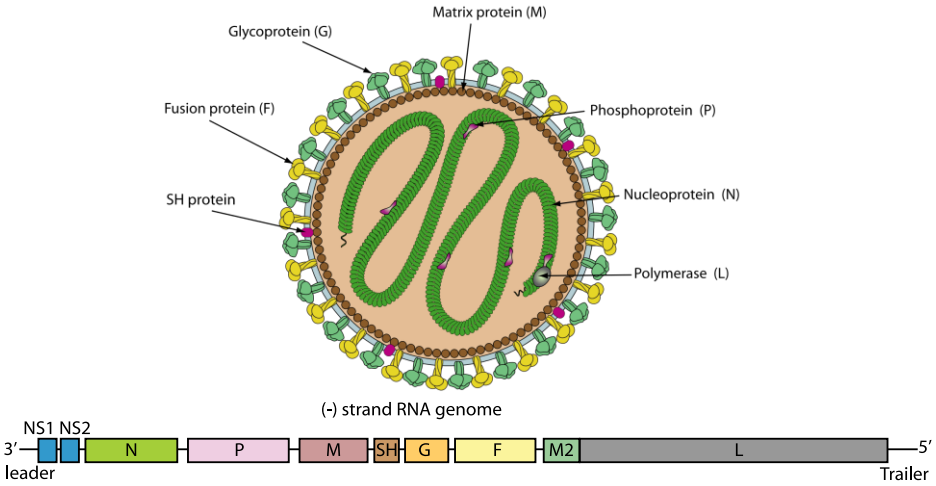


Figure 3 – *Orthopneumovirus*: structure, genome and encoded proteins. Font: Viral Zone, https://viralzone.expasy.org

1.1.1.3. Orbiviruses: Epizootic haemorrhagic disease virus (EHDV) and Bluetongue virus (BTV)

EHDV and BTV are considered Arboviruses since they are transmitted by arthropods, being their main vectors species *Culicoides* midges, that can vary according to the individual virus. The distribution of the viruses is intrinsically related to the distribution of their vectors, and it's known that midges can be dispersed through winds for long distances [18]. The infections are usually seasonal (from mid-summer to late autumn), but global warming can have a negative influence on the distribution of the vectors since higher temperatures benefit the circulation of the vector, and hence the transmission of vector-borne diseases such as EHDV for longer periods [18].

EDHV and BTV share many domestic and ruminant hosts, such as sheep, white tailed deer (WTD) and cattle, but the clinical symptoms vary greatly depending on the host species, the virus and its serotype, and on the individual animal. For now, EHDV is endemic in parts of North America, Australia, and certain countries of Asia and Africa and 7 serotypes of the virus are described [18]. BTV, on the other hand, has more than 29 serotypes known, and it has been found to circulate widely in Africa, the middle East, Australia, South Pacific, North and South America and Asia. However, in the last years and months there has been a growth in the circulation of EHDV and BTV throughout Spain and neighbouring countries: a report from the "Ministerio de agricultura, pesca y alimentación" showed a significant spread of EHDV in Spain between 2022 and 2023 [19]; some news reported the arrival of EHDV to Catalonia in the beginning of October of 2023 and some days later it was also detected in France [20, 21]; and the circulation of BTV in Community of Valencia in February of 2024 was confirmed [22]. Right now, BTV has recently been confirmed in Catalonia, with more expected focuses to be reported in the upcoming days. This current distribution of the viruses requires greater surveillance of their circulation to prevent further spread and new outbreaks.

In endemic regions, cattle susceptibility to the BTV is very low, causing mainly long periods of viraemia, with few or no clinical symptoms or sometimes mild disease. However, more severe symptoms in cattle began to emerge during outbreaks caused by serotype 8 (BTV-8) in northern Europe [23].

Symptoms observed in cattle included loss of body functions, hyperthermia, nasal discharge, ulcers on the oral mucosa and deformations on the foetus, since this serotype can cross the placenta; in WTD and sheep was also observed haemorrhages, oedema of the face, eyelids and ears, intravascular coagulation, anorexia, bloody diarrhoea and cyanotic tongue [23-25]. The mortality rate of some strains of BTV can be very high especially for infected sheep and deer [15, 23].

The symptoms caused by EHDV range from a sub-acute form that include ulcers in the oral cavity and in the gastrointestinal tract. It can evolve to an acute form characterized by a haemorrhagic disease that includes dilation and redness of the conjunctiva and the oral mucosa, pulmonary oedema, pleural effusion, and many haemorrhages in a variety of organs, until in some cases reach the per acute disease causing death due to pulmonary oedema [18]. In cattle the mortality rate is very low and it depends on the strain, while the morbidity varies between 1 and 18% [26]. The economic impact for the farmers and the population can be serious since this virus can also cause loss of appetite, reduction in the production of milk and abortion, besides the loss of animals to illness. A first outbreak of EHDV-2 in Japan, in 1959, caused 4000 deaths among cattle, and in 2006 Israel had an estimated loss ranging from 1.5 million to 3.4 million US dollars, due to infections in cattle [18].

The infection process of EHDV in an animal is described shortly in **Figure 4**. Although EHDV is mainly transmitted by the described path, oral and faecal shedding by infected WTD have also been demonstrated, as well as the transmission between these animals by direct and indirect contact [27]. This evidence leads to the possibility of a rapid faecal-oral transmission between animals in high density farms, which is the case of cattle, and therefore the possibility of detection of the virus in faecal samples.

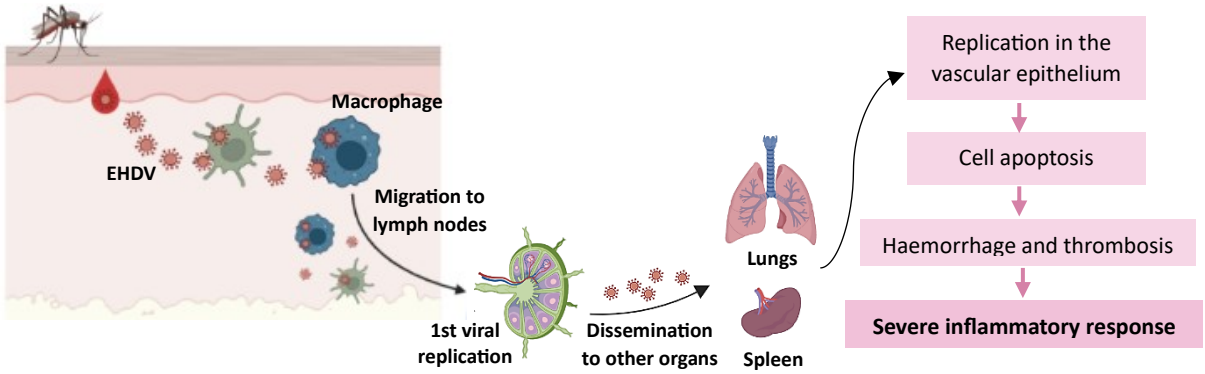


Figure 4- Pathway of infection o EHDV and BTV. Created with BioRender.

EHDV and BTV belong to the *Orbivirus* genus within the family *Sedoreoviridae.* They are non-enveloped viruses with an icosahedral capsid divided into 3 consecutive protein layers and their genomes are composed by 10 linear dsRNA segments that encode for 7 structural proteins (VP1-VP7) and at least 4 non-structural proteins (**Figure 5**) [15]. The protein VP2, encoded by segment 2, is the most variable in both viruses and it's the major determinant of serotype specificity, while segment 9, that encodes the protein VP6, presents a lot of conserved regions among serotypes [25]

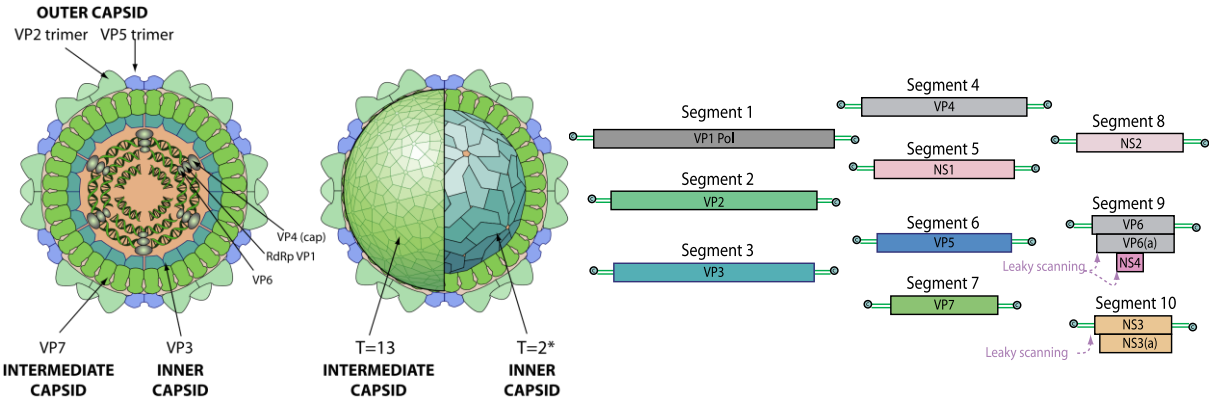


Figure 5- *Orbivirus* (EHDV and BTV): structure, genome and encoded proteins. Font: Viral Zone, https://viralzone.expasy.org

1.1.2. Zoonotic bovine viruses

1.1.2.1. Rotavirus (RoV)

Rotaviruses are a major cause of diarrhoea in intensive farmed animals all over the world and can infect a multitude of animal species, from cattle, goats and sheep to birds. The virus infects mainly enterocytes at the top of the villi of the small intestine and causes epithelial loss, intestinal malabsorption, maldigestion and crypt hyperplasia, leading to an outcome of acute gastroenteritis. In cattle, young animals with ages between 1 and 8 months are the most susceptible to the infection, showing more clinical symptoms, since the neonatal bowel has a slow epithelial turnover and a high quantity of terminally differentiated enterocytes. Some of these animals may die because of dehydration or a secondary bacterial infection, but the majority recovers in 3-4 days [28]. Besides being a virus of veterinarian concern, RoV also infects humans. In infants and young children until 5 years of age from USA and Europe, 5-10% of all cases of acute gastroenteritis are caused by RoV infections [29]. On the other hand, in developing countries, a study from 2004 estimates that severe diarrhoea caused by RoV reaches 39% of all cases [30] . The resulting clinical symptoms are vomiting, mild to severe diarrhoea, dehydration and moderately elevated body temperature. Death from RoV infection is due to severe dehydration and cardiovascular failure and it's rare in developed countries but frequent in non-developed countries, where people have no way to maintain a continuous rehydration [28, 29]. According to bibliography, sequencing of the 11 genome segments of human rotaviruses showed a close relation with porcine, bovine and feline rotaviruses, which suggests zoonotic events with viral interspecies transmission [28].

Excretion of RoV in faeces by infected animals is well documented, and high titters are usually found (up to 10^{11} viral particles per gram), considering that the maximum shedding happens on the 3rd or 4th days after infection. Rotaviruses can survive in faeces for months and are resistant to different disinfectants, including the process of chlorination in water, which facilitates its spreading in rearing pens by faecal-oral contamination [28]. This knowledge allows for greater reliance in this type of

environmental samples when the objective is to study the viral circulation in animals and in the environment.

The *Rotavirus* (RoV) genus belongs to the family *Sedoreoviridae* and it's a non-enveloped virus with a structure that resembles a wheel *(lat. rota)*. Its genome is composed by 11 dsRNA segment encoding 6 structural viral proteins (VP1-6) and 6 non-structural proteins (NSP1-6), represented in **Figure 6.** The rotavirus genus contains 9 species named from A until I (RoVA to RoVI) that are dependent on the serological reactivity and genetic variability of VP6 [31]. On the other hand, VP4 and VP7 are used for typification in a dual system defining P-genotype, dependent on VP4 (genome segment 4) and G-genotype, dependent on VP7(genome segment 9). However, an important distinction between serotype and genotype must be considered since the serotype is related with the virus response to specific antibodies while the genotype consists only of the genetic composition of the respective segment (the nucleotide sequence). For G type, serotype and genotype are the same, e.g. G1, G2, G3, etc. For P type, within the same serotype there are many distinct genotypes, so a more detailed double nomenclature can be used, e.g. P1A[8] represents the P serotype 1A and P genotype 8 [29, 31]. At the time, the RoV-A species comprises 39 P types and 28 G types.

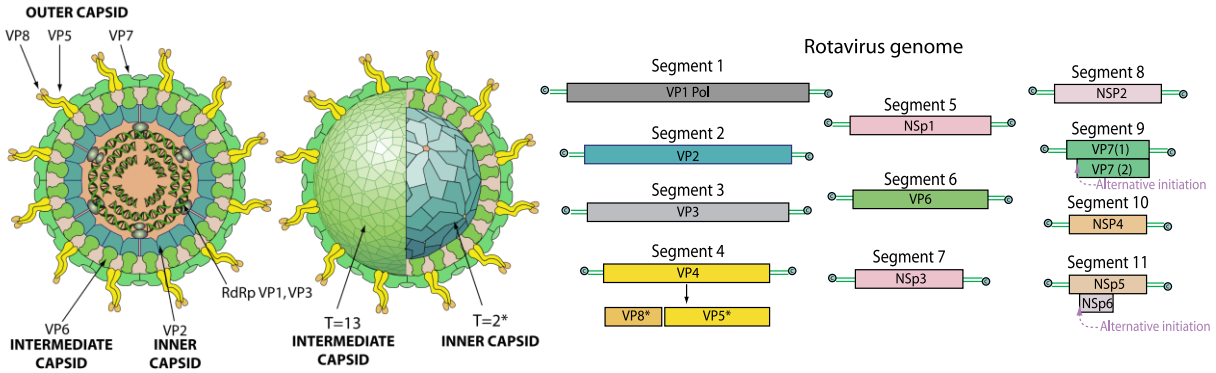


Figure 6 - Rotavirus: Structure, genome, and encoded proteins. Font: Viral Zone, https://viralzone.expasy.org

An important feature of viruses with segmented genomes, such as RoV, is the potential for reassortment after coinfection with more than one type of RoV. This reassortment causes the emergence of viruses with great variability since segments from different genotypes are assembled in the same viral particle. Thus, to obtain a better comprehension about the genome evolution and genetic diversity of RoVs, a complete genome classification system was created for RoV-A that defines genotypes for the 11 segments of the virus. This classification allowed the assignment of "genome constellations" that are reported as Gx-P[x]-Ix-RxCx-Mx-Ax-Nx-Tx-Ex-Hx, where "x" refers to the particular genotype [31] . **Table 1** presents a summary of all the RoV-A genotypes described until now and respective proteins associated.

Table 1- Rotavirus A genotypes. Updated from [29] using the NCBI Rotavirus resource [32].

RoV Protein	Number of genotypes	Protein function
VP7	28 G	Glycosylated
VP4	39 P	Protease-sensitive
VP6	21 I	Inner capsid
VP1	14 R	RNA-dependent RNA polymerase
VP2	14 C	Core protein
VP3	13 M	Methyltransferase
NSP1	24 A	Interferon Antagonist
NSP2	14 N	NTPase
NSP3	16 T	Translation enhancer
NSP4	21 E	Enterotoxin
NSP5	16 H	PHosphoprotein

1.1.2.2. Avian influenza virus (AIV)

The avian influenza viruses are a type A Influenza virus, a viral species known to infect a wide range of animal species. The natural reservoirs of the low pathogenic avian influenza virus (LPAIV) are wild waterbirds that can transmit the virus direct or indirectly to domestic birds, such as poultry, other wild animals, domestic animals and humans, a case of zoonosis. Once the subtypes H5 and H7 of LPAIV are introduced in poultry they can evolve to highly pathogenic avian influenza viruses (HPAIV) able to cause severe disease and mortality in these animals, with great losses for poultry industry due to decrease in egg and meat production [33]. Thus, H5 and H7 subtypes can be both LPAIV or HPAIV, depending on the infected animal – in wild birds they are low pathogenic viruses while in poultry they are highly pathogenic viruses. The HPAIV (H5N1) was detected recently in dairy cows and cats in the USA and in cattle a non-specific illness was reported, characterized by loss of appetite, less rumination and reduced milk production, while the fatal infection happened in cats fed with raw milk [34]. A human case of a farm worker was also reported, and the infection occurred after direct contact with dairy cows presumed to be infected with the H5N1 avian influenza virus.

The influenza A viruses are single-stranded, negative-sense RNA viruses composed by 8 genome segments, encoding at least 10 proteins (**Figure 7**). They are enveloped viruses belonging to the *Alphainfluenzavirus* genus within the *Orthomyxoviridae* family and they are classified according to the serological subtypes of the viral surface proteins hemagglutinin (HA) that gives the H type, and neuraminidase (NA) that gives the N type [35].

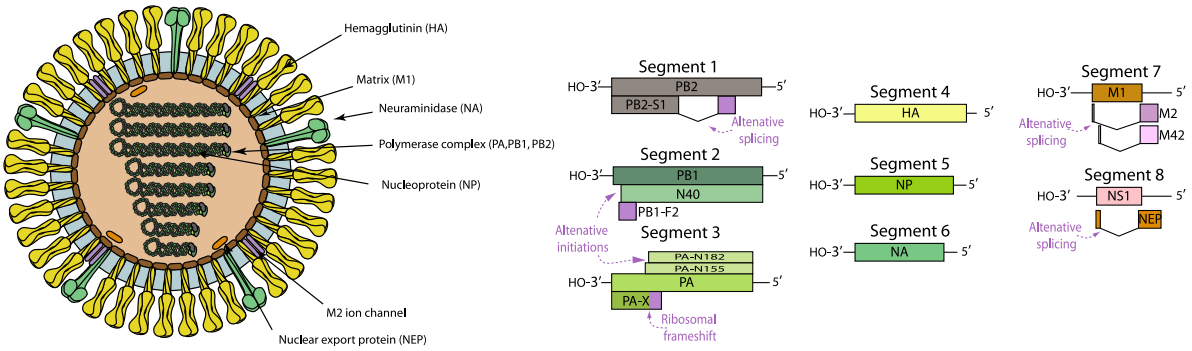


Figure 7 – *Influenza A virus*: Structure, genome, and encoded proteins. Font: Viral Zone, https://viralzone.expasy.org

1.1.2.3. Crimean-Congo haemorrhagic disease virus (CCHFV)

The first recognition of the CCHFV happened in 1944-1945 during an outbreak among 200 Soviet military personnel in Crimea. Some years later, in 1967 it was discovered that the virus detected in Crimea and the Congo virus, originally isolated in Congo and Uganda, were the same. Nowadays, the virus is geographically distributed across countries in Asia, Africa, Middle East and Southeastern Europe [36]. However, some factors like climate change and international trades have caused a shift in the epidemiology of the virus in the last years, with reported cases of infections in animals in Portugal and both in animals and humans in Spain, areas previously free of the virus [37-39].

The virus distribution is dependent on the circulation of its vector, a (hard) tick from the family *Ixodidae*, with the genus *Hyalomma* being its main reservoir and source of human infections, specifically *Hyalomma marginatum* in the mediterranean region [38, 40]. Nevertheless, a study showed that the tick specie *Rhipicephalus bursa* may also have an important role on the transmission and maintenance of the virus [41].

The transmission of the virus depends on the life cycle stage of the tick. It can happen by vertical transmission during copulation, during the different life stages of the tick (larva, nymph, adult) and from the female to her eggs (transovaric). It can also occur by horizontal transmission, between ticks involving other animals. Since the virus causes viraemia, it can be transmitted from an infected animal to a healthy tick through the bite. Humans can be infected with the virus by a tick's bite, by exposure to body fluids of infected animals (ie. ingestion of milk or milk products) or by an infected patient (nosocomial transmission) [40].

The CCHFV infection in animals is not a big concern since it's usually asymptomatic but humans infected with the virus can develop a febrile syndrome with vascular leak, multi-organ failure, shock and haemorrhage, leading to death in the worst cases [40]. In fact, CCHFV has been the aetiological agent associated with the death of 3 persons, in a total of 10 persons infected, in Spain during 2013-2021 [37]. In zones from Africa with high endemicity, it's an occupational disease affecting slaughterhouse workers, farmers and veterinarians [42].

A serologic study of the presence of Crimean Congo in different regions of Catalonia, performed in 6 types of wild animals, showed that *Capra pyrenaica (Iberian ibex)* presented the higher seropositivity (66 out of 84), followed by the wild boar (5 out of 156), leading to the possible conclusion that *Capra*

pyrenaica may be a potential host of the virus [43]. An interesting fact about this research is that the only area with seropositive animals corresponds to a wetland, which is a stopover for migratory birds from África, birds that can carry ticks infected with the virus. A subsequent study confirmed that the maintenance of the circulation of the virus is very dependent on the presence of *Capra pyrenaica*, sustaining the idea that this animal could be the main host of CCHFV in Spain [41].

CCHFV belongs to the genus *Nairovirus* in the family *Bunyaviridae,* it's an enveloped virus with a diameter of around 80-100nm and it is classified as a risk 4 pathogen. Its genome is composed by three distinct segments of single-stranded RNA negative sense: the S segment with ~1,7kb length that encodes for the viral nucleocapsid, the M segment with ~4,9kb encoding the precursor for the two glycoproteins Gc and Gn that form the viral envelope, and the L segment with ~12kb encodes a RNA-dependent RNA polymerase (**Figure 8**) [8, 40].

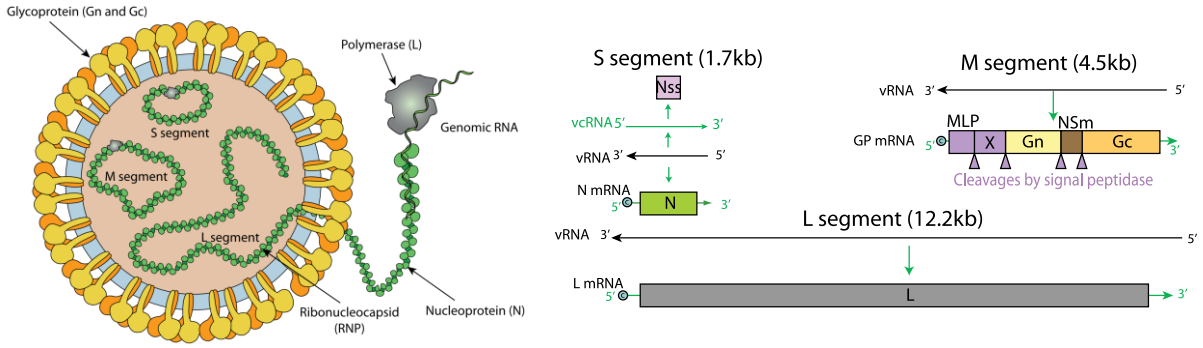


Figure 8- Crimean Congo haemorrhagic disease virus: structure, genome and encoded proteins. Font: Viral Zone, https://viralzone.expasy.org

Based on the Gn and Gc glycoproteins, the virus has 7 different clades distributed around the world: clades I, II and III circulate mainly in Africa, clade IV in Asia, clades V and VII in Eastern and Southeastern Europe and clade VI in Southeastern Europe. Until now, the detected strain in Spain was "clade III", with origin in Africa [44].

1.2. Routes of transmission of viruses and arrival to the environment

Viruses with zoonotic potential and with veterinarian interest can arrive to the environment through different routes, depending on its shedding mechanism, and from there they can infect other animals and humans in a continuous cycle (**Figure 9**).

Some viruses, such as enteric viruses, are excreted in faeces, urine and secretions by infected humans or animals and their survival in the environment is facilitated due to their extreme resistant nucleocapsids against environmental agents that could inactivate them. Besides, they are protected by the organic debris from the secretions, offering an easier route to infect other human or animal hosts by faecal-oral transmission [45]. The excretion concentrations of enteric viruses by infected individuals are usually around 10^7-10^9 viral particles per gram of faeces.

Respiratory viruses can be found in aerosols in intensive farms and, some aerosol particles can remain suspended in the air for more than 5 seconds at 1,5m height, as demonstrated in swine farms. Concentrations of 10^7 genomes/m^3 of airborne porcine-circovirus 2 were found in a Canadian swine confinement building [46]. Evidence shows that these aerosols containing airborne viruses are correlated with symptoms of respiratory illness in farm workers, which poses a threat to human health [47]. Surfaces in barns, also called fomites, like pen railings or door handles, can also retain viral particles because of the deposition of airborne particles, becoming other possible viral transmission route from the animals to humans [48].

Excretions and secretions of infected animals in transport trucks also represent a risk, not only for the animals but also for the workers who can contaminate their clothes and footwear with these excretions, creating a transmission route to humans [49].

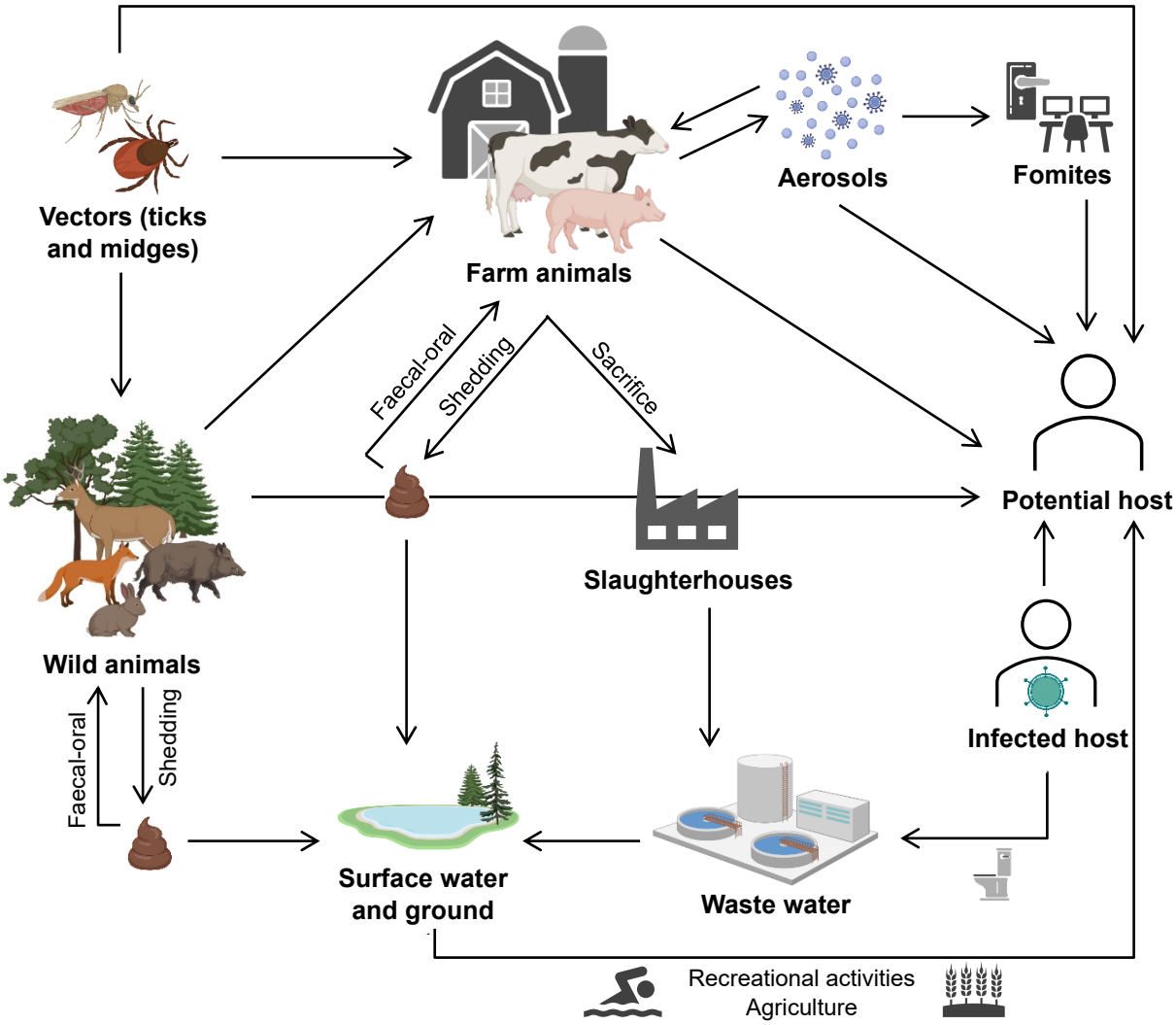


Figure 9 - Main transmission routes of viruses excreted by animals and humans to the environment. Created with Biorender.

1.2.1. The environment as a complementary tool for surveillance

Environmental samples have already been used to study the circulation of viruses that infects humans (i.e. Poliovirus [50]). Recently, the use of environmental samples has been used to infer the number of covid-19 cases within a population by measuring the RNA concentrations of the SARS-CoV-2 in wastewater [51]. In a similar manner, the use of collective animal samples such as slurry - the mixture of many individual faeces from different animals in a farm -, lixiviates - the result of cleaning up the animal excretions -, air, water, sewage and fomites give an overview of all the viruses circulation in a specific group of animals, like in a farm, or in large groups of animals, as is the case of sewage from slaughterhouses. In this case, the environmental samples from animal facilities would have the same role as the human sewage that is used to survey the circulation of human viruses in a population.

The complementation of clinical samples with environmental samples to perform viral surveillance could offer additional advantages, mentioned in **Figure 10**, for both researchers and animals. Their use would allow starting with a comprehensive analysis of the animal's population from several animal facilities which could then lead the need for analysis of individual samples if any pathogenic viruses were detected in the collective samples. This way, there would be a significant reduction on the number of samples collected, which would greatly reduce the analysis costs.

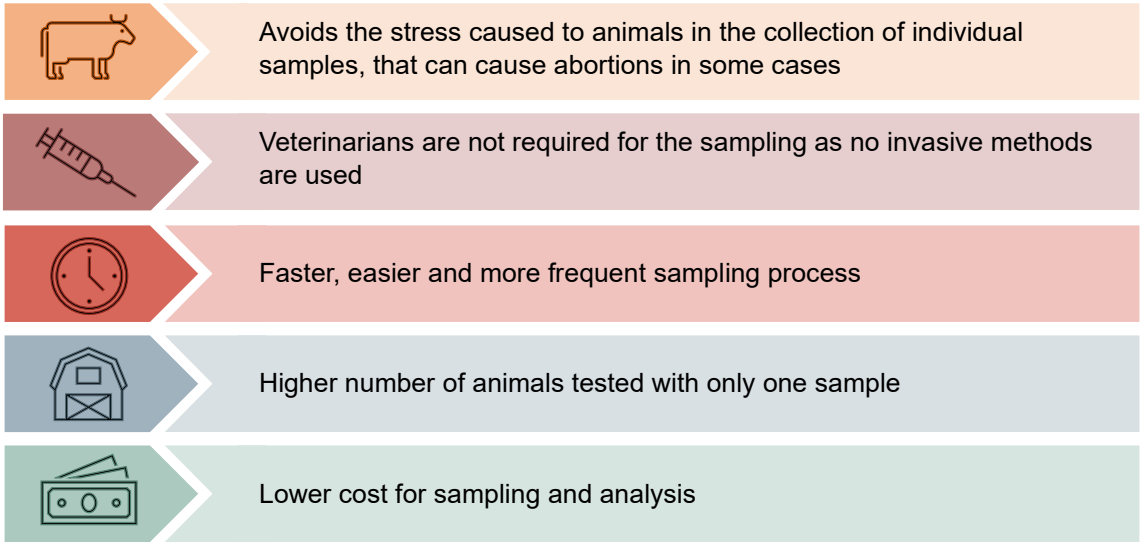


Figure 10 - Advantages on the use of environmental samples for surveillance of viral circulation.

However, it's also important to consider some disadvantages of the environmental samples when working with them so the detection of viruses could be the most efficient possible. These disadvantages include:

- **Low viral concentrations**: in water or air samples the viral particles are more diluted or scattered which makes the viral detection more challenging. Usually, to analyse these samples, a concentration of viral particles from the matrix is needed.

- **Matrix complexity**: organic and inorganic compounds present in the matrix can inhibit PCR reactions, hindering the detection of the virus. Virus fraction needs to be separated from other pathogens by specific concentration methods which tend to co-concentrate inhibitors.
- **External contamination**: viral contaminants could come from other sources, such as animals that enter the farms as plagues (ie. rats) and can excrete their viruses in the facilities
- **Virus degradation**: viruses can lose their integrity due to temperature variations, solar inactivation, and presence of chemical products such as disinfectants used in the farm.

As a consequence of their nature, environmental samples may require some complex and time-consuming processing, requiring additional steps of concentration and purification of the virus that are usually not necessary for clinical routine samples.

Even so, the advantages that environmental samples bring to epidemiological studies end up outweighing their disadvantages, in addition to being a very important complement to clinical samples for more economical, efficient and animal-friendly surveillance. The COVID-19 pandemic has shown that individual clinical observations can be too slow, and in some moments of the pandemic inaccurate, for health professionals to make an informed health management decision in times of uncertainty [52].

1.2.2. Waste-water based epidemiology (WBE)

Waste-water based epidemiology, also known as environmental surveillance, offers a broader perspective on the health status of entire communities. Wastewater contains various substances excreted by individuals within a community, such as pathogens and different chemical substances, so it serves as a collective sample that is much easily accessible than individually pooled clinical samples. On top of that, WBE enables to detect not only symptomatic infections as well as asymptomatic infections circulating in the population, which are often undervalued during clinical surveillance, a critical factor to estimate accurately case-fatality rates [52]. With all this information, researchers can effectively monitor the prevalence of diseases, track the spread of pathogens, understand the burden of infections in the population and even monitoring the beginning, spread and re-emergence of a pandemic, as in the case of COVID-19 [52].

Several studies already described the use of WBE as a very useful tool to analyse the circulation of viruses in a given population, showing cocirculation of Hepatitis A virus (HAV) strains [53], hepatitis E virus (HEV) in humans and pigs in industrialized countries [54] or leading to the adoption of a poliovirus surveillance in wastewater to accomplish the goal of the polio eradication program from the WHO [50] and the tracking of polio outbreaks in Israel [55] and Egypt [56].

As it's known that zoonotic viruses and viruses of veterinarian interest may also be transmitted via environmental pathways, the application of WBE to the animal industry is increasingly important and samples from wastewater, aerosols, lixiviates, slurry from farms and slaughterhouses can represent a huge source of information on viral pathogen circulation. Until now, scarce pathogen surveillance in these types of samples has been conducted on swine and cattle. The introduction of this surveillance

could represent an early-warning tool to detect human and animal pathogens with important health and economic impact.

1.3. Viral detection in environmental and faecal-derived samples

The detection of viruses in environmental samples requires the use of molecular methods since conventional cell-culture methods are not efficient enough and are less sensitive [57]. The workflow for the molecular detection of viruses in environmental samples is summarized in **Figure 11**

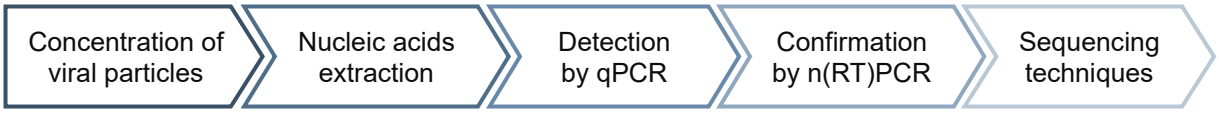


Figure 11- Workflow for the detection of viruses in environmental samples. qPCR is quantitative PCR and n(RT)PCR is nested PCR.

As mentioned before, environmental samples require a concentration step prior to molecular analysis. The most common techniques for viral concentration are based on adsorption-elution, where the viral particles are adsorbed in the filter by electrostatic interactions, or on ultrafiltration where the viral particles are retained in the filter depending on their size and the filter pores size, as is the case of an ultrafiltration protocol using the automatic Concentrating Pipette (CP-Select™) from Innovaprep with 150 kDa ultrafiltration. This last method relies on ultrafilter tips and has shown better recovery efficiencies than other concentration methods like Skimmed milk flocculation and centrifugal ultrafiltration [58]. This concentration step is not necessary for clinical samples, such as faeces, urine or blood, due to their high viral concentration, but they only represent one animal, while environmental samples represent a higher number of individuals despite having lower viral concentrations.

Nucleic acid extraction from their capsids and purification of their DNA/RNA is a mandatory step for molecular viral detection in all types of samples. Two of the major techniques used for nucleic acid extraction are the column-based methods and the magnetic-based methods and both start with a lysis of the viral particle. The column-based method uses a silica membrane with positive charge, within a spin column, to which the DNA or/and RNA bind, since they are negatively charged. Afterwards, the process includes several centrifugation steps for binding, washing and elution of the nucleic acids. The "QIAamp Viral RNA Mini Kit" from QIAGEN® is a widely used column-based commercial kit for extraction of nucleic acids and it can be used manually or automatized. The magnetic-based method uses magnetic beads coated with a material that binds nucleic acids in the presence of a binding buffer, followed by a separation step of the beads from the lysate using a magnetic field, washing steps and the nucleic acids elution in the end.

The presence and concentration of viruses is then assessed through different PCR techniques. The (RT)qPCR (reverse transcriptase quantitative polymerase chain reaction) allows the quantification of a given virus in a sample using specific primers and a probe. Usually, the target of the (RT)qPCR is a very conserved region of the genome of the virus, so all its strains are detected. When positive results are obtained in the (RT)qPCR, other PCR techniques can be used to confirm the presence of the virus

such as the nested or semi-nested PCR, which include two PCRs using different primers where the second PCR amplifies a smaller region within the amplicon amplified in the first PCR, presenting high specificity. The semi-nested PCR has the same principal but there's a common primer between the first and the second PCR. The products from these nested PCRs can later be purified and sequenced by Sanger sequencing, a process that determines the DNA sequence by synthesizing complementary DNA strands incorporating occasionally chain-terminating ddNTPs with a different fluorescent coloured label for each base, resulting on different fragments that are then separated by size by capillary electrophoresis where a laser reads the terminal fluorescent labels to identify the sequence of nucleotides.

In the analysis of environmental samples microbial source tracking (MST) tools may be used to enhance the accuracy and reliability of identifying viral contamination. Bovine polyomavirus (BPyV) has been used as an MST tool to detect bovine faecal contamination since BPyV it's a widespread virus in cattle. This virus is non-pathogenic, and it is excreted in high concentrations to the environment by faeces and urine [57]. The presence of bovine faecal contamination can be proven by the by the presence of the bovine indicator BPyV.

1.4. Study of the virome by Next generation sequencing (NGS)

The study of the virome can provide important information on virus circulation and emergence of new viruses within a given population. Next generation sequencing (NGS) tools have been successfully useful to study the microbial community present in a wide variety of samples. These techniques were easily applied to the study of bacterial communities but the lack of conserved regions between different viral genomes makes the study of the virome more challenging. However, this problem has been overcome by using random-primer-based sequencing methods such as SISPA (Sequence - independent single-primer amplification) which provides enough concentration of viral genomes for library preparation[59]. To perform SISPA, summarized in **Figure 12,** before the nucleic acid extraction a DNAse treatment is recommended. This treatment will degrade the free nucleic acids in the sample, thus reducing non-viral genetic material within the sample. After viral nucleic acid extraction, the following steps are performed: the viral DNA and RNA are tagged by a random nonameric primer A; cDNA is produced through retrotranscription of the RNA; treatment with RNase H to denaturate RNA-DNA hybrid structures; synthesis of cDNA complementary strand with sequenase enzyme; and finally an amplification of the dsDNA using a primer B complementary to the primer A tagged viral sequences [1].

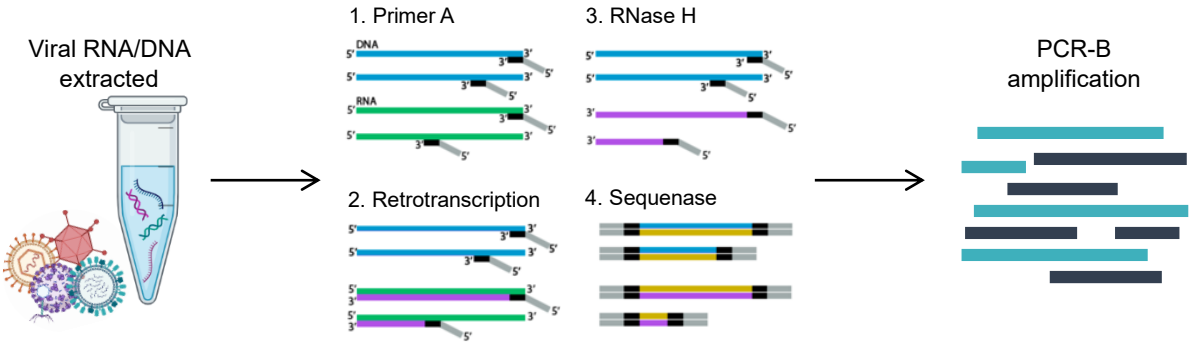


Figure 12 - Overview of the SISPA procedure. DNA in blue, RNA in green, cDNA in purple, double strains produced by sequenase enzyme in yellow, primer A in grey with random region in black and primer B in grey. Adapted from [1].

A major limitation of the application of NGS in any type of sample is that the viral genomes represent a lower proportion of the total DNA/RNA compared to the bacterial genomes and the host genome which tend to be much bigger in size. Even within the viral fraction, phages and plant viruses are dominant, which hamper the detection of animal and human viruses [2]. For this reason, methods such as Target enrichment sequencing (TES) were developed to allow an enrichment of a specific fraction of the viruses of interest prior to sequencing. An example of TES is the panel VirCapSeq (Virus capture sequencing) used for virome study that captures 207 viral species [59]. Better panels have been developed in the recent years such as the panel Twist NGS workflow protocol from TWIST Bioscience that captures 3153 viral species which contain at least a species member which infects vertebrates. TES is based on the use of millions of probes that cover the genome of several viruses that infect vertebrates. These probes are linked to biotin which has high affinity for the streptavidin that covers magnetic beads, that are then captured through a magnetic field, enabling the separation of the viral libraries of interest from the remaining genome. This technique considerably increases the genome coverage of the sequenced viruses allowing the detection of minoritarian viruses that would not be sequenced without the use of hybridization probes. The principle of TES method is represented in **Figure 13.**

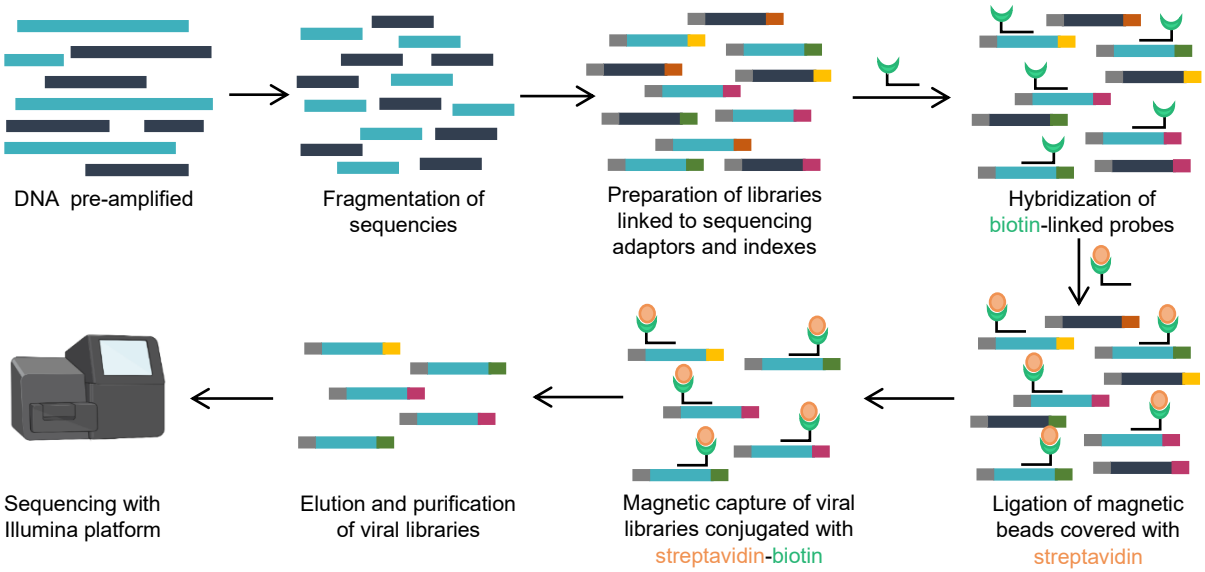


Figure 13 - Summarized procedure for Target enrichment sequencing using probes conjugated with biotin. Adapted from [2].

1.5. Objectives

Following the concept of the One-health approach, it's important to enhance preparedness and early warning of possible viral outbreaks, with potential to jeopardize the lives of animals and humans while maintaining the environment sustainability. Therefore, this work aims to develop a sampling campaign within cattle farms and slaughterhouses to evaluate the use of environmental samples for viral detection and to survey the circulation of several zoonotic and non-zoonotic bovine viruses. Thus, the concrete objectives of the work are listed below:

- Conduct a sampling campaign of collective samples: lixiviates and aerosols from cow farms and wastewater from cow slaughterhouses.
- Perform viral concentration techniques to improve viral detection in collective samples.
- Validate, optimize and compare different nucleic acid extraction methods for faecal samples.
- Evaluate the possible use of collective environmental samples to study the prevalence and circulation of viruses in animals as a complement to the use of clinical samples.
- Study of the circulation of zoonotic and non-zoonotic bovine viruses in the livestock industry in Catalonia, such as BPyV, RoV-A, BCoV, BRSV, CCHFV, EHDV, BTV and AIV and quantify them using qPCR and (RT)qPCR techniques.
- Development of new SOPs for the detection of emerging viral pathogens in cattle industry.
- Perform NGS techniques to study the virome excreted by cows throughout Catalonia.

2. MATERIALS AND METHODS

2.1. Sampling

2.1.1. Description of the sampling sites

In the present study, aerosol, lixiviate (or manure) and slaughterhouse wastewater samples were collected once per month. Aerosol and lixiviate samples were collected from bovine farms. In these farms the animals are raised in semi-open spaces with natural ventilation, with storage of the manure in manure sheds since they are mainly raised with straw litter on the ground to improve the animal comfort and absorption of stools.

Wastewater samples from slaughterhouses were collected monthly. To select the sampling sites, we ensured that the slaughterhouse only killed cows or at least no other animal was sacrificed 48h before the sampling date. The two sampling sites selected sacrificed animals from farms distributed throughout Catalonia and nearby regions.

In addition to environmental collective samples, individual faeces and saliva animal samples from some farms were collected. These samples were analysed to optimize the methods to be used in this study by using samples where viruses are expected to be in high concentrations.

More information about the mentioned farms and slaughterhouses is provided in **Table 2.**

Table 2 - Description of sampling sites in Catalunya: cow farms and cow slaughterhouses.

Farm	Location	No. of animals	Type of production	Sample type
A	Torregrossa	No data	Semi-intensive	**Individual** Faeces and saliva
B	Sucs	No data		
C	Montgai	No data		
D	Lleida	No data		
Can Bordois	Cardedeu	200	Semi – intensive Production of milk and other lactic products	**Collective** Lixiviates/manure and aerosols
La Pasiega	Olesa de Montserrat	190 (90 for reproduction)		
Slaughterhouses	**Location**	**No. of animals**	**Animals' origin**	**Sample type**
Escorxadors Sabadell	Sabadell	500 cows 50 sheep	Several farms throughout Catalonia and Aragon	**Collective** Wastewater
Torrents i Viñals	Argentona	800 cows		

2.1.2. Sampling of veterinarian clinical samples and collective samples from cow farms

A subset of clinical samples, also described here as individual samples, and some collective samples, including drinking water and bed straws, were collected between May and June of 2023 by veterinarians from the Grup de Sanejament Porcí (SP) and the University of Lleida (UdL), as partnerships within this project. **Table 3** shows the details of the collected samples from the four semi-intensive production cattle farms. From each cow randomly selected, a saliva and rectal swab were collected. A total of 50 saliva swabs, 49 rectal swabs and 7 collective samples were collected, transported to the laboratory at 4ºC and maintained at -20ºC until further analysis.

Table 3- Individual samples collected in 4 cow farms from Catalunya.

Farm	Sample type	No. of samples	Collection date
A	Faeces	10	03/05/2023
	Saliva	10	03/05/2023
B	Faeces	9	03/05/2023
	Saliva	10	03/05/2023
	Collective (water)	1	03/05/2023
C	Faeces	10	03/05/2023
	Saliva	10	03/05/2023
	Collective (straw)	2	03/05/2023
	Collective (water)	1	03/05/2023
D	Faeces	20	12/06/2023
	Saliva	20	12/06/2023
	Collective (straw)	2	12/06/2023

2.1.3. Sampling campaign of collective samples from cow farms and slaughterhouses

Collective samples of lixiviates/manure and aerosols from 2 cattle farms, Can Bordois and La Pasiega, were collected once per month between September and April (2023-2024).

The manure, a pasty mixture of faeces **(Figure 14),** and the lixiviates, that present a liquid form because of the cleaning of faecal and urine excretions from the farms **(Figure 15)**, were collected with a 50,0 ml pot at the site of each farm suitable for the accumulation of these residues.

The air sampling was performed using a Coriolis µ air-sampler from Bertin Technologies **(Figure 16)** located at selected points of the farms where the animals are kept together during the sampling time. The Coriolis air sampler can be used to collect airborne viruses outdoors or indoors thanks to its high flow rate – up to 300L/min and it's compatible with molecular techniques such as (RT)qPCR and NGS analysis. It uses a unique liquid cyclonic technology paired with a high suction rate, enabling the collection of large volumes of air in a less time-consuming manner. The viral particles are kept in a chosen collection liquid. For this sampling, 15 ml of PBS were placed in the collection cone and the instrument was programmed to have a flow rate of 300L/min for 50-60 min. After collection, the samples

were transported in cold conditions to the UB facilities and concentrated. **Table 4** contains more information about these samples.

Figure 14 - Farm Can Bordois: collection site of manure.

Figure 15 - Farm La Pasiega: collection site of lixiviates.

Figure 16 – Coriolis µ air-sampler (Bertin Technologies).

Table 4 - Collective samples from the 2 cow farms collected from September 2023 until April 2024.

Farm Can Bordois						
Lixiviate sample			**Aerosol sample**		**Collection date**	**Season of the year**
Sample	**Volume (ml)**	**Sample type**	**Sample**	**Volume (m^3)**		
F3S1	3,0	Manure	F3A1	16,5	12/09/2023	Summer
F3S2	3,0		F3A2	16,5	11/10/2023	Autumn
F3S3	6,0		F3A3	16,5	30/11/2023	
F3S4	1,5		F3A4	16,5	13/12/2023	
F3S5	5,0		F3A5	16,5	09/01/2024	Winter
F3S6	5,0		F3A6	16,5	13/02/2024	
F3S7	5,0		F3A7	15,0	11/03/2024	
F3S8	5,0		F3A8	15,0	10/04/2024	Spring
Farm La Pasiega						
Lixiviate sample			**Aerosol sample**		**Collection date**	**Season of the year**
Sample	**Volume (ml)**	**Sample type**	**Sample**	**Volume (m^3)**		
F4S1	--	--	F4A1	16,5	28/09/2023	Summer
F4S2	30	Lixiviate	F4A2	16,5	31/10/2023	Autumn
F4S3	5,0	Manure	F4A3	16,5	23/11/2023	
F4S4	40	Lixiviate	F4A4	16,5	18/12/2023	
F4S5	40		F4A5	16,5	18/01/2024	Winter
F4S6	40		F4A6	16,5	20/02/2024	
F4S7	40		F4A7	15,0	21/03/2024	
F4S8	40		F4A8	15,0	19/04/2024	Spring

The collection of waste-water samples from bovine slaughterhouses was done for 3 months and the samples correspond to wastewater from the first storage tank of the slaughterhouse's wastewater treatment plant before undergoing any chemical decontamination treatment. The samples were transported in cold conditions to the UB facilities to be further processed. **Table 5** presents more detailed information about these samples.

Table 5- Waste-water samples collected in the 2 bovine slaughterhouses between February and April 2024

Slaughterhouse	Sample	Collected volume (ml)	Collection date	Season of the year
Sabadell	E1D1	100	02/02/2024	Winter
	E1D2	100	20/02/2024	
	E1D3	100	15/03/2024	
	E1D4	100	18/04/2024	Spring
Argentona	E2D1	100	02/02/2024	Winter
	E2D2	100	20/02/2024	
	E2D3	100	15/03/2024	
	E2D4	100	19/04/2024	Spring

The Laboratory is a P2 facility with all equipment needed to safely perform several procedures with viral samples.

2.2. Concentration of viral particles

The first step of the sample processing performed was the concentration of viral particles to make viral detection more sensitive, since in these types of samples viral particles are usually considerably diluted. All collective samples were submitted to a viral concentration protocol depending on the sample type, as described in **Figure 17.**

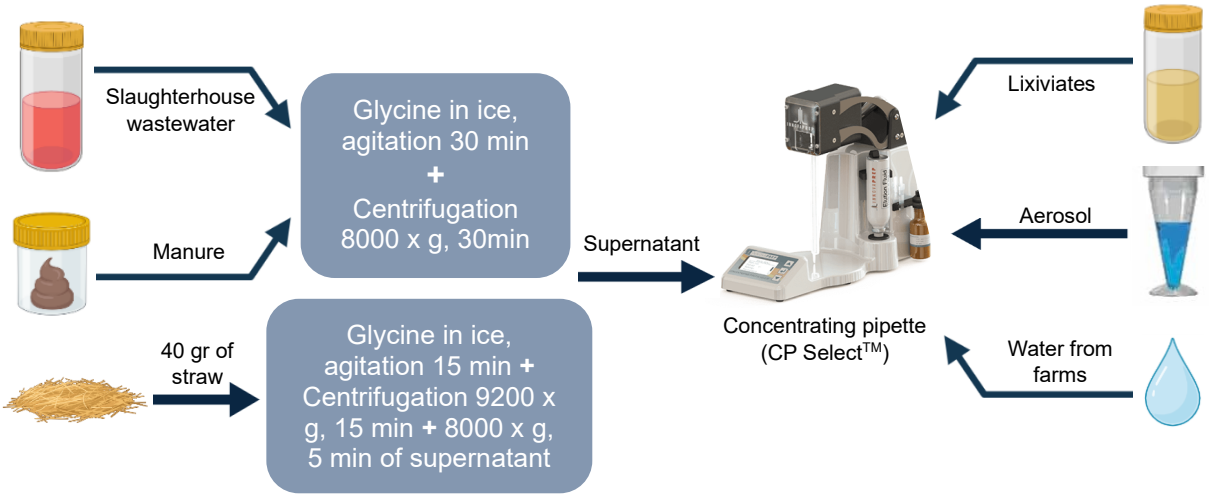


Figure 17 - Summarized protocol for concentration of viral particles of collective samples. Created with BioRender.

The individual samples (faeces and saliva) were not subjected to any concentration method, and they were processed accordingly to the procedures employed by a veterinarian diagnostics lab.

For the samples that have undergone a glycine treatment prior to the concentrating pipette step:

- A 1:1 v/v ratio of glycine and sample was prepared, except for straw samples where 20 ml of glycine (0,25 N, pH 9.5) was added.
- The centrifugation was performed in an Avanti JXN-26 centrifuge from Beckman Coulter, using a suitable rotor for the tubes.
- The supernatant was transferred to a new tube and the pellet discarded.

The last concentration step was performed using a concentrating Pipette that uses ultrafiltration tips composed by hollow fibres (CP-Select™ from Innovaprep®) with 150KDa pores and that allows to concentrate a starting volume of 0,5 mL - 5L to a final volume of 100-400 µl. The Pipette uses vacuum to pull up the sample through the tip where the viral particles are captured onto the surface of these porous membrane filters so then, they can be eluted by a Wet Foam Elution™ process using a elution fluid composed of water, a low concentration surfactant (> 0.1%), and a pH buffer, solution to which carbon dioxide (CO_2) is added in order to allow the formation of microbubbles, contributing to an efficient elution. The concentration was performed following the instructions on the user manual. In **Figure 17**, the operational steps of the CP-Select™ are summarized.

Figure 17 – Summary of the operational steps of CP-Select™.

The eluted volume varies from sample to sample, so PBS was added to all the samples until reach a final volume of 350 µL for wastewater, lixiviates and manure and a final volume of 150 µL for aerosol samples.

2.3. Nucleic acid extractions

Before beginning with the nucleic acid extractions of the samples, some optimizations and comparisons between different methods were done to procced with the most efficient method to extract nucleic acids from faecal samples.

2.3.1. Optimization of Maxwell AS1600 extraction method for faecal samples

The nucleic acid extraction of 5 replicates of a porcine faecal sample was performed following an adaptation to the protocol “PureFood GMO and Authentication Kit” (Catalogue No. AS1600; Promega Corporation, Madison, WI) developed by Promega [60] that includes a specific pre-processing for faecal samples prior to the automated procedure done in a Maxwell® RSC instrument (Catalogue No. AS4500;

Promega Corporation), by using Maxwell® RSC cartridges that were prepared as described by the manufacturer. The adapted protocol is schematized in **Figure 18.**

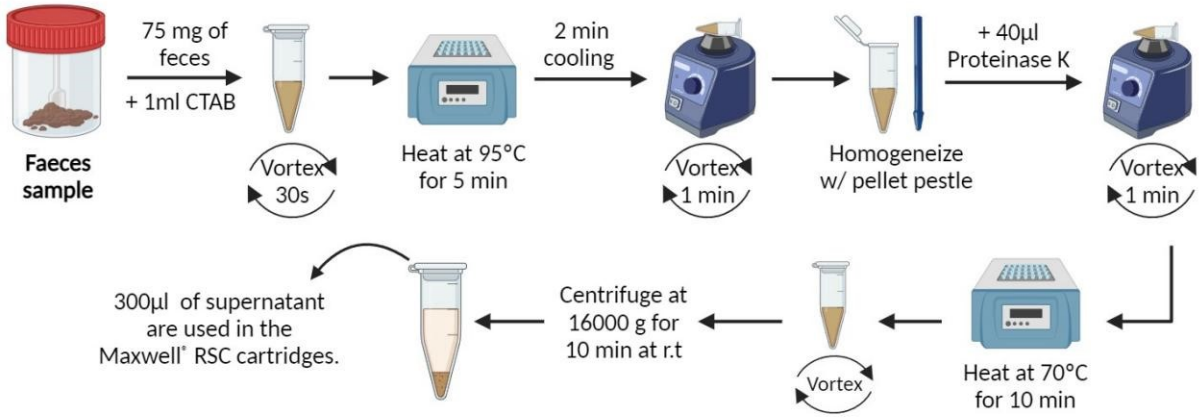


Figure 18- Nucleic acid extraction protocol for faecal samples. Created with BioRender.

All samples were eluted in 100 µl of provided Elution buffer. The extraction products were kept at -80ºC until further analysis.

A qPCR for these extraction products for PAdV, a widely excreted virus by swine population considered an indicator of porcine faecal contamination [57], was performed to validate the nucleic acid extraction protocol. The qPCR protocol is described in **Annex 1** as an SOP. The undiluted extract and 2 replicates of a 10-fold dilution of the nucleic acid extracts were analysed.

2.3.2. Comparison between two extraction methods for faeces samples: Maxwell AS1600 and "QIAamp Viral RNA Mini Kit"

After the validation described previously, the optimized Maxwell protocol for nucleic acid extraction was compared to other nucleic acid extraction method used as the standard extraction method in the laboratory, the "QIAamp Viral RNA Mini Kit" from QIAGEN®, a column-based method where the viral DNA/RNA adhere to a silica membrane. The protocol followed for this extraction is explained in detail in **Annex 2,** based on the spin procedure from QIAGEN®. Some adjustments to this protocol were made to be possible to compare accurately both methods, especially regarding the volumes used. **Figure 19** summarizes the procedure for the comparison of these protocols. For each method 2 sample replicates (Q1, Q2 and M1, M2) and 1 negative extraction control (QNC and MNC) were used. It's important to consider that for the QIAGEN® method, 300ml of sample was extracted, to match the Maxwell method, so the volumes of reagents used were adapted and the protocols were totally comparable in terms of sample analysed.

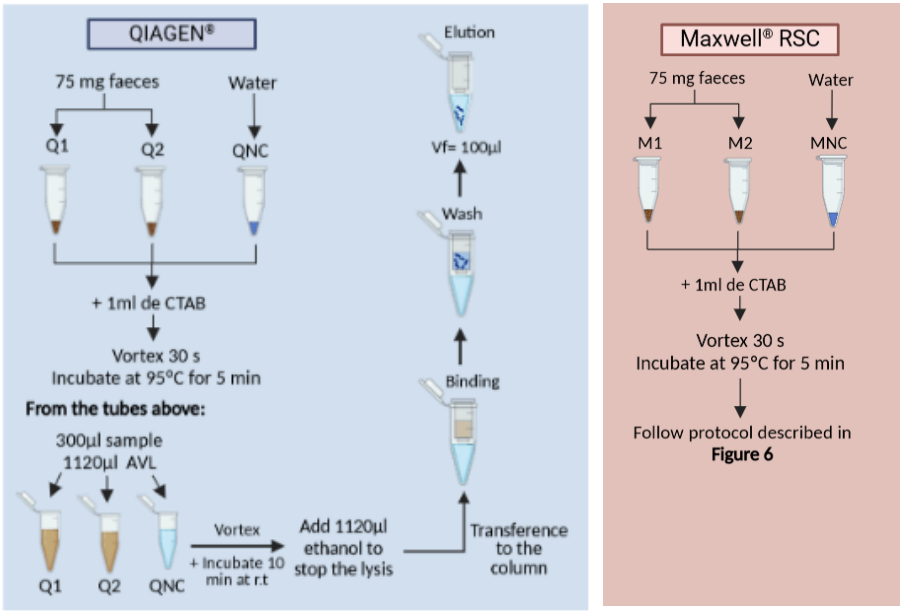


Figure 19 – Summarized procedure of the two nucleic acid extraction methods: QUIAGEN vs Maxwell. Created with BioRender. *QNC and MNC are the respective negative controls.

2.3.3. Nucleic acid extraction from individual and collective animal samples

The nucleic acid extraction method applied to each sample varied depending on the type of sample as well as the volume used for the extraction, as shown in **Figure 20**. Once the Maxwell AS1600 proved to be more efficient for faeces samples than the QIAGEN method, this was used for the individual cow samples. For the collective samples of lixiviates, manure, aerosols and wastewater, the standard method widely used for wastewater samples in the laboratory – QIAGEN - was the one selected.

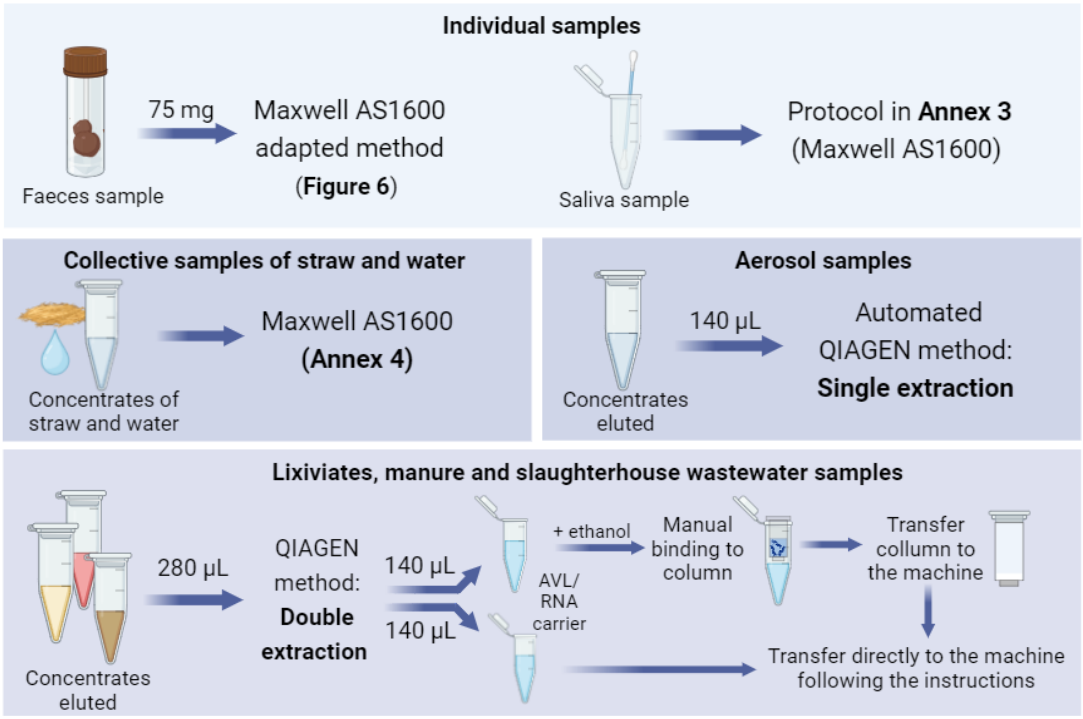


Figure 20 - Nucleic acid extraction methods used for each type of bovine sample. Created with BioRender.

2.4. Molecular detection by PCR and (RT)qPCR methods

PCR is the most widely used technique for viral molecular detection. In this work, (RT)qPCRs and nested PCRs were performed to detect the viruses of interest circulating in cow farms. Some of the protocols were already implemented in the laboratory while other protocols were created 'in-house' or commercially purchased.

2.4.1. Development of a n(RT)PCR to detect the EHDV

A new n(RT)PCR to detect the EHDV was developed as one of the objectives of this work. The target of this n(RT)PCR is the segment 9 (Seg-9) of the EHDV, the most conserved segment of the virus that encodes for the VP6 protein. **Figure 21** represents the workflow of the development of the n(RT)PCR and each step is explained in more detail below.

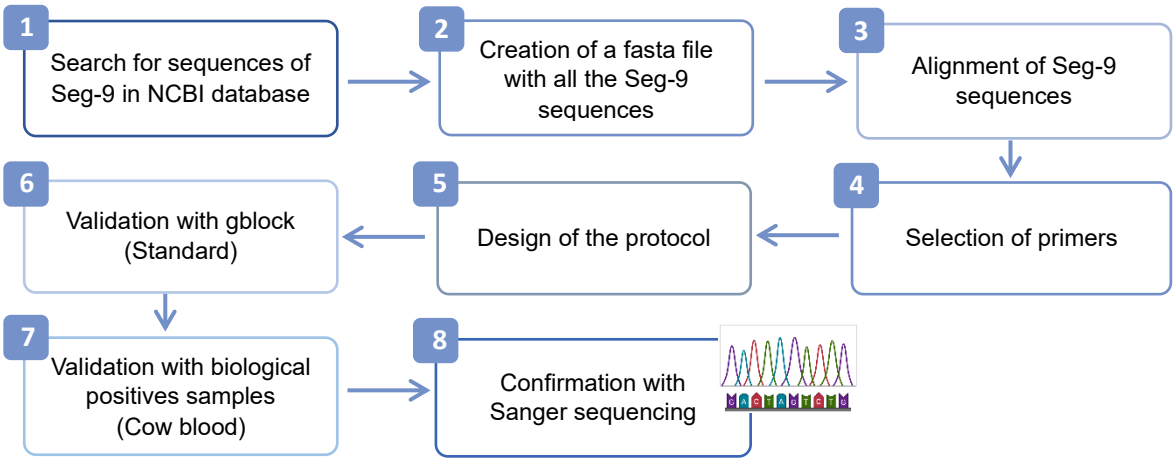


Figure 21 - Workflow of the n(RT)PCR of EHDV development.

1. In NCBI database the title "Epizootic haemorrhagic disease virus segment 9" was introduced in the search field and the Nucleotide database was selected.

2. Fasta files from Seg-9 sequences of different EHDV serotypes were transferred into the same file, with a total of 72 sequences. **Table A4 - Annex 5** compacts the information about the sequences used.

3. The alignment of the sequences was done using MUSCLE within Genious R 11.1 and the distance matrixes were obtained.

4. Conserved regions in Seg-9 were identified across all eight serotypes, as targets for the design of primers, but the selection of the primers prioritized the detection of serotypes 6 and 8, the ones circulating in Catalonia.

5. The protocol design for the detection of EHDV is a two-step n(RT)PCR, since the forward primer is the same for both PCRs, varying only the reverse primers. For the cDNA synthesis, the Superscript IV enzyme is used followed by two PCRs with DreamTaq Green Enzyme. After the n(RT)PCR, the products were run in an agarose gel. All the protocol is described in **Annex 6**.

6. The first protocol designed had some differences from the one described in **Annex 6**: Volume of "Primer pool" = 2 µL; Annealing temperature = 50ºC; Extension time = 1 min. The validation of this protocol was performed with the Gblock (positive control - standard) using two replicates of each of the concentrations 100 GC/rx and 200 GC/rx and a negative template control for each PCR (NTC 1 and NTC 2, respectively).

7. A second validation was performed with **two EHDV positive cow blood samples (A and B)** sent from a farm located in the province of Lleida (north-west Catalonia), from the first outbreak of the virus in that region around September of 2023. These samples were highly concentrated, so additional precautions were taken during their processing. **Figure 22** shows the steps performed with these samples for the validation and optimization of the protocol.

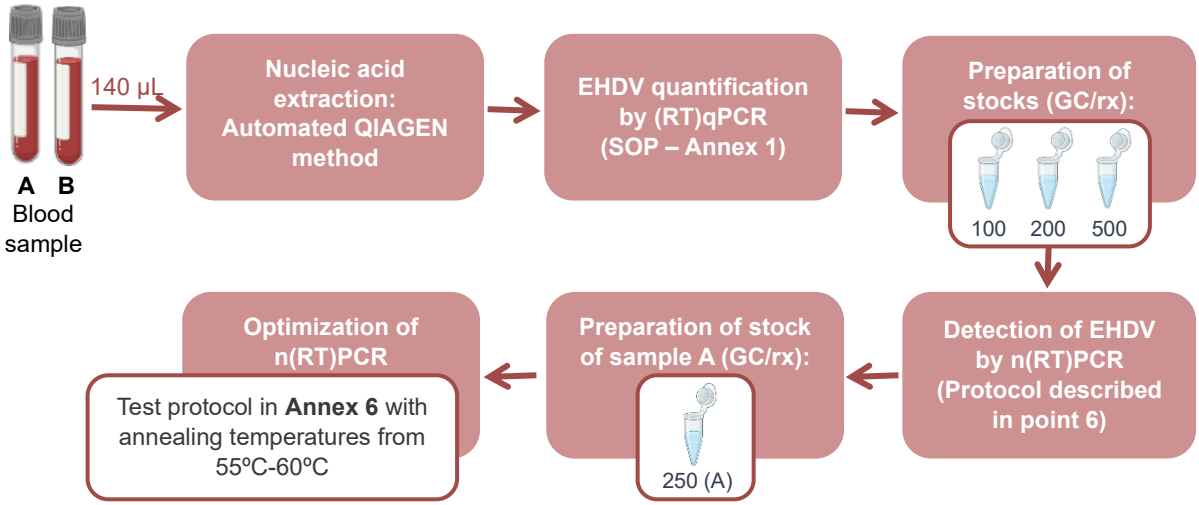


Figure 22 - Summarized protocol for the optimization of the n(RT)PCR for EHDV with positive bovine blood samples .from cow.

8. The preparation of the sample for Sanger sequencing was done from the PCR product: The purification of the DNA was performed with a DNA purification kit ("DNA Clean & Concentrator™-5 , Zymo Research"), followed by DNA quantification using the "Qubit™ 1x dsDNA HS Assay kit" with the fluorometer Qubit 3.0 instrument and preparation of the sample with the right concentration of DNA and primers to send to the Technical and Scientific Services of the University of Barcelona (SCiT) for Sanger sequencing. The detailed protocol is described in **Annex 7**.

2.4.2. Quantification by (RT)qPCR of different bovine viruses

Several bovine viruses were quantified in the samples collected, from the individual to the collective ones, to study the prevalence and circulation of these viruses in cow farms around Catalonia. This study included the following viruses: Bovine polyomavirus (BPyV), Rotavirus A (RoVA), Influenza-A virus (IAV), Bovine Coronavirus (BCoV), Bovine respiratory syncytial virus (BRSV), Crimean-Congo haemorrhagic disease virus (CCHFV), Epizootic haemorrhagic disease virus (EHDV) and the Bluetongue virus (BTV). The quantification of BPyV, RoV-A and IAV, currently analysed in the laboratory, was performed following the respective SOP described in **Annex 1**, but for the other viruses, commercial kits were used, or a new (RT)qPCR protocol was validated and included in the general SOP, as is the case of EHDV and BTV. **Table 6** presents information on the protocol used for each virus and different colours were attributed to each assay depending on the type of protocol.

Table 6 - Information on the qPCR and (RT)qPCR protocols of the bovine viruses quantified.

Virus	qPCR / (RT)qPCR assay	Target	Internal control	Protocol	Colour
BPyV	In house - SOP	VP1 gene	X	**Annex 1**	
RoV-A	In house - SOP	Segment 7	X	**Annex 1**	
IAV	In house - SOP	Segment 7	X	**Annex 1**	
BCoV	Comercial kit	No data	✓ (gene in the mix)	**Annex 8**	
BRSV	Comercial kit	No data	✓ (gene in the mix)	**Annex 8**	
CCHFV	Comercial kit modified	Segment S	X	**Annex 9**	
EHDV	Comercial kit	No data	✓ (mammal gene in the sample)	**Annex 10**	
	In house - SOP	Segment 9	X	**Annex 1**	
BTV	In house - SOP	Segment 10	X	**Annex 1**	

Table 7 identifies the viruses quantified in each sample using the colours scheme of **Table 6**. The selection of the viruses that would be analysed in each of the samples took several factors into account:

- The virus infection pathway, that is, whether it is usually present in the respective type of sample or not. For example, EHDV and BTV are not respiratory viruses, so they were not expected in high concentration in aerosol samples.
- The representativeness and complexity of the samples combined with the probability of the presence of the virus.
- The entire cost involved in each analysis, especially regarding the commercial kits.

However, in some cases, even if it's not documented the presence of the virus in a certain type of sample, the analysis was performed to verify the possibility of an alternative infection pathway.

BPyV was used as a marker to verify the quality of the samples, since it's a frequently bovine excreted virus.

Table 7 - Bovine viruses quantified in each sample and respective protocol used distinguished by colours based on Table 5. NA stands for not analysed.

		Bovine viruses							
Samples		**BPyV**	**RoVA**	**IAV**	**BCoV**	**BRSV**	**CCHFV**	**EHDV**	**BTV**
Individuals	**Faeces**			**NA**					**NA**
	Saliva			**NA**					**NA**
Collectives	**Straw and water**			**NA**					**NA**
Collectives	**Lixiviates / manure**					**NA**	**NA**	**NA**	**NA**
	Aerosols						**NA**	**NA**	**NA**
	Slaughterhouse wastewater					**NA**	**NA**		

IAV was only tested in the samples from march and April due to the outbreak of a highly pathogenic avian Influenza A virus strain – HPAIV (H5N1), that occurred in dairy cows during that time in the USA [34].

2.4.3. Standards used for viral quantification and quality control

In the case of "in-house" (RT)qPCRs, standard curves used in qPCR and (RT)qPCR are generated by using serial dilutions of known amounts of a synthetic DNA construct, also called Gblocks, containing the target region of the PCR, where one Gblock can contain target regions for more than one virus. The Gblocks sequences used in this work are shown in **Annex 11,** with the respective viruses they include. It's recommended to store the reference suspensions into DNA low binding tubes previously prepared with the concentrations ready-to-use for the PCR.

In the analysis of the results, a quality control shall be always performed considering some parameters: DNA suspensions containing less than 1 GC/reaction shouldn't present Cq values; Decimal dilutions of standard DNA or RNA suspensions should present Cq values within approximately 3 Cqs of difference between one dilution and the next ten-fold dilution. However, this may not be the case in environmental samples where direct dilutions sometimes show enzymatic inhibition (higher Cq values than expected considering the 1/10 dilution results); these results presenting inhibition should not be considered for quantification, considering only the assays with 1/10 dilutions. The slope of the regression curve should be between 3.10 – 3.60 (corresponding to amplification efficiencies of ~90-110%). Points of a minimum of 3 dilutions of standard must be included in the regression curve. Curves with r^2 values of <0.98 or with slopes out of the range 3.10-3.60 weren't used for calculations.

2.5. Characterization of the virome of collective animal samples by NGS

The samples of aerosols and lixiviates from the farms Can Bordois and La Pasiega and the wastewater samples from the slaughterhouses Sabadell and Argentona were sequenced by Illumina, a next generation sequencing (NGS) platform, previously subjected to a Target enrichment process. The complete workflow of the sequencing procedure from sample preparation until results analysis is summarized in **Figure 23**.

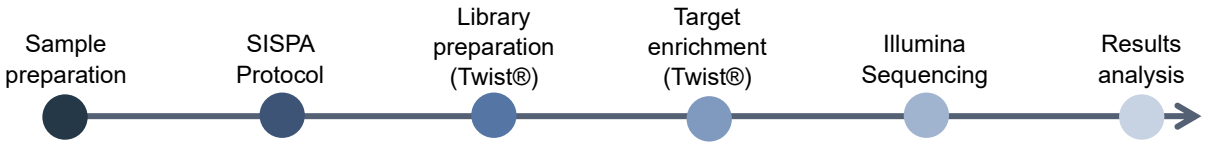


Figure 23 - NGS workflow. Includes a Sequence-independent single primer amplification (SISPA) followed by 2 steps based on protocols from Twist Bioscience ®.

The detailed protocol of the entire procedure from the DNase treatment until the target enrichment is described in **Annex 12.** Here, it's presented a summary of the protocol.

Sample preparation

Sample pools were prepared with 50µl of the samples collected between October and March as described in **Table 8**. Pools of lixiviates and wastewater samples were prepared from the concentrates of each sample without nucleic acids extracted, while the pools of aerosol samples were prepared from the nucleic acid extraction product once all concentrate had been extracted.

Table 8 - Sample pools prepared for NGS procedure.

	Lixiviate samples				**Aerosol samples**				**Wastewater samples**			
	Can Bordois		**La Pasiega**		**Can Bordois**		**La Pasiega**		**Sabadell**		**Argentona**	
Sampling months	Sample	Pool	Sample	Pool	Sample	Pool	Sample	Pool	Sample	Pool	Sample	Pool
October-December	F3S2	1	F4S2	3	F3A2	5	F4A2	7	E1D1	9	E2D1	11
	F3S3		F4S3		F3A3		F4A3		E1D2		E2D2	
	F3S4		F4S4		F3A4		F4A4		E1D3		E2D3	
January-March	F3S5	2	F4S5	4	F3A5	6	F4A5	8	E1D4	10	E2D4	12
	F3S6		F4S6		F3A6		F4A6		---		---	
	F3S7		F4S7		F3A7		F4A7		---		---	

After preparing the pools, a protocol for amplification of nucleic acids prior to library preparation and target enrichment was performed, involving a DNase treatment for free-DNA removal from the samples, followed by nucleic acid extraction and the SISPA method, described in **Figure 9** from introduction [1]. The aerosol samples weren't treated with DNase since their nucleic acids were already extracted, so their pools were directly used for SISPA.

Library preparation

The steps for the library preparation are summarized in **Figure 24**. This procedure aims to tag the viral sequences with adaptors, that enable their ligation to the sequencing platform, and with dual indexes that allow the recognition of each sample during the sequencing. The dual indexation avoids sample recognition errors due to possible index cross-links when using single adaptors.

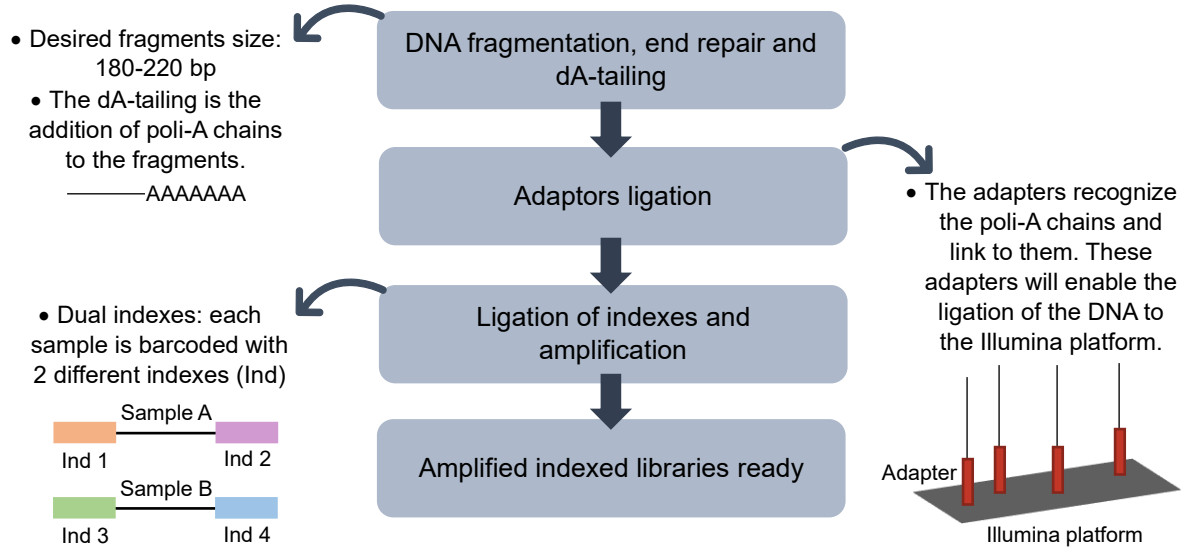


Figure 24 - Library preparation workflow.

Target enrichment

The steps of the target enrichment procedure are summarized in **Figure 25** and a more detailed explanation is described in **Figure 13** from the introduction.

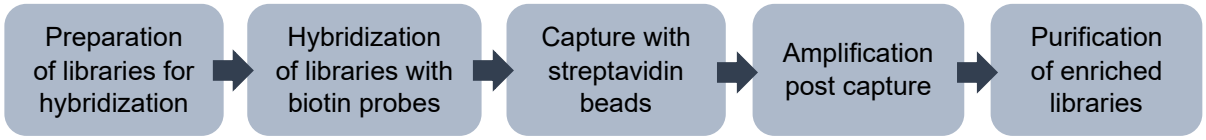


Figure 25 - Target enrichment workflow

Sequencing

The enriched libraries were polled and sent for sequencing in Parc de Recerca Biomèdica de Barcelona (PRBB). The sequencing was performed in an Illumina NextSeq 550 (2x 150bp) platform.

Bioinformatics – Analysis of results

The results from the Target enrichment sequencing were bioinformatically analysed using CZ ID portal (https:// czid.org/, accessed on June 10, 2024), an open-source cloud-based pipeline that allows to perform a metagenomic analysis and specific pathogen detection and monitoring [61]. In short, the portal starts by doing quality filtration steps and, if appropriate, host sequence filtration, then it performs an alignment pipeline based on assembly for the reads obtained which results in contigs that

are assigned to taxonomic categories. The portal also gives information on the coverage percentage of the assembled contigs in the complete genome or in a specific gene of the organisms and it enables to Blast the reads and contigs obtained to get the specific viral strain detected.

For the analysis in CZ ID portal, the results of the same type of pools (lixiviates, aerosols and wastewater) from the same farm or slaughterhouse were grouped so it's possible to do a general analysis of the virome over the sampled months from each sampled site. Despite carrying out target enrichment with a panel of viruses that infect vertebrates, sequences from bacteria or bacteriophages can still be found. For this reason, the data was filtered to only display sequences corresponding to the Viruses- Non-Phage category and with more than 10 reads. The Blasts were performed for the viral taxonomies that include viruses of interest for this work to obtain the specific viral species present in the sample: *Polyomaviridae, Rotavirus, Betacoronavirus* and *Orthopneumovirus*.

3. RESULTS AND DISCUSSION

3.1. Nucleic acid extractions

3.1.1. Optimization of Maxwell AS1600 extraction method for faeces samples

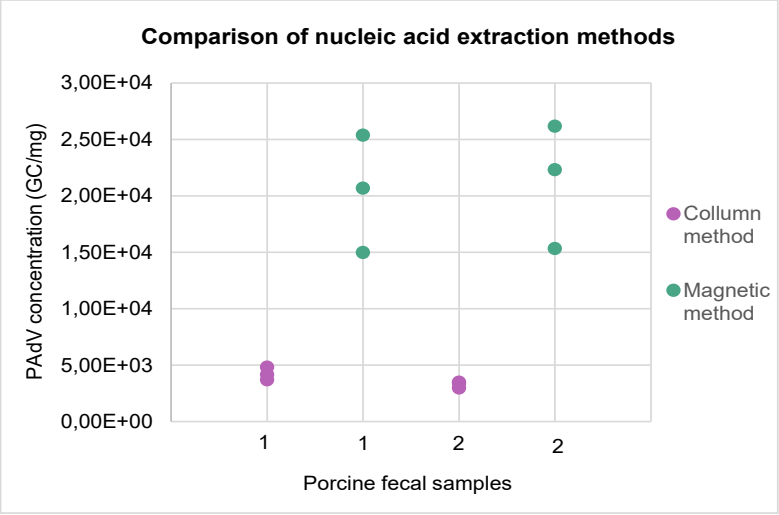
All nucleic acid extracts were positive for PAdV with high concentrations of the virus, ranging from 5.05 x 10^3 to1,09 x 10^4 genomic copies per mg of samples (**Table 9**) and no significant inhibition of the reaction was detected when comparing the concentrations between the direct extractions and the diluted ones as shown in **Table A5- Annex 13**. These results enable the validation of the adapted Maxwell AS1600 protocol for faecal samples.

Table 9 - Concentration of PAdV DNA in feacal samples extracted with the adapted Maxwell method.

Samples	GC/mg of sample (Mean values)
Sample 1	6,75 x 10^3
Sample 2	8.58 x 10^3
Sample 3	1,09 x 10^4
Sample 4	5.05 x 10^3
Sample 5	5.92 x 10^3

3.1.2. Comparison between two extraction methods for faeces samples: Maxwell AS1600 and "QIAamp Viral RNA Mini Kit"

The comparison between the two nucleic acid extraction methods - Maxwell AS1600 and "QIAamp Viral RNA Mini Kit" - was performed to select the best method to use for the faecal samples. **Table A6 – Annex 14** shows the detailed quantifications for both methods. In **Graph 1** is possible to verify that the nucleic acid extraction using the magnetic method (Maxwell) results in considerably higher viral concentrations for all replicates of both porcine faecal samples than the column-based method (Quiagen), on average 4 times greater, although it presents also higher variability.



Graph 1 - Comparison of two nucleic acid extraction methods efficiency.

Taking these results into account, the adapted Maxwell AS1600 method was selected to perform the nucleic acid extractions of the 49 individual faeces samples in this work.

3.2. Molecular detection by PCR based methods

3.2.1. Development of a n(RT)PCR for the detection of EHDV

Alignment of Segment 9 (Seg-9) sequences

The distance matrixes between all the sequences were obtained, with the lowest homology being 66% and the highest homology being 100% between 2 sequences with different accession numbers. Considering that the sequences correspond to all the 7 serotypes described until now, it was expected to obtain some low homologies between some serotypes.

Selection of primers

The primers selected for the EHDV n(RT)PCR (**Table 10**) have great homology with Seg-9 sequences from serotypes 6 and 8, but they can also anneal with other serotypes with less efficiency.

Table 10 - EHDV n(RT)PCR selected primers. The positions mentioned refer to the annealing positions of the primers in the target segment of the virus.

Primers	Sequence (3'-5')	Position (bp)
nestedEHDV-S9-F	AAAGAGCTGGGGTTCTCACG	543-562
nestedEHDV-S9-R	GTCGCATGTGCCACATCATC	973-954
nestedEHDV-S9-R2	TTCATCACGCTTCTCACCCC	905-886

When using these primers with the Gblock specifically designed as a positive control for this assay (Gblock Viralert 2 – sequence in **Annex 11),** the first-round fragment has a size of 213 bp and the second-round fragment has a size of 178 bp, since it wasn't included in the Gblock the complete sequence amplified in the virus. When using the primers to detect the virus, the first-round fragment is 430 bp long and the second-round fragment is 363 bp long. This amplicon size difference allows a quick identification of potential contamination with a positive control without any additional step.

Validation and optimization of the protocol

The n(RT)PCR for EHDV was first validated with the Gblock Viralert 2 (**Annex 11**), and it detects concentrations as low as 100 GC/rx with visible bands and without non-specificities (**Figure 26**). The high intensity and thickness of the bands may be explained because of the high concentration of the fragments produced not only in the 2nd PCR (178 bp) but also the products from the 1st PCR (213 bp), creating a thick band around the 200 bp.

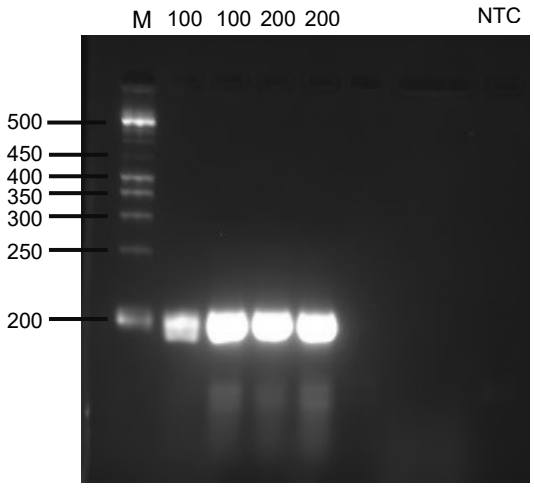


Figure 26 - Successful amplification of the gblock fragments by the n(RT)PCR for EHDV. The numbers attributed to the wells are in GC/rx. The vertical numbers correspond to the MW in base pairs of the bands. M stands for DNA marker (50bp); NTC stands for negative template control. Gel with 2% agarose.

Proceeding to the validation with the EHDV positive cow blood samples, both samples A and B tested positive by the EHDV (RT)qPCR, described in the SOP in **Annex 1**, and the viral concentrations are expressed in **Table 11**. No inhibition was detected.

Table 11 - EHDV quantification of sample A and B.

Samples	Concentration (GC/μL)
A	183,28
B	26,64

The stocks with different concentrations (100, 200 and 500 GC) to test the efficiency of the protocol were prepared only for sample A. Sample B was tested directly due to the lower viral concentration.

This validation required some steps of optimization during the process. On the first assay, where the protocol in **Annex 6** was followed with the same conditions used on the validation with the Gblock Viralert 2, as described in the materials and methods section, the desired fragment, with 363bp, is present in all samples except for one replicate of sample B, but non-specific products were also amplified in all the tested concentrations (**Figure 27-A**). In order to eliminate these non-specific products, some PCR conditions were changed: the final concentration of each primer was decreased from 0,8 μM to 0,4 μM, the extension time was decreased from 1 min to 45 seconds; and 6 different annealing temperatures were tested (55ºC, 56ºC, 57ºC, 58ºC, 59ºC and 60ªC) for the same concentration of sample A (250 GC/rx), considering that the melting temperature of the primers is 62ºC. The optimization was successful since all the non-specific products were eliminated for all the annealing temperatures tested (**Figure 27-B**). Considering that all the annealing temperatures used worked efficiently, the selected one for the final protocol was the 55ºC to prevent annealing of the primers from being too stringent, which can happen with very high annealing temperatures such as 60ºC.

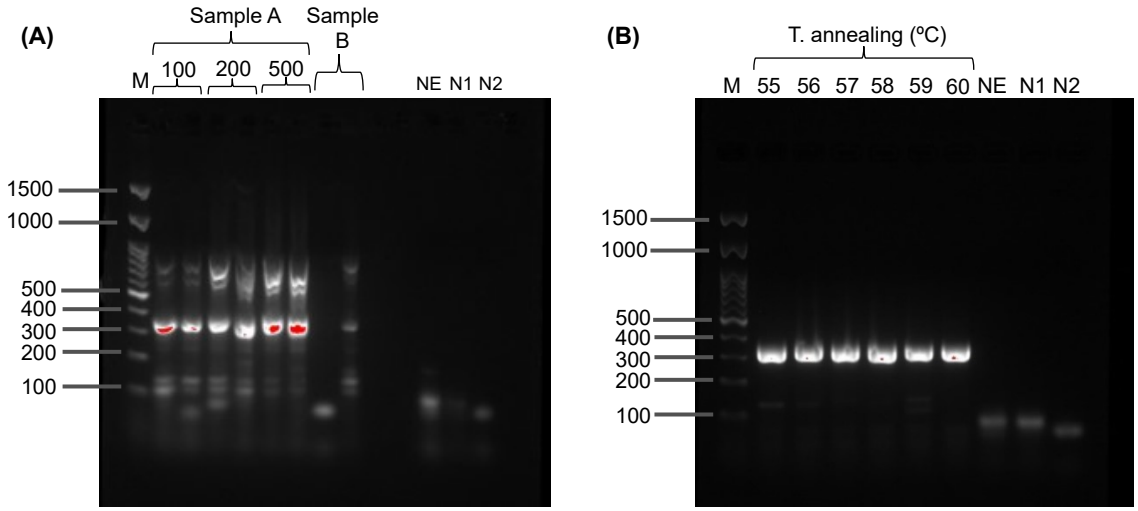


Figure 27 - Electrophoresis of the EHDV n(RT)PCR products: (A) Before optimization with different stocks of sample A and the direct concentration of sample B; and (B) After optimization with different annealing temperatures using the stock 250 GC/rx of sample A. M stands for DNA marker (100 bp); NE stands for negative control of extraction; N1 and N2 stand for negative template control of the 1st and 2nd PCR, respectively. The vertical numbers correspond to the MW in base pairs of the bands. Gels with 2% agarose.

The final protocol for the EHDV n(RT)PCR is described in **Annex 6** and it's able to detect concentrations as low as 250 GC/rx in biological samples.

Sanger sequencing

Since the electrophoresis gel after the optimization of the n(RT)PCR is clear, it was decided to use the PCR product in the tube directly for Sanger sequencing. The sequences obtained for both DNA strands (positive and negative sense) after sequencing were analysed in Genious R11.1. Some bases from the extremes of the sequences were eliminated due to low quality of the peaks and the consensus sequence was generated by aligning the sequences correspondent to the positive and negative strands of the amplified fragment – Sequence present in **Annex 15**.

A BLAST was performed with the consensus sequence and the highest homology obtained was 99% with the segment 9 of the strain "EHDV-8/Deer1 TUN2022", an EHDV serotype 8 isolated from a deer species (*Cervus elaphus barbarous*) in Tunisia in 2022, with the accession number OP971155. From this result is possible to conclude that:

- The EHDV n(RT)PCR is indeed amplifying the desired fragment in segment 9 and that it can be used as an efficient confirmation assay of the EHDV infection, mainly for serotypes 6 and 8 present in the mediterranean region.

- The EHDV serotype that caused the outbreak in cattle in September of 2023 in Catalonia is suspected to come from Tunisia based on its Seg-9 sequence, suggesting that the transportation of the virus through long distances by its vector could have happened.

3.2.2. Prevalence of bovine viruses in individual animal samples

The percentage of positives for each of the tested bovine viruses, in the individual samples of faeces and saliva and in the collective samples of straw and water from the same farms, is represented in **Table 12** as a heatmap. It's notorious the low percentages of positives among the different farms, being RoV-A the most prevalent virus among cows from different farms, mainly in farm 2 and 3. EHDV and CCHFV weren't detected in any sample, an expected result since they are not endemic viruses but emergent bovine viruses.

It's also important to notice that the BPyV was used as a bovine viral marker, since it's a widespread virus in these animals. However, its prevalence was very low among all the samples, and in some cases, it was negative even when other viruses were detected, such as RoV-A and BCoV (farm 2 and 3), but since the virus it's mostly excreted in urine the low viral loads or absence in faeces can naturally happen. For this reason, in future works, a more suitable marker for faecal and saliva bovine samples should be selected. One of the objectives of the viral analysis of these samples was to prove the hypothesis that the collective samples of straw and water could give us similar information on viral prevalence as the individual samples. Despite the low number of collected samples, some of the collective samples showed positive results for some viruses that were not detected in the individual samples, so we can assume that collective samples may be a better option to do a surveillance study.

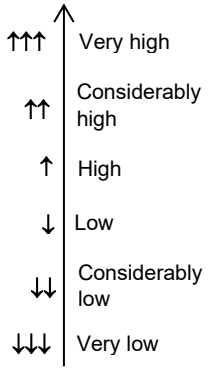
Table 12 - Heatmap representing the percentage of detection of 6 bovine viruses in individual and collective samples from 4 different cow farms (1,2,3 and 4).

			MARKER	PATHOGENIC VIRUSES				
Farm	**Sample type**	**N**	**BPyV**	**RoV - A**	**BCoV**	**BRSV**	**EHDV**	**CCHFV**
1	**Individual (Faeces)**	10	0	0	0	0	0	0
	Individual (Saliva)	10	0	0	0	0	0	0
	Collectives	0	-------	-------	-------	-------	-------	-------
2	**Individual (Faeces)**	9	0	0	0	0	0	0
	Individual (Saliva)	10	0	30	0	0	0	0
	Collectives	3	0	0	0	0	0	0
3	**Individual (Faeces)**	10	0	10	20	0	0	0
	Individual (Saliva)	10	0	10	0	0	0	0
	Collectives	2	50	50	0	0	0	0
4	**Individual (Faeces)**	20	10	0	0	0	0	0
	Individual (Saliva)	20	0	0	0	0	0	0
	Collectives	2	0	0	0	0	0	0

Since it was not possible to draw a reliable conclusion regarding the most appropriate type of sample for surveillance studies, other factors were also considered when choosing the types of samples to be used in the remaining work, which are elaborated in **Table 13.**

Table 13 - Balance of different characteristics between individual and collective samples.

Characteristics	Individual samples	Collective samples
Difficulty of sampling	↑↑↑	↓↓↓
Cost of sampling	↑↑	↓↓
Specialized personnel	↑↑↑	↓↓↓
Sampling time	↑↑	↓↓
Tested animals	↓↓	↑↑↑
Cost of analysis per farm	↑↑↑	↓↓
Concentration of sample	↑↑↑	↓↓↓
Presence of inhibitors	↓	↑↑
Viral integrity	↑↑↑	↓

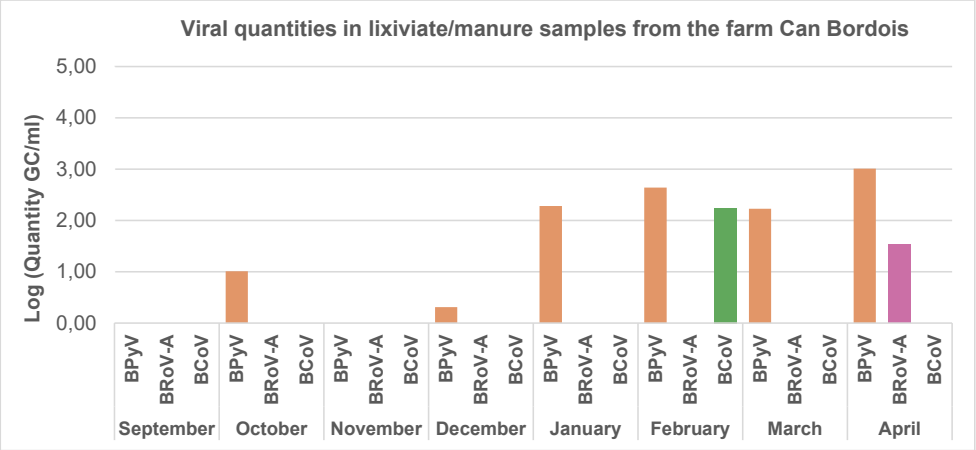


Considering all the advantages and disadvantages of each type of sample, the most appropriate for the purpose of this work were the collective samples due to all the negative points (red arrows) of the sampling process of individual samples, as well as the fact that they don't provide an overview as comprehensive as the collective samples can give of the viruses circulating on a farm, given the low representation of the total number of animals.

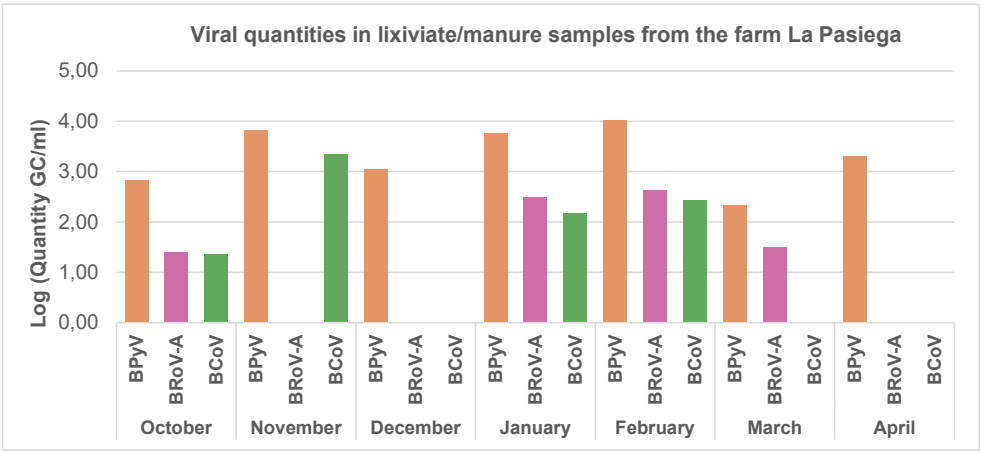
3.2.3. Surveillance of bovine viruses in collective animal samples

The viruses IAV, BRSV, EHDV and BTV weren't detected in any of the collective samples tested by (RT)qPCR. The concentrations of each sample together with the mean values for each of these viruses in each farm and slaughterhouse are shown in **Annex 16 - Tables A7, A8** and **A9**.

The results related to the surveillance of the circulation of BPyV, RoV-A and BCoV in lixiviates and manure samples in the farms Can Bordois over 8 months and La Pasiega over 7 months are represented in **Graph 2** and **Graph 3,** respectively, in logarithm of base 10 of the quantity of virus in GC/ml, to normalize the results for the same scale. For the farm La Pasiega, the sampling campaign started on October and BCoV wasn't analysed in the samples from March and April due to lack of commercial kit.



Graph 3 – Graphical representation of the logarithm of base 10 of the quantities in GC/ml of BPyV, BRoV-A and BCoV in lixiviate/manure samples from the farm Can Bordois.



Graph 2 - Graphical representation of the logarithm of base 10 of the quantities in GC/ml of BPyV, BRoV-A and BCoV in lixiviate/manure samples from the farm La Pasiega.

The viral distribution visible in **Graph 2 and Graph 3** allow us to draw the following conclusions:

- BPyV is the most abundant virus in all samples over the 8 months, except in Can Bordois samples from September and November where no virus was detected. This shows that the virus can be a good bovine viral urine marker in lixiviates and manure (that also contain urine, where the virus is mostly excreted).

- It's notorious that La Pasiega presents in general more viral diversity and higher viral loads than Can Bordois along the year, mainly for the pathogenic viruses RoV-A and BCoV, so it would be expected that cows from La Pasiega could present more clinical symptoms associated with the infection of these viruses or at least have higher circulation of these viruses throughout the sampled months.

- It's not possible to stablish a reliable association between the season of the year and the presence of the virus but it can be suggested that winter (including January, February and

March) is the season with the greatest variety of viruses in circulation in both farms, and February is the first month in which the presence of a pathogenic virus- BCoV- was detected in Can Bordois. Previous studies showed that BCoV is more prevalent in winter probably because colder conditions favour the stability of the virus, which is according to the results, and that outbreaks occur yearly in the same farm [62].

- The detection of RoV-A in La Pasiega between January and March and its absence in the remaining months except October is in agreement with the seasonality of the virus, that is known to typically cause infections during the winter in temperate regions, as studies have shown [63], being more present in La Pasiega mainly between January and March. On the other hand, in Can Bordois RoV-A was only detected in April. The low detection of viruses in general between September and December 2023 in Can Bordois can be related with changes in the texture of the slurry collected over the months. During these months the slurry was dry probably because of the higher temperatures registered and lack of rain during that period, while between January and April the slurry has a more pasty or liquid texture which can provide more stability to the viruses. This theory is reinforced by the fact that the BPyV quantification were much lower in these 3 months.

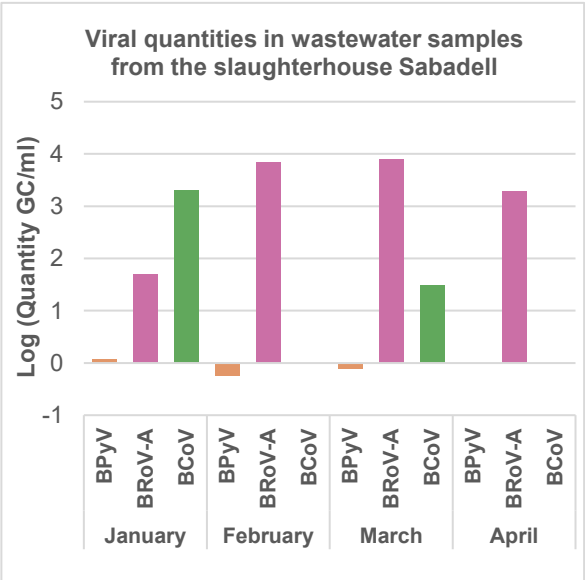
- Since BCoV was detected in several months in La Pasiega, it would be a good safety procedure for the workers to use protection masks when working near the animals, to avoid possible future spillovers of the virus even if for now it's a non-zoonotic virus.

Regarding the aerosol samples, all of them were negative for BRSV in both farms. For BCoV all aerosol samples from the farm Can Bordois were negative. In the farm La Pasiega four lixiviate and two aerosol samples were positive, so it's interesting to do a comparison between the presence of the virus in both type of samples collected in the same day since they give us different information. Lixiviates stay long periods in the farms so when a virus is circulating some days before the sampling it can be detected in the lixiviate sample. On the other hand, the aerosol samples capture only viruses that are infecting the animals at the time of sampling or that are aerosolized from the slurry. **Table 14** shows that when the virus is detected in the aerosol sample, it's also detected in the lixiviate sample in higher concentrations, but the opposite it's not verified. From these results, it's concluded that BCoV was circulating among the cows form La Pasiega farm before the sampling day of October, November, January and February and that some cows were infected in the day of sampling in October and November.

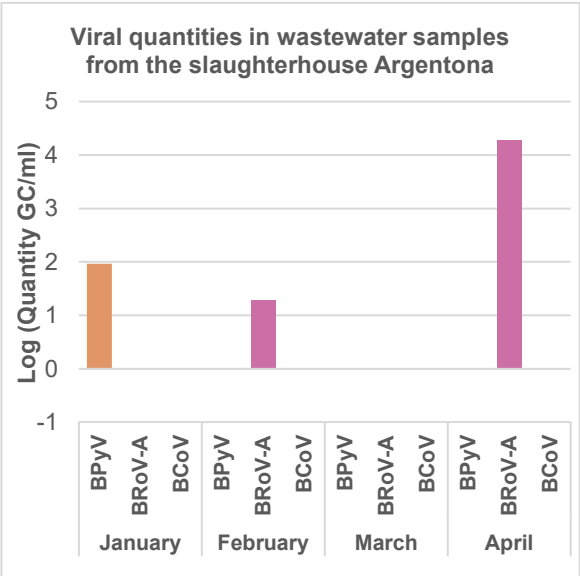
Table 14 - Concentration in GC/ml of BCoV in collective samples of lixiviates and aerosols from the farm La Pasiega, over 6 months – a comparative approach.

	Sample type	
Month	**Lixiviates**	**Aerosol**
October	2,29E+01	1,82E+01
November	2,21E+03	1,75E+00
December	Negative	Negative
January	1,47E+02	Negative
February	2,67E+02	Negative

Finaly, the wastewater samples from cow slaughterhouses presented negative results for EHDV and BTV over the 4 months tested. On the other hand, BPyV, RoV-A and BCoV were detected in some of the samples and the viral distribution in the slaughterhouses Sabadell and Argentona is represented in **Graph 4** and **Graph 5,** respectively**,** as the logarithm of base 10 of the quantity of viruses in GC/ml.



Graph 4- Graphical representation of the Log_{10} of the quantities in GC/ml of BPyV, BRoV-A and BCoV in wastewater samples from Sabadell slaughterhouse.



Graph 5 - Graphical representation of the Log_{10} of the quantities in GC/ml of BPyV, BRoV-A and BCoV in wastewater samples from Argentona slaughterhouse.

By analysing the graphs, it's possible to conclude the following:

- The wastewater samples from Sabadell present higher viral loads than Argentona regarding these three viruses, where RoV-A and BCoV prevail.

- BPyV appears only once in a significant amount in January in Argentona and is otherwise absent or present in minimal quantities in the other months for both slaughterhouses. These results contrast greatly with the ones from lixiviates. It can be suggested that BPyV may be a good bovine viral marker in lixiviate/manure samples but not in wastewater samples from slaughterhouses. A reasonable explanation is that BPyV is mainly excreted in urine which ends

up in the lixiviates and manure that were collected, but in slaughterhouses the animals are kept fasting until sacrifice which reduces greatly the excretions produced, so the wastewater will be rich in blood instead of animal excretions, like faeces and urine.

- RoV-A was detected in high quantities in all samples from Sabadell from January until April which is according to its winter seasonality as mentioned before, and it shows the virus is circulating greatly among cows from different farms around Catalonia and Aragon. In Argentona, RoV-A was detected in only half of the samples, but it was the most prevalent virus in this slaughterhouse.

- The high abundance of BCoV in Sabadell in January and March, followed by its absence in the other months, is according to its winter seasonality as mentioned previously, although there are no summer samples to confirm this seasonality.

Slaughterhouse's wastewater samples are more representative both in number (500-800 vs. 200 animals in farms) and in geographical distribution, compared to the collective farm samples. Therefore, it's reasonable that pathogenic viruses are commonly detected in higher viral loads in these samples.

3.3. Characterization of the virome of collective bovine samples

The massive sequencing of the viral nucleic acids extracted from aerosols and slurry from cow farms and of the bovine slaughterhouse's wastewater resulted in a list of viral sequences which provides information on the viruses excreted or circulating in cows grown in different places in Catalonia and nearby regions. Despite carrying out target enrichment with a panel of viruses that infect vertebrates, sequences from bacteria or bacteriophages are still found. For this reason, the data was filtered to only display sequences corresponding to the Viruses - Non-Phage category and with more than 10 reads observed.

The heatmap represented in **Table 15** gives an overview of the diversity of bovine viruses found in the collective samples. It's notable that the wastewater samples present the highest richness and viral diversity, followed by the slurry samples and finally the aerosol samples. Aerosol samples are known for presenting less nucleic acids compared to other environmental samples (such as soil or water). Additionally, it's important to consider that the aerosol samples were sequenced by NGS with the presence of the RNA-carrier used in their nucleic acid extraction, unlike the other samples, which could have jeopardized the sequencing of other viral nucleic acids present in lower quantities, so their viral diversity can be greater than the observed even if lower than in lixiviates.

Table 15 - Heatmap based on reads per million representing the diversity of bovine viruses in aerosol and slurry samples of 2 cow farms - Can Bordois and La Pasiega - and in wastewater samples from 2 cow slaughterhouses - Sabadell and Argentona. WW stands for wastewater.

Genus	Viral species	Aerosol Can bordois	Aerosol La Pasiega	Slurry Can Bordois	Slurry La Pasiega	WW Sabadell	WW Argentona
Betacoronavirus	*Betacoronavirus 1*						
Betapapillomavirus	*Betapapillomavirus 1*						
Iotapapillomavirus	*Iotapapillomavirus 1*						
Xipapillomavirus	*Bos taurus papillomavirus 28*						
Bocaparvovirus	*Bocaparvovirus ungulate 1*						
	Bocaparvovirus ungulate 6						
Copiparvovirus	*Copiparvovirus ungulate 1*						
	Copiparvovirus ungulate 5						
Erythroparvovirus	*Erythroparvovirus ungulate1*						
Cyclovirus	*Cyclovirus sp.*						
Enterovirus	*Enterovirus E*						
	Bovine enterovirus JS20-1						
	Enterovirus sp.						
Epsilonpolyomavirus	*Epsilonpolyomavirus bovis*						
Kobuvirus	*Aichivirus B*						
	Aichivirus D						
	Kobuvirus sp.						
	Kobuvirus cattle/BV253/2008/BEL						
Mamastrovirus	*Bovine astrovirus*						
	Bovine astrovirus B76-2/HK						
	Bovine astrovirus B18/HK						
	Bovine astrovirus B76/HK						
	Bovine astrovirus B34/HK						
	Bovine astrovirus B170/HK						
Norovirus	*Norwalk virus*						
Orthopneumovirus	*Orthopneumovirus bovis*						
Picobirnavirus	*Picobirnavirus sp.*						
	Bovine picobirnavirus						
Rotavirus	*Rotavirus B*						
	Rotavirus A						
Torovirus	*Bovine torovirus*						

0 69895

A more careful analysis of **Table 15** provides interesting information about the distribution of the bovine viruses detected:

- The *Erythroparvovirus ungulate 1* is mainly present in the blood from infected cattle, and it was detected in both wastewater samples, standing out in Sabadell wastewater for the highest number of reads per million among viruses and samples. Despite being very abundant, the virus is not associated with any disease [64].

- Other viruses widely present in several samples are from the genus *Bocaparvovirus* where the species *Bocaparvovirus ungulate 1* is present in all samples with exception of the aerosol from La Pasiega. This virus is associated with diarrhoea in calves and spontaneous abortions in

adult cattle but it seems to be widespread in healthy and ill cows which explains its detection [64].

- It's also important to notice the presence in several samples of other viruses which cause diarrhoea in calves such as viruses from the genus *Norovius, Mamastrovirus, Betacoronavirus, Enterovirus, Rotavirus* and *Torovirus* [65]. The specific Norovirus sp. detected in the wastewater samples and in slurry from La Pasiega was *Norovirus GIII*, a strain that infects cattle.

  .

  It's interesting to see again that the bovine polyomavirus, corresponding to the *Epsilonpolyomavirus bovis sp.,* used as a viral marker is not detected in the slaughterhouses' wastewater as observed in the (RT)qPCR results. The lack of detection by NGS might be explained by the low concentration of this virus, probably due to the low excretion from cattle before being processed in the slaughterhouses (short time in the slaughterhouse facilities before sacrifice or use of fasting practices). However, the virus is detected in the lixiviates and aerosols for both farms where positive (RT)qPCR were obtained.

- *Betacoronavirus 1 sp*. and the *Orthopneumovirus bovis sp*. which include the BCoV and the BRSV, respectively, and that cause respiratory diseases in cattle were found in some of the samples. The *BRSV* was only detected by NGS throughout the work, while the BCoV was also detected in some samples by (RT)qPCR.

After a general overview of the samples' virome, a more specific analysis of the viral families of interest for this work was performed. Using the CZ ID portal, Blasts searches for the obtained contigs were run for the following viral families: *Betacoronovirus, Orthopneumovirus* and *Rotavirus*. The results of the Blasts are shown in **Annex 17 - Table A10** and **Table A11**, which contain information about the viral species, host, genome coverage, sequenced gene corresponding to the best hit of the blast with the larger contig among other additional information.

In addition to bovine viruses, NGS has the potential to identify unexpected viruses from domestic or wild animals as well as human viruses. **Table 16** gives an overview in the form of a heatmap of the diversity of these viruses and respective hosts.

The wastewater samples are again the ones harbouring a higher number of viral species compared to lixiviates and aerosol samples. Noteworthy, the aerosol samples present a high number of reads associated to viral species that infect non-bovine viruses. Some of the conclusions drawn from the analysis of the **Table 16** are as follows:

- The detection of human viruses in both wastewater samples may be explained by the possibility that the wastewater from the toilets used by the workers is mixed with the wastewater coming from the slaughterhouse itself, since these viruses are widely excreted by humans.

Table 16 - Heatmap based on reads per million representing the diversity other domestic and wild animal viruses in aerosol and slurry samples of 2 cow farms - Can Bordois and La Pasiega - and in wastewater samples from 2 cow slaughterhouses - Sabadell and Argentona. Viral species are ordered by their associated host. WW stands for wastewater.

Host	Viral species	Aerosol Can Bordois	Aerosol La Pasiega	Slurry Can Bordois	Slurry La Pasiega	WW Sabadell	WW Argentona
Human	*Aichivirus A*						
	Betapolyomavirus secuhominis						
	Deltapapillomavirus 4						
	Human mastadenovirus D						
Ovine animals	*Ovine enterovirus 2019-00927*						
	Ovine mastadenovirus A						
	Tetraparvovirus ungulate4						
Birds	*Aveparvovirus galliform 1*						
	Circovirus starling						
Bats	*Bat circovirus*						
Rodents	*Lucheng Rn rat coronavirus*						
	Norway rat kobuvirus 1						
	Protoparvovirus rodent						
	Rat kobuvirus GZ85						
	Rat kobuvirus MM33						
	Rat minute virus						
	Rodent bocavirus						
Goat	*Goat enterovirus*						
Dog	*Canine astrovirus*						

- The high amount of a Human papillomavirus (*Deltapapillomavirus 4*) in the wastewater and aerosol samples can have an epithelial or faecal origin from the hands or toilets of the workers, respectively. A published work (Itarte *et al.* 2024) showed the presence of human papillomaviruses in surfaces from common spaces of workers from a wastewater treatment plant, such as the kitchen [66].

- The slaughterhouses from Sabadell and Argentona also sacrifice sheep and goats. Despite they are sacrificed in lower proportions compared to cows (1:10, sheep/goat vs cows) and in very specific dates during the week, their presence in wastewater is possible (i.e. *Goat enterovirus)*.

- The detection of bat viruses in slurry samples from La Pasiega indicates the presence or circulation of these mammals on the farm or nearby. This may also represent a threat of viral interspecies transmission from the bats to the cows that can result in dangerous outcomes for the cattle and, eventually, humans. The *Bat circovirus sp*. detected in slurry from La Pasiega

presents 100% of genome coverage and 93,5% of identity with this species. The presence of bats in farms and their contribution to spillover events has been previously investigated [67].

- Viruses associated to wild animals such as rats were only detected in slaughterhouse's wastewater. If rats are present nearby the slaughterhouses' wastewater treatment plant (WWTP), they can easily reach the wastewater tanks. Therefore, the presence of rat associated viruses is not surprising.

3.3.1. Comparison of results from qPCR or (RT)qPCR and NGS

The qPCR or (RT)qPCR and NGS techniques have different sensitivities and objectives so it would be interesting to compare the results obtained from these methods to assess the sensitivity of each method for the bovine viruses analysed. **Table 17** shows the summarized results of (RT)qPCR and NGS for BPyV, BCoV, RoV-A and BRSV.

Table 17 - Comparison of the detection of BPyV, BCoV, RoV-A and BRSV between qPCR or (RT)qPCR and NGS

Sample pool	Virus	Positive by qPCR	Mean quantification by qPCR (GC/ml)	Result NGS
Lixiviates Can Bordois	BPyV	5/6	3,05E+02	Detected
	BCoV	1/5	1,76E+02	Detected
	RoV-A	1/8	3,40E+01	Not detected
Lixiviates La Pasiega	BPyV	6/6	3,89E+03	Detected
	BCoV	4/5	6,62E+02	Detected
	RoV-A	4/8	1,97E+02	Not detected
Aerosols Can Bordois	BPyV	NA	NA	Detected
	BCoV	0/5	0,00E+00	Not detected
	BRSV	0/6	0,00E+00	Not detected
Aerosols La Pasiega	BPyV	NA	NA	Detected
	BCoV	2/5	9,98E+00	Not detected
	BRSV	0/6	0,00E+00	Not detected
Wastewater Sabadell	BPyV	3/4	8,51E-01	Not detected
	BCoV	1/2	1,01E+03	Detected
	RoV-A	4/4	4,12E+03	Detected
	BRSV	NA	NA	Not detected
Wastewater Argentona	BPyV	2/4	4,68E+01	Not detected
	BCoV	0/4	0,00E+00	Not detected
	RoV-A	2/4	9,51E+03	Not detected
	BRSV	NA	NA	Detected

Regarding the BPyV, the main highlight is its detection by NGS in both pools of aerosols in large quantities, verified by the number of reads obtained, and with genome coverages that reach 100% in the case of *Bovine polyomavirus 2* **(Annex 17 - Table A10).** This virus wasn't tested by (RT)qPCR in aerosols since it's not a respiratory virus, however these results show that it has the potential to be a good bovine viral marker in this type of samples. On the other hand, BPyV wasn´t detected by NGS in any of the wastewater pools. This lack of detection goes in line with the low quantities of the virus detected by (RT)qPCR. When analysing **Table 14** and **Table 15**, it's verified that both wastewater samples have large amounts of different viruses from other families which can jeopardize the detection of a virus that is present in a much smaller quantity, as the BPyV.

For the BCoV, only in the aerosols from La Pasiega we have two positive results by (RT)qPCR but a negative result by NGS. It's important to consider the very low concentration of virus in this sample (mean of 9,98 GC/ml) that can easily be masked by other viruses in higher quantities. In the lixiviate samples, with higher concentrations of the virus, many reads were obtained, confirming its circulation in the farms.

The BRSV was only detected by NGS in the wastewater of Argentona, but this sample wasn't tested by (RT)qPCR. The detection of BRSV in the slaughterhouse suggests that the virus can be circulating on farms other than the ones sampled in this study.

Finally, the discussion around the RoV-A deserves more attention. The NGS barely detected RoV-A in most of the samples that were positive by (RT)qPCR with considerable high viral concentrations. Only in the wastewater of Sabadell was possible to sequence Rotavirus A and Rotavirus B, where the latter appears to be present in greater quantities (80 reads of RoV-B against 14 reads of RoV-A) with a 65,5% of genome coverage. Knowing that both viruses are of bovine origin, this may indicate that RoV-B is circulating more among cows than RoV-A. The (RT)qPCR used in this work is specific for RoV-A, so maybe samples that showed to be negative for RoV-A could eventually be positive for RoV-B by (RT)qPCR using a specific assay for this RoV species. Regarding the very low detection of RoV by NGS, there are several studies that demonstrate the difficulty in capturing double-stranded RNA genomes, even with high viral loads detected by (RT)qPCR [68], which is verified in **Table 16.** This may be due to an incomplete denaturation and tagging of the double-stranded chains during cDNA production [69]. This should be taken into account if other dsRNA viruses such as EHDV and BTV want to be analysed by NGS in this type of samples.

Therefore, an improvement in the SISPA protocol (**Annex 12** – step 1) can be suggested to favour the detection of dsRNA genomes. In step 1.3. from **Annex 12**, related with the retro transcription to cDNA and tagging of viral RNA genomes, for the denaturation of the double stranded chains we could prepare for each sample duplicates of sample tubes so we could incubate one of the tubes at 65ºC for 5 min as mentioned in the protocol and the other tube at 98ºC for 3 min, as it's done for samples tested for dsRNA viruses by (RT)qPCR to try to increase the tagging of these genomes in the cDNA production. By doing this, it would be possible to compare the viruses found using each of the incubation temperatures. It would be expected to find more dsRNA viruses in the case of the 98ºC incubation.

Additionally, and very briefly, some of the samples present in the pools for NGS, that were positive for RoV-A by (RT)qPCR, were tested by (RT)nPCR with RoV-A human primers used in a study from 2021 targeting the complete segment encoding the VP7 protein [70]. It was interesting to see that the lixiviates from La Pasiega tested positive for this (RT)nPCR, suggesting the possible circulation of human strain RoV-A among cows in this farm. These RoV-A species were not detected by NGS and the obtained amplicons will merit further analysis to confirm or discard this hypothesis.

4. CONCLUSIONS

- The Maxwell AS1600 “PureFood GMO and Authentication Kit” including an additional pre-processing for faecal samples, that eliminates organic debris and lysate the viral particles, developed by Promega showed to be efficient extracting nucleic acids of these samples and better when compared with the “QIAmp Viral Mini kit” from Qiagen®.

- The n(RT)PCR developed “in house” for the detection of EHDV, targeting mainly the serotypes 6 and 8 of the virus, can detect as low as 250GC/rx in biological samples and as low as 100 GC/rx with synthetic DNA (Gblock).

- BPyV did not appear to be a good bovine viral marker in saliva or rectal clinical samples nor in wastewater samples from slaughterhouses, contrary to what is seen in collective samples of lixiviates where the virus is detected in 92% of the samples with a mean concentration of $2{,}10x10^3$ GC/ml.

- The viruses AIV, CCHFV, BRSV, EHDV and BTV weren’t detected in any of the individual and collective samples tested by (RT)qPCR.

- RoV-A was detected in 31,25% of the lixiviates from the cow farms and in 75% of the wastewater samples from slaughterhouses presenting mean concentrations of $1{,}15 \times 10^2$ GC/ml and $5{,}32 \times 10^3$ GC/ ml, respectively.

- BCoV was detected in all types of samples, standing out in La Pasiega farm where was present in both lixiviates and aerosol samples. In Sabadell slaughterhouse, BCoV was detected in 50% of the samples with a mean of $1{,}01 \times 10^3$ GC/ml, proving the circulation of the virus among cattle in Catalonia.

- Using NGS coupled with target enrichment, several bovine viruses from the genus *Mamastrovirus, Betacoronavirus, Enterovirus, Rotavirus* and *Torovirus,* causing diarrhoea in calves were found in the collective samples.

- The BPyV was detected by NGS presenting a high number of viral reads and good genome coverage in aerosol samples. This suggests that BPyV could be a good bovine viral marker for this type of samples.

- BRSV was only detected by NGS throughout the work, while BCoV was detected by NGS and (RT)qPCR in some samples.

- Besides bovine viruses, other animal viruses were widely detected in cows' collective samples, such as viruses from humans, ovine animals, birds, bats, rodents, goat and dogs. The detection of bat viruses indicates a circulation of bats in the cow farms, which may represent a threat to cows, and later to humans, in the case of viral interspecies transmission. Similarly, other animals such as rodents might contribute to the viral composition of slaughterhouse wastewater.

- Target enrichment sequencing enabled the detection of several bovine and non-bovine viruses in the collective samples of lixiviates, aerosols and wastewater, including viruses not detected by (RT)qPCR techniques. However, it was observed the difficulty in capturing double-stranded RNA genomes, such as RoV, even with high viral loads detected by (RT)qPCR, which can also jeopardize the detection of EHDV and BTV, other dsRNA viruses.

- Considering factors like animal comfort, economic costs and viral detection, collective samples revealed to be suitable for surveillance of viral circulation in cows to complement and reduce the use of clinical samples.

5. FUTURE WORK

As future work, it would be important to continue the sampling campaign of collective environmental samples and the detection of the studied viruses to get an overview of the viral circulation in cattle for 1 year, so it would be possible to analyse also the seasonality of the viruses. It would be worth it to start analysing the BPyV by qPCR in the aerosol samples since the NGS showed that this virus can be a good bovine viral marker in these samples. The next step of the project is the analysis of individual faecal samples from wild animals collected nearby the sampled farms to verify if there are common viruses circulating among them and cattle. Furthermore, due to the emergence of BTV in Catalonia that has been happening during the last months and the expected resurgence of EHDV due the vectors peak activity, it would be important to increase the sampling in cow farms and slaughterhouses to prove the suitability of collective sample testing to identify and control the outbreaks on time. It would be also important to implement studies of viral surveillance, like the one developed in this project, in cattle farms from other countries to achieve a global One health approach.

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ANNEXES

Annex 1 – Quantitative PCR standard operating procedure for quantification of viruses in the samples studied in the project

This SOP describes the performance of specific qPCR for 2 DNA viruses (porcine adenovirus (PAdV) and bovine polyomavirus (BPyV)) and 5 RNA viruses (rotavirus (RoV), influenza A virus (IAV), Crimean-Congo haemorrhagic fever virus (CCHFV), epizootic haemorrhagic disease virus (EHDV) and bluetongue virus (BTV)). amplification and quantification were performed in a QuantStudio™ 1 Real-Time PCR System from ThermoFisher. The reactions take place in a 96-well Optical reaction plate (Applied Biosystems Cat. No. 4306737 for 20 units) covered with ABI Prism™ adhesive film (Applied Biosystems Cat. No. 4311971 (100 units)). Standard curves used in qPCR and qRT-PCR are generated by using serial dilutions of known amounts of a synthetic DNA construct containing the target region of the PCR.

The qPCRs always included a non-template control to demonstrate that the mix did not produce fluorescence and an extraction negative control in duplicate to assess possible contamination during the extraction procedure. For viral quantification, 10-fold dilutions of the standards were prepared from 10^2 until 10^5 copies per reaction.

Reagents

- **For qPCR:** TaqMan Environmental PCR Master Mix (Applied Biosystems. Part nº 4396838) supplied in a 2x concentration.
- **For qRT-PCR:** RNA Ultrasense™ One-Step Quantitative RT-PCR System (Invitrogen Cat.No.: 11732-927).

Master mix preparation

Table A1- Reagents volume per reaction in the qPCR mix.

qPCR	DNA	RNA Ultrassense
Environmental Mix 2x	12.5 µl	------
5xUltrasense Mix	------	5 µl
Primer Forward	1 µl	1 µl
Primer Reverse	1 µl	1 µl
Probe	0.5 µl	0.25 µl
ROX (dilution 1/10) as reference dye	------	0.5 µl
BSA (1mg/ml)	------	1 µl
Enzyme (last)	------	1.25 µl
H_2O (DNase/RNase free)	------	10 µl
Total volume of mix	**15 µl**	**20 µl**
Sample	**10 µl**	**5 µl**

Notes: The master mix is prepared in a clean separated area and considering the total number of reactions to be performed and then distributing the correct volume of mix per well. The samples tested for dsRNA viruses must include an incubation of 98ªC for 3 min before charging it in the PCR plate, to denature the double-stranded chains

Primers and probes for qPCR and RT-qPCR

Table A2 - Primers and probes for each qPCR and respective concentrations. *Volume of a starting solution of 100 µM needed to prepare 100 µl of working solution to be used directly in the master mix.

Virus	Ref	Name	Sequence (5'- 3')	Working solution*	Final PCR conc.
PAdV	[71]	Q-PAdV-F	AACGGCCGCTACTGCAAG	22,5 µl	0,9 µM
		Q-PAdV-R	AGCAGCAGGCTCTTGAGG	22,5 µl	0,9 µM
		Q-PAdV-P	CACATCCAGGTGCCGC	11,25 µl	0,225 µM
BPyV	[72]	QP-F1-1	CTAGATCCTACCCTCAAGGGAAT	10 µl	0,4 µM
		QB-R1-1	TTACTTGGATCTGGACACCAAC	10 µl	0,4 µM
		QB-P1-2	GACAAAGATGGTGTGTATCCTGTTGA	6 µl	0,12 µM
RoV	[73]	NSP3-F	ACCATCTWCACRTRACCCTCTATGAG	10 µl	0,4 µM
		NSP3-R	GGTCACATAACGCCCCTATAGC	10 µl	0,4 µM
		NSP3-P	FAM-AGTTAAAAGCTAACACTGTCAAA-MGB	10 µl	0,1 µM
IAV	CDC	InfA Forward	GACCRATCCTGTCACCTCTGAC	20 µl	0,8 µM
		InfA Reverse	AGGGCATTYTGGACAAAKCGTCTA	20 µl	0,8 µM
		InfA Probe	FAM- TGCAGTCCTCGCTCACTGGGCACG - BHQ1	20 µl	0,2 µM
EHDV	[74]	EHDV/Seg-9/F	ATGTCAGCTGCGGTYTTG	15 µl	0,6 µM
		EHDV/Seg-9/R	TCCCAATCAACTAARTGRATYTGVATCT	15 µl	0,6 µM
		EHDV/Seg-9/P	FAM-CCTCGGTCGAACGTTGGATCAC-BHQ-1	10 µl	0,1 µM
CCHFV	[75]	ForwardCCHFV	GGAVTGGTGVAGGGARTTTG	25 µl	1,0 µM
		ReverseCCHFV	CADGGTGGRTTGAARGC	25 µl	1,0 µM
		ProbeCCHFV	6FAM-CAARGGCAARTACATMAT-MGBNGQ	20 µl	0,2 µM
BTV	[25]	BTV-qPCR-F	TGGAYAAAGCRATGTCAAA	10 µl	0,4 µM
		BTV-qPCR-R	ACRTCATCACGAAACGCTTC	10 µl	0,4 µM
		BTV-qPCR-P	6-FAM-ARGCTGCATTCGCATCGTACGC-BHQ-1	1,25 µl	0,2 µM

Thermocycling

Table A3 - Cycles for each qPCR assay

Virus	Genetic material	Cycles program
PAdV	DNA	95ºC – 10min; 45 cycles (95ºC – 15s; 55ºC – 20s; 60ºC – 20s)
BPyV	DNA	95ºC – 10min; 45 cycles (95ºC – 15s; 60ºC – 30s)
RoV	dsRNA	48ºC – 30 min; 95ºC - 10 min; 40 cycles (95ºC - 15s; 60ºC – 1min)
IAV	ssRNA	50ºC - 30min; 95ºC - 5 min; 45 cycles (95ºC - 15s; 55ºC – 30s)
EHDV	dsRNA	55ºC - 1h; 95ºC - 10 min; 50 cycles (95ºC - 30s; 60ºC - 1min)
CCHFV	ssRNA	55ºC - 1h; 95ºC - 5min; 50 cycles (95ºC - 15s; 55º - 20s; 68ºC - 5s)
BTV	dsRNA	48ºC - 30min; 95ºC - 2 min; 50 cycles (95ºC - 15s; 56ºC - 30s; 72ºC - 30s)

Once the run is completed the data was analysed using the Thermocloud app from Thermofisher (https://apps.thermofisher.com/apps/quantigene).

Annex 2 – Column-based nucleic acid extraction protocol using the "QIAamp® Viral RNA Mini Kit" from QIAGEN

Protocol adapted from "QIAamp® Viral RNA Mini Handbook", Quiagen:

- In a 2 ml eppendorf tube, put 75 mg of faecal sample and add 1 ml of CTAB.
- Vortex for 30 s and incubate at 95ºC for 5 min. After that, let it cool on the bench.
- In a new tube pipet 1120 ml of Buffer AVL containing carrier RNA (AVL- carrier RNA) and 300 ml of the sample supernatant. (If the AVL-carrier RNA shows a precipitate, incubate it at 80ºC for no re than 5 min)
- Vortex for 15 s and incubate at r.t for 10 min. Briefly centrifuge the tube to remove drops from the walls and lid.
- Add 1120 ml of ethanol (96-100%) and vortex for 15 s to stop the lysis.
- Put 640 ml of the solution from previous step to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap and centrifuge at 6000g (or 8000rpm) for 1 min.
- Discard the filtrate in the collection tube and repeat the previous step until there's no solution left.
- Add 500 ml of Buffer AW1, close the cap and centrifuge at 6000g for 1 min. Put the column in a new collection tube.
- Add 500 ml of Buffer AW2, close the cap and centrifuge at 20000g for 3 min.
- Optional step: Put the column in a new collection tube and centrifuge at 20000g for 1 min.
- Put the column in a clean tube of 1,5 ml and add 50 ml of Buffer AVE. Incubate for 1 min at r.t. and then centrifuge at 6000g for 1 min.
- Add again 50 ml of Buffer AVE, so the final elution volume is 100 ml, and repeat the incubation and centrifugation.
- Discard the column and keep the extracted nucleic acids at -80ºC until further analysis.

Annex 3 – Protocol of nucleic acid extraction for cow saliva samples

The saliva samples were taken with a swab that was kept in an Eppendorf at -20ºC until perform the nucleic extraction. It was used the PureFood GMO and Authentication Kit (Catalogue No. AS1600; Promega Corporation, Madison, WI) with adaptations for saliva samples as described below:

- Add 300μl of Lysis buffer and 30μl of Protease K to the swab in the tube.
- Vortex and then incubate in the thermoblock at 56ºC for 10 min. Vortex again after the incubation.
- With a spatula, squeeze the swab as much as possible so the content retained in the swab remains in the tube and discard the swab.
- Briefly centrifuge the tube to remove drops from the wall and lid.
- Prepare the cartridges in a Maxwell® RSC instrument as described by the manufacturer: transfer 300 μl of sample to the well 1 and 100μl of elution buffer to the elution tube.

Annex 4 – Protocol of nucleic acid extraction of straw and water samples from farms A, B, C and D

The concentrate of straw and water samples were directly transferred to the cartridges in the Maxwell® instrument following the manufacturer instructions. Briefly:

- Transfer 300μl of Lysis buffer and 300μl of the concentrate to well 1
- Transfer 100μl of Elution buffer to the elution tube
- Place the plungers in the wells 8
- Place the cartridges in the machine, select the cartridges with samples and choose the correct kit.

Annex 5 – Information on the segment 9 sequences of EHDV used for the construction of the fasta file for the development of the n(RT)PCR

Table A4 - EHDV Seg-9 sequences information, including accession numbers.

Isolate	Serotype	Year	Country	Host	seg-9
OV208	6	2016	USA	white-tailed deer	MG886405
M44/96	6	1996	South Africa	cattle	HM636915
CC 304-06	6r	2006	USA	white-tailed deer	HM641780
C/cervidae/Ohio/12-3437-8/2012	6	2012	USA	white-tailed deer	KF570138
C/cervidae/Texas/10-0235-1/2010	1	2010	USA	white-tailed deer	KF570118
C/cattle/Indiana/13-04197/2013	2	2013	USA	cattle	KJ125195
AUS1979/05 (CSIRO 439)	2	1979	Australia	Sentinel Cattle	AM744995
AUS1981/06 (CSIRO 775)	7	1981	Australia	Sentinel Cattle	AM745055
AUS1981/07 (CSIRO 753)	6	1981	Australia	Sentinel Cattle	AM745045
AUS1982/06 (DPP59)	8	1982	Australia	Sentinel Cattle	AM745065
CAN1962/01 (Alberta)	2	1962	Canada	white-tailed deer	AM745005
JAP1959/01 (Ibaraki virus)	2	1959	Japan	cattle	AM745085
NIG1968/01 (Ib Ar 33853)	4	1968	Nigeria	Culicoides spp.	AM745025
NIG1967/01 (Ib Ar 22619)	1	1967	Nigeria	Culicoides spp.	AM745015
USA1955/01 (New Jersey)	1	1955	USA	white-tailed deer	AM744985
OV215	2	2016	USA	white-tailed deer	MF688821
OV202	1	2015	USA	white-tailed deer	MF688831
CC177-04	2	2004	USA	white-tailed deer	KU140905
CC131-93	2	1993	USA	not given	KU140907
CC87-90	2	1990	USA	not given	KU140912
76-5460-M-32313-75	2	1975	USA	white-tailed deer	KU140897
SS-65-DEER-137	2	1971	USA	white-tailed deer	KU140898
CC211-06	1	2006	USA	white-tailed deer	KU140917
EHD-74-297B-1	1	1974	USA	cattle	KU140900
HD Windsor	1	1972	USA	Culicoides spp.	KU140901
Parker-A	1	2008	USA	white-tailed deer	KU140916
ISR2006/02	7	2006	Israel	cattle	KM391736
TAT2013-02	6	2013	Trinidad and Tobago	Bovine	MK919262
EHDV-2 Cuba (3139)	2	2022	Cuba	Bovine	OR611904
EHDV-8/Deer1 TUN2022	8	2022	Tunisia	Cervus elaphus barbarus	OP971155
EHDV-8/Cattle Bizerte TUN2022	8	2022	Tunisia	Bos taurus	OP971135
EHDV-8/Cattle Jendouba TUN2022	8	2022	Tunisia	Bos taurus	OP971125
EHDV-8/Culicoides sp/2 TUN2022	8	2022	Tunisia	Culicoides sp.	OP937339
YNV/03-2	7	2020	China	Culicoides orientalis	OM953821
YNV/KM3	1	2019	China	Culicoides sp.	OM953799
USA2012/IL 12-38993-2	6	2012	USA	cattle	MH845397
USA2012/OH 12-43618	2	2012	USA	cattle	MH845387
USA2007/IN CC372-07b	2	2007	USA	cattle	MH845377
USA2012/SD 12-39713	2	2012	USA	bison	MH845357
USA2012/SD 12-150871	2	2012	USA	cattle	MH845347
USA2012/NE 12-36542-8	2	2012	USA	cattle	MH845337
USA2012/SD 12-15085	2	2012	USA	cattle	MH845317
USA2012/IA 12-39007	2	2012	USA	cattle	MH845307
USA2012/MN 12-41125	2	2012	USA	cattle	MH845297
USA2012/NE 12-35934	2	2012	USA	cattle	MH845277

USA2012/SD 12-035472-4	2	2012	USA	cattle	MH845267
USA2012/IA 12-36567	2	2012	USA	cattle	MH845257
USA1998/MO Cow17	2	1998	USA	cattle	MH845247
Kawanabe 525	1	1985	Japan	Bos taurus	LC552739
KSB-14/E/97	7	1997	Japan	Bos taurus	LC552746
ON-4/B/98	no data	1998	Japan	Bos taurus	LC552753
N-11/E/16	5	2016	Japan	Bos taurus	LC757721
ON-3/E/14	6	2014	Japan	Bos taurus	LC757731
HG-1/E/15	6	2015	Japan	Bos taurus	LC757739
C/cervidae/Texas/14-04039-2/2014 cervidae	no data	2014	USA	no data	MG737829
YN09-04	7	2013	China	cattle	MK656461
IL41747	2	2018	USA	Cervidae sp.	MK754244
IL42218	2	2018	USA	Cervidae sp.	MK754254
OV610	2	2017	USA	Odocoileus virginianus	MK958992
OV617	2	2017	USA	Odocoileus virginianus	MK959002
OV862	2	2018	USA	Odocoileus virginianus	MK959012
OV867	2	2018	USA	Odocoileus virginianus	MK959022
JC13C673	10	2013	China	Culicoides	MT013322
JC13C644	10	2013	China	Culicoides	MT013332
OV1248_FL_EHDV6	6	2019	USA	Odocoileus virginianus	OK500225
ISR-2096/16	1	2016	Israel	Cattle	OM502371
EHDV-8/Cattle Guspini SAR2022	8	2022	Italy	Bos taurus	OP897283
EHDV-8/60 TUN2021	8	2021	Tunisia	Bos taurus	OP897548
EHDV-8/Deer TUN2021	8	2022	Tunisia	Cervus elaphus barbarus	OP897558
reindeer/Minnesota/20-026082/2020	6	2020	USA	Reindeer	OQ679901
deer/Iowa/21-025321/2021	6	2021	USA	Deer	OQ679911
white-tailed deer/Minnesota/21-027870/2021	6	2021	USA	White-tailed deer	OQ679928

Annex 6 – Protocol of the two-step n(RT)PCR for EHDV detection

EHDV – n(RT)PCR (dsRNA)			
RT: Synthesis of cDNA – V_{final} = 20μL			**Superscript IV**
Mix 1 : Volume / Reaction		__ Reactions	
Random hexamers (50 μM)	1 μL		95 ºC for 5 min, then put in ice.
dNTP (10mM)	1μL		
In each tube: 2 μL mix 1 + 11μL sample		**V_{total} = 13 μL**	
Mix 2 : Volume / Reaction		__ Reactions	
SSIV Buffer	4 μL		23ºC - 10’ 50ºC - 30’ 80ºC - 10’ Keep at 4ºC
DTT	1 μL		
Rnase out	1 μL		
Superscript IV Enzyme	1 μL		
Add 7 μL mix 2 to each previous tube		**V_{total} = 20 μL**	
1st PCR – Primers: nestedEHDV-S9-F and nestedEHDV-S9-R			**DreamTaq Green**
“Primer pool” = 20 μL F + 20 μL R + 60μL H_2O		__ Reactions	95ºC – 5´
Ultrapure H_2O	19 μL		40x { 95ºC – 30” 55ºC – 30” 72ºC – 45”
DreamTaq Green Enzyme	25 μL		
Primer pool	1 μL		
In new tubes: 45 μL mix + 5 μL cDNA		**V_{total} = 50 μL**	72ºC – 10’ Keep at 4ºC
2nd PCR – Primers: nestedEHDV-S9-F and nestedEHDV-S9-R2			**DreamTaq Green**
“Primer pool” = 20 μL F + 20 μL R2 + 60μL H_2O		__ Reactions	95ºC – 5´
Ultrapure H_2O	22 μL		40x { 95ºC – 30” 55ºC – 30” 72ºC – 45”
DreamTaq Green Enzyme	25 μL		
Primer pool	1 μL		
In new tubes: 48 μL mix + 2 μL DNA product 1st PCR		**V_{total} = 50 μL**	72ºC – 10’ Keep at 4ºC
Electrophoresis: Agarose: 2 %; GelRed: 1 μL/10ml of agarose; DNA Marker: 3-5 μL ; Sample:15 μL; Positive control: 5-10μL; DNA ladder (MW) = 100 bp			
Run the electrophoresis at 90 Volts, for around 50 min. → See at UV light			
Expected fragment: Sample 1st PCR: 431 pb Expected fragment: Sample 2nd PCR: 363 pb		Pos. cont. gblock 1st PCR = 213 pb Pos. cont. gblock 2nd PCR = 178 pb	
Primers sequences	nestedEHDV-S9-F: AAAGAGCTGGGGTTCTCACG		
	nestedEHDV-S9-R: GTCGCATGTGCCACATCATC		
	nestedEHDV-S9-R2: TTCATCACGCTTCTCACCCC		

Annex 7 – DNA purification protocol and preparation of sample for Sanger sequencing

DNA purification with "DNA Clean & Concentrator™-5, Zymo Research:

- Transfer the 38 μL of the PCR product to a 1,5 ml eppendorf and add 190 μL of Binding buffer (ratio of 1:5 v/v of PCR product and Binding buffer). Mix by vortexing and do up and down.
- Transfer all the mixture to a column placed in a collector tube, close the cap and centrifuge at 13000 x g for 1 min. Discard the filtrate.
- Add 200 μL of Washing buffer to the column and centrifuge at 13000 x g for 30 seconds. Discard the filtrate and repeat the wash one more time.
- Place the column in a new 1,5 ml Eppendorf and add 30 μL of Elution buffer. Incubate at r.t for 5 min and centrifuge at 13000 x g for 30 seconds.
- The purified DNA is in the filtrate. Keep at 4ºC until further analysis.

Quantification in QUBIT:

- To the tube with 99 μL of QUBIT reagent kept at r.t for 30 min before its use, add 1 μL of purified DNA. Vortex and pulse spin.
- Place the tube in the QUBIT device to measure the concentration with high sensitivity, after performing the standard curve.

Preparation of sample:

For Sanger sequencing the amount of DNA should be 100 ng per 1kb fragment size. Since the fragment has 363 bp, approximately 36 ng are needed.

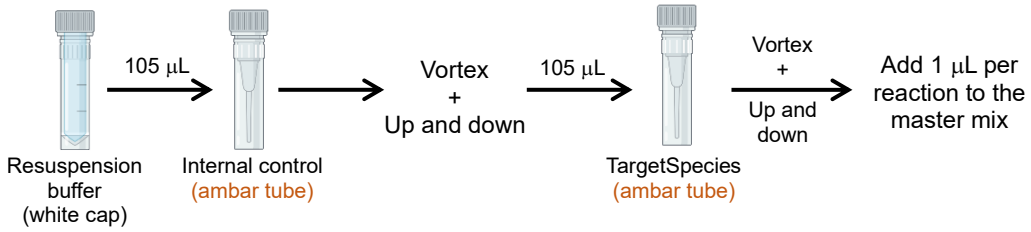
- Prepare working solutions of the primers nestedEHDV-S9-F and nestedEHDV-S9-R2 with a concentration of 5 μM. In 0,2 ml tubes, add 1 μL of forward primer to one of the tubes and 1 μL of reverse primer to other tube.
- Add 1 μL of purified DNA (volume calculated considering the concentration measured in QUBIT) to both tubes and label properly.
- Incubate in the thermocycler at 80ºC for 10-15 min with the thermocycler lid and tube caps open, to evaporate the liquid.
- Keep at 4ºC until sending for Sanger sequencing.

Annex 8 – Protocol of BCoV and BRSV (RT)qPCR commercial kits Eurovet (BCOV-RTqPCR-F100+IC) and Eurovet (BRSV-RTqPCR-F100) (Summarized version)

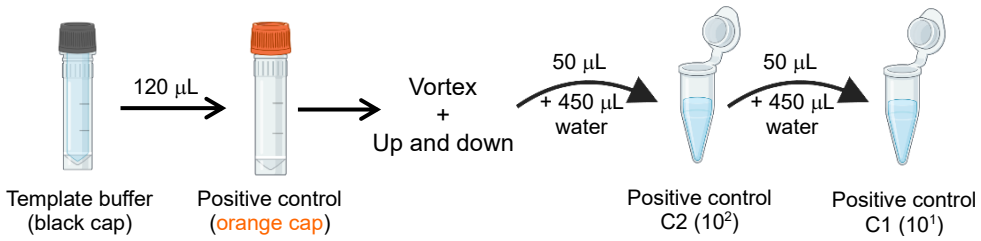
1. Prepare first part of the master mix calculating the total volume of each reagent for the total of reactions (In a clean zone):

Reagent	Volume / reaction
GPS-RT mix (blue cap)	5 µL
Water (green cap)	9 µL

2. Prepare Target species mix with Internal control and finish master mix (In a different zone):



3. Transfer 15 µL of master mix + 5 µL of sample to each well in the PCR plate.
4. Prepare positive controls (standards):



Add 5 µL of the respective positive control to the corresponding wells.

5. Thermocycles: 50ºC - 10min; 95ºC - 2 min; 40 cycles (95ºC – 5 s; 60ºC – 20 s).
6. Collect the results with FAM channel for the virus and with HEX channel for the internal control, with NFQ as the quencher.

Annex 9 – Modified protocol of CCHFV (RT)qPCR commercial kit – Genesig® Standard kit by Primerdesign™ Ltd (Summarized version)

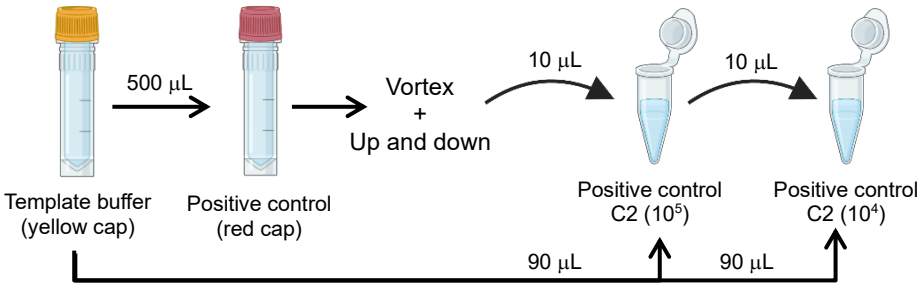
For this qPCR, instead of the master mix recommended by the manufacturer, the Ultrassense kit reagents were used together with the primer/probe mix provided by the CCHFV commercial kit.

1. Prepare mix primer/probe: Add 165 µL of ultra-pure water (white cap) to the primer/probe mix (brown cap), vortex and do up and down.
2. Prepare the master mix calculating the total volume of each reagent for the total of reactions (In a clean zone):

Reagent	Volume / reaction
5xUltrasense Mix	3,75 µL
Mix primer/probe (brown cap)	1 µL
Enzyme	1 µL
Water (white cap)	9,25 µL
Total mix	**15 µL**

Add 15 µL of mix to each well + 5 µL of sample.

3. Prepare positive controls (standards):



Add 5µL of the respective positive control to the corresponding wells.

4. Thermocycles: 55ºC – 60 min; 95ºC - 5 min; 50 cycles (95ºC – 15 s; 60ºC – 1 min).
5. Collect the results with FAM channel for the virus.

Annex 10 - Protocol of EHDV (RT)qPCR commercial kit "VETMAX EHDV kit, applied biosystems" (Summarized version)

This assay is only qualitative since the positive control doesn't present its concentration. The mix from this kit contains primers and probe for a mammal gene present in the samples, working as an internal control.

1. Add 20 μL of the Mix EHDV to each well in a clean zone.
2. Transfer 7 μL of sample to a PCR tube and incubate it at 98ºC for 3 min. When finished transfer directly to ice.
3. Transfer 5 μL of the previously tube to the respective wells.
4. Repeat the step 2 and 3 for the positive control (EPC EHDV) in a different zone.
5. Thermocycles: 45ºC – 10 min; 95ºC - 10 min; 40 cycles (95ºC – 15 s; 60ºC – 45 s).
6. Collect the results with FAM channel for the virus and with VIC channel for the internal control, with NFQ as the quencher.

Annex 11 – Gblocks sequences

Note: Some Gblocks include sequences of viruses not analysed in this study

1. Gblock 1: qPCR Porcine Adenovirus – qPCR Ovine Polyomavirus

TCCCCGGATGTCATGGACAACGTCAACCCCTTCAACCACCACCGGCTCCTGGGCAACGGCCGCTACTGCAAGTTCCACATC
CAGGTGCCGCAAAAGTTCTTTGCCCTCAAGAGCCTGCTGCTCCTGCCGGGGGCGACCGGCCAACGTCACCAACCTGCCCA
TCTCCATTCCCAGCCGCAACTGGCCGCATCAGCATCCAGTTCGACTCCTCCGTGGCCTGCACACCCATGGACACAGAGATG
GCCTCTTTCTCACAGCTGCAGACATTGTGGGCATGATTACCAAGCCAGACAGTGGGCACATGGCTTTTCGTGGGCTCCCAA
GGTACTTCAATGTCACTCTAAGAAAGAGAGTGGTTAAGAACCCATATCCTGTTAGCACATTGCTGAACTCTCTCTTTTCAAATC
TTATGCCCAGATTGGAAGGCCAGGCCATGACTGGAGATAAATCC

2. Gblock 2: qPCR Bovine Polyomavirus- qPCR Chicken-Turkey Parvovirus

GAAAAGGTATTCGCCCTCTGCTGGTCAAGGCTCAGCAATATGTGAGGATTTCAAAGCCCCCTATCATCCCACAGTACCCAGG
TGCTAGATCCTACCCTCAAGGGAATTCTAGACAAAGATGGTGTGTATCCTGTTGAGTGTTGGTGTCCAGATCCAAGTAAAAAT
GAACCGCTTCTTGATGAGAATGGCGTAGGCCCGCAGACTGGTGAAGAACCCATACCCCATTGCCAGCATACTCACACCCAT
GGACACGGTAATTGGAATTGTGATACTATATGGGCTGAAAATAAGCCGCCATTGTGTCTGTCTTATGCGTGACATGGATCAGC
GTGCACTTTAGTCCACGAGATTGGCAACAACTAGTTAATAATTATTCGAGATGGCGCCCACGCGCCCACACACAGTACACGT
GAAAATCTTTAACCTGCAAGTCATCCAGAAAACCGTAACAGATAGCTGACAGGAACAATCCAAATATTTGCAGATCAGGAAGG

3. Gblock Viralert 1: qPCR Reovirus – qPCR RoV A – qPCR Hepatitis E virus – qPCR Sendai vírus – pan qPCR Poliovirus – qPCR BTV

TTACTATACAGTGAGTTGCTGAACGCAAATTATTTTGGTCATTTATTGCGACTAAAAATACCATATATTACAGAGGTTAATCTGAT
GATTCGCAAGAATGTCACATTAACCATCTACACATGACCCTCTATGAGCACAATAGTTAAAAGCTAACACTGTCAAAAACCTAC
ACACACAAATGGCTATAGGGGCGTTATGTGACCGTATCCGGTGGTTTCTGGGGTGACCGGGCTGATTCTCAGCCCTTCGCA
ATCCCCTATATTCATCCAACCAACCCCTTCGAAGAGGTATAGGAGTCCCTGAAGTCTTAGAAGATTGGGATCCCGAGGCAGAT
AATGCACTGTTAGATGGTATCGCGGCAGAAATACAACAGAATATTCCTTTGGGACATCAGACTAGAGCCCCTGACACATTGGA
GTTCTTCACTTATTCTAGATTTGATATGGAATTTACCTTTGTGGTTACTGCAAATTTCACTGAGACTAACAATGGACATGCCTTA
AATCAAGTATACCAAATTATGTACGTCCCACCCGGAGCTCCAGTGCATTGATCCTTATGTGGACAAAGCGATGTCAAACACAA
CTGGTGCAACGCAAACACAGAAGGCGGAGAAGGCTGCATTCGCATCGTACGCAGAAGCGTTTCGTGATGATGTTAGGATCC
GTTAAATGCCGTA

4. Gblock Viralert 2: qPCR BHerpes vírus – qPCR EHDV – n(RT)PCR CCHFV – n(RT)PCR EHDV

ACCTTTGTGGACCTAAACCTCACGGTTCTGGAGGACCGCGAGTTCTTGCCGCTAGAAGTGTACACGCGCGCCGAGCTCGC
CGACACGGGTCTGCTCGACTACAGCGCATGTCAGCTGCGGTCTTGCTCGCACCCGGTGACGTGATCCAACGTTCGACCGA
GGAACTAAAACAAAGACAGATCCAGATTCATTTAGTTGATTGGGATAAAAAAGGAAATGGACTTGTGGATACCTTCACACACA
CACACACATTACCACACACACTCCTATTCCTTTTGCGAGAGTGTTCCAAATTTGGACAAGTTTGTGTAAAAATAGAACAACTTG
CCAATTACCAACAAGCTGCCTTGAAATGGAGAAAGGACATAGGTTTCCGTGTCACACACATGTGACACACACAATGCAAACA
CAGCGGCTCTGAGCAACAAAGTCCGAATCCACGTGGCCCTGTGAGCCGTGAGCATGTGGACTGGTGCAGGGAGTTTGTCA
AAGAATATCAACAAGTCAGGCCGTTCAGGAATAGCACTTGTCGCAACAGGCCTTGCCAAGCTAGAAAAAGAGCTGGGGTTCT
CACGTGAGATGGCAGATTCGGAAAGTCTTTAGGGTCTTCTACCTTTTTCACACACATTTCTTTTTAGGTGGGGTAGAGTGTTG
GGATCCTGTGTTTTCATCATCACTGGCAAACATTTGAACGGAGCAGACAATGATAGGTGGGGGTGAGAAGCGTGATGAAACA
ATACGCGCAATCGATGATGTGGCACATGCGACTGC

Annex 12 – Target enrichment sequencing protocol

The target enrichment sequencing procedure involves several steps with different objectives and based on different protocols, as explained in the methods section, that are joined together in a continuous workflow. Each protocol is described below with the adaptations made for the tested samples.

1. Protocol of DNAse treatment and SISPA

Protocol reference: Fernandez-Cassi X, Rusiñol M, Martínez-Puchol S. (2018). Viral Concentration and Amplification from Human Serum Samples Prior to Application of Next-Generation Sequencing Analysis. Methods in molecular biology (clifton, N.J.), doi: 10.1007/978-1-4939-8682-8_13.

1.1. DNase treatment:

- Add 50 µL of each sample pool to a different 0.5 mL sterile plastic tubes – Pools with 3 samples are divided in 3 tubes and pools with 1 sample are kept in the same sterile plastic tubes
- Add to each tube the following reagents and vortex vigorously: 10 µL of TurboDNase® (2 U/µL) and 10 µL 10× TurboDNase® reaction Buffer.
- Incubate for 1 h at 37 °C by using a Thermoblock to ensure homogeneous heating of the sample. After the first 30 min of incubation add 3 µL of turbo DNase enzyme per tube. Incubate for another half an hour. Regular agitation during incubation is recommended.
- Collect in a single 1.5 mL sterile plastic tube the DNase-treated viral concentrate from the 3 tubes of the same pool.
- Inactivate the DNase treatment by adding 44 µL of the DNase inactivation buffer previously vortexed. Once the reagent is added, the tube must be inverted a couple of times in order to mix the reagent with the tube content. Do not vortex the sample once inactivation agent is added.
- Incubate the mixture for 5 min at room temperature (22–26 °C).
- Centrifuge the tubes at 10,000 × g for 1,5 min. After centrifugation a white pellet is observed. Keep the supernatant containing the viral particles without disturbing the pellet and place it in a new collection tube. Discard the visible pellet.

*Aerosol samples are not submitted to this process.

1.2. Nucleic acid extraction:

In this protocol “QIAmp® Viral RNA Mini Kit” from QIAGEN is used. To extract the nucleic acids 140 µL of the viral DNase-treated concentrate are used. A negative control must be included for the whole process.

- Pipet 560 µL of AVL Buffer (without RNA-carrier) into a 5.0 mL centrifuge tube.
- Add 140 µL of viral concentrate to the previous tube. Mix the tube by vortexing for 15 s. Incubate at room temperature (15–25 °C) for 10 min to disrupt the viral nucleocapsids.
- Briefly, spin the tubes to remove drops from inside of the lid.
- Add 560 µL of ethanol (96%–100%) to the sample. Mix by vortexing for 15 s. Spin the tubes briefly to remove drops from the inside of the lid.
- Carefully apply 630 µL of the solution from the previous step to a QIAamp® Mini spin column (in a 2 mL collection tube) without wetting the rim. Centrifuge at 6000 × g for 1 min.
- Place the QIAamp spin column into a clean 2 mL collection tube and discard the tube containing the filtrate. Repeat the previous step until all sample volume has gone through the filter.
- Add 500 µL of washing buffer AW1. Centrifuge at 6000 × g for 1 min. Keep the spin column and discard the collection tube and the filtrate.
- Add 500 µL of washing buffer AW2. Centrifuge at 20,000 × g for 3 min. Keep the spin column and discard the collection tube and the filtrate.
- Put a new collection tube and centrifuge at 20,000 × g for 1 min. Keep the spin column.
- Place the QIAamp® Mini Spin Column into a clean 1.5 mL centrifuge tube (collection tube). Open the column and add 60 µL of AVE. Incubate the Spin Column for 2 min and centrifuge at 6000 × g for 1 min.
- Store the eluted NA at −80 °C for further molecular analysis.

1.3. Retrotranscription to cDNA and tagging of viral RNA genomes:

The cDNA step is prepared in two tubes: the master mix tube and the sample tube. The master mix tube will contain all the common components for the retrotranscription reaction to minimize pipetting errors. The volumes of the samples mix will be different for lixiviates and wastewater samples and for aerosol samples, but it will always contain primers, dNTPs and the specific DNA/RNA extracted from the sample.

- For each lixiviates and wastewater samples prepare only 1 sample tube and for each aerosol sample prepare duplicates, according to the following reagents volumes:

Lixiviates and wastewater	
Primer A	1 µL
DNTPs	1 µL
DNase/RNase-free H_2O	3 µL
NA Extraction	8 µL
Final volume	**13 µL**

Aerosols	
Primer A	1 µL
DNTPs	1 µL
NA Extraction	11 µL
Final volume	**13 µL**

- Denature the RNA and primer by incubating for 5 min at 65 °C. Chill on ice for 5 min to break secondary structures of RNA.
- Prepare a master mix tube common for all types of samples. The enzyme used was different from the one used in the original protocol. Include a negative control for the whole process.

Add the following reagents per sample: 4 µL of RT buffer SuperScript™ IV, 1 µL of 0.1 M DTT, 1 µL of RNase™ Out, and 1 µL of SuperScript™ IV enzyme. Homogenize the master mix tube by vortexing.

- Add 7 µL of the master mix to each sample tube.
- Place the sample tubes into the PCR thermocycler and run the RT program as follows: 23 °C for 10 min, 50 °C for 10 min, 80 °C for 10 min. When finished, place the PCR tubes in a cooler.
- Add 1 µL of RNase H to each sample tube to denature RNA-DNA hybrid structures which could affect subsequent reactions. Then, incubate the PCR tubes in a PCR thermocycler for 20 min at 37 °C. Until further use, keep the tubes at 4 °C.

1.4. Retrotranscription to cDNA and tagging of viral RNA genomes:

Second strand cDNA is constructed in the PCR tubes resulting from the retrotranscription.

- Incubate the sample tubes at 95 °C for 5 min in a PCR thermocycler. Rapidly chill all the sample tubes in ice or in a cooler for 5 min.
- Prepare a second master mix tube (master mix 2). Master mix 2 contains the following reagents for each sample: 2 µL of sequenase buffer, 0.3 µL of sequenase enzyme, and 7.7 µL of UltraPure™ DEPC-Treated Water. Add 10 µL of master mix 2 into each sample tube.
- Place the PCR tubes in a thermocycler. The second strand synthesis needs a temperature ramp: Ramp 1 °C every 18 s starting at 10ºC until 37ºC, 37° for 8 min, 94 °C for 2 min, and 10 °C for 5 min.
- A second step using sequenase to entirely complete the second strand is performed. In this second reaction, the enzyme is diluted using the enzyme dilution reagent provided. Prepare a master mix 3: 0.3 µL sequenase enzyme and 0.9 µL sequenase dilution buffer. Add to each tube 1.2 µL of master mix.
- Repeat the program described in step 5, increasing the 94 °C time from 2 to 8 min.
- Keep the primer A tagged viral dsDNA sequences at 4 °C.

1.5. PCR-B amplification of viral randomly tagged dsDNA chains:

The PCR master mix should be prepared in a separate area to avoid contamination with other PCR amplicons. Template from PCR reaction should be added in a separate room. Keep the reagents and master mix chilled on ice. Amounts provided are per reaction tube. Prepare 2 reaction PCR tubes for each tested sample. A negative PCR reaction tube for this step should be included.

- Add the following reagents to obtain a 90 µL PCR-B master mix for the sample: 8 µL of $MgCl_2$, 10 µL of PCR Buffer 10×, 1 µL dNTPs, 1 µL of Taq DNA polymerase, 1 µL of Primer, and 69 µL of UltraPure™ DEPC-Treated Water.
- In a separate room add 10 µL of the Sequenase template. For each sample tested, two parallel PCR reactions are performed by using 20 out of 30 µL of sequenase template. Keep the spare volume for an extra PCR-B reaction, if needed.

- Place the tubes in the thermocycler and follow the program: 95 °C-10 min, followed by 30 cycles at 94 °C for 30 s, 40 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. Finally, an extension at 72 °C for 10 min and 4 °C-hold.
- In the end, we had 2 PCR-B products for each pool of lixiviates and wastewater samples, and 4 PCR-B products for each aerosol pool.

1.6. Cleaning Viral Randomly Amplified PCR Products and Concentration of Viral dsDNA

Excess of primers and dNTPs not used during the PCR amplification should be removed to obtain dsDNA clean PCR-B products.

- Pool the content of the PCR-B tubes of each sample to a 1.5 mL plastic tube (For lixiviates and wastewater pools the final volume was 200 µL and for aerosol pools was 400 µL)
- Add DNA binding buffer within the sample tube with a proportion of 5:1 with the sample (1ml for lixiviates and wastewater and 2 ml for aerosols).
- Briefly vortex the mixture and transfer the mix into a Zymo column. Centrifuge at 13000 × g for 1 min. Discard the eluate and place the column in a new collector tube.
- Add 200 µL of DNA wash buffer into the column. Centrifuge at 13000 × g for 1 min. Discard the eluate and place the column in a new collector tube. Repeat this washing step.
- Discard the eluate and place the column in a new collector tube.
- Add 15 µL pre-warmed at 70 °C water DNase/RNase free to the column and place a new plastic tube under the column. Incubate for 5 min. Centrifuge at 13000 × g for 1 min.
- Keep the eluate that contains the purified viral dsDNA for library preparation.

1.7. Fluorometric quantification:

- Prepare two assay tubes for the standard curve. Prepare as many assay tubes as there are samples to be tested.
- Prepare 200 µL of qubit working solution buffer for each standard and sample to be tested. The Qubit® working solution consists of 1 µL of the qubit reagent and 199 µL Qubit Buffer per reaction. Ensure the mixture of both components by vortexing 10 s.
- For the two standard tubes place 190 µL of Qubit® working solution per tube and add 10 µL of the standard solution 1 or 2.
- To quantify the DNA concentration from samples, place 199 µL of Qubit® working solution and 1 µL of purified PCR-B product. Vortex the tubes for 2–3 s and wait a couple of min at room temperature before taking the reading.
- Insert the tubes in the Qubit® Fluorometer and turn on the device. On the Home screen, select the assay for dsDNA measure. Select the *high sensitivity assay*. Select *read the standards*. Place standard 1 tube into the sample chamber and select read standard. Do the same with standard 2 tube. After observing the calibration curve the Qubit® device is ready to read the samples.
- On the *read standard* screen, select *run samples*. On the sample volume screen, select the sample volume as 1 µL. Select the units expressed as ng/µL. Insert a sample tube into the

sample chamber, close the lid, and then select read tube to get the concentration of the sample.

- Samples will be ready for library preparation with Enzymatic fragmentation and Twist universal Adapter system", Twist Bioscience ®

2. Protocol for Library preparation – "Library preparation EF 2.0 with Enzymatic fragmentation and Twist universal Adapter system", Twist Bioscience ® (DOC-001239)

Protocol reference: Twist Bioscience. "Library Preparation EF 2.0 with Enzymatic Fragmentation and Twist Universal Adapter System".
The protocol is summarized and with some adaptations to our samples.

2.1. Perform DNA Fragmentation, End Repair, and dA-tailing

- Program the thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C. Start the program to pre-chill the thermal cycler. The conditions in step 2 depend on the desired insert size, which is 180-220 bp in this case.

Step	Temperature	Time
1	4ºC	Hold
2	37ºC	10 min
3	65ºC	30 min
4	4ºC	Hold

- Mix gDNA by flicking the tube with a finger. Bring 50 ng of each gDNA sample in a total volume of 40 µl with water in thin-walled PCR 0.2-ml strip-tubes
- Pulse-spin to ensure all the solution is at the bottom of the tube and place on ice
- Vortex the Frag/AT Buffer for 5 seconds. Pulse-spin to collect all liquid in the bottom of the tube.
- Invert Frag/AT Enzymes a minimum of 10 times to homogenize or briefly vortex to ensure complete mixing. Pulse-spin to collect all liquid in the bottom of the tube.
- Prepare an enzymatic fragmentation mix in a 1.5 ml microfuge tube on ice. Use the volumes listed below. Homogenize the master mix with moderate vortexing for 5 seconds or pipetting a minimum of half the total volume up and down 10 times (avoid formation of bubbles).

Reagent	Volume per reagent
Frag/ AT Buffer	4 µl
Frag/AT Enzymes	6 µl
Total	**10 µl**

- Add 10 µl enzymatic fragmentation mix (from Step 1.8) to each 40 µl gDNA sample tube or well. Homogenize with moderate vortexing for 5 seconds or by pipetting a minimum of half

the total volume up and down 10 times (avoid formation of bubbles). Cap the tube(s) or seal the plate and keep the reaction on ice.

- Pulse-spin the sample plate or tube(s) and immediately transfer to the pre-chilled thermal cycler. Initiate steps 2 to 4 of the thermal cycler program
- When the thermal cycler program is complete and the sample block has returned to 4°C, remove the samples from the block and place them on ice.

2.2. Ligate Twist Universal Adapters and Purify

- Add 5 µl Twist Universal Adapters into each sample well or tube containing the dA-tailed DNA fragments from the last step. Mix gently by pipetting and keep on ice.
- Invert the Ligation Master Mix a minimum of 10 times until homogenized and place on ice. NOTE: Do not vortex the Ligation Master Mix.
- Add 20 µl of Ligation Master Mix to each sample from the first step. Pipette a minimum of half the total volume up and down 10 times to ensure complete mixing. Seal or cap the sample plate or tube(s) and pulse-spin to ensure all solution is at the bottom of the tube.
- Incubate the ligation reaction at 20°C for 15 minutes in the thermal cycler, then move the samples to the bench top.
- Proceed to the Purify step. Vortex the pre-equilibrated room temperature DNA Purification Beads until well mixed.
- Add 60 µl of homogenized (0.8x) DNA Purification Beads to each ligation. Mix well by vortexing. Incubate the samples for 5 minutes at room temperature.
- Place the samples on a magnetic plate for 1 minute or until the supernatant is clear. The DNA Purification Beads form a pellet, leaving a clear supernatant.
- Without removing plate or tube(s) from the magnetic plate, remove and discard the supernatant.
- Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.
- Repeat the wash once, for a total of two washes, while keeping the sample(s) on the magnetic plate. Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet. NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.
- Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.
- Remove the plate or tube(s) from the magnetic plate and add 17 µl water to each sample. Mix by pipetting until homogenized. Incubate at room temperature for 2 minutes.
- Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.
- Transfer 15 µl of the clear supernatant containing the ligated libraries to a clean thin- walled PCR 0.2- ml strip-tube, making sure not to disturb the bead pellet.

2.3. PCR Amplify Using Twist UDI Primers, Purify, and Perform QC

- Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C.

	Step	Temperature	Time	Number cycles
1	Initialization	98 ºC	45 s	1
2	Denaturation	98 ºC	15 s	7
	Annealing	60 ºC	30 s	
	Extension	72ºC	30 s	
3	Final extension	72 ºC	1 min	1
4	Final hold	4 ºC	Hold	---

- Add 10 µl of Twist UDI or Twist HT UDI Primer from the provided 96-well to each of the gDNA libraries and mix well by gentle pipetting.
- Add 25 µl of Equinox Library Amp Mix (2x) to the gDNA libraries and mix well by gentle pipetting. NOTE: Invert Equinox Library Amp Mix (2x) 5 times before use. Do not vortex.
- Pulse-spin sample tubes and immediately transfer to the thermal cycler. Start the program.
- When the thermal cycler program is complete proceed to purification.
- Vortex the pre-equilibrated DNA Purification Beads until mixed.
- Add 50 µl (1x) of homogenized DNA Purification Beads to each ligation sample. Mix well by vortexing. Incubate the samples for 5 minutes at room temperature.
- Place the samples on a magnetic plate for 1 minute. The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing plate or tubes from the magnetic plate, remove and discard the supernatant.
- Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.
- Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate.
- Carefully remove all remaining ethanol with a 10-µl pipet, making sure not to disturb the bead pellet. NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.
- Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not over dry the bead pellet.
- Remove the plate or tubes from the magnetic plate and add 22 µl water to each sample. Mix by pipetting until homogenized. Incubate at room temperature for 2 minutes.
- Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.
- Transfer 20 µl of the clear supernatant containing the Amplified Indexed Libraries to a clean thin-walled PCR 0.2-ml strip-tube, making sure not to disturb the bead pellet.
- Quantify each library using the Qubit® Fluorometer as described in step 1.7.

3. Protocol for Target enrichment – "Twist Target enrichment standard hybridization v1 Protocol", Twist Bioscience ® (DOC-001085)

Protocol reference: Twist Bioscience. "Twist Target Enrichment Standard Hybridization v1 Protocol". The protocol is summarized and with some adaptations to our samples. This protocol supports a single or multiplex (up to 8-plex) hybridization capture. The amount of indexed library to use depends on the number of indexed samples per pool.

3.1. Prepare libraries for hybridization

- Use the concentration of each amplified, indexed library to calculate the volume (in µl) of each library needed for hybridization. We multiplexed eight libraries per hybridization reaction, so the amount of each library must be 187.5 ng and the total mass of the pool will be 1500 ng. In the end total we had 3 pools.
- Transfer the calculated volumes from each amplified indexed library to an indexed library pool reaction tube for each hybridization being performed. In the end, we had 3 pools. NOTE: Clean thin-walled PCR 0.2-ml strip-tubes or well of a 96- well thermal cycling plate are recommended to avoid unnecessary transfers in downstream steps. Check for a proper seal on the tubes as evaporation may occur leading to decreased performance.
- Pulse-spin the indexed library pool tubes to minimize the amount of bubbles present.
- Proceed to the drying of the indexed library pools (The Alternate Pre-Hybridization DNA Concentration Protocol in Appendix was followed)
- Add 1.8x homogenized DNA Purification Beads to the tubes containing the DNA libraries. Mix well by vortexing. NOTE: For amplified, indexed library pools with a volume of less than 10 µl, bring volume up to 10 µl with water. Incubate for 5 minutes at room temperature.
- Pulse spin to ensure all the solution is at the bottom of the tubes and place the tubes on a magnetic plate or rack for 3 minute or until the solution is clear. The DNA Purification Beads form a pellet, leaving a clear supernatant.
- Without removing the plate or tubes from the magnetic plate or rack, remove and discard the clear supernatant.
- Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.
- Repeat this wash once, for a total of two washes, while keeping the tube on the magnetic plate.
- Carefully remove all remaining ethanol using a 10 µl pipette, making sure to not disturb the bead pellet. NOTE: Pulse spin if necessary to ensure complete removal of ethanol.
- Air-dry the bead pellet on a magnetic plate for 1–5 minutes or until the bead pellet is dry. Do not over dry the bead pellet.
- Remove the tube(s) from the magnetic plate or rack and add 7 µl Universal Blockers and 5 µl Blocker Solution. Mix by pipetting until homogenized.

3.2. Hybridize capture probes with pools

- Heat the Hybridization Mix at 65°C in the heat block for 10 minutes, or until all precipitate is dissolved, then cool to room temperature on the benchtop for 5 minutes.
- Prepare a probe solution in a clean thin-walled PCR 0.2-ml strip-tube as indicated in the table below. Mix by flicking the tubes.

Reagent	Volume
Hybridization mix	20 µl
Twist fixed or Custom panel	4 µl
Water	4 µl
Total	**28 µl**

- Heat the probe solution to 95°C for 2 minutes in a thermal cycler with the lid at 105°C, then immediately cool on ice for 5 minutes.
- While probe solution is cooling on ice, heat the tube containing the resuspended indexed library pool at 95°C for 5 minutes in a thermal cycler with the lid at 105°C, then equilibrate both the probe solution and resuspended indexed library pool to room temperature on the benchtop for 5 minutes.
- Vortex and spin down the probe solution, then transfer the entire volume to the resuspended indexed library pool. Mix well by vortexing. Pulse-spin the tubes to ensure all solution is at the bottom of the tubes.
- Add 30 µl Hybridization Enhancer to the top of the entire capture reaction. Pulse-spin the tubes to ensure there are no bubbles present.
- Incubate the hybridization reaction at 70°C for 16 hours in a thermal cycler with the lid at 85°C. NOTE: Seal the tubes tightly to prevent excess evaporation over the 16-hour incubation. Halting hybridization between 15–17 hours will not affect downstream capture quality.

3.3. Bind hybridized targets to streptavidin beads

Before beginning:

- Preheat the following tubes at 48°C until any precipitate is dissolved: Binding Buffer, Wash Buffer 1, Wash Buffer 2.
- For each hybridization reaction: Equilibrate 800 µl Binding Buffer to room temperature; Equilibrate 200 µl Wash Buffer 1 to room temperature: leave 700 µl Wash Buffer 2 at 48°C.
- Equilibrate the Streptavidin Binding Beads to room temperature for at least 30 minutes
- Thaw on ice: Equinox Library Amp Mix (2x) and Amplification Primers
- Equilibrate DNA Purification Beads (from the Twist Binding and Purification Beads or Twist Dry Down Beads) to room temperature for at least 30 minutes

Beginning:

- Vortex the pre-equilibrated Streptavidin Binding Beads until mixed.
- Add 100 µl Streptavidin Binding Beads to a 1.5-ml microcentrifuge tube. Prepare one tube for each hybridization reaction.
- Add 200 µl Binding Buffer to the tube(s) and mix by pipetting. Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.
- Repeat the wash with Binding buffer (previous step) two more times for a total of three washes.
- After removing the clear supernatant from the third wash, add a final 200 µl Binding Buffer and resuspend the beads by vortexing until homogenized.
- After the hybridization (Final step from point 3.2) is complete, open the thermal cycler lid and directly transfer the volume of each hybridization reaction into a corresponding tube of washed Streptavidin Binding Beads from the previous step. Mix by pipetting and flicking.
- Mix the tubes of the hybridization reaction with the Streptavidin Binding Beads for 30 minutes at room temperature on a shaker. NOTE: Do not vortex. Aggressive mixing is not required.
- Remove the tubes containing the hybridization reaction with Streptavidin Binding Beads from the mixer and pulse-spin to ensure all solution is at the bottom of the tubes.
- Place the tubes on a magnetic stand for 1 minute. Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet.
- Remove the tubes from the magnetic stand and add 200 µl Wash Buffer 1. Mix by pipetting. Pulse-spin to ensure all solution is at the bottom of the tubes. Transfer the entire volume from the previous step (~200 µl) into a new 1.5-ml microcentrifuge tube, one per hybridization reaction.
- Place the tubes on a magnetic stand for 1 minute. IMPORTANT: This step reduces background from non-specific binding to the surface of the tube.
- Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.
- Remove the tubes from the magnetic stand and add 200 µl of 48°C Wash Buffer 2. Mix by pipetting, then pulse-spin to ensure all solution is at the bottom of the tubes. Incubate the tubes for 5 minutes at 48°C.
- Place the tubes on a magnetic stand for 1 minute. Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.
- Repeat the wash described in the previous 2 steps two more times, for a total of three washes.
- After the final wash, use a 10 µl pipette to remove all traces of supernatant. Proceed immediately to the next step. Do not allow the beads to dry.
- Remove the tubes from the magnetic stand and add 45 µl water. Mix by pipetting until homogenized, then incubate this solution, hereafter referred to as the Streptavidin Binding Bead slurry, on ice.

3.4. Post-capture PCR amplify, purify, and perform QC

Before beginning: Prepare 500 µl 80% ethanol for each Streptavidin Binding Bead slurry to be processed.

- Program a thermal cycler with the following conditions. Set the heated lid to 105°C.

	Step	Temperature	Time	Number cycles
1	Initialization	98 ºC	45 s	1
2	Denaturation	98 ºC	15 s	8
	Annealing	60 ºC	30 s	
	Extension	72ºC	30 s	
3	Final extension	72 ºC	1 min	1
4	Final hold	4 ºC	Hold	---

- If the Streptavidin Binding Bead slurry has settled, mix by pipetting.
- Transfer 22.5 µl of the Streptavidin Binding Bead slurry to a 0.2-ml thin-walled PCR strip-tube(s). Keep on ice until ready to use in the next step. NOTE: Store the remaining 22.5 µl water/Streptavidin Binding Bead slurry at –20°C for future use.
- Prepare a PCR mixture by adding the following reagents to the tubes containing the Streptavidin Binding Bead slurry. Mix by pipetting.

Reagent	Volume per reagent
Streptavidin Binding Bead Slurry	22,5 µl
Amplification Primers, ILMN	2,5 µl
Equinox Library Amp Mix (2x)	25 µl
Total	**50 µl**

- Pulse-spin the tubes, transfer them to the thermal cycler and start the cycling program described in the first step.
- When the thermal cycler program is complete, remove the tubes from the block and immediately proceed to the Purify step.
- Vortex the pre-equilibrated DNA Purification Beads until well mixed.
- Add 50 µl (1.0x) homogenized DNA Purification Beads to the tubes removed from the thermocycler. Mix well by vortexing. NOTE: It is not necessary to recover supernatant or remove Streptavidin Binding Beads from the amplified PCR product.
- Incubate for 5 minutes at room temperature.
- Place the tube(s) on a magnetic plate for 1 minute or until the supernatant is clear. The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tubes from the magnetic plate, remove and discard the clear supernatant.

- Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.
- Repeat this wash once, for a total of two washes, while keeping the tube on the magnetic plate.
- Carefully remove all remaining ethanol using a 10 µl pipette, making sure to not disturb the bead pellet. NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.
- Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not over dry the bead pellet.
- Remove the tubes from the magnetic plate and add 14 µl water to each capture reaction (This volume was changed to try to concentrate the libraries). Mix by pipetting until homogenized. Incubate at room temperature for 2 minutes.
- Place the tubes on a magnetic plate and let stand for 3 minutes or until the beads fully pellet.
- Transfer 12 µl of the clear supernatant containing the enriched library to a clean thin-walled PCR 0.2-ml strip-tube making sure to not disturb the bead pellet.
- Quantify each enriched library using the Qubit® Fluorometer as described in step 1.7.

4. Preparation of final pool for sequencing

Pool the 12 µl of each enriched library in one clean thin-walled PCR 0.2-ml strip-tube and calculate the total amount of gDNA considering the quantification of the libraries to verify if it's enough for sequencing.

5. Sequencing

Sequence the enriched libraries on an Illumina NextSeq 550 (2x 150bp).

Annex 13 – Results of the qPCR of PAdV with samples extracted with the adapted Maxwell protocol

Table A5 - Quantification of genomic copies of PAdV by qPCR from porcine faecal samples extracted with the adapted Maxwell protocol.

		Cq value	GC/ 10 µL of sample (raw)	GC / 10 µL of sample (adjusted)	GC / 10µl of sample (mean)
Sample 1	Direct	27,057	1,42E+04	1,42E+04	1,45E+04
	Dilution 1:10	30,613	1,41E+03	1,41E+04	
	Dilution 1:10	30,507	1,51E+03	1,51E+04	
Sample 2	Direct	26,886	1,59E+04	1,59E+04	1,84E+04
	Dilution 1:10	30,486	1,53E+03	1,53E+04	
	Dilution 1:10	29,793	2,40E+03	2,40E+04	
Sample 3	Direct	26,542	1,98E+04	1,98E+04	2,33E+04
	Dilution 1:10	29,734	2,49E+03	2,49E+04	
	Dilution 1:10	29,714	2,53E+03	2,53E+04	
Sample 4	Direct	27,304	1,21E+04	1,21E+04	1,08E+04
	Dilution 1:10	31,131	1,01E+03	1,01E+04	
	Dilution 1:10	31,093	1,03E+03	1,03E+04	
Sample 5	Direct	27,335	1,19E+04	1,19E+04	1,27E+04
	Dilution 1:10	30,737	1,30E+03	1,30E+04	
	Dilution 1:10	30,714	1,32E+03	1,32E+04	

Annex 14 – Results of the qPCR of PAdV for the comparison between two extraction methods for faeces samples: Maxwell AS1600 and "QIAamp Viral RNA Mini Kit"

Table A6 - Quantification of genomic copies of PAdV by qPCR from porcine faecal samples extracted with two different methods.

Method	Samples	Cq	GC/ 10 µl (raw)	GC/ 10 µl (adjusted)	GC/mg of sample	GC/mg of sample (mean)
Column based (QIAGEN)	1	28,09	7,73E+03	7,73E+03	3,71E+03	4,25E+03
		31,84	8,74E+02	8,74E+03	4,19E+03	
		31,59	1,01E+03	1,01E+04	4,85E+03	
	2	28,19	7,33E+03	7,33E+03	3,52E+03	3,31E+03
		32,42	6,24E+02	6,24E+03	3,00E+03	
		32,19	7,12E+02	7,12E+03	3,42E+03	
	Negative Control	Undetermined	0,00E+00	0,00E+00	0,00E+00	0,00E+00
		Undetermined	0,00E+00	0,00E+00	0,00E+00	0,00E+00
Magnetic (Maxwell)	1	25,69	3,13E+04	3,13E+04	1,50E+04	2,04E+04
		29,10	4,31E+03	4,31E+04	2,07E+04	
		28,75	5,29E+03	5,29E+04	2,54E+04	
	2	25,65	3,20E+04	3,20E+04	1,54E+04	2,13E+04
		28,97	4,65E+03	4,65E+04	2,23E+04	
		28,69	5,45E+03	5,45E+04	2,62E+04	
	Negative Control	Undetermined	0,00E+00	0,00E+00	0,00E+00	0,00E+00
		Undetermined	0,00E+00	0,00E+00	0,00E+00	0,00E+00

Annex 15 – Consensus sequence obtained by Sanger sequencing of the EHDV n(RT)PCR product.

(3´)GTTCTCACGCGAGATGGCAGCGGAGCAAACGGAAGCGCTTCGCACCATTAAAAGTGAATTAAAGAAACGTAAGACGGCC
AGGCCCCACGCGATGAAGAGTGACGTAACGCGAAAGAATAACAAGAGAGGGGGAAAGGAAGAGAAAAATGATGGAGTTAA
GAAGGGAGAAGAGGAAAACACTGATAAGCGTGAAATTCAGGATGACGATGATGAYAAAGAGGAAAAGGAGAAAACAGAACC
ATCGGTGTCGTCATCAGAANCGTATCCCCAGATTTGGCTGTAGAGGATGTGATGAGCCAAAAGAAATTACTATCAATGATAGG
TGGGGGTGAGAA(5')

Annex 16 - Results of the quantification of collective samples of the 2 cow farms and 2 bovine slaughterhouses by qPCR and q(RT)PCR methods

Table A7 - Viral quantities in genomic copies per ml in lixiviate samples from the 2 cow farms. NEG stands for negative result.

		Quantification (GC/ml)		
Sample	**Farm**	**BPyV**	**RoV-A**	**BCoV**
F3S1	**Can Bordois Lixiviates**	NEG	NEG	NEG
F3S2		1,02E+01	NEG	NEG
F3S3		NEG	NEG	NEG
F3S4		2,04E+00	NEG	NEG
F3S5		1,91E+02	NEG	NEG
F3S6		4,39E+02	NEG	1,76E+02
F3S7		1,70E+02	NEG	----
F3S8		1,02E+03	3,40E+01	----
Mean quantity (GC/ml)		**3,05E+02**	**3,40E+01**	**1,76E+02**
F4S2	**La Pasiega Lixiviates**	6,86E+02	2,47E+01	2,29E+01
F4S3		6,64E+03	NEG	2,21E+03
F4S4		1,13E+03	NEG	NEG
F4S5		5,86E+03	3,09E+02	1,47E+02
F4S6		1,07E+04	4,22E+02	2,67E+02
F4S7		2,17E+02	3,20E+01	----
F4S8		2,00E+03	NEG	----
Mean quantity (GC/ml)		**3,89E+03**	**1,97E+02**	**6,62E+02**

Table A8 -Viral quantities in genomic copies per ml in aerosol samples from the 2 cow farms. NEG stands for negative result.

		Quantification (GC/ml)	
Sample	**Farm**	**BCoV**	**BRSV**
F3A1	**Can Bordois Aerosols**	NEG	NEG
F3A2		NEG	NEG
F3A3		NEG	NEG
F3A4		NEG	NEG
F3A5		NEG	NEG
F3A6		NEG	NEG
F3A7		no kit	NEG
F3A8		no kit	NEG
Mean quantity (GC/ml)		**0,00E+00**	**0,00E+00**
F4A1	**La Pasiega Aerosols**	NEG	NEG
F4A2		1,82E+01	NEG
F4A3		1,75E+00	NEG
F4A4		NEG	NEG
F4A5		NEG	NEG
F4A6		NEG	NEG
F4A7		no kit	NEG
F4A8		no kit	NEG
Mean quantity (GC/ml)		**9,98E+00**	**0,00E+00**

Table A9 -Viral quantities in genomic copies per ml in wastewater samples from the 2 cow slaughterhouses. NEG stands for negative result.

		Quantification (GC/ml)					
Sample	**Place**	**BPyV**	**RoV-A**	**BCoV**	**EHDV**	**BTV**	**IAV**
E1D1	**Sabadell**	1,17E+00	4,89E+01	1,99E+03	NEG	NEG	----
E1D2		5,85E-01	6,76E+03	NEG	NEG	NEG	----
E1D3		7,98E-01	7,71E+03	3,03E+01	NEG	NEG	NEG
E1D4		NEG	1,95E+03	NEG	NEG	NEG	NEG
Mean quantity (GC/ml)		**8,51E-01**	**4,12E+03**	**1,01E+03**	**0,00E+00**	**0,00E+00**	**0,00E+00**
E2D1	**Argentona**	9,25E+01	NEG	NEG	NEG	NEG	----
E2D1		1,03E+00	1,88E+01	NEG	NEG	NEG	----
E2D1		NEG	NEG	NEG	NEG	NEG	NEG
E2D1		NEG	1,90E+04	NEG	NEG	NEG	NEG
Mean quantity (GC/ml)		**4,68E+01**	**9,51E+03**	**0,00E+00**	**0,00E+00**	**0,00E+00**	**0,00E+00**

Annex 17 - Results from the Blast of the sequences of the families *Betacoronovirus, Orthopneumovirus* and *Rotavirus* obtained by NGS

Table A10 -Results of the Blasts performed: Viral species sequenced in lixiviate and aerosol samples.

Sample pool	Viral family	Viral specie	Host	Nr of reads	Nr of contigs	Coverage (%)	Gene	Protein
Lixiviates Can Bordois	Betacoronavirus	Betacoronavirus 1	Bovine	644	6	12,2	ORF1ab	Orf 1ab protein
	Polyomaviridae	Bovine polyomavirus 2	Cattle	8	1	11,4	Complete genome	All
Lixiviates La Pasiega	Betacoronavirus	Betacoronavirus 1	Bovine	101	1	6,6	Orf 1b	NSP 1b
	Polyomaviridae	Bovine polyomavirus 2	Cattle	11	1	4,9	Complete genome	All
Aerosols Can Bordois	Polyomaviridae	Bovine polyomavirus 2	Bovine	612	1	99,6	Complete genome	All
	Epsilon polyomavirus	Bovine polyomavirus 1	Cattle	473	4	46,2	Complete genome	All
Aerosols La Pasiega	Polyomaviridae	Bovine polyomavirus 2	Cattle	876	1	100	Complete genome	All
	Epsilon polyomavirus	Bovine polyomavirus 1	Cattle	139	1	25	Complete genome	All

Table A11 - Results of the Blasts performed: Viral species sequenced in slaughterhouse wastewater samples.

Sample pool	Viral taxon	Viral specie	Host	Nr of reads	Nr of contigs	Coverage (%)	Gene	Protein
WW Sabadell	Rotavirus	Rotavirus A	Bovine	14	0	no data	Segment 10	NSP4
		Rotavirus B	Bovine	80	1	65,5	Segment 8	NSP2
	Beta-polyomavirus	Betapolyomavirus secuhominis	Homo sapiens	35	2	15,8	VP2	VP2 capsid protein
	Betacoronavirus	Bovine coronavirus	Bovine	9	1	0,9	ORF1ab	Replicase polyprotein 1a
WW Argentona	Ortho-pneumovirus	Bovine orthopneumovirus (i.e. BRSV)	Bovine	27	1	3,6	Complete genome	All