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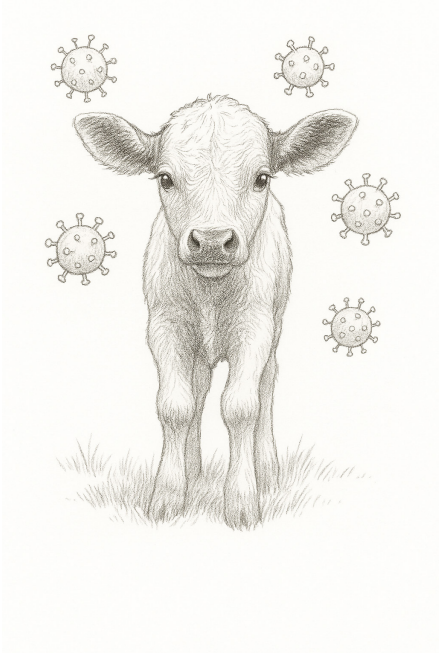
Role of Influenza D Virus in Bovine Respiratory Disease

Implications of coinfections and detection in Sweden

Ignacio Alvarez



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Abstract

This thesis investigates the role of Influenza D virus (IDV) in bovine respiratory disease (BRD), with a focus on detection of IDV in Sweden and interactions with other respiratory pathogens. BRD is a multifactorial disease with a significant impact on cattle health and productivity. The presence of IDV in cattle is increasingly detected worldwide and is considered a potential co-pathogen in BRD. The work included seroepidemiological surveillance using bulk tank milk and ELISA, phylogenetic characterization of Swedish IDV isolates by RT-qPCR and whole genome sequencing, and experimental models used to study coinfections with *Mycoplasmopsis (Mycoplasma) bovis*, bovine respiratory syncytial virus and bovine parainfluenza virus 3. Proteomic and lipidomic analyses of bronchoalveolar lavage samples from co-infected calves revealed alterations in host immune responses and inflammatory pathways. *In vitro* studies further demonstrated that IDV changed the expression of key innate immune regulators, particularly during coinfections. These findings support a modulatory role for IDV in BRD pathogenesis and highlight its importance in surveillance.

Keywords: Influenza D virus, Bovine Respiratory Disease, Coinfection, Sweden, Proteomic, Phylogenetic, Immune modulation.

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Abstract

Denna avhandling undersöker influensa D-virusets (IDVs) roll vid luftvägssjukdom hos nötkreatur, med särskilt fokus på detektion av IDV i Sverige och interaktioner med andra luftvägspatogener. Lunginflammationer har en betydande påverkan på nötkreaturs hälsa och produktivitet. IDV påvisas i ökande grad hos nötkreatur världen över och betraktas som en potentiellt saminfekterande patogen vid luftvägssjukdom. Arbetet omfattade seroepidemiologiska studier baserade på antikroppsdetektion i tankmjölk med hjälp av ELISA, fylogenetisk karakterisering av svenska IDV-isolat med RT-qPCR och helgenomsekvensering, samt experimentella modeller för analys av saminfektioner med *Mycoplasmopsis (Mycoplasma) bovis*, bovint respiratoriskt syncytialt virus och bovint parainfluenza virus typ 3. Analyser av proteiner och oxylipider i arkiverade lungsköljprover från saminfekterade kalvar visade förändringar i immunsvar och inflammation. *In vitro*-studier indikerade att IDV ändrar uttrycket av viktiga regulatorer inom det medfödda immunförsvaret, särskilt vid saminfektioner. Dessa resultat tyder på att IDV kan ha en modulerande roll vid luftvägssjukdom och understryker betydelsen av virusövervakning.

Keywords: Influensa D-virus, Luftvägssjukdom, Saminfektion, Sverige, Proteomik, Fylogeni, Immunmodulation

Dedication

"Positions are temporary. Ranks and titles are limited. But the way you treat people will always be remembered."
Unknown

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This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Alvarez, I*., Hägglund, S., Näslund, K., Eriksson, A., Ahlgren, E., Ohlson, A., Ducatez, M. F., Meyer, G., Valarcher, J. F., & Zohari, S. (2023). Detection of Influenza D-Specific Antibodies in Bulk Tank Milk from Swedish Dairy Farms. *Viruses*, *15*(4), 829. https://doi.org/10.3390/v15040829

II. Alvarez, I*., Banihashem, F., Persson, A., Hurri, E., Kim, H., Ducatez, M., Geijer, E., Valarcher, J. F., Hägglund, S., & Zohari, S. (2024). Detection and Phylogenetic Characterization of Influenza D in Swedish Cattle. *Viruses*, *17*(1), 17. https://doi.org/10.3390/v17010017

III. Alvarez, I*., Ducatez, M., Guo, Y., Lion, A., Widgren, A., Dubourdeau, M., Baillif, V., Saias, L., Zohari, S., Bergquist, J., Meyer, G., Valarcher, J. F., & Hägglund, S. (2024). Proteomic and Lipidomic Profiling of Calves Experimentally Co-Infected with Influenza D Virus and *Mycoplasma bovis*: Insights into the Host-Pathogen Interactions. *Viruses*, *16*(3), 361. https://doi.org/10.3390/v16030361

IV. Alvarez, I*, Pfaff, F., Guo, Y., Zohari S., Blaise-Boisseau S., Ducatez M., Meyer G., Valarcher J.F. & Hägglund S. (2025). Understanding the Impact of Influenza D Virus in Bovine Respiratory Coinfections: A Comprehensive *In Vitro* Approach. (Manuscript)

Papers I, II and III are reproduced with the permission of the publishers.

The contribution of Ignacio Alvarez to the papers included in this thesis was as follows:

I. Performed interpretation of the data; was responsible for writing the manuscript and preparing the final version of the article.

II. Performed the IDV-PCR part of the laboratory work. Organized and analyzed the diagnostic part of the data. Was responsible for drafting the manuscript and preparing the final version of the article.

III. Performed data analysis and statistical analyses of the results. Wrote the first draft of the manuscript and prepared the final version of the article.

IV. Performed experimental design and planning. Executed part of the sample collection and laboratory work. Performed statistical analysis of the results. Was responsible for writing the manuscript and preparing the final version of the article.

Other papers that are not included in this thesis were as follows:

I. Alvarez, I*., Canton, G., Streitenberger, N., Macias Rioseco, M., Uzal, F., Diab, S. Anthrax in Cattle: A Retrospective Analysis of 116 Cases. (Manuscript)

II. Diab, S*., Alvarez, I., Ramirez-Barrios, R., Reddy, A., & Carvallo, F. Infectious causes of interstitial and bronchointerstitial pneumonia in cattle: A review of etiologies, pathogenesis, and diagnostic algorithm (Submitted for publication). Journal of Veterinary Diagnostic Investigation.

III. López, S., Álvarez, I., Andreoli, V., Delgado, S., Pérez, S., Pereyra, S., Romeo, F., Grolli, S., & Verna, A*. Gene expression modulation in bovine endometrial cells infected with Gammaherpesvirus type 4 and exposed to lipopolysaccharide in the presence of platelet-rich plasma (Submitted for publication). Viruses.

IV. Ovelar, M. F., Cantón, G. J., García, J. P., Riccio, M. B., Rodríguez, A. R., Farace, M. I., & Alvarez, I*. Botulism in waterfowl: Case report in Argentina (Submitted for publication). Acta Veterinaria Scandinavica.

V. Hägglund, S., Laloy, E., Alvarez, I., Guo, Y., Hallbrink Ågren, G., Yazdan Panah, H., Widgren, A., Bergquist, J., Hillström, A., Baillif, V., Saias, L., Dubourdeau, M., Timsit, E., & Valarcher, J. F. (2024). Effects of early treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) on the bronchoalveolar lavage proteome and oxylipids during bovine respiratory syncytial virus (BRSV) infection. *PloS one*, *19*(11), e0309609. https://doi.org/10.1371/journal.pone.0309609

VI. Michaud, C., Alvarez, I., Litz, B., Landmesser, A., Romey, A., Bernelin-Cottet, C., Salomez, A.-L., Relmy, A., Huet, H., Zientara, S., Pfaff, F., Valarcher, J.-F., Bakkali Kassimi, L., Hägglund, S., Eschbaumer, M., & Blaise-Boisseau, S. Leaderless FMDV O does not establish a persistent infection in multilayered cells derived from bovine dorsal soft palate (Manuscript)

1. Introduction

1.1 Bovine respiratory disease

Bovine respiratory disease (BRD) is a generic term that applies to a number of respiratory disorders that are caused by infectious agents in cattle. Respiratory disease is one of the most prevalent diseases in the cattle industry and presents significant challenges beyond animal health and welfare, including potential human health risks associated with the inappropriate use of antibiotics, the development of antimicrobial resistance, and the economic impact. Respiratory disease in cattle is multifactorial, resulting from a complex interplay between pathogens, host immunity and environmental factors. The onset of BRD, which often occurs at young age, is largely due to disturbances in this sensitive multifactorial balance. Young calves rely on passive immunity obtained from colostrum, which provides essential antibodies during the early stages of life. Any deficiency in this passive transfer can dramatically increase their susceptibility to infection. As calves grow, their active immunity begins to develop, but this can be compromised by environmental stressors such as extreme weather conditions, humidity, poor housing and overcrowding. These stressors not only weaken the immune response, but also facilitate the spread of pathogens, making effective management and prevention strategies critical to maintaining herd health (Taylor *et al.* 2010).

The presence of BRD is influenced by several factors, including geographic location, animal age, herd management practices, and specific pathogens. One way to measure the prevalence of BRD worldwide is through the assessment of lung lesions in slaughterhouses. In the United States, researchers found that 32.3% of slaughtered veal calves (n=19.229) had BRD

lesions in the lungs, while in Europe, a study carried out in France, the Netherlands and Italy demonstrated that 21.6% of veal calves (n=100) had pneumonic lesions (Brscic *et al.* 2012; Rezac *et al.* 2014). In Spain, 17.9% of culled cattle (n=1101) had lung lesions consistent with BRD (Fernandez *et al.* 2020).

Despite implementing biosecurity measures and policies, along with successful disease eradication programs for diseases like Bovine Viral Diarrhea, Brucellosis, Enzootic Bovine Leukosis, Infectious Bovine Rhinotracheitis and Tuberculosis, as well as not having detected a case of Paratuberculosis since 2005, Sweden continues to face concerns related to BRD (Emmi Andersson 2022). In 2023, BRD lesions were identified at slaughter in 9% (n = 128,908) of young bulls (Jonasson 2023). In Sweden, only healthy animals are permitted for slaughter and consequently, the lesions observed at slaughter represent subclinical disease or healing processes rather than active disease, which may partly explain the lower prevalence compared to other countries. Respiratory infections accounted for 57% and 58% of antibiotic treatments administered to heifer and bull calves respectively, aged 0–6 months in 2021. This positions BRD as the primary reason for antibiotic use within this age group (VÄXA 2021).

1.2 Pathogen diversity and interaction in bovine respiratory disease

Bovine respiratory disease is influenced by a wide number of factors, including the presence of microorganisms in the respiratory tract. The respiratory microbiome plays a crucial role in cattle health and their susceptibility to BRD. The microbiota, consisting of commensal microbes, some of which are potential pathogens, inhabit the mucosal surface of the respiratory tract (Man *et al.* 2017; Perez-Cobas *et al.* 2021). These microbial communities are established early, strongly influenced by maternal microbiota at birth, and undergo changes throughout the life (Timsit *et al.* 2020). Particularly during stressful events like transport or changes in the environment, these microorganism communities can be disrupted (Holman *et al.* 2015a; Holman *et al.* 2015b; Lima *et al.* 2019; Timsit *et al.* 2020). It appears that a higher bacterial load of *Pasteurella (P.) multocida*, *Manhemmia (M.) haemolytica* and M*ycoplasmopsis (M). bovis* in the

nasopharynx, increases susceptibility to BRD in steers (Timsit *et al.* 2018). In contrast, in healthy steers the microbial community was enriched with *Mycoplasma dispar*, *Lactococcus lactis* and *Lactobacillus casei*, suggesting that the presence of these bacterial genera could have a protective effect against opportunistic pathogens. This understanding has opened the door to potential BRD prevention strategies, including the use of probiotics to maintain or restore healthy microbiota, thereby reducing BRD incidence (Amat *et al.* 2017). This knowledge of the bovine respiratory microbiome in healthy cattle is important for understanding the complex pathogen interactions in the respiratory tract, and for targeting specific pathogens involved in BRD. Several pathogens can induce BRD, including viruses, bacteria, parasites, and fungi. However, not all these pathogens are equally pathogenic, and viruses and bacteria have a much more established role and prevalence than parasites and fungi. In addition, the complexity of the pathogenesis of BRD is reflected in the fact that not all potential pathogens identified in the respiratory tract, are always considered to be either causative or contributory. Among viruses, bovine respiratory syncytial virus (BRSV), bovine coronavirus (BCoV), bovine herpesvirus 1 (BoHV-1), bovine parainfluenza virus 3 (BPIV-3) and bovine viral diarrhea virus (BVDV) are generally well-established pathogens, while influenza D virus (IDV) emerged as a new potential player of BRD in recent years (Fulton 2020). Bacteria are present mainly in the upper respiratory tract as normal commensal organisms, but when the immunity of the animal declines, they take the opportunity to colonise the lower respiratory tract causing lesions in the lung and evident respiratory clinical signs, leading to the use of antibiotics. The main bacterial components of BRD include *P. multocida*, *M. haemolytica*, *Histophilus (H.) somni* and *M. bovis* (Griffin *et al.* 2010).

The dynamics within the disease process are further complicated by interactions among multiple pathogens. The advance of molecular diagnostic tools has further expanded our understanding of the polymicrobial nature of BRD, revealing that the presence of multiple pathogens is more strongly associated with disease severity than single-pathogen infections. Surveillance studies show that at least two pathogens are detected simultaneously in 41-58% of BRD cases, highlighting the significance of pathogen interactions in disease pathogenesis (Murray *et al.* 2017; Headley *et al.* 2018; Saegerman *et al.* 2022). The most common BRD scenario involves a primary viral infection followed by a secondary bacterial

infection, a combination that often results in severe disease (Gaudino *et al.* 2022b). Experimental studies have shown that animals exposed to a virus-bacteria combination have more extensive and severe lung lesions and increased clinical signs than single viral infections (Ganheim *et al.* 2003; Gershwin *et al.* 2005; Prysliak *et al.* 2011; Lion *et al.* 2021). Several *in vitro* studies have been carried out to elucidate the involved mechanism underlying the enhanced clinical signs and pathological lesions in the field. One of the most accepted mechanisms is the enhancement of bacterial adherence resulting from prior viral infection. Through different studies Sudaryatma et al. have demonstrated that BRSV infection significantly increases the adherence of *P. multocida* to cells in the trachea, bronchi, and lungs. This enhanced bacterial adherence is facilitated by the upregulation of IL-6 mRNA and the platelet-activating factor receptor (PAFR). IL-6, a key pro-inflammatory cytokine, likely modifies the cellular environment to favor bacterial attachment. Simultaneously, increased concentration of of PAFR, a receptor known to mediate bacterial binding, were detected, suggesting an enhanced potential for bacterial adherence. Additionally, the same researchers found that the adherence of *P. multocida* increased viral replication during subsequent infection with BCoV. This increase correlated with elevated levels of PAFR and Intercellular Adhesion Molecule-1 (ICAM-1), suggesting a common mechanism among viruses that cause BRD. ICAM-1, a cell surface glycoprotein, plays a crucial role in enhancing cell-cell interactions by facilitating the binding and stabilization of these connections. This includes promoting the attachment of pathogens such as *P. multocida* to host cells, thereby increasing the complexity of the infection (Sudaryatma *et al.* 2018; Sudaryatma *et al.* 2019; Sudaryatma *et al.* 2020; Fahkrajang *et al.* 2021). An *in vivo* study with BRSV and *H. somni* resulted in significantly increased cytotoxicity in alveolar epithelial cells. This interaction also facilitated greater transmigration of *H. somni* across the alveolar barrier. Additionally, there was an amplified expression and activity of the matrix metalloproteinases MMP1 and MMP3. These enzymes broke extracellular matrix components, compromising the structural integrity of the epithelial barrier. Consequently, the barrier became more permeable, making it more vulnerable to bacterial transmigration and invasion (Gershwin *et al.* 2005). The combination of BRSV and *M. haemolytica* increases IL-17 production compared to single infections, promoting neutrophil recruitment

and inflammation, which further intensifies lung damage (McGill *et al.* 2016).

Virus-virus interactions are also involved in the pathogenesis of BRD however these interactions are much less explored. Studies show that pre-infection with BVDV exacerbates the effects of subsequent viral infections, such as BoHV-1 or BRSV (Elvander *et al.* 1998; Chase 2013). Molecular mechanisms include increased TNFα secretion, reduced IL-10 production, delayed IFNγ response, and inhibited CD8+ and CD4+ T lymphocyte activity, which together impair local cell-mediated immunity (Risalde *et al.* 2011; Risalde *et al.* 2015; Romero-Palomo *et al.* 2017). This leads to more severe clinical signs, prolonged viral shedding, and greater lung damage. The timing of the secondary infection significantly influences disease severity. Experimental studies demonstrated that calves infected with BVDV followed by BoCV 6 days later exhibited more pronounced lung lesions compared to those infected after 3 or 9 days, indicating that an intermediate interval between viral exposures may result in more severe respiratory disease (Ridpath *et al.* 2020).

Bacterial-bacterial interactions in BRD are the least explored combination. However, studies indicate that coinfections can worsen disease outcomes. Calves simultaneously infected with *M. bovis* and *M. haemolytica* exhibited severe lung consolidation, up to 60%, compared to no lung consolidation in calves that had been single infected with *M. haemolytica* (Gourlay & Houghton 1985). Molecular mechanisms driving these severe outcomes likely involve enhanced inflammatory responses and tissue damage. *In vitro* studies suggest that some bacterial strains can enhance or inhibit the growth of others. For example, *M. haemolytica* growth was inhibited by physical separation from *P. multocida*, indicating a contact-dependent mechanism (Bavananthasivam *et al.* 2012). Additionally, *H. somni* and *P. multocida* were shown to co-exist in biofilms, potentially complicating treatment and management (Petruzzi *et al.* 2020). However, whether these interactions are consistently synergistic or antagonistic remains unclear, highlighting the need for further research.

1.3 Influenza viruses in cattle

Influenza viruses, members of the *Orthomyxoviridae* family, are negative-sense, single-stranded RNA viruses known for causing respiratory disease across a broad range of hosts worldwide, including humans. The viruses are categorised into four main types: Influenza A (IAV), B (IBV), C (ICV), and D (IDV) (Yu *et al.* 2021).

Influenza A and B viruses are characterized by their eight RNA segments that encode essential viral proteins such as the polymerase proteins (PB2, PB1, and PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), and matrix (M) and non-structural (NS) proteins (Figure 1) (Yu *et al.* 2021). Influenza A is particularly known for its ability to undergo high genetic reassortment, which contributes to its broad host range and pandemic potential. Highly pathogenic strains within the H5 and H7 subtypes of Influenza A can cause severe disease due to a HA protein that allows the virus to infect multiple organs, leading to systemic infections (Charostad *et al.* 2023). In contrast, Influenza B evolves more slowly and primarily causes seasonal epidemics in human populations.

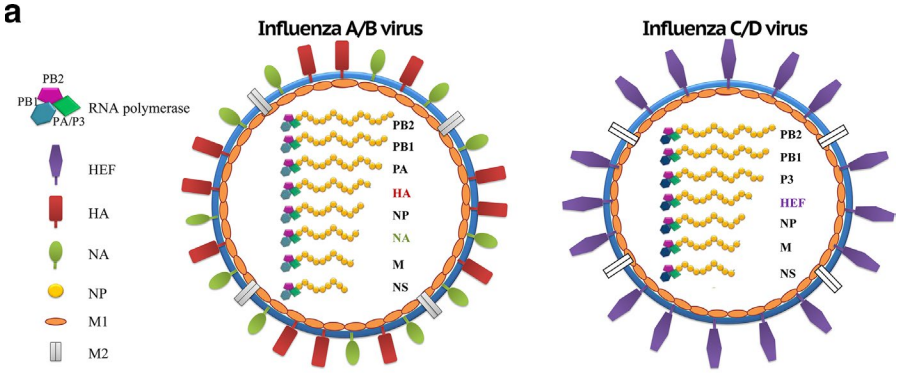

Figure 1. The structural differences of the four types of influenza viruses. Extracted from Yu *et al*., 2021.

Influenza C and D, with seven RNA segments, include polymerase proteins, nucleoprotein, matrix proteins, and a distinctive hemagglutinin-esterase-fusion (HEF) protein that combines receptor binding, membrane fusion, and esterase activity (Yu *et al.* 2021).

Historically, influenza has not significantly impacted cattle as it has for other species (Sreenivasan *et al.* 2019). Only sporadic and non-mortal natural outbreaks, mainly related with IAV, and some experimental studies were described in cattle (Fatkhuddinova *et al.* 1973; Tanyi *et al.* 1974; Lopez & Woods 1984; Sreenivasan *et al.* 2019) . This relatively low pathogenicity in cattle may stem from host-dependent restriction factors in the respiratory tract, which can hinder the replication and adaptation of influenza viruses (Sreenivasan *et al.* 2019). In addition to the restriction factors in the respiratory tract, studies have highlighted that certain serum components and secretory proteins in bovines exhibit significant anti-influenza properties. Among these protective agents are lactoferrin and antimicrobial peptides such conglutinin and collectin. These proteins are particularly effective at inhibiting viral replication by interfering with the ability of the virus to enter and replicate in the host cells. (Lanni *et al.* 1949; Curtain & Pye 1955; Ng *et al.* 2010; Ammendolia *et al.* 2012). Thus, until recently, it was believed that the limited susceptibility of cattle to influenza could be due to physiological, cellular, or immune-related factors inherent to this particular species. However, recent developments have challenged this understanding. The emergence of IDV in 2011, characterized by significant serological and molecular detection in cattle, alongside the recent outbreaks of Highly Pathogenic Avian Influenza Virus (HPAI) H5N1 in the US, has shown that cattle are less resilient to influenza virus than previously thought (Caserta *et al.* 2024; Sreenivasan *et al.* 2024; Lang *et al.* 2025). Regarding the HPAI outbreaks, the first detection was made in March 2024 and by April 2025, 1020 cases in 17 states have been affected (United States Department of Agriculture 2025). Affected cattle exhibited symptoms including fever, lethargy, dry/tacky feces or diarrhea, mastitis and a marked decrease in milk production. In addition, the virus was detected at high concentrations in raw milk. The current hypothesis suggests transmission from wild birds, potentially facilitated by environmental contamination or using poultry manure/litter in cattle feed, a practice that is not heavily regulated in the United States. This situation, coupled with the frequent detection of IDV in cattle, underscores the need for continuous research into the impact of influenza viruses on cattle.

1.4 Influenza D virus

IDV was first isolated in April 2011 in Oklahoma, USA, from a pig exhibiting clinical signs similar to those of swine influenza (Hause et al., 2013). Initially thought to be ICV due to genetic similarity, further analysis revealed that the virus belonged to a new, distinct genus within the *Orthomyxoviridae* family, later classified as *Deltainfluenzavirus*.

While IDV shares structural similarities with ICV, particularly in the HEF protein, the HEF of IDV exhibits enhanced stability across a variety of environmental conditions, including temperature and pH fluctuations. Under acidic conditions with a pH of 3.0 for 30 minutes, IDV retains about 80% of its original infectivity, demonstrating a significant resistance. In contrast, IAV, IBV and ICV are completely inactivated when are exposed to the same conditions (Yu *et al.* 2017). This increased stability of the virus not only facilitates the survival outside its primary host but also broadens its tissue tropism. The ability of IDV to maintain the integrity of its HEF protein in different tissues allows it to successfully infect and replicate in diverse cell types, expanding its host range.

Serological studies have revealed that IDV is widespread in cattle populations around the world, having been retrospectively identified in U.S. cattle as early as 2003 (Luo et al., 2017). Since this first detection, IDV has been detected in several countries across North America, South America, Europe, and Asia, with varying prevalence rates (Table 1). In Europe, IDV was first identified in France in 2015, and subsequent studies have reported its presence in cattle herds across several European countries (Ducatez et al., 2015; Oliva et al., 2019; Chiapponi et al., 2019). The virus has also been detected in a range of other animals, including horses, small ruminants, wild boar, buffalo, camels, and even humans, particularly those with close contact with cattle (Sreenivasan et al., 2021).

Country, Year	Method	Prevalence BRD (n)[1]	Prevalence healthy (n)[2]	Total (n)[3]	Reference
FR, 2011–2014	HI	-	4.5% (134)	-	(Ducatez *et al.* 2015)
FR, 2014-2018	HI	-	-	47.2% (3326)	(Oliva *et al.* 2019)
IT 2014-2016	PCR	1.3% (150)	-	-	(Chiapponi *et al.* 2016)
IT 2018-2019	PCR	10.6% (936)	-	-	(Chiapponi *et al.* 2019)
IE, 2014–2016	HI	64.9% (1183)	94.6% (1219)	-	(O'Donovan *et al.* 2019)
IE, 2014-2016	PCR	5.62% (320)	-	-	(Flynn *et al.* 2018)
LU, 2012–2016	HI	-	80.2% (450)	-	(Snoeck *et al.* 2018)
CH, 2016	PCR	-	-	4.1% (764)	(Studer *et al.* 2021)
UK, 2017-2018	PCR	8.7% (104)	-	-	(Dane *et al.* 2019)

Table 1. Summary of bovine IDV prevalence across Europe. FR: France, IT: Italy, IE: Irland, LU: Luxemburg, CH: Switzerland, UK: United Kingdom, DK: Denmark. [1] Animals displaying respiratory clinical signs.[2] Animals without respiratory clinical signs.[3] Total count of animals, both with and without respiratory clinical signs

IDV is primarily transmitted among cattle through direct contact and aerosol routes, similar to other respiratory viruses. The virus can spread rapidly within cattle herds, particularly in confined environments such as feedlots or housing systems. The mean duration of IDV excretion in infected cattle is approximately 6 to 8 days, with peak viral replication occurring around day 4 post-infection (dpi). Recently, IDV has also been detected in feces, suggesting that fecal-oral transmission could be a possible route of spread (Ferguson et al., 2016; Salem et al., 2019). Calves develop detectable antibody titers as early as 6 dpi, but the duration of these antibodies remains unclear (Ferguson et al., 2016).

IDV generally causes mild to moderate respiratory disease in cattle, with clinical signs appearing from 4 to 10 dpi and peaking around 8 dpi. Clinical signs are typically resolved by 12 dpi. The most commonly observed clinical

signs include dry cough, nasal and ocular discharge, depression, and mild to moderate tachypnea. In more severe cases, dyspnea and abnormal lung sounds, such as wheezing, may occur (Ferguson et al., 2016; Salem et al., 2019).

IDV primarily affects the upper respiratory tract, though it can also infect the lower respiratory tract, particularly in cases complicated by coinfections. An *ex vivo* study conducted by Gaudino et al., using a precision-cut lung slice model, demonstrated that IDV can infect a broad range of cell types within bovine lungs. The authors observed that IDV primarily infects bronchial epithelial cells, alveolar pneumocytes, and bronchiolar exocrine (Club) cells. In addition, endothelial cells and MHC-II expressing cells were also infected, though less frequently (Gaudino *et al.* 2023a).

Macroscopically, IDV infection is characterized by red, patchy areas of mild interstitial pneumonia, often located in the right cranial lobe of the lung. Microscopically, infected tissues exhibit inflammatory lesions along the respiratory airways, including neutrophil infiltration in the trachea and bronchi, and bronchointerstitial pneumonia characterized by neutrophilic and macrophagic alveolitis, and peribronchial and septal lymphoplasmocytic infiltration in the lung (Ferguson et al., 2016; Salem et al., 2019; Lion et al., 2021).

IDV diagnosis relies on direct and indirect techniques. Serological assays, like hemagglutination inhibition (HI) and enzyme-linked immunosorbent assays (ELISA), are employed to detect antibodies against IDV. Molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR), which target conserved genes, like the PB1 gene, are used for virus detection. Nasal swabs are commonly used for sampling because they provide a high amount of viral material, enhancing the likelihood of detection. Additionally, bronchoalveolar lavage fluids, transtracheal washes, and lung tissue samples can be used for diagnosis. While viral isolation is a valid option, its use has been primarily restricted to research purposes due to its labour intensive, time-consuming process as well as its lower sensitivity and specificity compared to other techniques. For diagnostic purposes, RT-PCR is the most suitable and widely used test. Histopathological examination using haematoxylin and eosin staining, combined with immunohistochemistry, can identify microscopic lesions and confirm the presence of the virus in tissue samples (Ferguson et al., 2016; Salem et al., 2019).

1.5 Phylogenetic diversity of IDV

Phylogenetic analyses have revealed that IDV circulates globally In two major lineage D/OK-2011 and D/660-2013, and eight minor Clade D/France-2012, D/Yamagata-2016, D/Texas-2017, D/Yamagata-2019, D/Australia-2019, D/Michigan-2019 and D/California-2019 based on the HEF phylogeny and additional genotype based on the reassortant of the internal genes. (Murakami *et al.* 2020; Yu *et al.* 2021; Molini *et al.* 2022; Ruiz *et al.* 2022; Yu *et al.* 2022) (Figure 2). Additionally, new viral strains from potentially novel lineages continue to be identified, as seen in recent cases from California, Brazil, or Turkey (Huang *et al.* 2021; da Silva *et al.* 2022; Yesilbag *et al.* 2022)

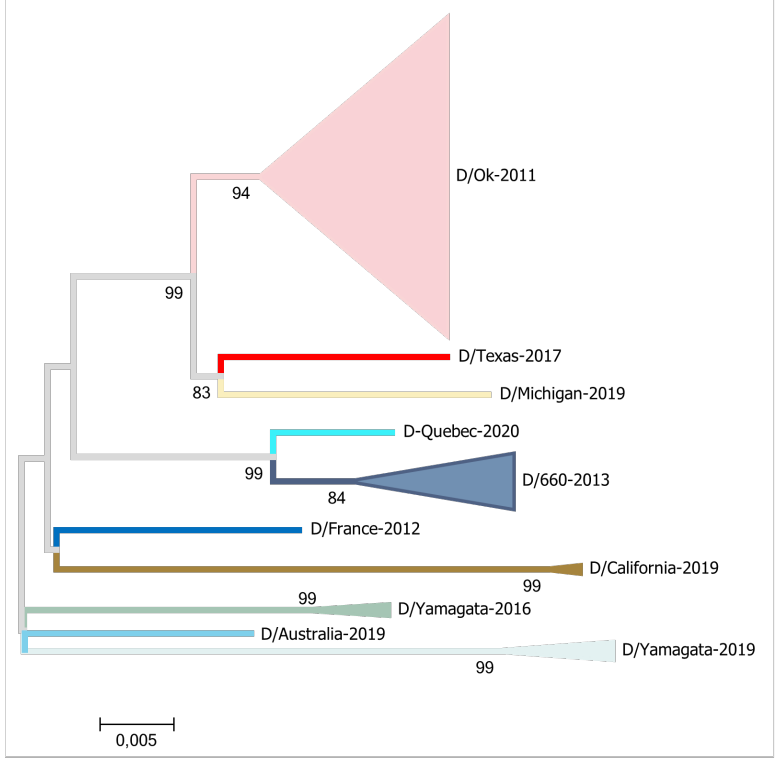

Figure 2. Phylogenetic analyses based on the hemagglutinin-esterase-fusion gene.

The D/OK lineage, first identified in North America, remains commonly present in both swine and cattle in that region. In Europe, the D/OK clade was also the first to be identified through virus surveillance (Ducatez *et al.*

2015; Chiapponi *et al.* 2016; Foni *et al.* 2017; Flynn *et al.* 2018). In contrast, the D/660 clade was only recently detected in Europe, with its first appearance in Italy in 2019, suggesting a more recent introduction of this lineage (Chiapponi *et al.* 2019).

In *in vitro* studies on tissue tropism within the respiratory tract of farmed and wild animals, Nemanichvili et al. discovered that receptors for both D/OK and D/660 strains are present in all farm animals. However, they proposed that Bovidae species contain either a specific subset of 9-O-Ac sialic acid receptors that D/OK is unable to recognize or a subset that is highly preferred by D/660. (Nemanichvili *et al.* 2021; Nemanichvili *et al.* 2022). Serum from cattle infected with either the D/OK or D/660 lineage exhibits different hemagglutination inhibition (HI) titers when tested against homologous and heterologous virus strains, indicating that the immune response is only partially cross-reactive. This suggests that even though these lineages can reassort, their antigenic profiles remain distinct, influencing the immune system ability to recognize and react to each lineage effectively.

The nucleotide substitution rate of the IDV HEF glycoprotein was found to be significantly higher than that of the HEF in ICV (Gaudino *et al.* 2022a). Viral strains from lineages D/OK and D/660 have been reported to frequently reassort with each other, and recent research has highlighted the emergence of reassortant strains within the D/660 lineage, incorporating gene segments from the D/OK lineage (Goecke *et al.* 2022). This suggests active co-circulation and reassortment between these lineages in certain European regions.

1.6 Immune response during bovine respiratory disease

During BRD, a complex interplay is activated between the host immune system, including both innate and adaptive immune responses, and the diverse range of pathogens that challenge the host. The mucosal defense serves as the initial barrier against these pathogens, with airway epithelial cells playing a crucial role. These cells control the mucociliary system, which effectively clears a significant number of respiratory pathogens through ciliary action (Lopez *et al.* 1982)

Additionally, bovine airway epithelial cells secrete antimicrobial peptides such as tracheal antimicrobial peptide (TAP), lingual antimicrobial peptide

(LAP), and bovine myeloid antimicrobial peptide (BMAP-28) (Al-Haddawi *et al.* 2007; Berghuis *et al.* 2014). These peptides exhibit bactericidal activities against key pathogens like *M. haemolytica*, *H. somni* and *P. multocida* (Taha-Abdelaziz et al. 2013). The production of these peptides is upregulated in response to pathogen-associated molecular patterns (PAMPs) through Toll-like receptors (TLRs), which are found on epithelial cells, macrophages, and dendritic cells. These triggers signaling pathways that lead to the production of pro-inflammatory cytokines such as IL-1, TNF-α, and IL-6, promoting the recruitment of neutrophils and other immune cells to the site of infection (Ackermann et al., 2010). The adaptive immune system is activated subsequently, involving antigen presentation and the activation of T and B cells, which leads to a more targeted response through the production of antibodies and the activation of cytotoxic T cells (Czuprynski, 2009).

The specific lung response to bacterial infections in BRD is characterized by a robust neutrophilic infiltration. Neutrophils play a critical role in controlling bacterial infections by releasing antimicrobial peptides and forming neutrophil extracellular traps (NETs) that capture and kill bacteria (Czuprynski, 2009). Similarly, an overreaction of neutrophils has been observed in BRSV infections (Cortjens *et al.* 2016; Hagglund *et al.* 2017) , which lead to excessive tissue damage, contributing to the pathology observed in BRD. Bacteria such as *M. haemolytica* and *H. somni* are major pathogens in BRD, known for their ability to induce severe inflammatory responses. *M. haemolytica*, for instance, produces leukotoxin (LKT) that binds to β2 integrins on the surface of bovine leukocytes, leading to cell death and the release of additional inflammatory mediators, which exacerbate lung tissue damage (Czuprynski, 2009). This intense inflammation often results in fibrinous pleuropneumonia, a characteristic lesion of severe BRD. Additionally, *H. somni* infection is associated with vasculitis and thrombosis due to its ability to alter endothelial cell function, further contributing to the severity of lung pathology in BRD (Czuprynski, 2009).

During viral infection, type I interferons (IFN-α and IFN-β) are quickly produced by epithelial and immune cells in the respiratory tract. These cytokines are important for the control of viral replication, the upregulation of interferon-stimulated genes (ISGs), and the activation of immune effectors like natural killer (NK) cells and cytotoxic T lymphocytes (Ackermann *et al.* 2010; McGill & Sacco 2020). However, the interferon response can also act

by amplifying inflammation. IFN-α has been shown to enhance the release of pro-inflammatory cytokines (e.g., IL-1, TNF-α, IL-8) by alveolar macrophages, which can exacerbate lung damage in the presence of pathogens such as *M. haemolytica* (Ellis 2001). Some pathogens, including BVDV and BRSV, have developed mechanisms to suppress type I IFN signaling, facilitating immune evasion (Srikumaran *et al.* 2007; McGill & Sacco 2020). Coinfections can modulate IFN effects. For example, *H. somni* has been shown to induce a type I IFN response that protects epithelial cells from BRSV infection, suggesting a complex role for interferons in BRD pathogenesis (McGill & Sacco 2020).

Another key mechanism in the host response is the complement system, which triggers a series of reactions that opsonize pathogens, enhance phagocytosis, and lyse pathogens or infected cells through the membrane attack complex. These processes are essential for preventing significant infections in the respiratory tract (Detsika *et al.* 2024). A recently published study highlights that calves with severe signs of disease have compromised complement activity, increasing their susceptibility to infections and leading to worse outcomes (Flores *et al.* 2023). These calves struggle to counteract the damaging effects of extracellular histones released during cell injury, which can intensify lung tissue damage. This inability to manage histone-related damage indicates that a strong complement response is vital for controlling the severity and progression of respiratory diseases, underscoring the role of the complement system not only in defense against pathogens but also in regulating lung inflammation.

An important group of immune mediators related to lung inflammation are the lipid mediators (LM) (Basil & Levy 2016; Sandhaus & Swick 2021). These mediators are a crucial group of bioactive lipids derived from the oxidation of polyunsaturated fatty acids (PUFAs). They have a key role in inflammation, immune responses, and tissue homeostasis. Lipid mediators can be pro-inflammatory or pro-resolving, influencing distinct physiological and pathological functions. Pro-inflammatory LM, such as certain prostaglandins (PG), leukotrienes, and thromboxanes can enhance vascular permeability, recruit immune cells, induce bronchoconstriction, and promote inflammatory responses while pro-resolving PM like PGE2, prostacyclin, lipoxins, resolvins, protectins, and maresins play key roles in the resolution phase of inflammation in the lung. They inhibit neutrophil recruitment, stimulate the clearance of apoptotic cells, and reduce cytokine production

and leukocyte infiltration (Heller *et al.* 1998; Levy *et al.* 2001). These actions help restore tissue integrity and function, promoting healing and tissue regeneration.

1.7 The role of IDV as a co-pathogen and its impact on the host immune response

Initially, the main concern with IDV was its impact on BRD as a single pathogen. However, recent studies have increasingly highlighted its potential role as an important co-pathogen. Coinfections between IDV and other pathogens in cattle, such as BVDV, *M. bovis* and *M. haemolytica*, have demonstrated the complex interactions and diverse outcomes that these coinfections can produce. For example, a study by *Meyer et al.* (2023) demonstrated that calves previously exposed to BVDV-2 had prolonged IDV shedding and elevated IDV RNA quantity. These calves also exhibited thymic atrophy and weakened T-cell responses, particularly reduced CD8+ and IFN-γ-expressing cells following IDV stimulation. Such immunosuppression is likely to exacerbate the course of IDV infection, as reflected by increased viral loads and impaired immune defenses. These findings highlight how BVDV-2 coinfection can compromise host immunity and exacerbate respiratory disease severity, potentially making IDV an emergent co-pathogen in cattle.

In terms of IDV-bacteria interactions, the effects of coinfection with *M. bovis* and *M. haemolytica* have been extensively studied both in cattle and *in vitro* (Zhang et al. 2019; Lion et al. 2021; Gaudino et al. 2023). Coinfection with IDV and *M. bovis* resulted in more severe clinical signs and increased lung lesions. This increased severity was attributed to IDV's ability to deviate the host's innate immune response, in particular by downregulating pro-inflammatory cytokines and chemokines essential for bacterial clearance(Lion *et al.* 2021). In *in vitro* studies using precision-cut lung slices (PCLS), IDV interfered with the NF-κB pathway controlling the cytokine production. In addition, activation of the Retinoic Acid-Inducible Gene I (RIG-I) and Melanoma Differentiation-Associated Protein 5 (MDA5) pathway by IDV likely desensitized the TLR2 pathway, which is critical for fighting bacterial infections, thereby facilitating bacterial proliferation and exacerbating disease severity (Gaudino *et al.* 2023a).

Contrasting results have been observed with *M. haemolytica*. Under experimental conditions, IDV infection preceding *M. haemolytica* did not result in increased clinical disease severity or lung pathology compared to *M. haemolytica* infection alone in calves. Although IDV caused mild respiratory symptoms and tracheal inflammation, the expected synergistic effect was not observed. These findings suggest that IDV may not significantly enhance the virulence of *M. haemolytica* in BRD, at least under the controlled conditions of this study (Zhang et al. 2019). A recent study by Saipinta *et al* (2022) investigated the role of IDV in calves with BRD and found that IDV may play a protective role in reducing the severity of the disease (Saipinta *et al.* 2022). The study demonstrated that calves infected with IDV had a lower risk of culling or death compared to those without IDV infection. This was against the general expectation that viral infections would worsen the outcome of BRD. The results suggest that IDV may reduce susceptibility to viral or bacterial coinfections or secondary infections, possibly by inducing antiviral immune responses that protect the host from severe outcomes (Saipinta et al. 2022).

2. Aim

The general aim of this thesis was to investigate the role of Influenza D Virus as a co-pathogen in bovine respiratory disease, as well as to detect and characterize the virus in Sweden.
The specific objectives were to:

Study I: Determine the presence and spatio-temporal distribution of IDV antibodies in selected Swedish dairy herds using an in-house indirect ELISA on bulk tank milk samples.

Study II: Detect IDV in respiratory samples of cattle with respiratory disease and perform a phylogenetic characterization of IDV strains that are circulating in Sweden

Study III: Investigate the early protein response and the kinetics of the lipidomic profile in calves infected with IDV, *M. bovis*, or both, to contribute to the elucidation of the role of IDV as a co-pathogen in BRD.

Study IV: Begin to evaluate the impact of IDV on replication, cytopathic effects and innate immune responses of bovine nasal turbinate cells when co-infected with BRSV and BPIV-3 *in vitro*.

3. Materials and methods

A general review of material and methods is described in this section. For more information on specific studies, please see papers I-IV.

3.1 Study design and sample collection (study I, II, III, IV)

Study I: A total of 461 and 338 bulk tank milk (BTM) samples were collected in the spring of 2019 and 2020, respectively, as part of the Swedish surveillance program for bovine viral diarrhea virus conducted by the Swedish Veterinary Agency (SVA). These samples were collected according to a risk-based and blinded design in dairy herds throughout the country.

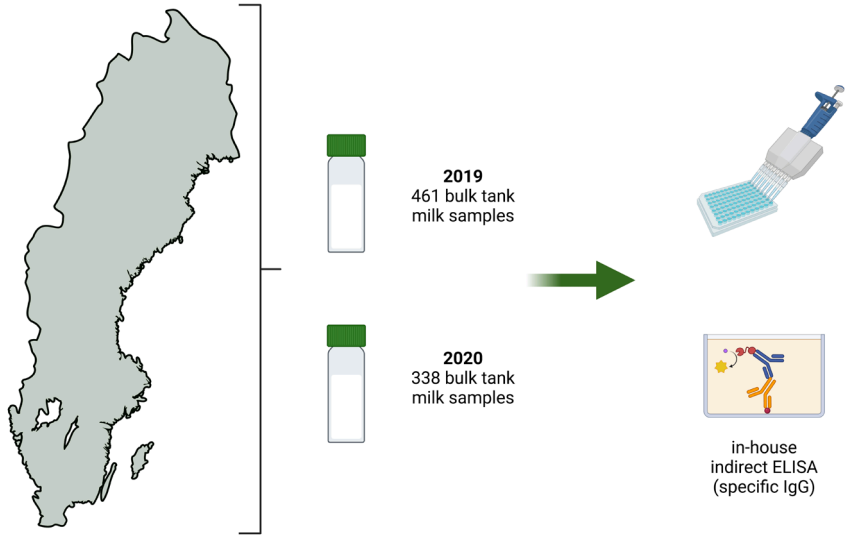

Figure 2. Study Design for Study I. A total of 799 bulk tank milk samples were collected from different regions in Sweden between 2019 and 2020 and analyzed using an in-house indirect ELISA.

Study II: A total of 1,763 archived respiratory samples from cattle with clinical signs of respiratory disease were analyzed. These samples, collected from January 1, 2021, to June 30, 2024, and submitted by field veterinarians to the routine diagnostics within the Swedish Veterinary Agency, included nasal swabs and lung tissues initially tested for other respiratory pathogens. Pooled samples from the same herd, were first screened for IDV using RT-qPCR targeting the NP-coding gene. If IDV was detected, individual samples within the positive pools were tested again and subsequently used for phylogenetic analysis. Positive samples were retested in triplicate using a second RT-qPCR assay targeting the polymerase-coding gene (PB1) to confirm results. IDV-RNA-positive samples with cycle threshold (Ct) values <30 was selected for whole genome sequencing using Illumina MiSeq platform.

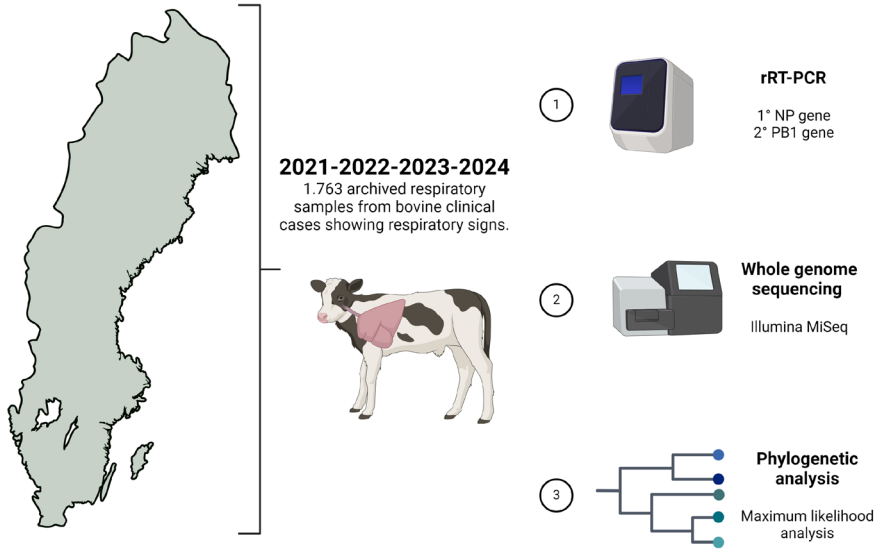

Figure 3. Study Design for Study II. A total of 1,763 samples were collected from different regions in Sweden from 2021 to 2024 and analyzed by RT-PCR for the detection of IDV, followed by sequencing.

Study III: The study was based on archived samples from an experiment including 29 Normand and Holstein calves aged 3 to 8 weeks (Lion *et al.* 2021). The calves were screened for *M. haemolytica*, *P. multocida*, *M. bovis*, *H. somni*, BCoV, IDV, BRSV, and BPIV-3 in nasal swab samples using real-time PCR (Bio-T respiratory qPCR kits; BioSellal, France). The absence of BVDV was confirmed by analysis of the non-structural protein 3 (NS3) antigen in serum by ELISA (SERELISA BVDV-BD; Synbiotics, Lyon, France). Before the experimental challenge, the calves were also confirmed negative for *M. bovis*- and IDV-specific antibodies using ELISA (Bio K 302; BioX diagnostics, Belgium) and a HI assay, respectively.

The calves were divided into four groups, with age distributions harmonized across groups, and housed in separate rooms: control (uninfected), IDV-infected, *M. bovis*-infected, and co-infected (IDV and *M. bovis*), with eight calves in each infected group and five in the control group. Infections were administered via nebulization using 10^7 $TCID_{50}$ of IDV and/or 10^{10} CFU of *M. bovis* in 10 ml of DMEM, whereas calves in the control group received DMEM only. Clinical examinations were performed,

and nasal swabs, blood samples and bronchoalveolar lavage (BAL) fluid samples were collected at 2, 7, and 14 dpi, as described previously (Lion *et al.* 2021). The BAL samples were filtered through two layers of sterile gauze, and the BAL cells and supernatant were separated by centrifugation, as previously described. The supernatants were stored at −75 ◦C, prior to mass spectrometric analyses for proteomic and lipidomic profiling (Hagglund *et al.* 2017). Samples from three calves per group, which were sacrificed on day 6, were excluded from the se analyses.

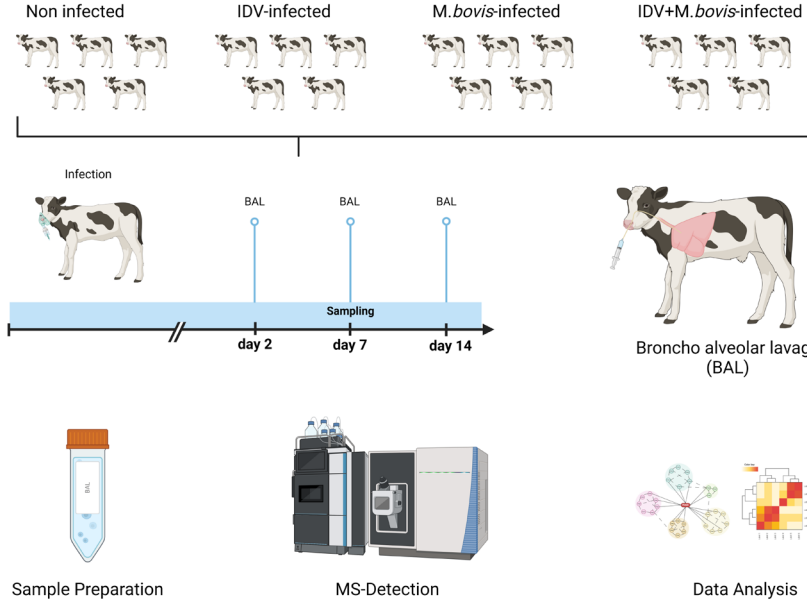

Figure 4. Study design for study III. Experimental setup involving 20 calves categorized into four groups: control (non-infected), IDV-infected, M. *bovis*-infected, and co-infected (IDV and M. *bovis*). Calves in each group were housed separately and subjected to infections with IDV, M. *bovis*, or DMEM (for control). Bronchoalveolar lavage samples were collected on days 2, 7, and 14 post-infections. The BAL supernatants were then analyzed using mass spectrometry for both proteomic and lipidomic profiling

Study IV: *In vitro* infections were performed in 12-well plates using bovine turbinates cells (ATCC 3190). Each pathogen (IDV, BRSV, BPIV-3), including single and coinfections were assigned to separate plates, with an additional plate reserved for uninfected cells (control). Three wells per plate were sampled daily for each pathogen combination (replicate (R)1-R3).

Supernatants were used for viral titration and qPCR analysis. For transcriptomic studies, cell samples were collected and preserved in QIAzol Lysis reagent (Qiagen, Cat. No. 79306, Hilden, Germany).

In the IDV and BPIV-3 coinfection experiment, supernatant samples were collected at 12-, 48-, and 72-hours post-infection (hpi) for titration and qPCR analysis. In contrast, for the IDV and BRSV coinfection experiment, samples were collected at 12, 24, 48, 72, and 96 hpi. Transcriptomic sampling times were adjusted based on the extent of cytopathic effects observed. For the IDV and BPIV-3 experiment, cells were collected at 12 and 24 hpi, while for the IDV and BRSV experiment, cells for transcriptomic analysis were collected at 12, 24, 48, and 72 hpi. This strategy ensured that transcriptomic analyses were performed on samples without significant loss of cells.

The morphology of the cell monolayers was examined using brightfield microscopy at 4× and 20× magnification. Confocal microscopy was used to determine the localization of viruses within the cells.

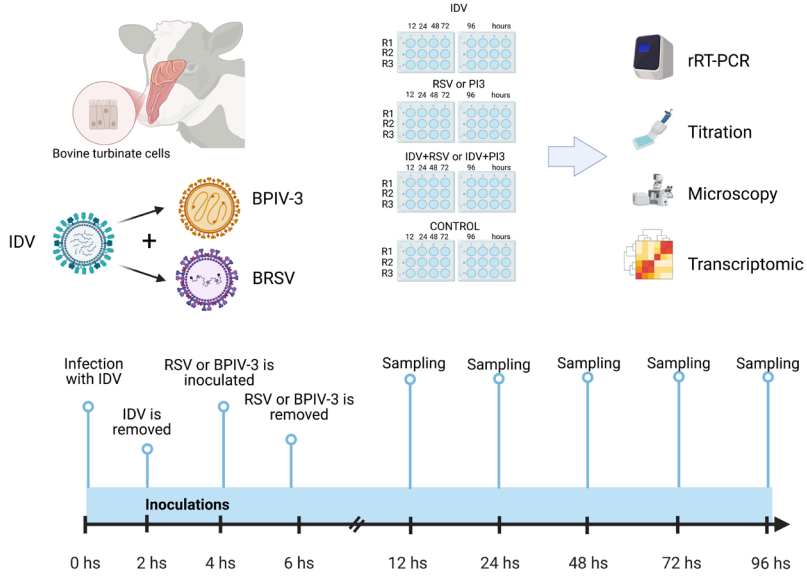

Figure 5. Experimental design of *in vitro* infections in bovine turbinate cells with IDV, BRSV, BPIV-3, and coinfections. Samples were collected at multiple time points for RT-PCR, virus titration, microscopy, and transcriptomic analyses. R1, R2, and R3 indicate biological replicates for each condition.

3.2 Indirect ELISA (study I)

The ELISA used was previously described by Salem *et* al. (Salem *et al.* 2019). Briefly, 96-well plates were coated with lysates from cells infected with a French IDV strain (D/bovine/France/5920/2014) and uninfected cells using 0.05 M sodium carbonate-bicarbonate buffer. This coating was followed by an overnight incubation at 4°C. The plates were then washed twice with a wash buffer containing phosphate-buffered saline (PBS) and 0.05% Tween 20 (PBS-T), before blocking with the same buffer for 1 h at room temperature. Milk samples and control sera were diluted 1:50 with wash buffer and 100 µL of the diluted samples were added to each well. Plates were then incubated at 37°C for 1 hour and washed three times with PBS-T. A conjugate (BRSV-Ab kit; INDICAL, Svanova) was diluted 1:2 in PBS-T and 100 µL was added to each well, followed by incubation at 37°C for 1 hour. After incubation, the plates were washed as described above. Then 100 µL of 3,3',5,5'-tetramethylbenzidine substrate (INDICAL Svanova) was added per well and incubated for 10 min at room temperature. Finally, 50 µL of Stop Solution (INDICAL, Svanova) was added per well and the optical density (OD) was measured at 450 nm using a spectrophotometer. Corrected OD values (COD) were determined by subtracting the OD values of sera tested on the control antigen from those obtained when the same sera were tested on the IDV antigen. The positive control used in the present study was an IDV antibody positive serum obtained from a calf experimentally infected with IDV strain D/bovine/France/5920/2014 (HI titer: 1/1024).

3.3 Sequencing and phylogenetic analysis (study II)

Samples included nasal swabs, tracheal and lung tissues that were initially tested for other respiratory pathogens as part of routine diagnostics. RNA was extracted using the IndiMag Pathogen Kit (INDICAL Bioscience). For each extraction, 200 µL of sample and 20 µL of Proteinase K were processed with 500 µL of lysate mixture. Extraction was performed using a Maelstrom-9600 extraction robot (TANBead). IDV detection was performed by rRT-PCR targeting the NP gene, with positive samples retested by a second rRT-PCR targeting the PB1, as previously described (Hause *et al.* 2013; Henritzi *et al.* 2019). Briefly, RNA was amplified using the AG-Path-ID™ One-Step RT-PCR Kit (Ambion). The primer sequences for the NP gene were as

follows: forward primer 5'-CTTGAAAGATTGCAAATGCAG-3', reverse primer 5'-GTTGGGTTTCAGTGCCATTC-3', and probe 5'-HEX-CACTACATTTCCCAGCTGTTGACTCC-BHQ1-3'. The thermocycling conditions for the NP assay included an initial reverse transcription at 45°C for 10 minutes, denaturation at 95°C for 10 minutes, followed by 42 cycles of 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 30 seconds.
For the PB1 gene, the primers used were: forward primer 5'-GCTGTTTGCAAGTTGATGGG-3', reverse primer 5'-TGAAAGCAGGTAACTCCAAGG-3', and probe 5'-TTCAGGCAAGCACCCGTAGGATT-3'. The thermocycling conditions for the PB1 assay consisted of reverse transcription at 45°C for 10 minutes, denaturation at 95°C for 10 minutes, followed by 47 cycles of 95°C for 15 seconds and 60°C for 45 seconds. Positive samples with Ct values between 20 and 30 were selected for whole genome sequencing (WGS) using the Illumina MiSeq instrument. Library construction was performed using the NEXTERA-XT kit (Illumina Inc.), and quality assessment was performed using the Agilent 2100 Bioanalyzer. Sequencing was performed using a MiSeq Reagent Kit v3 in a 600-cycle paired-end run. Data was analyzed using CLC Genomics Workbench v21. Phylogenetic trees were constructed for each gene segment using relevant sequences from GenBank, with phylogenetic analysis performed using maximum likelihood (ML) in MEGA7, and robustness assessed by bootstrap analysis with 2000 replicates.

3.4 Proteomic and lipidomic analysis (study III)

Proteins in filtered BAL supernatant collected at 2 dpi were identified and semi-quantified using a MS/MS analysis performed with a Thermo Fisher Scientific QExactive Plus Orbitrap mass spectrometer at the Science for Life Laboratory, Mass Spectrometry Based Proteomics Facility, Uppsala, Sweden. Prior to mass spectrometric analysis, sample volumes were reduced using a Speedvac (Thermo Fisher Scientific, Waltham, MA, USA), and the total protein concentration was measured by the Bradford Protein Assay, using bovine serum albumin as the standard. Samples were subjected to reduction, alkylation and trypsin treatment. After purification, peptides were reconstituted and analysed by Nano Liquid Chromatography-Tandem Mass Spectrometry (nano-LC-MS/MS). Protein identification was performed using MaxQuant software against a combined database of the *Bos taurus*

proteome, IDV and *M. bovis*. The proteins were semi-quantified by using label-free intensity, which allows for comparison of the quantity of the same protein in different samples. Data filtering retained only proteins identified and quantified in at least four calves in at least one of the two groups compared. Label-free protein quantities were log2 transformed, normalized and missing values imputed using a minimum probabilistic imputation technique according to Aguilan et al. (2020) (Aguilan *et al.* 2020). Proteins with a differential fold change of less than -1 or greater than 1 and a p-value of 0.05 were further analyzed using Ingenuity Pathway Analysis (IPA) software.

Lipid mediators (LM) were analyzed in BAL filtered samples collected at 2-, 7- and 14-days dpi at Ambiotis SAS, Toulouse, France. In summary, protein precipitation with methanol was followed by sample extraction using Oasis HLB 96-well plates (Waters) via solid phase extraction. Lipid mediators were then eluted with a combination of methanol and methyl formate. Quantification of results was expressed in picograms per milliliter (pg/ml) of fluid.

3.5 *In vitro* infection (study IV)

Bovine turbinates cells (ATCC, CRL-1390) were cultured in 12- and 24-well plates at a seeding density of 30,000 cells/cm² and inoculated with IDV, BRSV, BPIV-3 and coinfections with these viruses at a multiplicity of infection (MOI) of 0.1. An additional plate was left uninfected to serve as a control. For coinfection studies, cells initially infected with IDV were co-infected with either BRSV or BPIV-3 after two hours of incubation. After inoculation, all cells were incubated at 37°C in a 5% CO_2 atmosphere in Dulbecco's minimum essential medium (DMEM, Lonza, Belgium) supplemented with 2% gamma-irradiated heat-inactivated fetal calf serum (FCS, Sigma-Aldrich, F3885), 20 mL 1 M HEPES buffer solution (VWR, BioWhittaker BE17-737F), 10 mL 200 mM L-glutamine (VWR, BioWhittaker BE17-605F), 60 mg penicillin G (Sigma-Aldrich, P3032), and 100 mg streptomycin sulfate salt (Sigma-Aldrich, S6501-5G). The virus strains used were isolated from naturally infected calves. These included the influenza D virus strain D/bovine/France/5920/2014, the BRSV strain

BRSV/Sweden/HPIG-SLU-620-Lovsta/2016, and the BPIV-3 strain BPIV-3/France/ICSA-11/2013(Ducatez et al. 2015; Gaudino et al. 2023b) .

3.6 Viral quantification and fluorescence microscopy (study IV)

The following methods were used for virus quantification and fluorescence microscopy:
Virus titration was performed using the 50% tissue culture infection dose (TCID50) assay in a 96-well plate and confirmed by immunostaining and fluorescence microscopy (Kärber 1931). Immunostainings were conducted using virus-specific antibodies targeting the HEF protein of IDV and the F protein of BRSV, which were kindly provided by Dr. Ana Moreno from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy, and Dr. Geraldine Taylor from The Pirbright Institute, United Kingdom, respectively. For the IDV, the primary antibody used was the monoclonal 3G3, which targets the HEF protein, employed at a 1:10 dilution. For BRSV, the monoclonal antibody 16, targeting the F protein, was used at a 1:200 dilution. The secondary antibodies utilized included goat anti-mouse 488 (Abcam, Cat # ab15011) for IDV and IgG1 clone M1-14D12 FITC conjugate (Thermo Fisher, Cat # 11-4015-82) for BRSV, both diluted 1:400. For BPIV-3 detection, a monoclonal FITC-conjugated antibody from a commercial kit (BioX Diagnostics, Cat. #BIO 290) was used at a 1:20 dilution. Additionally, fluorescein isothiocyanate (FITC)-labeled polyclonal sheep anti-mouse immunoglobulin antibody (BioX Diagnostics, Cat. #BIO 407) was also used at a 1:20 dilution.
During the immunofluorescence staining, infected cells and controls were first washed with PBS and then fixed using 4% paraformaldehyde for 15 minutes at room temperature. After fixation, cells were washed three times with PBS and blocked for 45 minutes at room temperature in PBS containing 5% fetal calf serum. Primary antibodies were then added, and the cells were incubated for 1 hour at room temperature. Following this incubation, the cells were washed three times with PBS to remove any unbound primary antibodies. Secondary antibodies were subsequently applied, and the cells were incubated for another hour at room temperature. Finally, the cells were washed three times with PBS to remove the excess of secondary antibodies.

For viral quantification, RNA extraction was performed using the RNAeasy® Mini Kit (Qiagen, Sweden) according to the manufacturer's guidelines. A commercial TaqMan RT-qPCR kit (LSI VetMAX™ Screening Pack-Ruminants Respiratory Pathogens, Life technologies, France) was used for the detection and quantification of BRSV and BPIV-3. For IDV, an RT-qPCR protocol targeting the PB1 gene was used, described previously (Hause *et al.* 2013). Virus quantification was achieved by using a standard curve generated from an IDV, BRSV and BPIV-3 infected cell-lysate with known virus titer. The RT-qPCR experiments were performed on a BioRAD CFX96™ Touch (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the results were analyzed using the CFX Maestro™ software program. Results were expressed as equivalent TCID50.

3.7 Confocal microscopy (study IV)

Confocal microscopy was conducted using a Stellaris Leica confocal microscope equipped with LAS X software (version 4.6.1.27508). Imaging was done at various magnifications. For high-resolution imaging, a 63× oil-immersion objective (HC PL APO CS2 63×/1.40 OIL, numerical aperture 1.4) was utilized. Lower magnification images were taken with a 10× dry objective (HC PL APO CS2 10×/0.40 DRY, numerical aperture 0.4).

Cell cultures were set up in an 8-well chamber slide (Thermo Scientific Nunc Lab-Tek II). Infection was carried out using a GFP-tagged BRSV virus (replacing the previously mentioned BRSV/Sweden/HPIG-SLU-620-Lovsta/2016). Uninfected cells serve as control. The multiplicity of infection (MOI), as well as primary and secondary antibodies, were employed as outlined in prior descriptions (Section 3.6 Viral quantification and fluorescence microscopy).

Spectral imaging settings included capturing fluorescence signals across three channels at spectral ranges of 420–470 nm, 492–580 nm, and 595–750 nm. Each channel was exposed once to gather the imaging data.

Post-acquisition image processing was performed to adjust brightness and contrast uniformly using the Leica LAS X software, ensuring consistent data presentation

3.8 Transcriptomic analysis

For the investigation of transcriptomic alterations, cells infected with the virus were preserved in QIAzol Lysis reagent (Qiagen, Cat. No. 79306, Hilden, Germany) and stored at -80°C until analysis. Infected cells and controls were collected at 12- and 24-hip in triplicate for the IDV and BPIV-3 study. In the IDV and BRSV study, sample collections occurred at 12-, 24-, and 48- hpi. Total RNA was isolated from the cell culture supernatants using QIAzol Lysis reagent (Qiagen, Cat. No. 79306, Hilden, Germany) and the RNeasy Mini Kit (Qiagen, Hilden, Germany).

RNA integrity was verified using the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The transcriptomic analyses were performed by Novogene Europe, United Kingdom. mRNA was isolated using poly-T oligo-attached magnetic beads. Libraries for both strand-specific and non-strand-specific sequencing were prepared according to established protocols. Library quality and size distribution were assessed using the Qubit fluorometer, real-time PCR, and the Bioanalyzer. Sequencing was conducted on either the Illumina platform (Life Technologies) using the Ion 540 OT2 and Chip Kit.

Sequence data processing was performed with fastp for adapter removal and quality filtering. The cleaned, high-quality reads were then aligned to the reference genome with HISAT2 (v2.0.5). StringTie (v1.3.3b) was used for the identification of novel transcripts. Gene expression levels were quantified using featureCounts, and differential expression analysis was conducted with DESeq2 or edgeR. Enrichment analyses for Reactome were performed using the clusterProfiler R package, with significant terms defined as $p \leq 0.005$.

3.9 Statistical analyses (study III)

Study III: Lipidomic data were analyzed using Kruskal-Wallis test with Dunn's multiple comparison. For proteomic data, normality was assessed with Shapiro-Wilk test. If both replicates showed normal distribution, the F-test was used to test homogeneity of variance, and an appropriate t-test (equal or unequal variance) was selected for comparison. Proteins with significant deviation from normal distribution, determined by Shapiro-Wilk test, were

analyzed with Mann-Whitney test. Graphs were generated using GraphPad 9.5.0 (La Jolla, CA, USA).

4. Results and discussion

4.1 Study I – IDV Antibodies in Swedish Dairy Herds

In summary, the article presents the results of a study of IDV presence in Swedish dairy herds based on IDV-specific IgG antibody detection in BTM samples collected in 2019 and 2020. The results indicate the presence of IDV-specific antibodies in the northern region (Norrland) for the first time in 2020, while the southern region (Götaland), particularly Halland County, consistently exhibited high antibody prevalence in both years. Moreover, there was an increase in IDV-antibody positive BTM samples from 32% in 2019 to 40% in 2020.

Contrasting with international findings, the IDV seroprevalence in Sweden was relatively low. In Japan, Argentina, and Luxembourg, herd-level IDV-antibody positivity rates of 50%, 75%, and 97.7%, respectively, have been reported (Horimoto *et al.* 2016; Snoeck *et al.* 2018; Alvarez *et al.* 2020).

The higher prevalence in regions like Halland County suggests that herd density and farm proximity may play a critical role in the spread of IDV and other BRD viruses. Halland has been identified in previous studies as part of a southern Swedish cluster with consistently high seroprevalence of BRSV and BCV (Ohlson *et al.* 2014). These observations align with a study conducted by Saegerman et al, on IDV in Europe and other studies on bovine respiratory viruses showing that geographic location, farm density, and biosecurity measures significantly influence seroprevalence patterns. (Beaudeau *et al.* 2010a; Beaudeau *et al.* 2010b; Wolff *et al.* 2015; Saegerman *et al.* 2021). To establish definitive causal relationships, however, detailed

epidemiological studies that incorporate data on farm management practices, animal movement patterns, and biosecurity measures are required.

In terms of methodology, while BTM testing offers a practical, non-invasive, and cost-effective tool for herd-level surveillance, it may underestimate the true prevalence of IDV. This limitation is often due to dilution effects if only a small number of cows in a herd are seropositive, the antibody concentration in pooled milk may fall below the detection threshold (Sekiya *et al.* 2013; Lanyon *et al.* 2014). In addition, antibody levels in milk are typically 30 times lower than in serum and can be influenced by factors such as stage of lactation, maternal status, milk yield, and animal age (Sekiya *et al.* 2013; Milovanovic *et al.* 2020)

These variables complicate the interpretation of negative results and highlight the importance of considering within-herd seroprevalence when using BTM ELISA in surveillance contexts. Some studies suggest that a minimum within-herd seropositivity of 20-25% is often required before antibodies are detectable in bulk milk (Booth *et al.* 2013; Sekiya *et al.* 2013). Despite these limitations, regular BTM screening remains a valuable tool identifying high-risk herds, and supporting region-wide surveillance programs, especially when complemented by individual testing.

To achieve a nationwide determination of IDV seroprevalence, several considerations are essential. First, a statistically robust sampling design is needed to ensure representativeness across all regions, accounting for the total number of cattle in Sweden. This would involve calculating the required sample size based on herd-level prevalence estimates, expected variability, and desired confidence intervals.

The study highlights the use of BTM as a practical and non-invasive surveillance tool, although potential variations in antibody concentrations in milk suggest a need for follow-up testing. The observed shift in positive rates between years may be due to differences in sample size or that seasonality influences IDV prevalence. Overall, this study provides insights into the circulation of IDV in Swedish dairy cattle, highlighting possible regional differences and the importance of continued surveillance.

4.2 Study II- Detection and phylogenetic characterization of IDV in Sweden

A total of 51 animals tested positive for IDV in nasal swabs, with the majority detected during the 2020–2021 influenza season (1st of October-30th of September). These positive samples originated from 14 different farms and predominantly involved calves between 2 and 16 weeks of age, consistent with patterns observed for other respiratory pathogens, likely due to waning maternal antibodies, the mix of calves from different herds and the increased susceptibility to infections. Dual infections were common, with *P. multocida* and BCoV being the most frequent IDV co-infecting pathogens, highlighting the role of IDV as a potential co-pathogen in the BRD.

Geographical data from 2021 to 2024 showed variable detection across municipalities. In addition, notable spikes of positive samples during the 2020–2021 and 2023–2024 seasons were detected. Whereas a more extended study period is needed to assess the possibility of a cyclic or ”wave-like” pattern, the detection of positive animals during the colder months of 2021 and 2023/2024 suggests some degree of seasonality or annual occurrence, similarly to what has been observed in several influenza studies, including those on IDV (Horimoto *et al.* 2016; Trombetta *et al.* 2020; Yu *et al.* 2021). Positive cases were identified in municipalities such as Gotland, Klippan, and Varberg, reflecting a heterogeneous distribution of the virus.

Phylogenetic analysis revealed distinct genetic lineages circulating over time. While strains from 2021 belonged exclusively to the D/OK lineage, those from 2023–2024 exhibited reassortment with the D/660 lineage, particularly in gene segment 1 (PB2). The reassortment observed in the Swedish IDV strains between the D/OK and D/660 lineages in the PB2 gene segment shows an active viral evolution in the cattle population. This genetic mixing is likely due to coinfection with multiple lineages within individual cattle, especially in regions with high herd density and frequent animal movement. However, cross-species transmission may also play a role, as shown in studies from France where reassortant IDV strains were identified in pigs on mixed pig-cattle farms, suggesting that close interspecies contact may allow coinfection and reassortment in non-reservoir hosts (Gorin *et al.* 2024). Similar findings in Denmark further emphasize that animal trade and ecological interfaces contribute to the spread and evolution of reassortant strains (Goecke *et al.* 2022). Although IDV and IAV have segmented

genomes, reassortment between them is highly unlikely. This is primarily due to incompatible packaging signals that prevent the correct incorporation of gene segments during viral assembly. Studies of A-B reassortment have shown that even when segments are transcribed, incompatible signals block genome packaging (Baker *et al.* 2014). This, combined with different host ranges and protein incompatibility, makes reassortment between IDV and IAV almost impossible.

The geographical distribution of IDV-positive cases aligns with regions where serological data from BTM samples indicated high antibody prevalence, particularly in southern regions such as Halland County (study I). This alignment supports the reliability of the sampling strategy and diagnostic assays. Overall, these findings highlight the dynamic of IDV circulation in Swedish cattle, emphasizing the need for continued surveillance to monitor the evolution of the virus and the potential impact on bovine health.

4.3 Study III- Proteomic and lipidomic characterization of bronchoalveolar lavage from calves infected with IDV and *M.bovis*

4.3.1 Proteins

Our observations suggest that during infection with IDV, certain pathways of the innate immune response, particularly those related to the acute phase response and coagulation, are initially suppressed. This early suppression appears to counteract the activation of the host response to *M. bovis* and consequently the clearance of this pathogen during coinfection.

At 2 dpi, proteomic results revealed early host responses to the pathogens, showing significant changes in the expression of proteins directly involved in innate immunity. These proteins have a significant role in coagulation and the acute-phase response, highlighting the critical pathways affected by the infections.

While *M. bovis* infection alone significantly upregulated the coagulation pathway (represented by protein F2, FGB, FGG, KNG1 and SERPINC1), the presence of IDV in co-infected calves resulted in the downregulation of this response (represented by protein A2M, FGA, FGB, FGG, KNC1, PLC, SERPINC1). This suggests that IDV may have a modulatory effect on the

coagulation processes triggered by *M. bovis*, potentially affecting the progression and severity of clinical outcomes. These findings align with observations in human medicine, where respiratory pathogens such as *Mycoplasma pneumoniae* have been linked to enhanced coagulation responses in severe cases (Li *et al.* 2023).

In addition, proteins associated with the acute phase response showed differential expressions between infected calves. The single infection with IDV compared to uninfected calves resulted in a downregulation of the acute phase response (represented by protein A2M, AGT, APOA2, C4A, C4B, C5, FN1, HRG, ITIH2, SERPIND1), in contrast to the upregulation observed with *M. bovis* single infection (represented by protein C9, F2, FGB, FGG). Reviewed literature does not report significant downregulation of acute phase proteins in response to influenza infection, suggesting that the observed reduction in the IDV-infected calves may be specific (Perez 2019). The addition of IDV to the *M. bovis* infection showed a downregulation of the acute phase response (represented by protein A2M, AMBP, C4A, C4B, FGA, FGB, FGG, FN1, PLG). This suggests that IDV primarily downregulates innate immune pathways induced by *M. bovis* infection potentially impairing early host defense mechanisms. These findings align with those reported by Gaudino et al., where precision-cut lung slices served as an organotypic model to illustrate the inhibitory effect of IDV on the innate immune response to *M. bovis*. This inhibition was characterized as reduced levels of pro-inflammatory cytokines and chemokines, including IL-8, IL-1β, and IL-17 (Gaudino *et al.* 2023a)

4.3.2 Lipids mediators

Lipidomic analysis of infected calves and controls revealed the production of several key LM generated by COX-1, COX-2 and LOX enzymes across different time points. It should be noted that the lipidomic profile may vary depending on the viral strain studied and the affected host. For example, Tam et al. demonstrated different lipid expression patterns between high- and low-pathogenic strains of Influenza A virus infection in mice, where resolution-phase metabolites were delayed or dysregulated in more virulent infections (Tam *et al.* 2013).

At 2 dpi, levels of PGE2 were significantly elevated in IDV and *M. bovis* co-infected calves compared to controls. A comparable rise in PGE_2 was also

observed in a bovine BRSV model, where ibuprofen treatment led to decreased PGE_2 levels, partial clinical improvement, increased BRSV shedding and loss of one calf, further confirming its early involvement in infection-induced inflammation (Walsh *et al.* 2016)

In our study, at 7 dpi, calves co-infected with both pathogens exhibited the highest total LM expression, predominantly derived from arachidonic acid. This period closely aligns with the peak of clinical signs observed at 8.6 ± 1.3 dpi in these calves. However, the correlation between high LM concentrations and severity of clinical signs was not straightforward, indicating that LMs might not directly cause clinical signs. This aligns with the findings by Newson et al., who demonstrated that PGE_2 generated via COX-1/mPGES-1 during the resolution phase may suppress local immunity and sustain tolerance rather than drive acute symptoms (Newson *et al.* 2017). Instead, these variations suggest individual differences in immune response, regulatory feedback mechanisms, or compensatory anti-inflammatory processes, potentially reflecting specific immunological pathways or tissue responses rather than direct clinical manifestations. Mattmiller et al. (2013) suggested that these variations may also be influenced by selenium status, as selenoproteins such as glutathione peroxidase modulate eicosanoid biosynthesis, potentially explaining some of the inter-individual variation in LM levels (Mattmiller *et al.* 2013). In the double-infected calves by 7 dpi, increases in TXB, LTB4 and PGE2 were observed, suggesting the activation of COX-1, COX-2, and LOX-5 pathways. This suggests that the use of selective inhibition drugs of a single pathway may not entirely suppress inflammation. Some co-infected calves also exhibited elevated levels of RVD1, indicating ongoing tissue repair and resolution of inflammation. These observations are in line with Tam et al., where specialized pro-resolving mediators such as resolvins were elevated during the resolution phase, contributing to tissue repair and inflammatory clearance (Tam *et al.* 2013).

By day 14, a significant decrease in LM levels was noted, pointing to a reduction in the inflammatory process. These findings underscore the complex interplay of COX and LOX pathways in shaping the inflammatory response during coinfections.

4.4 Study IV-*In vitro* interaction of IDV and other bovine respiratory viruses

4.4.1 Viral replication

The replication profiles of IDV, BRSV and BPIV-3, either alone or in coinfection, revealed a clear viral interference suggesting that IDV can suppress the replication of BRSV and BPIV-3. In the IDV and BRSV coinfections, IDV RNA quantity were significantly reduced at 24, 48, and 72 hours hpi compared to in single infections, although infectious titers remained unchanged at all time points. The same trend was observed in IDV/BPIV-3 coinfections, with significantly reduced IDV RNA quantity at 24 and 48 hpi in the single infection, but no significant changes in infectious titers. In contrast, both BRSV and BPIV-3 showed a significant reduction in RNA abundance and infectious titers during coinfection with IDV. For BRSV, viral RNA quantity were significantly lower in co-infected scenarios at 24, 72, and 96 hpi, in addition to lower titers at 72 hpi Similar patterns were observed for BPIV-3, with RNA quantity and viral titers being lower in coinfected conditions compared to single infections. Single infections yielded higher RNA quantities at 24, 48, and 72 hpi, and significantly higher titers at 48 and 72 hpi. There was no evidence of increased replication of either BRSV or BPIV-3 during coinfection, neither by RT-PCR, nor by titer, reinforcing the suppressive effect of IDV. These data are strongly indicative of viral interference mechanisms, in which the presence of IDV appears to hinder the replication of other co-infecting respiratory viruses. This aligns with known scenarios in human virology, where interaction among respiratory viruses can impact on their replication (Babawale & Guerrero-Plata 2024). These results can be also linked to findings from animal surveillance data, such as the work of Saipinta et al., which reported lower BRD-related mortality in IDV-positive calves (Saipinta *et al.* 2022). This supports the hypothesis that IDV may contribute to altered pathogenesis by interfering with the replication of more pathogenic respiratory viruses, thereby modulating disease severity and progression in naturally co-infected hosts.

4.4.2 Cytopathic effect

Microscopic evaluation of the CPE provided further insight into the functional consequences of viral challenges. At 72 hpi, single BRSV infections induced pronounced morphological changes in approximately

30% of the cell monolayer, increasing to 50% at 96 hpi. In contrast, co-infected cultures (IDV and BRSV) showed only 10% CPE at 72 hpi and 20% at 96hpi, highlighting a significant attenuation of cellular damage in the presence of IDV. This attenuation correlates with the reduced replication of BRSV in coinfection and may reflect a broader modulation of host cellular responses. Similar protective effects have been observed in both experimental and clinical settings. In human airway epithelial models, prior rhinovirus infection reduces influenza A virus replication by stimulating interferon-mediated responses, particularly through the induction of interferon-stimulated genes (Wu *et al.* 2020; Babawale & Guerrero-Plata 2024). In the case of IDV and BPIV-3 coinfections, a similar pattern was observed. BPIV-3 affected approximately 30% of the monolayer at 48 hpi, while in IDV and BPIV-3 coinfections, the CPE was attenuated, to 10% at the same time point. This attenuation suggests that IDV also exerts a protective or moderating influence during BPIV-3 infection. These outcomes are consistent with the viral RNA data. The CPE data supports the hypothesis that IDV interferes with both viral replication and associated cellular pathology, possibly through mechanisms such as innate immune activation or altered host cell signaling. Prior studies in human respiratory coinfections support this concept. In particular, rhinovirus infection has been shown to protect airway epithelial cells from subsequent damage by more virulent pathogens such as influenza A virus. This protection is mediated through the robust induction of interferon-stimulated genes (ISGs), which establish an antiviral state in the epithelium and significantly inhibit IAV replication (Wu *et al.* 2020). Taking together, these results suggest that the degree of cytopathogenic attenuation by IDV may be virus-specific and shaped by the nature of the co-infecting virus and its interaction with the host antiviral machinery.

4.4.3 Confocal microscopy

Confocal microscopy of IDV and BRSV coinfected cultures confirmed that both viruses are capable of infecting the same cell population. While a quantitative analysis was not performed, visual examination suggested an approximate 10% coinfection rate at the cellular level. Dual infection was evident, with no dominance of one virus over the other, using equal MOI used for both. Based on the low number of co-infected cells, it can be hypothesized that viral interference is more likely driven by a host-mediated response to infection and potential intercellular communication, rather than direct intracellular competition for replication resources. In the study performed by Wu et al., researchers showed that rhinovirus infection of

airway epithelial cells significantly inhibited subsequent influenza A virus replication, even though most cells were not co-infected (Wu *et al.* 2020). The protective effect was lost when interferon signaling was pharmacologically blocked, underscoring the role of intercellular antiviral signaling rather than direct virus-virus interaction.

4.4.4 Transcriptomic

Transcriptomic analysis in single and coinfection models demonstrated different time and virus-specific patterns of host response regulation. At 12 hpi, BRSV significantly upregulated a subset of interferon-stimulated gene (ISG)-associated pathways, consistent with the known ability of the virus to induce a transient early innate immune response. In contrast, IDV alone did not activate these pathways at this time point. At 12 hpi, the addition of IDV to BRSV did not enhance ISG signaling but rather induced a significant upregulation in signaling through the receptor tyrosine kinase TrkA. The TrkA signaling, which is activated by its ligand NGF, has been implicated in promoting viral replication and driving inflammatory responses in respiratory infections, including influenza A virus (Verma *et al.* 2022). TrkA promotes proinflammatory cytokine production and viral RNA synthesis in airway epithelial cells, supporting its dual role in viral pathogenesis and immune modulation.

At 24 hpi, BRSV exhibited a marked downregulation of genes controlled by nuclear factor erythroid 2-related factor 2 (NFE2L2/NRF2), a key regulator of antioxidant defense (Komaravelli *et al.* 2017). Suppression of NRF2 reduces the expression of antioxidant enzymes, exacerbating oxidative stress and inflammation in infected airways. In contrast to BRSV, IDV showed a delayed but significant induction of interferon responses at 24 hpi. Furthermore, IDV coinfection enhanced interferon pathway activation compared to BRSV alone at the same time point, suggesting that IDV may temporally reprogram host antiviral signaling to inhibit BRSV replication. This modulation may underline the observed suppression of BRSV cytopathic effects in coinfection models, as late-phase interferon signaling has been shown to suppress viral replication. At 48 and 72 hpi, all infection models exhibited a strong activation of ISG pathways, although the coinfection condition induced a broader and stronger upregulation of several

DEGs compared to BRSV alone. These results suggest a synergistic enhancement of antiviral defenses over time in the presence of IDV.

The transcriptomic profile in the IDV and BPIV-3 coinfection model showed a distinct pattern. At 12 hpi, both viruses activated cytokine and interleukin signaling compared to uninfected controls. By 24 hpi, interferon responses were also enhanced. Downregulated pathways were primarily associated with cytoskeletal and structural remodeling, suggesting a suppression of cellular maintenance functions during active viral replication. There were few DEGs that distinguished coinfection from BPIV-3 alone, however, a downregulation of NGF-stimulated transcription occurred at 12 hpi. This may reflect a suppression of neurogenic inflammation by the virus, as NGF is known to enhance the biosynthesis of proinflammatory neurotransmitters such as substance P and to sensitize neural circuits associated with cough and bronchial hyperreactivity (Chiaretti *et al.* 2013).

Taken together, these results demonstrate how coinfection with IDV alters the dynamics of host immune and stress responses. The suppression of NRF2 by BRSV and the enhanced late-phase IFN response in coinfection suggest a complex interplay that may ultimately limit viral replication. The IDV-mediated modulation of NGF- and TrkA-associated pathways suggests an additional layer of immuno-neural regulation that may influence both disease severity and symptomatology.

Conclusion

This work provides new insights into the emerging role of IDV in the pathogenesis of bovine respiratory disease. The detection of IDV in Swedish cattle herds confirmed its circulation within the national livestock population and revealed a broader global epidemiological presence. Phylogenetic analyses indicated that several lineages of IDV, including reassortant strains, are actively co-circulating in Sweden, highlighting its genetic diversity and evolutionary dynamics. Previous experimental infection studies have shown that IDV can alter disease progression and immune response in calves, especially in coinfections with *M. bovis*. Proteomic and lipidomic profiling of bronchoalveolar lavage samples revealed alterations in key inflammatory pathways, suggesting a modulatory effect of IDV on host innate immune mechanisms. In addition, *in vitro* coinfection models with BRSV and BPIV-3 further confirmed the ability of IDV to enhance antiviral signaling and to attenuate replication and the cytopathic severity depending on the pathogen combination. This work highlights the importance of recognizing IDV as a contributing co-pathogen that can alter disease outcomes in BRD. These findings enhance our understanding of virus interactions and provide a basis for future research into BRD and immunomodulation strategies. Given the demonstrated prevalence and biological impact of IDV, its inclusion in diagnostic panels for BRD is reasonable.

5. Future perspectives

Future research should focus on further dissecting the immunomodulatory mechanisms by which IDV alters the host-pathogen interaction, particularly in the context of mixed infections. Longitudinal field studies are needed to determine the seasonal dynamics and transmission patterns of IDV in different cattle production systems. In addition, the reassortment ability and the zoonotic potential of the virus should be prioritized, given the sporadic detection in humans. IDV surveillance actions could help to better understand and follow the impact of IDV.

References

Ackermann, M.R., Derscheid, R. & Roth, J.A. (2010). Innate immunology of bovine respiratory disease. *Vet Clin North Am Food Anim Pract,* 26(2), 215-28. https://doi.org/10.1016/j.cvfa.2010.03.001

Aguilan, J.T., Kulej, K. & Sidoli, S. (2020). Guide for protein fold change and p-value calculation for non-experts in proteomics. *Mol Omics,* 16(6), 573-582. https://doi.org/10.1039/d0mo00087f

Al-Haddawi, M., Mitchell, G.B., Clark, M.E., Wood, R.D. & Caswell, J.L. (2007). Impairment of innate immune responses of airway epithelium by infection with bovine viral diarrhea virus. *Vet Immunol Immunopathol,* 116(3-4), 153-62. https://doi.org/10.1016/j.vetimm.2007.01.007

Alvarez, I.J., Fort, M., Pasucci, J., Moreno, F., Gimenez, H., Naslund, K., Hagglund, S., Zohari, S. & Valarcher, J.F. (2020). Seroprevalence of influenza D virus in bulls in Argentina. *Journal of Veterinary Diagnostic Investigation,* 32(4), 585-588. https://doi.org/Artn 1040638720934056
10.1177/1040638720934056

Amat, S., Subramanian, S., Timsit, E. & Alexander, T.W. (2017). Probiotic bacteria inhibit the bovine respiratory pathogen Mannheimia haemolytica serotype 1 in vitro. *Lett Appl Microbiol,* 64(5), 343-349. https://doi.org/10.1111/lam.12723

Ammendolia, M.G., Agamennone, M., Pietrantoni, A., Lannutti, F., Siciliano, R.A., De Giulio, B., Amici, C. & Superti, F. (2012). Bovine lactoferrin-derived peptides as novel broad-spectrum inhibitors of influenza virus. *Pathog Glob Health,* 106(1), 12-9. https://doi.org/10.1179/2047773212Y.0000000004

Babawale, P.I. & Guerrero-Plata, A. (2024). Respiratory Viral Coinfections: Insights into Epidemiology, Immune Response, Pathology, and Clinical Outcomes. *Pathogens,* 13(4). https://doi.org/10.3390/pathogens13040316

Baker, S.F., Nogales, A., Finch, C., Tuffy, K.M., Domm, W., Perez, D.R., Topham, D.J. & Martinez-Sobrido, L. (2014). Influenza A and B virus intertypic reassortment through compatible viral packaging signals. *Journal of Virology,* 88(18), 10778-91. https://doi.org/10.1128/JVI.01440-14

Basil, M.C. & Levy, B.D. (2016). Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. *Nat Rev Immunol,* 16(1), 51-67. https://doi.org/10.1038/nri.2015.4

Bavananthasivam, J., Dassanayake, R.P., Kugadas, A., Shanthalingam, S., Call, D.R., Knowles, D.P. & Srikumaran, S. (2012). Proximity-dependent inhibition of growth of Mannheimia haemolytica by Pasteurella multocida.

Appl Environ Microbiol, 78(18), 6683-8. https://doi.org/10.1128/AEM.01119-12

Beaudeau, F., Bjorkman, C., Alenius, S. & Frossling, J. (2010a). Spatial patterns of bovine corona virus and bovine respiratory syncytial virus in the Swedish beef cattle population. *Acta Vet Scand,* 52, 33. https://doi.org/10.1186/1751-0147-52-33

Beaudeau, F., Ohlson, A. & Emanuelson, U. (2010b). Associations between bovine coronavirus and bovine respiratory syncytial virus infections and animal performance in Swedish dairy herds. *J Dairy Sci,* 93(4), 1523-33. https://doi.org/10.3168/jds.2009-2511

Berghuis, L., Abdelaziz, K.T., Bierworth, J., Wyer, L., Jacob, G., Karrow, N.A., Sharif, S., Clark, M.E. & Caswell, J.L. (2014). Comparison of innate immune agonists for induction of tracheal antimicrobial peptide gene expression in tracheal epithelial cells of cattle. *Vet Res,* 45(1), 105. https://doi.org/10.1186/s13567-014-0105-8

Booth, R.E., Cranwell, M.P. & Brownlie, J. (2013). Monitoring the bulk milk antibody response to BVDV: the effects of vaccination and herd infection status. *Vet Rec,* 172(17), 449. https://doi.org/10.1136/vr.101195

Brscic, M., Leruste, H., Heutinck, L.F., Bokkers, E.A., Wolthuis-Fillerup, M., Stockhofe, N., Gottardo, F., Lensink, B.J., Cozzi, G. & Van Reenen, C.G. (2012). Prevalence of respiratory disorders in veal calves and potential risk factors. *J Dairy Sci,* 95(5), 2753-64. https://doi.org/10.3168/jds.2011-4699

Caserta, L.C., Frye, E.A., Butt, S.L., Laverack, M., Nooruzzaman, M., Covaleda, L.M., Thompson, A.C., Koscielny, M.P., Cronk, B., Johnson, A., Kleinhenz, K., Edwards, E.E., Gomez, G., Hitchener, G., Martins, M., Kapczynski, D.R., Suarez, D.L., Alexander Morris, E.R., Hensley, T., Beeby, J.S., Lejeune, M., Swinford, A.K., Elvinger, F., Dimitrov, K.M. & Diel, D.G. (2024). Spillover of highly pathogenic avian influenza H5N1 virus to dairy cattle. *Nature,* 634(8034), 669-676. https://doi.org/10.1038/s41586-024-07849-4

Charostad, J., Rezaei Zadeh Rukerd, M., Mahmoudvand, S., Bashash, D., Hashemi, S.M.A., Nakhaie, M. & Zandi, K. (2023). A comprehensive review of highly pathogenic avian influenza (HPAI) H5N1: An imminent threat at doorstep. *Travel Med Infect Dis,* 55, 102638. https://doi.org/10.1016/j.tmaid.2023.102638

Chase, C.C. (2013). The impact of BVDV infection on adaptive immunity. *Biologicals,* 41(1), 52-60. https://doi.org/10.1016/j.biologicals.2012.09.009

Chiapponi, C., Faccini, S., De Mattia, A., Baioni, L., Barbieri, I., Rosignoli, C., Nigrelli, A. & Foni, E. (2016). Detection of Influenza D Virus among Swine and Cattle, Italy. *Emerging Infectious Diseases,* 22(2), 352-354. https://doi.org/10.3201/eid2202.151439

Chiapponi, C., Faccini, S., Fusaro, A., Moreno, A., Prosperi, A., Merenda, M., Baioni, L., Gabbi, V., Rosignoli, C., Alborali, G.L., Cavicchio, L., Monne,

I., Torreggiani, C., Luppi, A. & Foni, E. (2019). Detection of a New Genetic Cluster of Influenza D Virus in Italian Cattle. *Viruses-Basel,* 11(12). https://doi.org/ARTN 1110
10.3390/v11121110
Chiaretti, A., Pulitano, S., Conti, G., Barone, G., Buonsenso, D., Manni, L., Capozzi, D., Ria, F. & Riccardi, R. (2013). Interleukin and neurotrophin up-regulation correlates with severity of H1N1 infection in children: a case-control study. *International Journal of Infectious Diseases,* 17(12), e1186-93. https://doi.org/10.1016/j.ijid.2013.07.006
Cortjens, B., de Boer, O.J., de Jong, R., Antonis, A.F., Sabogal Pineros, Y.S., Lutter, R., van Woensel, J.B. & Bem, R.A. (2016). Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease. *J Pathol,* 238(3), 401-11. https://doi.org/10.1002/path.4660
Curtain, C.C. & Pye, J. (1955). A mucoprotein from bovine submaxillary glands with restricted inhibitory action against influenza virus haemagglutination. *Aust J Exp Biol Med Sci,* 33(3), 315-22. https://doi.org/10.1038/icb.1955.32
da Silva, M.S., Mosena, A.C.S., Baumbach, L., Demoliner, M., Gularte, J.S., Pavarini, S.P., Driemeier, D., Weber, M.N., Spilki, F.R. & Canal, C.W. (2022). Cattle influenza D virus in Brazil is divergent from established lineages. *Archives of Virology,* 167(4), 1181-1184. https://doi.org/10.1007/s00705-022-05416-8
Dane, H., Duffy, C., Guelbenzu, M., Hause, B., Fee, S., Forster, F., McMenamy, M.J. & Lemon, K. (2019). Detection of influenza D virus in bovine respiratory disease samples, UK. *Transboundary and Emerging Diseases,* 66(5), 2184-2187. https://doi.org/10.1111/tbed.13273
Detsika, M.G., Palamaris, K., Dimopoulou, I., Kotanidou, A. & Orfanos, S.E. (2024). The complement cascade in lung injury and disease. *Respir Res,* 25(1), 20. https://doi.org/10.1186/s12931-023-02657-2
Ducatez, M.F., Pelletier, C. & Meyer, G. (2015). Influenza D Virus in Cattle,. France, 2011-2014. *Emerging Infectious Diseases,* 21(2), 368-371. https://doi.org/10.3201/eid2102.141449
Ellis, J.A. (2001). The immunology of the bovine respiratory disease complex. *Vet Clin North Am Food Anim Pract,* 17(3), 535-50, vi-vii. https://doi.org/10.1016/s0749-0720(15)30005-0
Elvander, M., Baule, C., Persson, M., Egyed, L., Ballagi-Pordany, A., Belak, S. & Alenius, S. (1998). An experimental study of a concurrent primary infection with bovine respiratory syncytial virus (BRSV) and bovine viral diarrhoea virus (BVDV) in calves. *Acta Vet Scand,* 39(2), 251-64. https://doi.org/10.1186/BF03547797
Emmi Andersson, M.A., Charlotte Axén, Anna Bonnevie, Ioana Bujila, Erika Chenais, Mariann Dahlquist, Leigh Davidsson, Rikard Dryselius, Helena Eriksson, Linda Ernholm, Charlotta Fasth, Malin Grant, Gittan Gröndahl, Gunilla Hallgren, Anette Hansen, Marika Hjertqvist, Mia Holmberg,

Cecilia Hultén, Hampus Hällbom, Helena Höök, Karoline Jakobsson, Désirée Jansson, Tomas Jinnerot, Jonas Johansson Wensman, Jerker Jonsson, Oskar Karlsson Lindsjö, Sara Kjellsdotter, Ulrika König, Elina Lahti, Emelie Larsdotter, Neus Latorre-Margalef, Mats Lindblad, Anna Lundén, Anna Nilsson, Oskar Nilsson, Maria Nöremark, Anna Omazic, Anna Ordell, Ylva Persson, Emelie Pettersson, Ivana Rodriguez Ewerlöf, Thomas Rosendal, Marie Sjölund, Karl Ståhl, Lena Sundqvist, Robert Söderlund, Magnus Thelander, Karin Troell, Henrik Uhlhorn, Anders Wallensten, Stefan Widgren, Camilla Wikström, Ulrika Windahl, Beth Young, Nabil Yousef, Siamak Zohari, Erik Ågren, Estelle Ågren (2022). *Surveillance of infectious diseases in animals and humans in Sweden*. (SVA:s rapportserie 89 1654-7098). (SVA), N.V.I.

Fahkrajang, W., Sudaryatma, P.E., Mekata, H., Hamabe, S., Saito, A. & Okabayashi, T. (2021). Bovine respiratory coronavirus enhances bacterial adherence by upregulating expression of cellular receptors on bovine respiratory epithelial cells. *Veterinary Microbiology,* 255, 109017. https://doi.org/10.1016/j.vetmic.2021.109017

Fatkhuddinova, M.F., Kir'ianova, A.I., Isachenko, V.A. & Zakstel'skaia, L. (1973). [Isolation and identification of the A-Hong Kong (H3N2) virus in respiratory diseases of cattle]. *Vopr Virusol,* 18(4), 474-8. https://www.ncbi.nlm.nih.gov/pubmed/4274331

Fernandez, M., Ferreras, M.D.C., Giraldez, F.J., Benavides, J. & Perez, V. (2020). Production Significance of Bovine Respiratory Disease Lesions in Slaughtered Beef Cattle. *Animals (Basel),* 10(10). https://doi.org/10.3390/ani10101770

Flores, V.V., Hernandez Gifford, J.A., Soto-Navarro, S.A., Matera, J., Wilson, B.K., Hartson, S., Byrum, S.D. & Gifford, C.A. (2023). Calves severely affected by bovine respiratory disease have reduced protection against histone toxicity and exhibit lower complement activity. *Journal of Animal Science,* 101. https://doi.org/10.1093/jas/skad221

Flynn, O., Gallagher, C., Mooney, J., Irvine, C., Ducatez, M., Hause, B., McGrath, G. & Ryan, E. (2018). Influenza D Virus in Cattle, Ireland. *Emerging Infectious Diseases,* 24(2), 389-391. https://doi.org/10.3201/eid2402.170759

Foni, E., Chiapponi, C., Baioni, L., Zanni, I., Merenda, M., Rosignoli, C., Kyriakis, C.S., Luini, M.V., Mandola, M.L., Bolzoni, L., Nigrelli, A.D. & Faccini, S. (2017). Influenza D in Italy: towards a better understanding of an emerging viral infection in swine. *Scientific Reports,* 7. https://doi.org/ARTN 11660 10.1038/s41598-017-12012-3

Fulton, R.W. (2020). Viruses in Bovine Respiratory Disease in North America Knowledge Advances Using Genomic Testing. *Veterinary Clinics of North America-Food Animal Practice,* 36(2), 321-+. https://doi.org/10.1016/j.cvfa.2020.02.004

Ganheim, C., Hulten, C., Carlsson, U., Kindahl, H., Niskanen, R. & Waller, K.P. (2003). The acute phase response in calves experimentally infected with bovine viral diarrhoea virus and/or Mannheimia haemolytica. *J Vet Med B Infect Dis Vet Public Health,* 50(4), 183-90. https://doi.org/10.1046/j.1439-0450.2003.00658.x

Gaudino, M., Chiapponi, C., Moreno, A., Zohari, S., O'Donovan, T., Quinless, E., Sausy, A., Oliva, J., Salem, E., Fusade-Boyer, M., Meyer, G., Hubschen, J.M., Saegerman, C., Ducatez, M.F. & Snoeck, C.J. (2022a). Evolutionary and temporal dynamics of emerging influenza D virus in Europe (2009-22). *Virus Evolution,* 8(2), veac081. https://doi.org/10.1093/ve/veac081

Gaudino, M., Lion, A., Sagne, E., Nagamine, B., Oliva, J., Terrier, O., Errazuriz-Cerda, E., Scribe, A., Sikht, F.Z., Simon, E., Foret-Lucas, C., Gausseres, B., Lion, J., Moreno, A., Dordet-Frisoni, E., Baranowski, E., Volmer, R., Ducatez, M.F. & Meyer, G. (2023a). The Activation of the RIG-I/MDA5 Signaling Pathway upon Influenza D Virus Infection Impairs the Pulmonary Proinflammatory Response Triggered by Mycoplasma bovis Superinfection. *Journal of Virology,* 97(2), e0142322. https://doi.org/10.1128/jvi.01423-22

Gaudino, M., Nagamine, B., Ducatez, M.F. & Meyer, G. (2022b). Understanding the mechanisms of viral and bacterial coinfections in bovine respiratory disease: a comprehensive literature review of experimental evidence. *Vet Res,* 53(1), 70. https://doi.org/10.1186/s13567-022-01086-1

Gaudino, M., Valarcher, J.F., Hagglund, S., Naslund, K., Zohari, S., Ducatez, M.F. & Meyer, G. (2023b). Molecular and genetic characterization of bovine parainfluenza type 3 European field and vaccine strains. *Infect Genet Evol,* 113, 105483. https://doi.org/10.1016/j.meegid.2023.105483

Gershwin, L.J., Berghaus, L.J., Arnold, K., Anderson, M.L. & Corbeil, L.B. (2005). Immune mechanisms of pathogenetic synergy in concurrent bovine pulmonary infection with Haemophilus somnus and bovine respiratory syncytial virus. *Vet Immunol Immunopathol,* 107(1-2), 119-30. https://doi.org/10.1016/j.vetimm.2005.04.004

Goecke, N.B., Liang, Y., Otten, N.D., Hjulsager, C.K. & Larsen, L.E. (2022). Characterization of Influenza D Virus in Danish Calves. *Viruses-Basel,* 14(2). https://doi.org/ARTN 423
10.3390/v14020423

Gorin, S., Richard, G., Herve, S., Eveno, E., Blanchard, Y., Jardin, A., Rose, N. & Simon, G. (2024). Characterization of Influenza D Virus Reassortant Strain in Swine from Mixed Pig and Beef Farm, France. *Emerging Infectious Diseases,* 30(8), 1672-1676. https://doi.org/10.3201/eid3008.240089

Gourlay, R.N. & Houghton, S.B. (1985). Experimental pneumonia in conventionally reared and gnotobiotic calves by dual infection with Mycoplasma bovis and Pasteurella haemolytica. *Research in Veterinary Science,* 38(3), 377-82. https://www.ncbi.nlm.nih.gov/pubmed/4012041

Griffin, D., Chengappa, M.M., Kuszak, J. & McVey, D.S. (2010). Bacterial pathogens of the bovine respiratory disease complex. *Vet Clin North Am Food Anim Pract,* 26(2), 381-94. https://doi.org/10.1016/j.cvfa.2010.04.004

Hagglund, S., Blodorn, K., Naslund, K., Vargmar, K., Lind, S.B., Mi, J., Arainga, M., Riffault, S., Taylor, G., Pringle, J. & Valarcher, J.F. (2017). Proteome analysis of bronchoalveolar lavage from calves infected with bovine respiratory syncytial virus-Insights in pathogenesis and perspectives for new treatments. *Plos One,* 12(10), e0186594. https://doi.org/10.1371/journal.pone.0186594

Hause, B.M., Ducatez, M., Collin, E.A., Ran, Z., Liu, R., Sheng, Z., Armien, A., Kaplan, B., Chakravarty, S., Hoppe, A.D., Webby, R.J., Simonson, R.R. & Li, F. (2013). Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. *Plos Pathogens,* 9(2), e1003176. https://doi.org/10.1371/journal.ppat.1003176

Headley, S.A., Okano, W., Balbo, L.C., Marcasso, R.A., Oliveira, T.E., Alfieri, A.F., Negri Filho, L.C., Michelazzo, M.Z., Rodrigues, S.C., Baptista, A.L., Saut, J.P.E. & Alfieri, A.A. (2018). Molecular survey of infectious agents associated with bovine respiratory disease in a beef cattle feedlot in southern Brazil. *Journal of Veterinary Diagnostic Investigation,* 30(2), 249-251. https://doi.org/10.1177/1040638717739945

Heller, A., Koch, T., Schmeck, J. & van Ackern, K. (1998). Lipid mediators in inflammatory disorders. *Drugs,* 55(4), 487-96. https://doi.org/10.2165/00003495-199855040-00001

Henritzi, D., Hoffmann, B., Wacheck, S., Pesch, S., Herrler, G., Beer, M. & Harder, T.C. (2019). A newly developed tetraplex real-time RT-PCR for simultaneous screening of influenza virus types A, B, C and D. *Influenza and Other Respiratory Viruses,* 13(1), 71-82. https://doi.org/10.1111/irv.12613

Holman, D.B., McAllister, T.A., Topp, E., Wright, A.D. & Alexander, T.W. (2015a). The nasopharyngeal microbiota of feedlot cattle that develop bovine respiratory disease. *Veterinary Microbiology,* 180(1-2), 90-5. https://doi.org/10.1016/j.vetmic.2015.07.031

Holman, D.B., Timsit, E. & Alexander, T.W. (2015b). The nasopharyngeal microbiota of feedlot cattle. *Sci Rep,* 5, 15557. https://doi.org/10.1038/srep15557

Horimoto, T., Hiono, T., Mekata, H., Odagiri, T., Lei, Z., Kobayashi, T., Norimine, J., Inoshima, Y., Hikono, H., Murakami, K., Sato, R., Murakami, H., Sakaguchi, M., Ishii, K., Ando, T., Otomaru, K., Ozawa, M., Sakoda, Y. & Murakami, S. (2016). Nationwide Distribution of Bovine Influenza D Virus Infection in Japan. *Plos One,* 11(9). https://doi.org/ARTN e0163828
10.1371/journal.pone.0163828

Huang, C., Yu, J.S., Hause, B.M., Park, J.Y., Sreenivasan, C., Uprety, T., Sheng, Z.Z., Wang, D. & Li, F. (2021). Emergence of new phylogenetic lineage of

Influenza D virus with broad antigenicity in California, United States. *Emerging Microbes & Infections,* 10(1), 739-742. https://doi.org/10.1080/22221751.2021.1910078

Jonasson, A.R.A. (2023). *FarmStat-rapporten 2023*. Djurhälsan, G. https://www.gardochdjurhalsan.se/wp-content/uploads/2024/01/9140-FarmStat-rapporten-2023_SLUTVERSION_v3.pdf

Kärber, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Naunyn-Schmiedebergs Archiv für experimentelle Pathologie und Pharmakologie,* 162(4), 480-483. https://doi.org/10.1007/BF01863914

Komaravelli, N., Ansar, M., Garofalo, R.P. & Casola, A. (2017). Respiratory syncytial virus induces NRF2 degradation through a promyelocytic leukemia protein - ring finger protein 4 dependent pathway. *Free Radic Biol Med,* 113, 494-504. https://doi.org/10.1016/j.freeradbiomed.2017.10.380

Lang, Y., Shi, L., Roy, S., Gupta, D., Dai, C., Khalid, M.A., Zhang, M.Z., Zhang, S., Wan, X.F., Webby, R. & Ma, W. (2025). Detection of antibodies against influenza A viruses in cattle. *Journal of Virology*, e0213824. https://doi.org/10.1128/jvi.02138-24

Lanni, F., Lanni, Y.T. & Beard, J.W. (1949). Inhibitory effect of cow's milk on influenza virus hemagglutination. *Proc Soc Exp Biol Med,* 72(1), 227-32. https://doi.org/10.3181/00379727-72-17389

Lanyon, S.R., McCoy, R., Bergman, E. & Reichel, M.P. (2014). Milk as a diagnostic sample for a commercially available ELISA to identify bovine viral diarrhoea (BVD) antibodies in dairy herds. *Aust Vet J,* 92(7), 269-73. https://doi.org/10.1111/avj.12188

Levy, B.D., Clish, C.B., Schmidt, B., Gronert, K. & Serhan, C.N. (2001). Lipid mediator class switching during acute inflammation: signals in resolution. *Nat Immunol,* 2(7), 612-9. https://doi.org/10.1038/89759

Li, Y.T., Zhang, J., Wang, M.Z., Ma, Y.M., Zhi, K., Dai, F.L. & Li, S.J. (2023). Changes in coagulation markers in children with Mycoplasma pneumoniae pneumonia and their predictive value for Mycoplasma severity. *Ital J Pediatr,* 49(1), 143. https://doi.org/10.1186/s13052-023-01545-1

Lima, S.F., Bicalho, M.L.S. & Bicalho, R.C. (2019). The Bos taurus maternal microbiome: Role in determining the progeny early-life upper respiratory tract microbiome and health. *Plos One,* 14(3), e0208014. https://doi.org/10.1371/journal.pone.0208014

Lion, A., Secula, A., Rancon, C., Boulesteix, O., Pinard, A., Deslis, A., Hagglund, S., Salem, E., Cassard, H., Naslund, K., Gaudino, M., Moreno, A., Brocchi, E., Delverdier, M., Zohari, S., Baranowski, E., Valarcher, J.F., Ducatez, M.F. & Meyer, G. (2021). Enhanced Pathogenesis Caused by Influenza D Virus and Mycoplasma bovis Coinfection in Calves: a Disease Severity Linked with Overexpression of IFN-gamma as a Key Player of the

Enhanced Innate Immune Response in Lungs. *Microbiology Spectrum,* 9(3). https://doi.org/ARTN e01690-21
10.1128/Spectrum.01690-21
Lopez, A., Maxie, M.G., Savan, M., Ruhnke, H.L., Thomson, R.G., Barnum, D.A. & Geissinger, H.D. (1982). The pulmonary clearance of Pasteurella haemolytica in calves infected with bovine virus diarrhea or Mycoplasma bovis. *Can J Comp Med,* 46(3), 302-6. https://www.ncbi.nlm.nih.gov/pubmed/7127194
Lopez, J.W. & Woods, G.T. (1984). Influenza virus in ruminants: a review. *Res Commun Chem Pathol Pharmacol,* 45(3), 445-62. https://www.ncbi.nlm.nih.gov/pubmed/6390588
Man, W.H., de Steenhuijsen Piters, W.A. & Bogaert, D. (2017). The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol,* 15(5), 259-270. https://doi.org/10.1038/nrmicro.2017.14
Mattmiller, S.A., Carlson, B.A. & Sordillo, L.M. (2013). Regulation of inflammation by selenium and selenoproteins: impact on eicosanoid biosynthesis. *J Nutr Sci,* 2, e28. https://doi.org/10.1017/jns.2013.17
McGill, J.L., Rusk, R.A., Guerra-Maupome, M., Briggs, R.E. & Sacco, R.E. (2016). Bovine Gamma Delta T Cells Contribute to Exacerbated IL-17 Production in Response to Coinfection with Bovine RSV and Mannheimia haemolytica. *Plos One,* 11(3), e0151083. https://doi.org/10.1371/journal.pone.0151083
McGill, J.L. & Sacco, R.E. (2020). The Immunology of Bovine Respiratory Disease: Recent Advancements. *Vet Clin North Am Food Anim Pract,* 36(2), 333-348. https://doi.org/10.1016/j.cvfa.2020.03.002
Milovanovic, M., Milicevic, V., Radojicic, S., Valcic, M., Hoffmann, B. & Dietze, K. (2020). Suitability of individual and bulk milk samples to investigate the humoral immune response to lumpy skin disease vaccination by ELISA. *Virology Journal,* 17(1). https://doi.org/ARTN 28
10.1186/s12985-020-01298-x
Molini, U., Curini, V., Jacobs, E., Tongo, E., Berjaoui, S., Hemberger, M.Y., Puglia, I., Jago, M., Khaiseb, S., Cattoli, G., Dundon, W.G., Lorusso, A. & Di Giallonardo, F. (2022). First influenza D virus full-genome sequence retrieved from livestock in Namibia, Africa. *Acta Tropica,* 232. https://doi.org/ARTN 106482
10.1016/j.actatropica.2022.106482
Murakami, S., Sato, R., Ishida, H., Katayama, M., Takenaka-Uema, A. & Horimoto, T. (2020). Influenza D Virus of New Phylogenetic Lineage, Japan. *Emerging Infectious Diseases,* 26(1), 168-171. https://doi.org/10.3201/eid2601.191092
Murray, G.M., More, S.J., Sammin, D., Casey, M.J., McElroy, M.C., O'Neill, R.G., Byrne, W.J., Earley, B., Clegg, T.A., Ball, H., Bell, C.J. & Cassidy, J.P. (2017). Pathogens, patterns of pneumonia, and epidemiologic risk factors associated with respiratory disease in recently weaned cattle in Ireland.

Journal of Veterinary Diagnostic Investigation, 29(1), 20-34. https://doi.org/10.1177/1040638716674757

Nemanichvili, N., Berends, A.J., Tomris, I., Barnard, K.N., Parrish, C.R., Grone, A., Rijks, J.M., Verheije, M.H. & de Vries, R.P. (2022). Influenza D binding properties vary amongst the two major virus clades and wildlife species. *Veterinary Microbiology,* 264, 109298. https://doi.org/10.1016/j.vetmic.2021.109298

Nemanichvili, N., Berends, A.J., Wubbolts, R.W., Grone, A., Rijks, J.M., De Vries, R.P. & Verheije, M.H. (2021). Tissue Microarrays to Visualize Influenza D Attachment to Host Receptors in the Respiratory Tract of Farm Animals. *Viruses-Basel,* 13(4). https://doi.org/ARTN 586
10.3390/v13040586

Newson, J., Motwani, M.P., Kendall, A.C., Nicolaou, A., Muccioli, G.G., Alhouayek, M., Bennett, M., Van De Merwe, R., James, S., De Maeyer, R.P.H. & Gilroy, D.W. (2017). Inflammatory Resolution Triggers a Prolonged Phase of Immune Suppression through COX-1/mPGES-1-Derived Prostaglandin E(2). *Cell Reports,* 20(13), 3162-3175. https://doi.org/10.1016/j.celrep.2017.08.098

Ng, W.C., Wong, V., Muller, B., Rawlin, G. & Brown, L.E. (2010). Prevention and treatment of influenza with hyperimmune bovine colostrum antibody. *Plos One,* 5(10), e13622. https://doi.org/10.1371/journal.pone.0013622

O'Donovan, T., Donohoe, L., Ducatez, M.F., Meyer, G. & Ryan, E. (2019). Seroprevalence of influenza D virus in selected sample groups of Irish cattle, sheep and pigs. *Irish Veterinary Journal,* 72(1). https://doi.org/ARTN 11
10.1186/s13620-019-0150-8

Ohlson, A., Blanco-Penedo, I. & Fall, N. (2014). Comparison of Bovine coronavirus-specific and Bovine respiratory syncytial virus-specific antibodies in serum versus milk samples detected by enzyme-linked immunosorbent assay. *Journal of Veterinary Diagnostic Investigation,* 26(1), 113-6. https://doi.org/10.1177/1040638713509377

Oliva, J., Eichenbaum, A., Belin, J., Gaudino, M., Guillotin, J., Alzieu, J.P., Nicollet, P., Brugidou, R., Gueneau, E., Michel, E., Meyer, G. & Ducatez, M.F. (2019). Serological Evidence of Influenza D Virus Circulation Among Cattle and Small Ruminants in France. *Viruses-Basel,* 11(6). https://doi.org/ARTN 516
10.3390/v11060516

Perez-Cobas, A.E., Baquero, F., de Pablo, R., Soriano, M.C. & Coque, T.M. (2021). Altered Ecology of the Respiratory Tract Microbiome and Nosocomial Pneumonia. *Frontiers in Microbiology,* 12, 709421. https://doi.org/10.3389/fmicb.2021.709421

Perez, L. (2019). Acute phase protein response to viral infection and vaccination. *Arch Biochem Biophys,* 671, 196-202. https://doi.org/10.1016/j.abb.2019.07.013

Petruzzi, B., Dickerman, A., Lahmers, K., Scarratt, W.K. & Inzana, T.J. (2020). Polymicrobial Biofilm Interaction Between Histophilus somni and Pasteurella multocida. *Frontiers in Microbiology,* 11, 1561. https://doi.org/10.3389/fmicb.2020.01561

Prysliak, T., van der Merwe, J., Lawman, Z., Wilson, D., Townsend, H., van Drunen Littel-van den Hurk, S. & Perez-Casal, J. (2011). Respiratory disease caused by Mycoplasma bovis is enhanced by exposure to bovine herpes virus 1 (BHV-1) but not to bovine viral diarrhea virus (BVDV) type 2. *Can Vet J,* 52(11), 1195-202. https://www.ncbi.nlm.nih.gov/pubmed/22547839

Rezac, D.J., Thomson, D.U., Bartle, S.J., Osterstock, J.B., Prouty, F.L. & Reinhardt, C.D. (2014). Prevalence, severity, and relationships of lung lesions, liver abnormalities, and rumen health scores measured at slaughter in beef cattle. *Journal of Animal Science,* 92(6), 2595-602. https://doi.org/10.2527/jas.2013-7222

Ridpath, J.F., Fulton, R.W., Bauermann, F.V., Falkenberg, S.M., Welch, J. & Confer, A.W. (2020). Sequential exposure to bovine viral diarrhea virus and bovine coronavirus results in increased respiratory disease lesions: clinical, immunologic, pathologic, and immunohistochemical findings. *Journal of Veterinary Diagnostic Investigation,* 32(4), 513-526. https://doi.org/10.1177/1040638720918561

Risalde, M.A., Molina, V., Sanchez-Cordon, P.J., Pedrera, M., Panadero, R., Romero-Palomo, F. & Gomez-Villamandos, J.C. (2011). Response of proinflammatory and anti-inflammatory cytokines in calves with subclinical bovine viral diarrhea challenged with bovine herpesvirus-1. *Vet Immunol Immunopathol,* 144(1-2), 135-43. https://doi.org/10.1016/j.vetimm.2011.07.022

Risalde, M.A., Molina, V., Sanchez-Cordon, P.J., Romero-Palomo, F., Pedrera, M. & Gomez-Villamandos, J.C. (2015). Effects of Preinfection With Bovine Viral Diarrhea Virus on Immune Cells From the Lungs of Calves Inoculated With Bovine Herpesvirus 1.1. *Vet Pathol,* 52(4), 644-53. https://doi.org/10.1177/0300985814551579

Romero-Palomo, F., Risalde, M.A. & Gomez-Villamandos, J.C. (2017). Immunopathologic Changes in the Thymus of Calves Pre-infected with BVDV and Challenged with BHV-1. *Transboundary and Emerging Diseases,* 64(2), 574-584. https://doi.org/10.1111/tbed.12406

Ruiz, M., Puig, A., Bassols, M., Fraile, L. & Armengol, R. (2022). Influenza D Virus: A Review and Update of Its Role in Bovine Respiratory Syndrome. *Viruses,* 14(12). https://doi.org/10.3390/v14122717

Saegerman, C., Bianchini, J., Snoeck, C.J., Moreno, A., Chiapponi, C., Zohari, S. & Ducatez, M.F. (2021). First expert elicitation of knowledge on drivers of emergence of influenza D in Europe. *Transboundary and Emerging Diseases,* 68(6), 3349-3359. https://doi.org/10.1111/tbed.13938

Saegerman, C., Gaudino, M., Savard, C., Broes, A., Ariel, O., Meyer, G. & Ducatez, M.F. (2022). Influenza D virus in respiratory disease in Canadian, province

of Quebec, cattle: Relative importance and evidence of new reassortment between different clades. *Transboundary and Emerging Diseases,* 69(3), 1227-1245. https://doi.org/10.1111/tbed.14085

Saipinta, D., Panyamongkol, T., Chuammitri, P. & Suriyasathaporn, W. (2022). Reduction in Mortality of Calves with Bovine Respiratory Disease in Detection with Influenza C and D Virus. *Animals (Basel),* 12(23). https://doi.org/10.3390/ani12233252

Salem, E., Hagglund, S., Cassard, H., Corre, T., Naslund, K., Foret, C., Gauthier, D., Pinard, A., Delverdier, M., Zohari, S., Valarcher, J.F., Ducatez, M. & Meyer, G. (2019). Pathogenesis, Host Innate Immune Response, and Aerosol Transmission of Influenza D Virus in Cattle. *Journal of Virology,* 93(7). https://doi.org/ARTN e01853-18
10.1128/JVI.01853-18

Sandhaus, S. & Swick, A.G. (2021). Specialized proresolving mediators in infection and lung injury. *Biofactors,* 47(1), 6-18. https://doi.org/10.1002/biof.1691

Sekiya, M., Zintl, A. & Doherty, M.L. (2013). Bulk milk ELISA and the diagnosis of parasite infections in dairy herds: a review. *Ir Vet J,* 66(1), 14. https://doi.org/10.1186/2046-0481-66-14

Snoeck, C.J., Oliva, J., Pauly, M., Losch, S., Wildschutz, F., Muller, C.P., Hubschen, J.M. & Ducatez, M.F. (2018). Influenza D Virus Circulation in Cattle and Swine, Luxembourg, 2012-2016. *Emerging Infectious Diseases,* 24(7), 1388-1389. https://doi.org/10.3201/eid2407.171937

Sreenivasan, C.C., Li, F. & Wang, D. (2024). Emerging Threats of Highly Pathogenic Avian Influenza A (H5N1) in US Dairy Cattle: Understanding Cross-Species Transmission Dynamics in Mammalian Hosts. *Viruses,* 16(11), 1703. https://www.mdpi.com/1999-4915/16/11/1703

Sreenivasan, C.C., Thomas, M., Kaushik, R.S., Wang, D. & Li, F. (2019). Influenza A in Bovine Species: A Narrative Literature Review. *Viruses,* 11(6). https://doi.org/10.3390/v11060561

Srikumaran, S., Kelling, C.L. & Ambagala, A. (2007). Immune evasion by pathogens of bovine respiratory disease complex. *Anim Health Res Rev,* 8(2), 215-29. https://doi.org/10.1017/S1466252307001326

Studer, E., Schonecker, L., Meylan, M., Stucki, D., Dijkman, R., Holwerda, M., Glaus, A. & Becker, J. (2021). Prevalence of BRD-Related Viral Pathogens in the Upper Respiratory Tract of Swiss Veal Calves. *Animals (Basel),* 11(7). https://doi.org/10.3390/ani11071940

Sudaryatma, P.E., Mekata, H., Kubo, M., Subangkit, M., Goto, Y. & Okabayashi, T. (2019). Coinfection of epithelial cells established from the upper and lower bovine respiratory tract with bovine respiratory syncytial virus and bacteria. *Veterinary Microbiology,* 235, 80-85. https://doi.org/10.1016/j.vetmic.2019.06.010

Sudaryatma, P.E., Nakamura, K., Mekata, H., Sekiguchi, S., Kubo, M., Kobayashi, I., Subangkit, M., Goto, Y. & Okabayashi, T. (2018). Bovine respiratory syncytial virus infection enhances Pasteurella multocida adherence on

respiratory epithelial cells. *Veterinary Microbiology,* 220, 33-38. https://doi.org/10.1016/j.vetmic.2018.04.031

Sudaryatma, P.E., Saito, A., Mekata, H., Kubo, M., Fahkrajang, W., Mazimpaka, E. & Okabayashi, T. (2020). Bovine Respiratory Syncytial Virus Enhances the Adherence of Pasteurella multocida to Bovine Lower Respiratory Tract Epithelial Cells by Upregulating the Platelet-Activating Factor Receptor. *Frontiers in Microbiology,* 11, 1676. https://doi.org/10.3389/fmicb.2020.01676

Taha-Abdelaziz, K., Perez-Casal, J., Schott, C., Hsiao, J., Attah-Poku, S., Slavic, D. & Caswell, J.L. (2013). Bactericidal activity of tracheal antimicrobial peptide against respiratory pathogens of cattle. *Vet Immunol Immunopathol,* 152(3-4), 289-94. https://doi.org/10.1016/j.vetimm.2012.12.016

Tam, V.C., Quehenberger, O., Oshansky, C.M., Suen, R., Armando, A.M., Treuting, P.M., Thomas, P.G., Dennis, E.A. & Aderem, A. (2013). Lipidomic profiling of influenza infection identifies mediators that induce and resolve inflammation. *Cell,* 154(1), 213-27. https://doi.org/10.1016/j.cell.2013.05.052

Tanyi, J., Romvary, J., Aldasy, P. & Mathe, Z. (1974). Isolation of influenza a virus strains from cattle. Preliminary report. *Acta Vet Acad Sci Hung,* 24(3), 341-3. https://www.ncbi.nlm.nih.gov/pubmed/4456999

Taylor, J.D., Fulton, R.W., Lehenbauer, T.W., Step, D.L. & Confer, A.W. (2010). The epidemiology of bovine respiratory disease: What is the evidence for predisposing factors? *Can Vet J,* 51(10), 1095-102. https://www.ncbi.nlm.nih.gov/pubmed/21197200

Timsit, E., McMullen, C., Amat, S. & Alexander, T.W. (2020). Respiratory Bacterial Microbiota in Cattle: From Development to Modulation to Enhance Respiratory Health. *Vet Clin North Am Food Anim Pract,* 36(2), 297-320. https://doi.org/10.1016/j.cvfa.2020.03.001

Timsit, E., Workentine, M., van der Meer, F. & Alexander, T. (2018). Distinct bacterial metacommunities inhabit the upper and lower respiratory tracts of healthy feedlot cattle and those diagnosed with bronchopneumonia. *Veterinary Microbiology,* 221, 105-113. https://doi.org/10.1016/j.vetmic.2018.06.007

Trombetta, C.M., Marchi, S., Manini, I., Kistner, O., Li, F., Piu, P., Manenti, A., Biuso, F., Sreenivasan, C., Druce, J. & Montomoli, E. (2020). Influenza D Virus: Serological Evidence in the Italian Population from 2005 to 2017. *Viruses-Basel,* 12(1). https://doi.org/ARTN 30
10.3390/v12010030

United States Department of Agriculture, A.a.P.H.I.S. (2025). *Highly Pathogenic Avian Influenza (HPAI) Confirmed Cases in Livestock*. https://www.aphis.usda.gov/livestock-poultry-disease/avian/avian-influenza/hpai-detections/hpai-confirmed-cases-livestock [April 16, 2025]

VAXA (2021). *Antibiotikastatistik 2021*. https://vxa.qbank.se/mb/?h=3fb6d74d47ca02f4f86b10e5bc2e1465&p=dc#View%20document

Verma, V., Dileepan, M., Huang, Q., Phan, T., Hu, W.S., Ly, H. & Liang, Y. (2022). Influenza A virus activates cellular Tropomyosin receptor kinase A (TrkA) signaling to promote viral replication and lung inflammation. *Plos Pathogens,* 18(9), e1010874. https://doi.org/10.1371/journal.ppat.1010874

Walsh, P., Behrens, N., Carvallo Chaigneau, F.R., McEligot, H., Agrawal, K., Newman, J.W., Anderson, M. & Gershwin, L.J. (2016). A Randomized Placebo Controlled Trial of Ibuprofen for Respiratory Syncytial Virus Infection in a Bovine Model. *Plos One,* 11(4), e0152913. https://doi.org/10.1371/journal.pone.0152913

Wolff, C., Emanuelson, U., Ohlson, A., Alenius, S. & Fall, N. (2015). Bovine respiratory syncytial virus and bovine coronavirus in Swedish organic and conventional dairy herds. *Acta Vet Scand,* 57(1), 2. https://doi.org/10.1186/s13028-014-0091-x

Wu, A., Mihaylova, V.T., Landry, M.L. & Foxman, E.F. (2020). Interference between rhinovirus and influenza A virus: a clinical data analysis and experimental infection study. *Lancet Microbe,* 1(6), e254-e262. https://doi.org/10.1016/s2666-5247(20)30114-2

Yesilbag, K., Toker, E.B. & Ates, O. (2022). Recent strains of influenza D virus create a new genetic cluster for European strains. *Microb Pathog,* 172, 105769. https://doi.org/10.1016/j.micpath.2022.105769

Yu, J., Li, T., Wen, Z., Wu, S., Wang, Z., Zheng, J., Chen, M., Chen, F., Wei, W.K., Zhai, S.L. & Liao, M. (2022). Identification of D/Yama2019 Lineage-Like Influenza D Virus in Chinese Cattle. *Frontiers in Veterinary Science,* 9, 939456. https://doi.org/10.3389/fvets.2022.939456

Yu, J.S., Hika, B., Liu, R.X., Sheng, Z.Z., Hause, B.M., Li, F. & Wang, D. (2017). The Hemagglutinin-Esterase Fusion Glycoprotein Is a Primary Determinant of the Exceptional Thermal and Acid Stability of Influenza D Virus. *Msphere,* 2(4). https://doi.org/ARTN e00254-17
10.1128/mSphere.00254-17

Yu, J.S., Li, F. & Wang, D. (2021). The first decade of research advances in influenza D virus. *Journal of General Virology,* 102(1). https://doi.org/ARTN 001529
10.1099/jgv.0.001529

Popular science summary

Cattle often suffer from respiratory diseases caused by a combination of viruses and bacteria. One of the more recently identified viruses, known as Influenza D virus (IDV), appears to contribute to the development of such diseases. This research investigates the impact of IDV on cattle health, particularly when it occurs with other respiratory pathogens such as *Mycoplasmopsis (Mycoplasma) bovis*, bovine respiratory syncytial virus and bovine parainfluenza virus type 3. By detecting antibodies in milk samples collected from Swedish farms, this work demonstrated that IDV is present and widespread in the Swedish cattle population. In addition, using preliminary laboratory studies, we showed that the virus can enhance the immune responses and interfere with the effects of other respiratory pathogens. These findings contribute to a better understanding of the complexity of the development of bovine respiratory disease in cattle and to considering the presence of IDV as a modulating factor in the disease.

Populärvetenskaplig sammanfattning

Nötkreatur drabbas ofta av luftvägssjukdomar som orsakas av en kombination av virus och bakterier. Ett av de mer nyligen identifierade virusen, influensa D-virus (IDV), verkar bidra till utvecklingen av sådana sjukdomar.

Denna forskning undersöker IDVs effekt på nötkreaturens hälsa, särskilt när viruset förekommer tillsammans med andra smittämnen såsom *Mycoplasmopsis (Mycoplasma) bovis*, bovint respiratoriskt syncytialt virus och bovint parainfluensavirus typ 3.

Genom att påvisa antikroppar i mjölkprover insamlade från svenska gårdar, visade detta arbete att IDV finns och är utbrett i den svenska nötkreaturspopulationen.

Dessutom visade vi, med hjälp av preliminära laboratoriestudier, att viruset kan förstärka immunresponsen och påverka effekterna av andra smittämnen.

Dessa resultat bidrar till en bättre förståelse av komplexiteten i utvecklingen av luftvägssjukdomar hos nötkreatur och till att beakta IDVs närvaro som en modulerande faktor i sjukdomsförloppet.

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To Sweden: understated yet vibrant, innovative yet conservative, reserved yet deeply welcoming, distant yet intimately close, minimalist yet rich.

I

viruses

MDPI

Brief Report

Detection of Influenza D-Specific Antibodies in Bulk Tank Milk from Swedish Dairy Farms

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Abstract: Influenza D virus (IDV) has been detected in bovine respiratory disease (BRD) outbreaks, and experimental studies demonstrated this virus's capacity to cause lesions in the respiratory tract. In addition, IDV-specific antibodies were detected in human sera, which indicated that this virus plays a potential zoonotic role. The present study aimed to extend our knowledge about the epidemiologic situation of IDV in Swedish dairy farms, using bulk tank milk (BTM) samples for the detection of IDV antibodies. A total of 461 and 338 BTM samples collected during 2019 and 2020, respectively, were analyzed with an in-house indirect ELISA. In total, 147 (32%) and 135 (40%) samples were IDV-antibody-positive in 2019 and 2020, respectively. Overall, 2/125 (2%), 11/157 (7%) and 269/517 (52%) of the samples were IDV-antibody-positive in the northern, middle and southern regions of Sweden. The highest proportion of positive samples was repeatedly detected in the south, in the county of Halland, which is one of the counties with the highest cattle density in the country. In order to understand the epidemiology of IDV, further research in different cattle populations and in humans is required.

Keywords: Influenza D; cattle; serology; bovine respiratory disease; ELISA; bulk tank milk

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1. Introduction

Bovine respiratory disease (BRD) is one of the most common and extensively studied diseases in the cattle industry worldwide, with a negative impact on production, animal welfare and health [1]. Several factors, such as host immunity, environmental conditions and a wide diversity of pathogens, interact and promote the onset of the disease [2]. Thanks to the extended use of molecular tools, Influenza D virus (IDV) was recently identified as a new virus involved in BRD [3,4]. This member of the Orthomyxoviridae family was first detected in pigs with respiratory symptoms, but subsequently to epidemiological analyses that revealed a high seroprevalence in cattle, bovidae was proposed to be the main reservoir [5].

Investigations based on the detection of both IDV-specific antibodies and virus demonstrated that IDV is present in cattle herds on several continents and in numerous cases with high infection rates [6–12]. The role of IDV in the pathogenesis of BRD is still unclear, but experimental infections of calves suggested that IDV alone can cause respiratory signs and lesions and that this virus has the ability to replicate in both the upper and lower respiratory tracts. However, IDV can also be detected in healthy animals, and the potential role of IDV in co-infections should be studied further [7,13–15].

To address the zoonotic role that IDV can represent, IDV was shown to replicate well in human airway epithelial cells, and seroepidemiological studies demonstrated IDV-specific antibodies in up to 95% of occupational workers in contact with cattle in USA, as well as in

the general human population in Italy [16–19]. On the other hand, 3300 archived human respiratory samples collected in Scotland were tested for IDV by PCR, and no IDV-RNA was detected [20].

The enzyme-linked immunosorbent assay (ELISA) is well recognized as a robust technique with a good performance for the detection of antibodies against different pathogens in serum. Furthermore, the combination of ELISA with the use of bulk tank milk (BTM) samples provides a powerful tool to detect and monitor the prevalence of pathogens. However, there are potential risks of false negative results caused, for example, by a low seroprevalence in milked cows and the dilution effect caused by including a high number of seronegative animals in the BTM. Nevertheless, since the assay is simple, rapid and cheap, performing ELISA on BTM is considered an excellent option to detect and monitor the presence of infections in dairy cattle for surveillance and control programs' purposes [21–23].

Based on the lack of information about the existence of IDV in Sweden and the potential importance that this pathogen might have for cattle production and human health, this study aimed to determine the presence and spatio-temporal distribution of IDV antibodies in the Swedish cattle population using an in-house indirect ELISA on bulk tank milk samples.

2. Materials and Methods

2.1. Sample Collection

During the spring of 2019 and 2020, 461 and 338 BTM samples, respectively, were collected following a risk-based design within the framework of the Swedish Surveillance Program for bovine viral diarrhea virus. The samples were collected from dairy herds across the country in a blinded manner and focused on counties with the highest cattle density. The counties were categorized into 3 regions: Norrland (North), Svealand (Central) and Götaland (South). The milk was collected from the bulk tank in vials containing bronopol as the preservative and transported to the National Veterinary Institute for further processing. At arrival, the vials were centrifuged at 3000 RPM to facilitate the removal of the cream fraction. Samples were stored at −20 °C until they were tested.

2.2. Indirect ELISA

An in-house indirect ELISA was performed to detect the presence of IDV-specific IgG antibodies, as described previously [15]. We previously demonstrated that the sensitivity and specificity of this test were 87% and 100%, respectively, when analyzing serum and using a haemagglutination inhibition assay as the gold standard [24]. The positive control used in the present study was an IDV-antibody-positive serum obtained from a calf that had been experimentally infected with IDVstrain D/bovine/France/5920/2014 (HI titer: 1/1024). This positive IDV control serum had been serially diluted tenfold in both IDV antibody-negative milk and sera from cattle and had been analyzed with both HI and ELISA. The dilution that resulted in an OD value 10% higher than the mean OD value of the negative sera, plus three standard deviations, was used as the positive control. Samples were considered IDV-antibody-positive if the corrected optical density (COD = OD_{sample} − OD_{blank}) was >10% of the COD of the positive control.

2.3. Map Design

Choropleth maps showing Sweden with the different counties and regions were created by using the software QGIS (3.26.0-Buenos Aires).

3. Results

Overall, 147 (32%) and 135 (40%) BTM samples collected in 2019 and 2020, respectively, were IDV-antibody-positive. In 2019, all the BTM samples from the region of Norrland were negative for IDV-specific antibodies; however, in 2020, the presence of two positive samples in the counties of Västerbotten and Jämtland within the region of Norrland allowed us to also confirm the presence of the virus in the most northern region of the country

(Table 1). No IDV-specific antibodies were detected in Norrbotten (Norrland), Gävleborg (Norrland), Västernorrland (Norrland), Västmanland (Svealand), Stockholm (Svealand), Uppsala (Svealand) or Kronoberg (Götaland). Counties located in the southern region of Sweden (Götaland), and in particular in the county of Halland, had the highest proportion of antibody-positive samples (84% and 77% in 2019 and 2020, respectively, Table 1, Figure 1). Whereas the proportion of positive samples increased by 22% between 2019 and 2020 in the southern region, it increased by only 8.1% in the country.

Table 1. Sample size, number and proportion of herds with IDV-specific antibody in bulk tank milk in different Swedish counties.

		2019		2020		Overall	
Region	**County**	**Sample Size**	**NPS [a] (%) [b]**	**Sample Size**	**NPS [a] (%) [b]**	**Sample Size**	**NPS [a] (%) [b]**
Norrland (North)	Gävleborg	13	0 (0)	7	0 (0)	20	0
	Norrbotten	5	0 (0)	6	0 (0)	11	0
	Västerbotten	14	0 (0)	41	1 (2.4)	55	1 (1.8)
	Västernorrland	14	0 (0)	6	0 (0)	20	0
	Jämtland	5	0 (0)	14	1 (7.1)	19	1 (5.3)
Total Norrland		51	0 (0)	74	2 (2.7)	125	2 (1.6)
Svealand (Center)	Örebro	8	2 (25)	13	3 (23.1)	21	5 (23.8)
	Västmanland	10	0 (0)	3	0 (0)	13	0
	Dalarna	14	0 (0)	12	1 (8.3)	26	1 (3.8)
	Värmland	16	2 (13)	10	1 (10)	26	3 (11.5)
	Stockholm	5	0 (0)	7	(0)	12	0
	Uppsala	13	0 (0)	12	(0)	25	0
	Södermanland	19	0 (0)	15	2 (13.3)	34	2 (5.9)
Total Svealand		85	4 (4.7)	72	7 (9.7)	157	11(7)
Götaland (South)	Östergötland	16	6 (38)	6	0 (0)	22	6 (27.3)
	Västra Götaland	76	51 (67)	57	39 (68.4)	133	90 (67.7)
	Gotaland	24	5 (21)	7	5 (71.4)	31	10 (32.2)
	Halland	37	31 (84)	13	10 (76.9)	50	41 (82)
	Blekinge	5	0 (0)	7	1 (14.3)	12	1 (8.3)
	Skåne	56	33 (59)	40	25 (62.5)	96	58 (60.4)
	Kalmar	47	15 (32)	61	46 (75.4)	108	61 (56.5)
	Jönköping	40	2 (5)	1	0 (0)	41	2 (4.9)
	Kronoberg	24	0 (0)	0	0	24	0
Total Götaland		325	143 (44)	192	126 (65.6)	517	269 (52)
Total Sweden		461	147 (31.8)	338	135 (39.9)	799	282 (35.2)

[a] Number of positive samples [b] Percentage of positive samples.

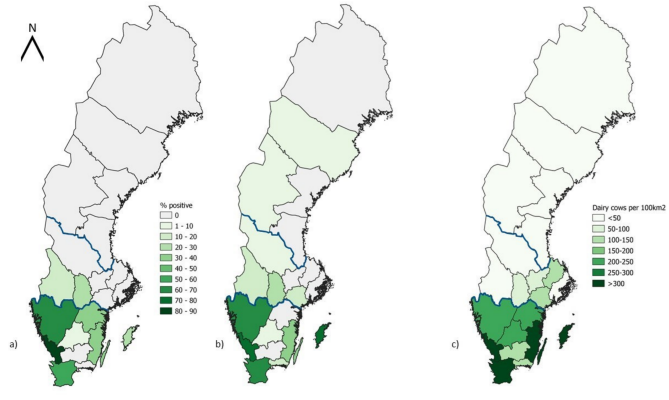

Figure 1. Maps of Sweden displaying the counties and the borders of the northern, central and southern regions. (**a**) The proportion of bulk milk samples in which IDV-specific IgG antibodies were detected in 2019, (**b**) the proportion of bulk milk samples in which IDV-specific IgG antibodies were detected in 2020, and (**c**) dairy cow density. The blue lines highlight the border of the different regions.

4. Discussion

These results demonstrate, for the first time, a circulation of IDV among Swedish dairy cattle. The southern region of the country (Götaland) consistently contained the highest number of antibody-positive herds, which suggests a high circulation of virus in that area. This was previously additionally demonstrated for bovine respiratory syncytial virus and bovine coronavirus and may be explained by the high density of animals [25]. Moreover, the shorter distance between farms may facilitate the transfer of animals between herds, more human contacts and animal professional visits in a higher number of herds per day. In addition, the connection with continental Europe might constitute an increased risk of transmission of infectious diseases. Halland, the county with the highest IDV-seroprevalence, has the highest density of dairy cows in Sweden (419 dairy cows per 100 km^2) and is situated very close to the border with Denmark. In contrast, no IDV-specific antibodies were detected in samples from the county of Norrbotten, which has the lowest dairy cow density in Sweden (4.59 dairy cows per 100 km^2) and which is situated at the opposite extreme of the border. Although not much information is available on the potential routes of transmission for IDV, experimental studies have shown that aerosol transmission within a building is possible [15]. Since IDV antibodies were detected in humans and in a wide range of animals, including wild animals such as wild boar, these may additionally contribute to the spread of the virus [26–29]. However, considering the fragility of influenza viruses in the environment, any human or animal species probably needs to be in the same building, and perhaps even in close contact with cattle, for the virus to spread.

Several serological studies conducted worldwide show a divergent seroprevalence in animals, but few of those studies report a seroprevalence at the herd level. Japan, Argentina and Luxembourg showed 50%, 75% and 97.7% IDV-antibody-positive farms among those sampled, respectively [8,12,24]. The broad sample design of this study, involving areas with a low and high animal density, and the high biosecurity level of the farms in Sweden may explain why the prevalence was not as high as in other countries.

To our knowledge, this is the first IDV-specific antibody survey performed by using BTM. Although adult cows are commonly less susceptible to respiratory viral infections than calves, the fact that they are kept in herds for a long period of time turns them into good sentinel animals for this kind of surveillance. Bulk tank milk samples can be obtained in a fast, inexpensive and non-invasive way and are an excellent option for the mass screening of diseases, as long as no classic vaccine has been used, as in the case of IDV. On the other hand, although immunoglobulin G is the most abundant immunoglobulin found in milk, the antibody concentrations are considerably lower than those found in serum [30]. The dilution effect that is generated when samples from pooled milk are analyzed does not allow one to exclude with precision the presence of a positive animal. In addition, several authors point out that antibody levels in milk may fluctuate depending on different factors, such as the stage of lactation in which the animal is at the time of sampling, subclinical mastitis, the season, the feeding system, the breed, and the age [31–33]. Regarding IDV, no studies have yet established the time during which the antibodies can be detected in milk. Consequently, it is possible that the rates reported in this study are underestimated. Therefore, once the herd has been identified as positive by BTM samples, follow-up tests with individual serum samples can be performed to further identify animals exposed to IDV. In addition, IDV-RTqPCR can be routinely carried out on nasal swabs to improve detection and confirm or exclude viral circulation in herds in which the virus is circulating.

The observed shifts in the positive rate between 2019 and 2020 could be explained by differences in the sample size of each county for each year. However, if we consider the seasonality of influenza cases in humans, it is possible to hypothesize that this same pattern also occurs for Influenza D in the cattle population. Therefore, a variation in positive rates can be expected depending on the season and the year in which the sample is collected.

5. Conclusions

The present study demonstrates that IDV is present in Swedish dairy herds and with a higher prevalence in the south of the country. Further studies should focus on the detection of IDV in BRD outbreaks and on the duration of IgG antibodies in milk and serum. In addition, it is necessary to study the prevalence of IDV in other species including humans in order to gain a deeper understanding of the epidemiological pattern of this virus.

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References

1. Blakebrough-Hall, C.; McMeniman, J.P.; González, L.A. An evaluation of the economic effects of bovine respiratory disease on animal performance, carcass traits, and economic outcomes in feedlot cattle defined using four BRD diagnosis methods. *J. Anim. Sci.* **2020**, *98*, skaa005. [CrossRef] [PubMed]
2. Deepak; Aly, S.S.; Love, W.J.; Blanchard, P.C.; Crossley, B.; Van Eenennaam, A.L.; Lehenbauer, T.W. Etiology and risk factors for bovine respiratory disease in pre-weaned calves on California dairies and calf ranches. *Prev. Vet. Med.* **2021**, *197*, 105506. [CrossRef] [PubMed]
3. Fulton, R.W. Viruses in Bovine Respiratory Disease in North America Knowledge Advances Using Genomic Testing. *Vet. Clin. N. Am. Food Anim. Pract.* **2020**, *36*, 321–332. [CrossRef] [PubMed]
4. Mitra, N.; Cernicchiaro, N.; Torres, S.; Li, F.; Hause, B.M. Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus. *J. Gen. Virol.* **2016**, *97*, 1771–1784. [CrossRef] [PubMed]
5. Hause, B.M.; Collin, E.A.; Liu, R.X.; Huang, B.; Sheng, Z.Z.; Lu, W.X.; Wang, D.; Nelson, E.A.; Li, F. Characterization of a Novel Influenza Virus in Cattle and Swine: Proposal for a New Genus in the *Orthomyxoviridae* Family. *mBio* **2014**, *5*. [CrossRef]
6. Alvarez, I.J.; Fort, M.; Pasucci, J.; Moreno, F.; Gimenez, H.; Näslund, K.; Hägglund, S.; Zohari, S.; Valarcher, J.F. Seroprevalence of influenza D virus in bulls in Argentina. *J. Vet. Diagn. Investig.* **2020**, *32*, 585–588. [CrossRef]
7. Ferguson, L.; Olivier, A.K.; Genova, S.; Epperson, W.B.; Smith, D.R.; Schneider, L.; Barton, K.; McCuan, K.; Webby, R.J.; Wan, X.-F. Pathogenesis of Influenza D Virus in Cattle. *J. Virol.* **2016**, *90*, 5636–5642. [CrossRef]
8. Horimoto, T.; Hiono, T.; Mekata, H.; Odagiri, T.; Lei, Z.; Kobayashi, T.; Norimine, J.; Inoshima, Y.; Hikono, H.; Murakami, K.; et al. Nationwide Distribution of Bovine Influenza D Virus Infection in Japan. *PLoS ONE* **2016**, *11*, e0163828. [CrossRef]
9. O'Donovan, T.; Donohoe, L.; Ducatez, M.F.; Meyer, G.; Ryan, E. Seroprevalence of influenza D virus in selected sample groups of Irish cattle, sheep and pigs. *Ir. Vet. J.* **2019**, *72*, 11. [CrossRef] [PubMed]
10. Oliva, J.; Eichenbaum, A.; Belin, J.; Gaudino, M.; Guillotin, J.; Alzieu, J.-P.; Nicollet, P.; Brugidou, R.; Gueneau, E.; Michel, E.; et al. Serological Evidence of Influenza D Virus Circulation Among Cattle and Small Ruminants in France. *Viruses* **2019**, *11*, 516. [CrossRef]
11. Sanogo, I.N.; Kouakou, C.; Batawui, K.; Djegui, F.; Byarugaba, D.K.; Adjin, R.; Adjabli, K.; Wabwire-Mangen, F.; Erima, B.; Atim, G.; et al. Serological Surveillance of Influenza D Virus in Ruminants and Swine in West and East Africa, 2017–2020. *Viruses* **2021**, *13*, 1749. [CrossRef] [PubMed]
12. Snoeck, C.J.; Oliva, J.; Pauly, M.; Losch, S.; Wildschutz, F.; Muller, C.P.; Hübschen, J.M.; Ducatez, M.F. Influenza D Virus Circulation in Cattle and Swine, Luxembourg, 2012–2016. *Emerg. Infect. Dis.* **2018**, *24*, 1388–1389. [CrossRef] [PubMed]

13. Lion, A.; Secula, A.; Rançon, C.; Boulesteix, O.; Pinard, A.; Deslis, A.; Hägglund, S.; Salem, E.; Cassard, H.; Näslund, K.; et al. Enhanced Pathogenesis Caused by Influenza D Virus and *Mycoplasma bovis* Coinfection in Calves: A Disease Severity Linked with Overexpression of IFN-γ as a Key Player of the Enhanced Innate Immune Response in Lungs. *Microbiol. Spectr.* **2021**, *9*. [CrossRef]
14. Mazzetto, E.; Bortolami, A.; Fusaro, A.; Mazzacan, E.; Maniero, S.; Vascellari, M.; Beato, M.S.; Schiavon, E.; Chiapponi, C.; Terregino, C.; et al. Replication of Influenza D Viruses of Bovine and Swine Origin in Ovine Respiratory Explants and Their Attachment to the Respiratory Tract of Bovine, Sheep, Goat, Horse, and Swine. *Front. Microbiol.* **2020**, *11*, 1136. [CrossRef]
15. Salem, E.; Hägglund, S.; Cassard, H.; Corre, T.; Näslund, K.; Foret, C.; Gauthier, D.; Pinard, A.; Delverdier, M.; Zohari, S.; et al. Pathogenesis, Host Innate Immune Response, and Aerosol Transmission of Influenza D Virus in Cattle. *J. Virol.* **2019**, *93*. [CrossRef]
16. Holwerda, M.; Kelly, J.; Laloli, L.; Stürmer, I.; Portmann, J.; Stalder, H.; Dijkman, R. Determining the Replication Kinetics and Cellular Tropism of Influenza D Virus on Primary Well-Differentiated Human Airway Epithelial Cells. *Viruses* **2019**, *11*, 377. [CrossRef]
17. Song, H.; Qi, J.; Khedri, Z.; Diaz, S.; Yu, H.; Chen, X.; Varki, A.; Shi, Y.; Gao, G.F. An Open Receptor-Binding Cavity of Hemagglutinin-Esterase-Fusion Glycoprotein from Newly-Identified Influenza D Virus: Basis for Its Broad Cell Tropism. *PLoS Pathog.* **2016**, *12*, e1005411. [CrossRef]
18. Trombetta, C.M.; Marchi, S.; Manini, I.; Kistner, O.; Li, F.; Piu, P.; Manenti, A.; Biuso, F.; Sreenivasan, C.; Druce, J.; et al. Influenza D Virus: Serological Evidence in the Italian Population from 2005 to 2017. *Viruses* **2020**, *12*, 30. [CrossRef]
19. White, S.K.; Ma, W.; McDaniel, C.J.; Gray, G.C.; Lednicky, J.A. Serologic evidence of exposure to influenza D virus among persons with occupational contact with cattle. *J. Clin. Virol.* **2016**, *81*, 31–33. [CrossRef]
20. Smith, D.B.; Gaunt, E.R.; Digard, P.; Templeton, K.; Simmonds, P. Detection of influenza C virus but not influenza D virus in Scottish respiratory samples. *J. Clin. Virol.* **2016**, *74*, 50–53. [CrossRef]
21. Ohlson, A.; Blanco-Penedo, I.; Fall, N. Comparison of Bovine coronavirus-specific and *Bovine respiratory syncytial virus*-specific antibodies in serum versus milk samples detected by enzyme-linked immunosorbent assay. *J. Vet. Diagn. Investig* **2014**, *26*, 113–116. [CrossRef]
22. Pautienius, A.; Dudas, G.; Simkute, E.; Grigas, J.; Zakiene, I.; Paulauskas, A.; Armonaite, A.; Zienius, D.; Slyzius, E.; Stankevicius, A. Bulk Milk Tank Samples Are Suitable to Assess Circulation of Tick-Borne Encephalitis Virus in High Endemic Areas. *Viruses* **2021**, *13*, 1772. [CrossRef] [PubMed]
23. Chanlun, A.; Näslund, K.; Aiumlamai, S.; Björkman, C. Use of bulk milk for detection of Neospora caninum infection in dairy herds in Thailand. *Vet. Parasitol.* **2002**, *110*, 35–44. [CrossRef] [PubMed]
24. Alvarez, I.J.; Fort, M.; Pasucci, J.; Moreno, F.; Gimenez, H.; Naeslund, K.; Haegglund, S.; Zohari, S.; Valarcher, J.F. Seroprevalence of influenza D virus in bulls in Argentina—Authors' response. *J. Vet. Diagn. Investig* **2020**, *32*, 633–634. [CrossRef]
25. Beaudeau, F.; Ohlson, A.; Emanuelson, U. Associations between bovine coronavirus and bovine respiratory syncytial virus infections and animal performance in Swedish dairy herds. *J. Dairy Sci.* **2010**, *93*, 1523–1533. [CrossRef] [PubMed]
26. Murakami, S.; Odagiri, T.; Melaku, S.K.; Bazartseren, B.; Ishida, H.; Takenaka-Uema, A.; Muraki, Y.; Sentsui, H.; Horimoto, T. Influenza D Virus Infection in Dromedary Camels, Ethiopia. *Emerg. Infect. Dis.* **2019**, *25*, 1224–1226. [CrossRef]
27. Nedland, H.; Wollman, J.; Sreenivasan, C.; Quast, M.; Singrey, A.; Fawcett, L.; Christopher-Hennings, J.; Nelson, E.; Kaushik, R.S.; Wang, D.; et al. Serological evidence for the co-circulation of two lineages of influenza D viruses in equine populations of the Midwest United States. *Zoonoses Public Health* **2018**, *65*, E148–E154. [CrossRef] [PubMed]
28. Ferguson, L.; Luo, K.; Olivier, A.K.; Cunningham, F.L.; Blackmon, S.; Hanson-Dorr, K.; Sun, H.; Baroch, J.; Lutman, M.W.; Quade, B.; et al. Influenza D Virus Infection in Feral Swine Populations, United States. *Emerg. Infect. Dis.* **2018**, *24*, 1020–1028. [CrossRef]
29. Quast, M.; Sreenivasan, C.; Sexton, G.; Nedland, H.; Singrey, A.; Fawcett, L.; Miller, G.; Lauer, D.; Voss, S.; Pollock, S.; et al. Serological evidence for the presence of influenza D virus in small ruminants. *Vet. Microbiol.* **2015**, *180*, 281–285. [CrossRef]
30. Hägglund, S.; Näslund, K.; Svensson, A.; Lefverman, C.; Enül, H.; Pascal, L.; Siltenius, J.; Holzhauer, M.; Delabouglise, A.; Österberg, J.; et al. Longitudinal study of the immune response and memory following natural bovine respiratory syncytial virus infections in cattle of different age. *PLoS ONE* **2022**, *17*, e0274332. [CrossRef]
31. Król, J.; Litwińczuk, Z.; Brodziak, A.; Barłowska, J. Lactoferrin, lysozyme and immunoglobulin G content in milk of four breeds of cows managed under intensive production system. *Pol. J. Vet. Sci.* **2010**, *13*, 357–361. [PubMed]
32. Milovanović, M.; Milićević, V.; Radojičić, S.; Valčić, M.; Hoffmann, B.; Dietze, K. Suitability of individual and bulk milk samples to investigate the humoral immune response to lumpy skin disease vaccination by ELISA. *Virol. J.* **2020**, *17*, 28. [CrossRef] [PubMed]
33. Morin, D.E.; Nelson, S.V.; Reid, E.D.; Nagy, D.W.; Dahl, G.E.; Constable, P.D. Effect of colostral volume, interval between calving and first milking, and photoperiod on colostral IgG concentrations in dairy cows. *J. Am. Vet. Med. Assoc.* **2010**, *237*, 420–428. [CrossRef] [PubMed]

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Brief Report

Detection and Phylogenetic Characterization of Influenza D in Swedish Cattle

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Abstract: Increased evidence suggests that cattle are the primary host of Influenza D virus (IDV) and may contribute to respiratory disease in this species. The aim of this study was to detect and characterise IDV in the Swedish cattle population using archived respiratory samples. This retrospective study comprised a collection of a total 1763 samples collected between 1 January 2021 and 30 June 2024. The samples were screened for IDV and other respiratory pathogens using real-time reverse transcription quantitative PCR (rRT-qPCR). Fifty-one IDV-positive samples were identified, with a mean cycle threshold (Ct) value of 27 (range: 15–37). Individual samples with a Ct value of <30 for IDV RNA were further analysed by deep sequencing. Phylogenetic analysis was performed by the maximum likelihood estimation method on the whole IDV genome sequence from 16 samples. The IDV strains collected in 2021 (n = 7) belonged to the D/OK clade, whereas samples from 2023 (n = 4) and 2024 (n = 5) consisted of reassortants between the D/OK and D/660 clades, for the PB2 gene. This study reports the first detection of IDV in Swedish cattle and the circulation of D/OK and reassortant D/OK-D/660 in this population.

Keywords: influenza D virus; cattle; bovine; PCR; Sweden; co-infection; phylogeny; sequencing; reassortment

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1. Introduction

In 2011, a virus similar to influenza C was identified in pigs with respiratory symptoms in the USA [1]. Due to its distinct genetic profile and lack of cross-reactivity with influenza C, it was reclassified as a new genus, now recognised as influenza D virus (IDV).

IDV can infect and transmit across several species, with cattle identified as the primary host. The presence of IDV in both healthy and sick animals suggests limited virulence in cattle [2,3]. However, metagenomic studies have implicated IDV in bovine respiratory disease (BRD) [4,5]. While experimental intranasal infections in calves have induced mild lesions in the respiratory tract, co-infections with *Mycoplasma bovis* have resulted in more

severe lesions and clinical signs, suggesting a potential role of IDV as a co-pathogen in BRD [6].

Despite the limited or null virulence of IDV reported in other animal species to date, there is still reason to be concerned about the potential role of IDV in humans as a zoonotic disease. Several studies have shown the presence of IDV antibodies in human sera, with a wide range of positivity among different geographical and professional populations [7–9]. In Florida, a seroprevalence of 97% was reported among cattle workers, compared to 18% among individuals without cattle contact, while in Italy, 46% of serum samples were IDV antibody-positive by HI assay in 2014 [7,9]. In addition to serological tests, IDV has been detected once in a bioaerosol from a hospital and in an airport, and in vitro studies demonstrate the ability of IDV to replicate in human respiratory epithelial cells and also to infect and transmit in ferrets, mice, and guinea pigs, which are common models used to study influenza infection in humans [1,10–14].

The virulence potential of influenza viruses is strongly linked to genetic characteristics allowing spreading between different hosts. Since their discovery, IDV strains have been divided based on the hemagglutinin-esterase fusion (HEF) gene into four major lineages. In Europe, two major lineages have been detected so far, D/OK and D/660 [15]. In 2022, Gaudino et al. demonstrated that the HEF glycoprotein of IDV has a significantly higher nucleotide substitution rate compared to the HEF of human influenza C virus, suggesting the possibility for reassortment and the emergence of new strains [15]. Genetic reassortant patterns in IDV, comprising gene segments from both D/OK and D/660, have previously been observed in Italy, USA, Canada, and France [16–19].

Previously in Sweden, the circulation of IDV was indicated by the detection of IDV-specific antibodies in 282 out of 799 (35%) bulk milk samples from Swedish dairy farms, but the IDV strains circulating in the cattle population were not detected or phylogenetically characterised [20]. This study aimed to screen respiratory samples collected from cattle with respiratory signs by IDV real-time reverse transcription quantitative PCR (RT-qPCR), to determine the presence of IDV in sampled animals and to perform a phylogenetic characterisation to improve the understanding of the evolutionary dynamics of IDV in Sweden.

2. Materials and Methods

A total of 1763 respiratory samples collected from bovine clinical cases exhibiting respiratory signs were retrospectively analysed for the presence of IDV. These samples, submitted to the Swedish Veterinary Agency between 1 January 2021, and 30 June 2024, consisted of lung tissue and individual or pooled nasal swabs, with each pool containing samples from up to five animals. Samples were collected in 248, 258, 248, and 239 herds in 2021, 2022, 2023, and 2024, respectively. The farms were geographically spread through the whole country and were located in 172 municipalities from the Övertorneå Municipality (66°23′17″ N 23°39′13″ E) in the North to the Trelleborg Municipality (55°22′ N 13°10′ E) in the Southern part of the country. The samples were initially screened for a range of the most common respiratory pathogens, including bovine coronavirus (BCoV), bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus type 3, *Mycoplasma bovis*, *Pasteurella multocida*, *Histophilus somni*, and *Mannheimia haemolytica*, as part of routine diagnostics and veterinary care investigation and stored at −80 °C until tested for IDV. If IDV was detected in any pooled samples, a confirmatory PCR test was performed on individual samples from that pool. This step not only reconfirmed the presence of IDV, but also determined the extent of positive animals among that pool. Positive samples for IDV were then used for phylogenetic analysis.

RNA was extracted using the IndiMag Pathogen Kit (INDICAL Bioscience, Leipzig, Germany). For each extraction, 200 µL of the sample and 20 µL of proteinase K were

transferred to a Deep Well-96 plate, followed by the addition of 500 µL of lysate mix, prepared according to the manufacturer's instructions. The extraction process was carried out using a Maelstrom-9600 (TANBead, Taoyuan City, Taiwan) extraction robot. The presence of IDV was detected using RT-qPCR, targeting the NP gene of IDV, as previously described [21]. Positive samples were retested in triplicate using a second RT-qPCR assay targeting the polymerase gene of influenza D viruses to confirm the results [1]. IDV-RNA-positive samples with Ct values <30 were selected for whole-genome sequencing. These samples underwent metagenomic next-generation sequencing using the Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA). Library construction was performed with the NEXTERA-XT kit (Illumina Inc., San Diego, CA, USA), according to the manufacturer's instructions. The quality of the libraries was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Sequencing was conducted on a MiSeq instrument using a MiSeq Reagent Kit v3 in a 600-cycle paired-end run. The generated data were analysed with CLC Genomics Workbench v21 (CLC bio, Aarhus, Denmark).

Phylogenetic trees were constructed for each gene segment using relevant nucleotide sequences from GenBank. Blast homology searches were performed to retrieve the top 100 homologous sequences for the sequenced gene segments. Phylogenetic analysis was conducted using the maximum likelihood method implemented in MEGA7: Molecular Evolutionary Genetics Analysis version 7.0.26. The robustness of the ML trees was evaluated by bootstrap analysis with 2000 replicates.

Choropleth maps showing Sweden with the positive municipalities and regions were created by using the R version 4.4.1.

3. Results

A total of 51 animals were positive for IDV. Of these, 36 positive samples were identified during the 2020–2021 influenza season (1st of October–30th of September), while the remaining 15 were detected during the 2023–2024 season (Figure 1). The mean Ct value for IDV-positive samples was 27, with a range from 15 to 37.

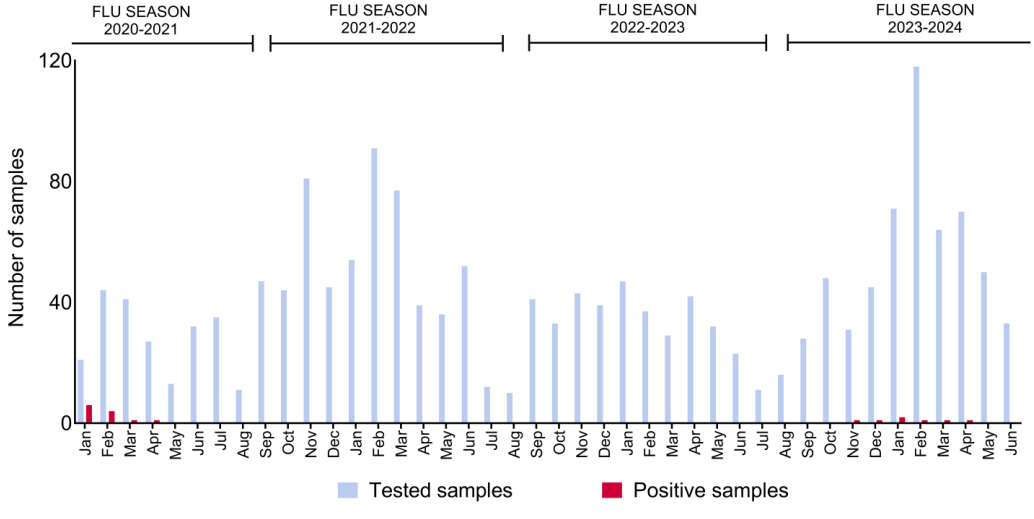

Figure 1. Number of tested and positive samples from 2021 to 2024, categorised by influenza seasons (October–September). Positive samples were detected during the 2020–2021 and 2023–2024 seasons.

Positive samples were collected from 14 different farms, 9 of which were fattening farms (Table 1). Regarding the age distribution, 38/51 (75%) positive samples were from calves younger than 16 weeks (Table 1).

Table 1. Laboratory results and sample information, including farm production type, sample type, and age of sampled calves. F = fattening farm, S = suckler cow, and D = dairy farm n.d = no data. NS = Nasal swabs and Lung= lung tissue. W = Week, Y = year. The results are presented as the Pos (+) and Neg (−) of the corresponding RT-qPCR for IDV.

Farm ID	Production Type	Sample Type	Age Group	Number of Tested Animals	Number of IDV-Positive Animals
1	F	NS	3–6 w	4	4
2	S	NS	1 y	4	4
3	F	NS	12–14 w	4	4
		NS	3–5 w	3	3
		NS	8–28 w	3	3
		NS	4 w	4	4
		NS	8 w	3	3
		NS	4 w	3	3
4	F	NS	8–12 w	3	1
5	n.d	NS	n.d	1	1
6	F	NS	28 w	1	1
7	F	NS	n.d	4	2
8	F	NS	4–14 w	4	4
9	F and S	Ns	49 w	1	1
10	D	NS	2–6 w	4	4
11	n.d	NS	n.d	1	1
12	F	NS	4–14 w	3	3
13	n.d	Lung	4 y	1	1
14	F	NS	8–16 w	4	4

Among the 51 samples that tested positive for IDV, only one animal was likely single infected with IDV, while the remaining 50 showed co-infections with at least two other pathogens from the respiratory panel. Specifically, *P. multocida* was the most common bacteria, detected in 45 IDV-positive samples (88%), whereas BCoV was the most common virus, detected in 31 IDV-positive samples (60%, Table S1). Regarding the simultaneous detection of several viruses, nine samples were positive for IDV, BCoV, and PIV-3, eight samples were positive for IDV, BCoV, and BRSV, and six samples were positive for IDV, BRSV, and PIV-3.

In 2021, positive samples were detected in six municipalities: Mjölby, Falköping, Mellerud, Vänersborg, Kristinehamn, and Gotland. In 2023, Tomelilla and Klippan were the only two municipalities with positive samples. However, in 2024, the number increased again, with positive samples detected in four different municipalities: Gotland, Varberg, Klippan, and Sjöbo (Figure 2).

Phylogenetic analysis of the seven gene segments of IDV revealed distinct patterns. Strains collected in 2021 (n = 7) clustered within the D/OK lineage. In contrast, strains collected in 2023–2024 (n = 9) were reassortants between the D/OK and D/660 lineages. Specifically, gene segment 1, which encodes the PB2 protein, clustered within the D/660 lineage (Figure 3), while the rest of the gene segments remained within the D/OK gene pool (Figure S1).

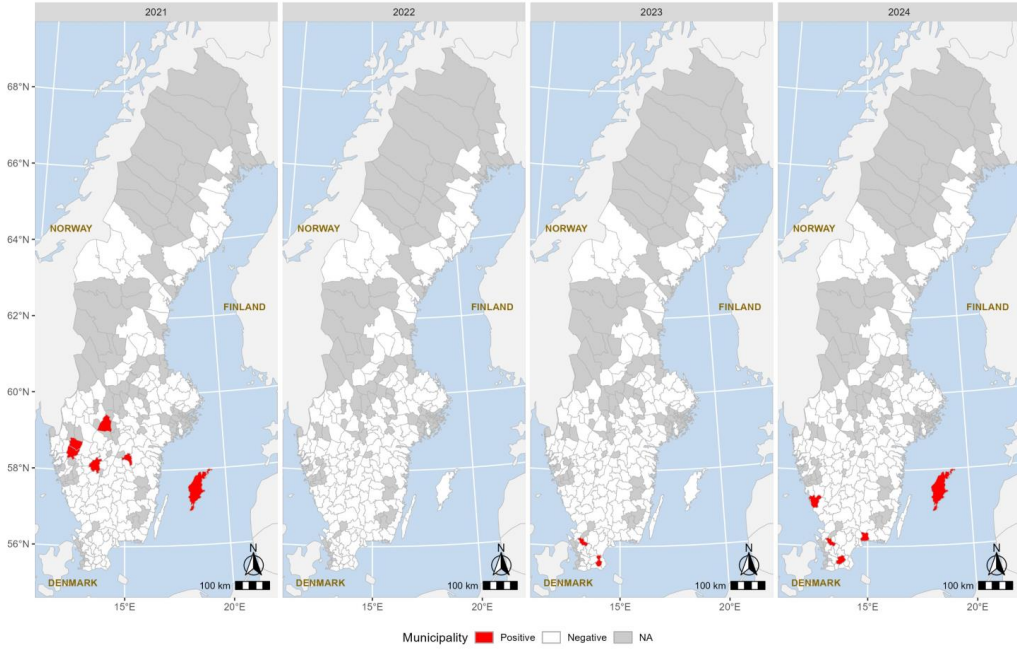

Figure 2. Choropleth maps showing municipalities with positive samples (in red) from 2021 to 2024.

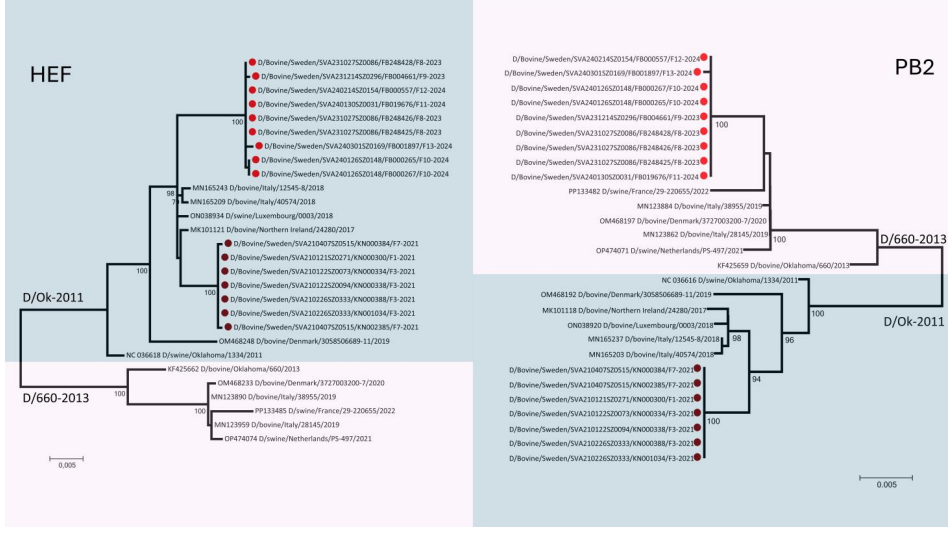

Figure 3. Phylogenetic trees of Influenza D virus collected in Sweden. The trees illustrate the relationships of the gene segments encoding the HEF and PB2 proteins with reference strains from the D/OK and D/660 lineages. Strains collected analysed based on HEF protein analysis clustered within the D/OK lineage. However, samples collected in 2023 and 2024 showed reassortment, with genetic material from both the D/OK and D/660 lineages, specifically with gene segment 1 clustering within the D/660 lineage.

4. Discussion

In this study, we confirmed the presence of IDV by detecting the virus genome in samples from animals with respiratory disease and identified two genetic variants of influenza D viruses among cattle in Sweden. The viruses circulating in 2021 had their complete genome constructed of segments of the D/OK lineage gene pool, while segment 1 (Polymerase Basic 2-PB2 gene) of the detected viruses in the 2023–2024 season belonged to the D/660 lineage gene pool, representing a novel genotype and reassortants between the D/OK and D/660 lineages. The low detection rate suggests that IDV is not a major health concern in Swedish cattle production. Regarding age and based on our results, it seems that IDV follows the same pattern as other respiratory pathogens, being predominantly detected in calves younger than 4 months. This coincides with the time when maternal antibodies have decreased and calves are exposed to stressors [22]. The higher detection rate of positive calves in fattening farms may be linked to common conditions in this type of production, such as the mixing of calves from different farms, transportation stress, changes in diet, new housing environment, and high animal density.

Only 1 out of 51 IDV-positive animals was found to be single infected with IDV, whereas the remaining 50 were positive for at least two other pathogens from the respiratory panel. Several of the detected bacteria are part of the normal nasal flora and may not contribute to clinical signs. This underscores the challenges in distinguishing between commensal presence and pathogenic impact and highlights the limitation in the use of the molecular detection of pathogens in nasal secretions for diagnostics. Several studies have identified IDV in nasal swabs of asymptomatic animals, suggesting that subclinical infections are common. While much research on BRD focuses on interactions between viruses and bacteria, our findings indicate that co-infections involving multiple established respiratory viruses are also common. This aspect of BRD has been relatively understudied, and our results emphasise the need for further investigation into the implications of such viral interactions.

While a more extended study period is needed to assess the possibility of a cyclic or "wave-like" pattern, the detection of positive animals during the colder months of 2021 and 2023/2024 suggests some degree of seasonality or annual occurrence, similarly to what has been observed in several influenza studies, including those on IDV [7,23–25]. In this study, it is challenging to distinguish between the effects of the winter season on immune status and the increased presence of the virus. During winter, when temperatures drop and daylight is limited, especially in a country like Sweden, several factors could contribute to the higher detection rates. The use of indoor housing systems, more common in colder seasons, likely increases viral transmission due to closer animal proximity, reduced ventilation, and increased viral survival in the environment [26].

The discrepancy between the prevalence of IDV antibodies in the previous serological study and the limited detection of the pathogen by RT-qPCR in the present study may be due to the use of different approaches (indirect vs. direct virus detection), different sample types (bulk milk vs. respiratory samples), and differences in the study population (cows vs. calves). Bulk milk reflects the antibody status of the adult cow population, the group with the longest pathogen exposure on a dairy farm. Although the duration of antibody responses against IDV is not well defined, it is reasonable to assume that IDV-specific antibodies can remain detectable for several years, as was demonstrated for IAV virus in humans and BRSV in cattle [27–29]. In addition, limited information is available regarding the persistence or clearance of IDV in different tissues and samples. Based on three studies with experimental infections, the clinical signs of IDV are mild and peak between 5 and 8 days post-infection (dpi) [6,30,31]. In one of these studies, Ferguson et al. demonstrated that at 4 dpi, IDV RNA was present in both the upper and lower respiratory tracts of

infected calves, but by 6 dpi, it was only detected in nasal tissue [31]. Moreover, Lion et al. reported that five out of five animals infected with IDV tested positive in nasal swabs at 8 dpi, but only two out of those five were positive at 10 dpi, and only one was at 12 dpi [30]. These results suggest that the detection window for IDV in the respiratory tract is very short. It is important to note that these studies involved experimental infections, where the viral load and the direct induction of the infection in the upper respiratory tract were probably different than under natural field conditions. The inability to detect mild clinical signs, combined with delays in sampling until more severe clinical signs, likely due to secondary infections, could explain the limited detection of animals infected with IDV. The detection of positive samples in 2021, followed by their re-emergence in 2023–2024, might serve as an example of the infection–immunity–reinfection model that can explain the cyclic pattern of IDV. After an IDV outbreak, a significant portion of the population likely develops a partial and temporary immunity, which reduces the viral circulation in the following years. As this immunity declines or new susceptible individuals are introduced into the population, the risk of viral re-emergence increases, facilitating the viral return. Sweden is characterised by a lower cattle density and higher biosecurity standards compared to other countries, which help to limit and prevent the introduction and spread of various pathogens [32].

Monitoring the genetic characteristics of IDV is crucial for tracking viral variation and understanding its impact on the clinical expression of infections, as well as tissue and animal species tropism. Phylogenetic analysis of the sequenced samples revealed that IDV strains collected in 2021 belonged to the D/OK lineage, whereas the sequenced samples from 2023 consisted of reassortants between the D/OK and D/660 lineages. Although a limited number of strains were identified, this suggests an ongoing genetic reassortment and evolution of the virus in the Swedish cattle population. Similar results have been observed in Denmark, where a shift in the circulating IDV lineage from D/OK to D/660 has been detected in recent years [33]. This shift is particularly significant when discussing influenza viruses because, in the case of IDV, it has been shown that the D/OK and D/660 lineages do not generate a fully cross-reactive immune response [16,34]. Although it is difficult to predict the exact impact of the PB2 gene reassortment, this could significantly influence the behaviour and epidemiology of the virus, potentially altering its replication and hence its virulence and transmission dynamics [35,36].

Given the current understanding of the role of IDV in bovine respiratory disease, it would be recommended to include IDV in routine diagnostic respiratory panels. This would provide opportunities to follow the prevalence and virulence, gain a more comprehensive insight into the respiratory disease complex of cattle, and improve disease management strategies in the field.

In conclusion, although IDV currently appears to have a limited impact on cattle health in Sweden, its potential role as a co-pathogen and the ongoing genetic evolution highlights the need for continued surveillance.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/v17010017/s1, Table S1. Results of the laboratory analyses. Samples of nasal swabs (nasal swabs-NS, pooled nasal swabs-pNS-each pool containing three to five individual samples) and lung tissue. The production type is F = Fattening farm, S = Suckler cow and D = Dairy farm. Positive results of pooled and individual samples are presented as the Pos/Neg of the corresponding PCR; BCoV = Bovine Coronavirus, BRSV = Bovine Respiratory Syncitia virus, PIV-3 = Bovine Parainfluenza 3, M.Bovis = Mycoplasma Bovis, P. multocida = Pasturella multocida, M. haem = Mannheimia haemolytica, H.somnia = Histophilus somni, IDV = Influenza D virus, Pos. = positive qPCR result, Neg. = Negative qPCR. Table S2. Accession Number for the Sequenced Strain. Figure S1. Phylogenetic trees illustrating the relationships of the gene segments encoding the PB1, P3, NP, P42, and NS proteins with reference strains from the D/OK and D/660 lineages.

Figure S2. Philogenetic trees illustrate the relationships of the gene segments encoding the HEF and PB2 proteins with reference strains from the D/OK and D/660 lineages.

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Institutional Review Board Statement: This study used biological samples collected for diagnostic reasons as a part of routine veterinary care, without any direct actions from the authors' side influencing the management of each submitted case. The terms and conditions of submission samples to the Swedish Veterinary Agency permit the use of submitted samples for research purposes without further consent. Thus, approval from an ethical committee was not required according to national regulations.

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequences are available at www.ncbi.nlm.nih.gov/genbank (accessed on 1 December 2024), and the accession numbers are presented in Table S2.

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References

1. Hause, B.M.; Ducatez, M.; Collin, E.A.; Ran, Z.; Liu, R.; Sheng, Z.; Armien, A.; Kaplan, B.; Chakravarty, S.; Hoppe, A.D.; et al. Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. *PLoS Pathog.* **2013**, *9*, e1003176. [CrossRef]
2. Mekata, H.; Yamamoto, M.; Hamabe, S.; Tanaka, H.; Omatsu, T.; Mizutani, T.; Hause, B.M.; Okabayashi, T. Molecular epidemiological survey and phylogenetic analysis of bovine influenza D virus in Japan. *Transbound. Emerg. Dis.* **2018**, *65*, e355–e360. [CrossRef]
3. Wan, X.F.; Ferguson, L.; Oliva, J.; Rubrum, A.; Eckard, L.; Zhang, X.J.; Woolums, A.R.; Lion, A.; Meyer, G.; Murakami, S.; et al. Limited Cross-Protection Provided by Prior Infection Contributes to High Prevalence of Influenza D Viruses in Cattle. *J. Virol.* **2020**, *94*, e00240-20. [CrossRef] [PubMed]
4. Mitra, N.; Cernicchiaro, N.; Torres, S.; Li, F.; Hause, B.M. Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus. *J. Gen. Virol.* **2016**, *97*, 1771–1784. [CrossRef] [PubMed]
5. Ng, T.F.; Kondov, N.O.; Deng, X.; Van Eenennaam, A.; Neibergs, H.L.; Delwart, E. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. *J. Virol.* **2015**, *89*, 5340–5349. [CrossRef] [PubMed]
6. Salem, E.; Hagglund, S.; Cassard, H.; Corre, T.; Naslund, K.; Foret, C.; Gauthier, D.; Pinard, A.; Delverdier, M.; Zohari, S.; et al. Pathogenesis, Host Innate Immune Response, and Aerosol Transmission of Influenza D Virus in Cattle. *J. Virol.* **2019**, *93*, e01853-18. [CrossRef]
7. Trombetta, C.M.; Marchi, S.; Manini, I.; Kistner, O.; Li, F.; Piu, P.; Manenti, A.; Biuso, F.; Sreenivasan, C.; Druce, J.; et al. Influenza D Virus: Serological Evidence in the Italian Population from 2005 to 2017. *Viruses* **2020**, *12*, 30. [CrossRef]
8. Trombetta, C.M.; Montomoli, E.; Di Bartolo, I.; Ostanello, F.; Chiapponi, C.; Marchi, S. Detection of antibodies against influenza D virus in swine veterinarians in Italy in 2004. *J. Med. Virol.* **2022**, *94*, 2855–2859. [CrossRef]
9. White, S.K.; Ma, W.; McDaniel, C.J.; Gray, G.C.; Lednicky, J.A. Serologic evidence of exposure to influenza D virus among persons with occupational contact with cattle. *J. Clin. Virol.* **2016**, *81*, 31–33. [CrossRef]

10. Bailey, E.S.; Choi, J.Y.; Zemke, J.; Yondon, M.; Gray, G.C. Molecular surveillance of respiratory viruses with bioaerosol sampling in an airport. *Trop. Dis. Travel. Med. Vaccines* **2018**, *4*, 11. [CrossRef]
11. Choi, J.Y.; Zemke, J.; Philo, S.E.; Bailey, E.S.; Yondon, M.; Gray, G.C. Aerosol Sampling in a Hospital Emergency Room Setting: A Complementary Surveillance Method for the Detection of Respiratory Viruses. *Front. Public Health* **2018**, *6*, 174. [CrossRef] [PubMed]
12. Oliva, J.; Mettier, J.; Sedano, L.; Delverdier, M.; Bourges-Abella, N.; Hause, B.; Loupias, J.; Pardo, I.; Bleuart, C.; Bordignon, P.J.; et al. Murine Model for the Study of Influenza D Virus. *J. Virol.* **2020**, *94*, e01662-19. [CrossRef]
13. Sreenivasan, C.; Thomas, M.; Sheng, Z.Z.; Hause, B.M.; Collin, E.A.; Knudsen, D.E.B.; Pillatzki, A.; Nelson, E.; Wang, D.; Kaushik, R.S.; et al. Replication and Transmission of the Novel Bovine Influenza D Virus in a Guinea Pig Model. *J. Virol.* **2015**, *89*, 11990–12001. [CrossRef] [PubMed]
14. Holwerda, M.; Kelly, J.; Laloli, L.; Sturmer, I.; Portmann, J.; Stalder, H.; Dijkman, R. Determining the Replication Kinetics and Cellular Tropism of Influenza D Virus on Primary Well-Differentiated Human Airway Epithelial Cells. *Viruses* **2019**, *11*, 377. [CrossRef] [PubMed]
15. Gaudino, M.; Chiapponi, C.; Moreno, A.; Zohari, S.; O'Donovan, T.; Quinless, E.; Sausy, A.; Oliva, J.; Salem, E.; Fusade-Boyer, M.; et al. Evolutionary and temporal dynamics of emerging influenza D virus in Europe (2009-22). *Virus Evol.* **2022**, *8*, veac081. [CrossRef]
16. Collin, E.A.; Sheng, Z.Z.; Lang, Y.K.; Ma, W.J.; Hause, B.; Li, F. Cocirculation of Two Distinct Genetic and Antigenic Lineages of Proposed Influenza D Virus in Cattle. *J. Virol.* **2015**, *89*, 1036–1042. [CrossRef]
17. Chiapponi, C.; Faccini, S.; Fusaro, A.; Moreno, A.; Prosperi, A.; Merenda, M.; Baioni, L.; Gabbi, V.; Rosignoli, C.; Alborali, G.L.; et al. Detection of a New Genetic Cluster of Influenza D Virus in Italian Cattle. *Viruses* **2019**, *11*, 1110. [CrossRef] [PubMed]
18. Gorin, S.; Richard, G.; Herve, S.; Eveno, E.; Blanchard, Y.; Jardin, A.; Rose, N.; Simon, G. Characterization of Influenza D Virus Reassortant Strain in Swine from Mixed Pig and Beef Farm, France. *Emerg. Infect. Dis.* **2024**, *30*, 1672–1676. [CrossRef]
19. Saegerman, C.; Gaudino, M.; Savard, C.; Broes, A.; Ariel, O.; Meyer, G.; Ducatez, M.F. Influenza D virus in respiratory disease in Canadian, province of Quebec, cattle: Relative importance and evidence of new reassortment between different clades. *Transbound. Emerg. Dis.* **2022**, *69*, 1227–1245. [CrossRef]
20. Alvarez, I.; Hagglund, S.; Naslund, K.; Eriksson, A.; Ahlgren, E.; Ohlson, A.; Ducatez, M.F.; Meyer, G.; Valarcher, J.F.; Zohari, S. Detection of Influenza D-Specific Antibodies in Bulk Tank Milk from Swedish Dairy Farms. *Viruses* **2023**, *15*, 829. [CrossRef] [PubMed]
21. Henritzi, D.; Hoffmann, B.; Wacheck, S.; Pesch, S.; Herrler, G.; Beer, M.; Harder, T.C. A newly developed tetraplex real-time RT-PCR for simultaneous screening of influenza virus types A, B, C and D. *Influenza Other Resp.* **2019**, *13*, 71–82. [CrossRef]
22. Barrington, G.M.; Parish, S.M. Bovine neonatal immunology. *Vet. Clin. N. Am. Food Anim. Pract.* **2001**, *17*, 463–476. [CrossRef] [PubMed]
23. Yu, J.S.; Li, F.; Wang, D. The first decade of research advances in influenza D virus. *J. Gen. Virol.* **2021**, *102*, jgv001529. [CrossRef]
24. He, D.; Lui, R.; Wang, L.; Tse, C.K.; Yang, L.; Stone, L. Global Spatio-temporal Patterns of Influenza in the Post-pandemic Era. *Sci. Rep.* **2015**, *5*, 11013. [CrossRef]
25. Horimoto, T.; Hiono, T.; Mekata, H.; Odagiri, T.; Lei, Z.; Kobayashi, T.; Norimine, J.; Inoshima, Y.; Hikono, H.; Murakami, K.; et al. Nationwide Distribution of Bovine Influenza D Virus Infection in Japan. *PLoS ONE* **2016**, *11*, e0163828. [CrossRef]
26. Welliver, R.C., Sr. Temperature, humidity, and ultraviolet B radiation predict community respiratory syncytial virus activity. *Pediatr. Infect. Dis. J.* **2007**, *26*, S29–S35. [CrossRef] [PubMed]
27. Chen, J.; Zhu, H.; Horby, P.W.; Wang, Q.; Zhou, J.; Jiang, H.; Liu, L.; Zhang, T.; Zhang, Y.; Chen, X.; et al. Specificity, kinetics and longevity of antibody responses to avian influenza A(H7N9) virus infection in humans. *J. Infect.* **2020**, *80*, 310–319. [CrossRef]
28. Hagglund, S.; Naslund, K.; Svensson, A.; Lefverman, C.; Enul, H.; Pascal, L.; Siltenius, J.; Holzhauer, M.; Delabouglise, A.; Osterberg, J.; et al. Longitudinal study of the immune response and memory following natural bovine respiratory syncytial virus infections in cattle of different age. *PLoS ONE* **2022**, *17*, e0274332. [CrossRef] [PubMed]
29. Krammer, F. The human antibody response to influenza A virus infection and vaccination. *Nat. Rev. Immunol.* **2019**, *19*, 383–397. [CrossRef]
30. Lion, A.; Secula, A.; Rancon, C.; Boulesteix, O.; Pinard, A.; Deslis, A.; Hagglund, S.; Salem, E.; Cassard, H.; Naslund, K.; et al. Enhanced Pathogenesis Caused by Influenza D Virus and Mycoplasma bovis Coinfection in Calves: A Disease Severity Linked with Overexpression of IFN-gamma as a Key Player of the Enhanced Innate Immune Response in Lungs. *Microbiol. Spectr.* **2021**, *9*, e01690-21. [CrossRef]
31. Ferguson, L.; Olivier, A.K.; Genova, S.; Epperson, W.B.; Smith, D.R.; Schneider, L.; Barton, K.; McCuan, K.; Webby, R.J.; Wan, X.F. Pathogenesis of Influenza D Virus in Cattle. *J. Virol.* **2016**, *90*, 5636–5642. [CrossRef] [PubMed]
32. Noremark, M.; Frossling, J.; Lewerin, S.S. Application of routines that contribute to on-farm biosecurity as reported by Swedish livestock farmers. *Transbound. Emerg. Dis.* **2010**, *57*, 225–236. [CrossRef] [PubMed]

33. Goecke, N.B.; Liang, Y.; Otten, N.D.; Hjulsager, C.K.; Larsen, L.E. Characterization of Influenza D Virus in Danish Calves. *Viruses* **2022**, *14*, 423. [CrossRef]
34. Wan, Y.M.; Kang, G.B.; Sreenivasan, C.; Daharsh, L.; Zhang, J.F.; Fan, W.J.; Wang, D.; Moriyama, H.; Li, F.; Li, Q.S. A DNA Vaccine Expressing Consensus Hemagglutinin-Esterase Fusion Protein Protected Guinea Pigs from Infection by Two Lineages of Influenza D Virus. *J. Virol.* **2018**, *92*, e00110-18. [CrossRef] [PubMed]
35. Chauhan, R.P.; Gordon, M.L. An overview of influenza A virus genes, protein functions, and replication cycle highlighting important updates. *Virus Genes* **2022**, *58*, 255–269. [CrossRef] [PubMed]
36. Wang, Y.; Sun, W.; Wang, Z.; Zhao, M.; Zhang, X.; Kong, Y.; Wang, X.; Feng, N.; Wang, T.; Yan, F.; et al. Amino acid sites related to the PB2 subunits of IDV affect polymerase activity. *Virol. J.* **2021**, *18*, 230. [CrossRef]

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Proteomic and Lipidomic Profiling of Calves Experimentally Co-Infected with Influenza D Virus and *Mycoplasma bovis*: Insights into the Host–Pathogen Interactions

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Abstract: The role of Influenza D virus (IDV) in bovine respiratory disease remains unclear. An in vivo experiment resulted in increased clinical signs, lesions, and pathogen replication in calves co-infected with IDV and *Mycoplasma bovis* (*M. bovis*), compared to single-infected calves. The present study aimed to elucidate the host–pathogen interactions and profile the kinetics of lipid mediators in the airways of these calves. Bronchoalveolar lavage (BAL) samples collected at 2 days post-infection (dpi) were used for proteomic analyses by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Additionally, lipidomic analyses were performed by LC-MS/MS on BAL samples collected at 2, 7 and 14 dpi. Whereas *M. bovis* induced the expression of proteins involved in fibrin formation, IDV co-infection counteracted this coagulation mechanism and downregulated other acute-phase response proteins, such as complement component 4 (C4) and plasminogen (PLG). The reduced inflammatory response against *M. bovis* likely resulted in increased *M. bovis* replication and delayed *M. bovis* clearance, which led to a significantly increased abundance of oxylipids in co-infected calves. The identified induced oxylipids mainly derived from arachidonic acid; were likely oxidized by COX-1, COX-2, and LOX-5; and peaked at 7 dpi. This paper presents the first characterization of BAL proteome and lipid mediator kinetics in response to IDV and *M. bovis* infection in cattle and raises hypotheses regarding how IDV acts as a co-pathogen in bovine respiratory disease.

Keywords: Influenza D; *Mycoplasma bovis*; proteomics; lipidomics; oxylipids; eicosanoids; bovine respiratory disease; cattle; co-infection

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1. Introduction

The increased use of molecular technologies has allowed for the discovery of previously unknown viruses, such as Influenza D virus (IDV), in the upper respiratory tract of cattle [1–3]. In addition, the widespread detection of high IDV-specific antibody titers in cattle on different continents suggests that cattle are the primary reservoir for IDV [4–11]. Although the precise role of IDV in bovine respiratory disease (BRD) remains unclear, several epidemiologic and molecular studies indicated that this virus is associated with respiratory clinical signs in calves [1–3,7,12]. In addition, experimental studies demonstrated

that IDV induces respiratory clinical signs and replicates in both the upper and lower respiratory tract of calves [13,14]. To date, IDV has also been studied during co-infection with *S. aureus* in mice, *M. ovipneumoniae* in lambs, and *M. haemolytica* and *M. bovis* in cattle, with conflicting results [13,15–17]. In mice, a primary infection with IDV did not cause any signs of disease and did not increase the susceptibility to a secondary *S. aureus* infection. In lambs, IDV alone did not induce clinical signs, but previous exposure to *M. ovipneumoniae* enhanced the inflammatory response induced by IDV. Furthermore, Zhang et al. infected calves intranasally with IDV and five days later intratracheally with *M. haemolytica*. The co-infected calves developed fewer clinical signs and less severe lesions than those infected with *M. haemolytica* alone, suggesting that IDV can reduce disease as a co-pathogen [17].

Among the different co-infections studied so far, the interaction between IDV and *M. bovis* showed the most negative impact on the health of the animals. Compared with calves infected with IDV or *M. bovis* alone, the IDV and *M. bovis* co-infected calves developed more severe clinical signs and more extensive pathological lesions in the respiratory tract. In addition, IDV co-infection increased the replication of *M. bovis*. The interaction between IDV and *M. bovis* was additionally studied in ex vivo models using precision-cut lung slices by Gaudino et al. [18]. The results suggested that a primary IDV infection promoted *M. bovis* superinfection by increasing bacterial replication and by causing damage to lung pneumocytes. Additional experiments using cytosolic helicase and Toll-like receptor (TLR) agonists indicated that IDV increases the susceptibility of cattle to bacterial superinfections, such as *M. bovis*, by impairing the innate immune response [18].

As the interaction between IDV and *M. bovis* was demonstrated to be particularly detrimental, understanding the disease mechanisms involved in this process is crucial. Proteomic analysis provides insights into the immune response and allows us to identify proteins or pathways involved in host defense or pathogenic mechanisms of a novel virus such as IDV [19]. On the other hand, lipidomic profiling at multiple time points after infection enables us to describe the processes involved in the induction and resolution of inflammation during the course of infection. Anti-inflammatory drugs that are used to treat BRD act by inhibiting cyclooxygenase (COX) enzymes that oxidize lipids involved in both inflammatory and resolutive pathways. A better characterization of the oxylipid response in the context of infection would improve our understanding of these responses and can ultimately guide both the application of medication and drug development. When used in combination, proteomic and lipidomic techniques can provide a better picture of host–pathogen interactions and disease progression. In this study, we aimed to investigate the early protein response and the kinetics of the lipidomic profile in bronchoalveolar lavage (BAL) supernatants from calves infected with IDV, *M. bovis*, or both, to elucidate the role of IDV as a co-pathogen in BRD.

2. Materials and Methods

2.1. The Animal Experiment Design and Sample Collection

A total of 29 Normand and Holstein calves were included in an experimental infection study performed at the Research Platform of Infectious Disease (PFIE, National Institute for Agronomic Research, INRAE, Nouzilly, France) in accordance with an ethical agreement (number APAFIS 16364-2018080211232403; French Ministry of Agriculture, Ethics Committee no. 019, approval date October 31th, 2018), as described previously in detail [13]. Briefly, calves were transferred to the facilities when they were between 3 and 6 days old and were demonstrated to be negative for *M. haemolytica*, *P. multocida*, *M. bovis*, *H. somni*, bovine coronavirus, IDV, bovine respiratory syncytial virus, bovine parainfluenza virus type 3, and bovine viral diarrhea virus. The absence of *M. bovis* and IDV-specific serum antibodies was confirmed using ELISA (Bio K 302; BioX diagnostics, Belgium) and hemagglutination inhibition (HI) assays, respectively. The calves were divided into four separate groups according to age and were infected by nebulization with IDV (n = 8), *M. bovis* (n = 8), or IDV and *M. bovis* (n = 8) or were similarly inoculated with non-infected cell culture medium (n = 5). The inoculum consisted of 10^7 TCID50 of Influenza virus strain

D/bovine/France/5920/2014 and/or 10^{10} CFU of *M. bovis* strain RM16 in a 10 mL volume of Dulbecco modified eagle medium (DMEM). Daily clinical examinations were performed, and nasal swabs were collected daily from day 3 pre-infection until 21 days post-infection (−3 to 21 dpi). Blood samples were collected from all calves at −1, 3, 7, 10, 14, and 21 dpi, and bronchoalveolar lavage (BAL) fluid samples were obtained from five calves per group at −5, 2, 7, and 14 dpi, as described previously [13]. Three calves per group, which were sacrificed on day 6, were excluded from the BAL sampling.

The clinical and pathological findings are summarized in the introduction above, and these data were described previously in detail [13]. As additionally previously described, IDV-RNA was detected in all IDV-infected calves, in both nasal swabs and BAL samples, and *M. bovis* DNA was detected in all co-infected calves in nasal swabs. Moreover, *M. bovis* DNA was detected in BAL samples from two out of five single *M. bovis*-infected calves and four out of five co-infected calves. Significantly higher concentrations of *M. bovis* DNA copies were detected in co-infected than in single-infected calves—in nasal secretions 4 dpi and in BAL samples 7 dpi. All non-infected calves were found negative for both pathogens by RT-qPCR in both nasal swabs and BAL samples.

2.2. Sample Processing

The BAL samples were filtered through two layers of sterile gauze, and the BAL cells and supernatant were separated by centrifugation, as previously described [20]. The supernatants were stored at −75 °C, prior to mass spectrometric analyses.

2.3. Proteomics

Bronchoalveolar lavages collected at 2 dpi were analyzed by mass spectrometry. Prior to analysis, sample volumes were reduced using Speedvac (Thermo Fisher Scientific, Waltham, MA, USA), and the total protein concentration was measured by the Bradford Protein Assay using bovine serum albumin, as standard. Aliquots containing 7 µg of protein were digested by trypsin overnight at 37 °C after reduction and alkylation. Before MS analysis, the peptides were purified and desalted by Pierce C18 Spin Columns (Thermo Fisher Scientific). These columns were activated by 2 × 200 µL of 50% acetonitrile (ACN) and equilibrated with 2 × 200 µL of 0.5% trifluoroacetic acid (TFA). The tryptic peptides were adsorbed to the media using two repeated cycles of 40 µL sample loading and the column was washed using 3 × 200 µL of 0.5% TFA. Finally, the peptides were eluted in 3 × 50 µL of 70% ACN and dried.

The dried peptides were reconstituted in 50 µL of 0.1% formic acid and diluted threefold for nano-LC-MS/MS analysis. The samples were analyzed using a QExactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ion source. The peptides were separated by reversed-phase liquid chromatography using an EASY-nLC 1000 system (Thermo Fisher Scientific). A set-up of a precolumn and an analytical column was used. The precolumn was a 2 cm EASY-column (1D 100 µm, 5 µm C18) (Thermo Fisher Scientific), while the analytical column was a 10 cm EASY-column (ID 75 µm, 3 µm, C18; Thermo Fisher Scientific). Peptides were eluted with a 90 min linear gradient from 4% to 100% ACN at 250 nL min/L. The mass spectrometer was operated in positive ion mode, acquiring a survey mass spectrum with a resolving power of 70,000 (full-width half maximum), m/z = 400–1750 using an automatic gain control (AGC) target of 3 × 106. The 10 most intense ions were selected for higher-energy collisional dissociation (HCD) fragmentation (25% normalized collision energy), and MS/MS spectra were generated with an AGC target of 5 × 105 at a resolution of 17,500. The mass spectrometer worked in data-dependent mode.

The acquired data (.RAW files) were processed in MaxQuant version 1.5.3.30, and database searches were performed using the implemented Andromeda search engine. MS/MS spectra were correlated to a FASTA database containing proteins from the Bos taurus proteome, IDV, and *M. bovis*. A decoy search database, including common contaminants and a reverse database, was used to estimate the identification false-discovery rate

(FDR). An FDR of 1% was accepted. The search parameters included: maximum 10 ppm and 0.6 Da error tolerances for the survey scan and MS/MS analysis, respectively; the enzyme specificity was trypsin; a maximum of one missed cleavage site was allowed; and cysteine carbamidomethylation was set as static modification and oxidation (M) was set as variable modification. The search criteria for protein identification were set to at least two matching peptides. Label-free quantification was applied for comparative proteomics.

The results from all fractions were combined for a total label-free intensity analysis of each sample. The collected data were filtered to include only proteins that were identified and quantified in at least four calves within at least one of the two groups compared, as previously described [21]. As previously described by Aguilan et al., label-free protein quantities (LFQ) were log2-transformed and normalized by scaling each value against the average of all proteins within a given sample [21]. A probabilistic minimum imputation technique based on the normal distribution was used to address missing values. Proteins with a differential fold change of less than −1 or greater than 1 and a *p*-value of 0.05 (−log2 *p*-value > 4.3219) were further analyzed using Ingenuity Pathway Analysis (IPA) software (version 90348151, 2023 QIAGEN).

2.4. Lipidomics

Lipids mediators were analyzed in BAL samples collected at 2, 7 and 14 dpi. The extraction protocol and LC-MS/MS analysis were performed as described in Le Faouder et al. [22]. Briefly, after protein precipitation with methanol, the samples were extracted by solid phase extraction using Oasis-HLB 96-well plates (Waters). Lipids mediators were eluted with methanol and methyl formate. The results were expressed in pg/mL of fluids.

2.5. Statistics

Statistical analyses of the lipidomic results were performed using the Kruskal–Wallis test with Dunn's multiple comparisons. For proteomic data, the normality was assessed using the Shapiro–Wilk test. If both replicates showed a normal distribution, the F-test was used to determine the homogeneity of variance, and the appropriate *t*-test (with equal or unequal variance) was then chosen for statistical comparison. Proteins with a significant deviation from a normal distribution, as determined by the Shapiro–Wilk test, were analyzed using the Mann–Whitney test. All graphs were created by GraphPad 9.5.0 (La Jolla, San Diego, CA, USA).

3. Results

3.1. IDV Suppresses the Coagulation and the Acute-Phase Response Induced by M. bovis

In total, 1188 proteins were detected in the BAL samples of infected and non-infected calves. The average (range) number of semi-quantified proteins were 480 (321–590), 527 (371–796), 462 (304–618), and 489 (440–545) in controls, IDV-, *M. bovis*-, and IDV+*M. bovis*-infected calves, respectively.

In order to identify the variance of the total protein expression for each animal 2 dpi, a principal component analysis (PCA) was performed. Although individual variability between calves was observed, a tendency of clustering according to treatment was detected as early as 2 dpi, with a similar pattern observed in the control and co-infected calves. (Figure 1).

Next, we examined the differences in protein expression in BAL samples from infected calves and controls and analyzed the probability of the activation or inhibition of biological pathways using IPA. For each comparison, the top-five pathways with the highest *p*-values were illustrated (Figure 2). The pathway named "Role of Tissue Factor in Cancer" was excluded from the analysis comparing *M. bovis*-infected calves and control calves due to the lack of relevance to acute infectious respiratory disease in calves.

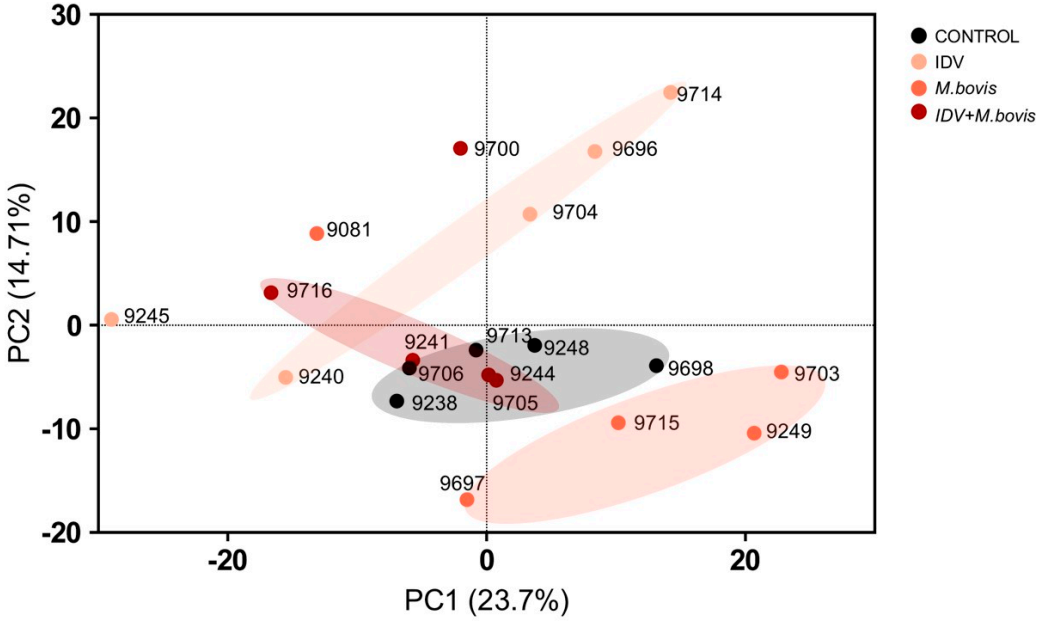

Figure 1. Proteomic principal component analysis (PCA) plot illustrating the distribution of bronchoalveolar proteome in calves at 2 dpi. The black, pink, orange, and red dots correspond to the BAL samples of the controls, IDV−, *M. bovis*− and IDV+*M. bovis*-infected calves, respectively, and are labeled with the calf identity number.

Four different comparisons were made to investigate the host response to IDV and *M. bovis* as single infections or the role of IDV and *M. bovis* as co-pathogens: IDV vs. control; *M. bovis* vs. control; IDV+*M. bovis* vs. *M. bovis;* and IDV+*M. bovis* vs. IDV. Overall, a total of eight canonical pathways were identified, which included the coagulation system, intrinsic prothrombin activation, extrinsic prothrombin activation, LXR/RXR activation, FXR/RXR activation, the neutrophil extracellular trap, acute-phase response signaling, and airway pathology in chronic obstructive pulmonary disease. Due to the small number of differentially expressed proteins in IDV+*M. bovis*- vs. IDV-infected calves, no pathways were identified in this comparison.

A total of 59 proteins were differentially expressed between IDV-infected calves and controls. Compared to the controls, the BAL samples of IDV-infected calves contained 22 downregulated proteins and 37 upregulated proteins (Figure 2, Supplement S1). Of these, nineteen proteins were found to be involved in the top-five most significant pathways. Among these 19 proteins, 7 were associated with the acute-phase response and 10 were downregulated. The acute-phase response pathway was the most significantly affected pathway, with a −log (*p*-value) of 12.2 (Figure 2). In particular, APOA2 was strongly downregulated by IDV, with a fold change of −5 and a −log 10 (*p*-value) of 6.12. On the other hand, seven proteins were implicated in the neutrophil extracellular trap pathway, six of which were upregulated (Figure 2).

Overall, 25 proteins were differentially expressed between *M. bovis*-infected calves and controls, with 15 proteins downregulated and 10 proteins upregulated by *M. bovis* (Figure 3). Of these, seven proteins were found to be involved in the top-five affected pathways. The coagulation system pathway emerged as the most significantly upregulated pathway, with

a −log (*p*-value) of 9.75 (Figure 3). Unlike in IDV-infected calves, the acute-phase response pathway was activated by the upregulation of four proteins: C9, F2, FGB, and FGG).

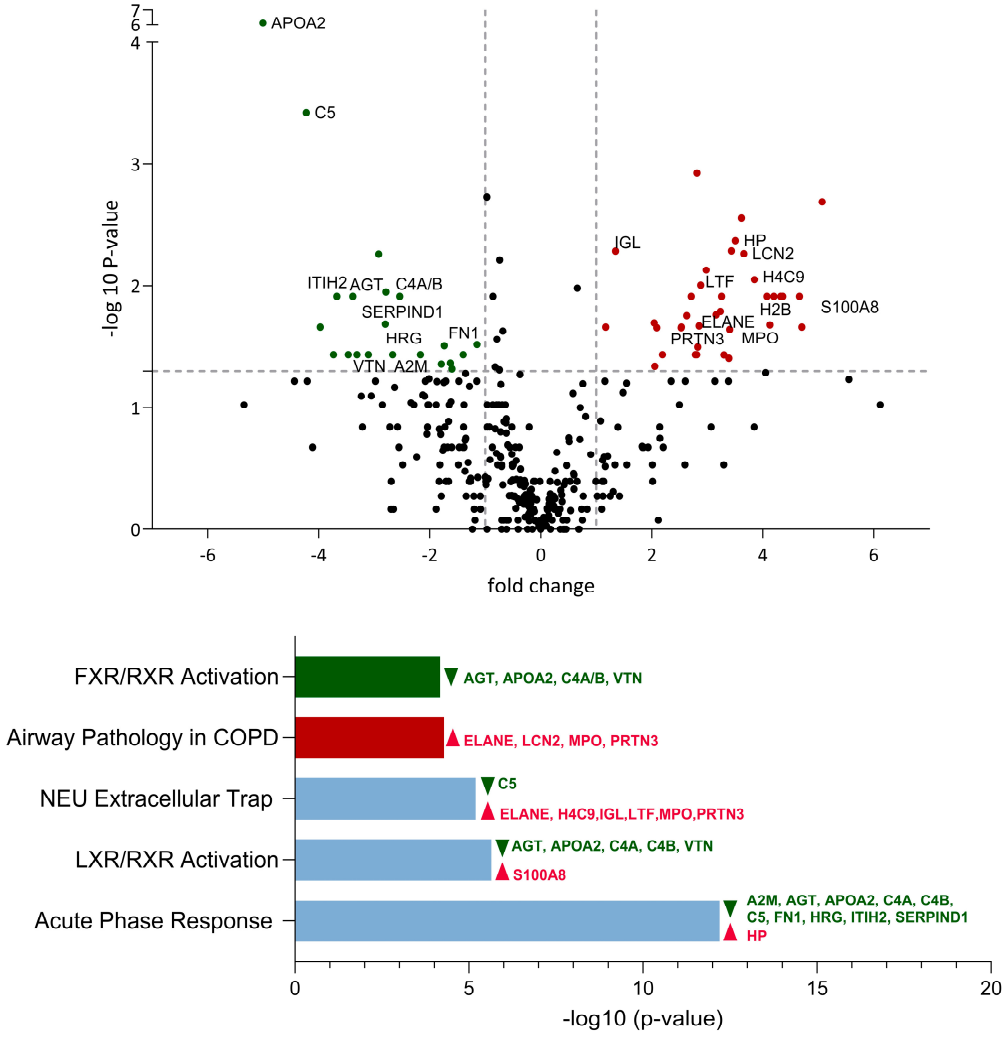

Figure 2. Comparative analysis of protein expression and associated biological pathways in bronchoalveolar lavage of IDV-infected calves vs. controls. Upregulated proteins and pathways are illustrated in red, whereas downregulated proteins and pathways are represented in green. Pathways exhibiting both upregulated and downregulated proteins are indicated in light blue. The proteins involved in the top-five significantly affected pathways are identified by their gene name. All illustrated proteins are listed in the supplementary information (Supplement S1).

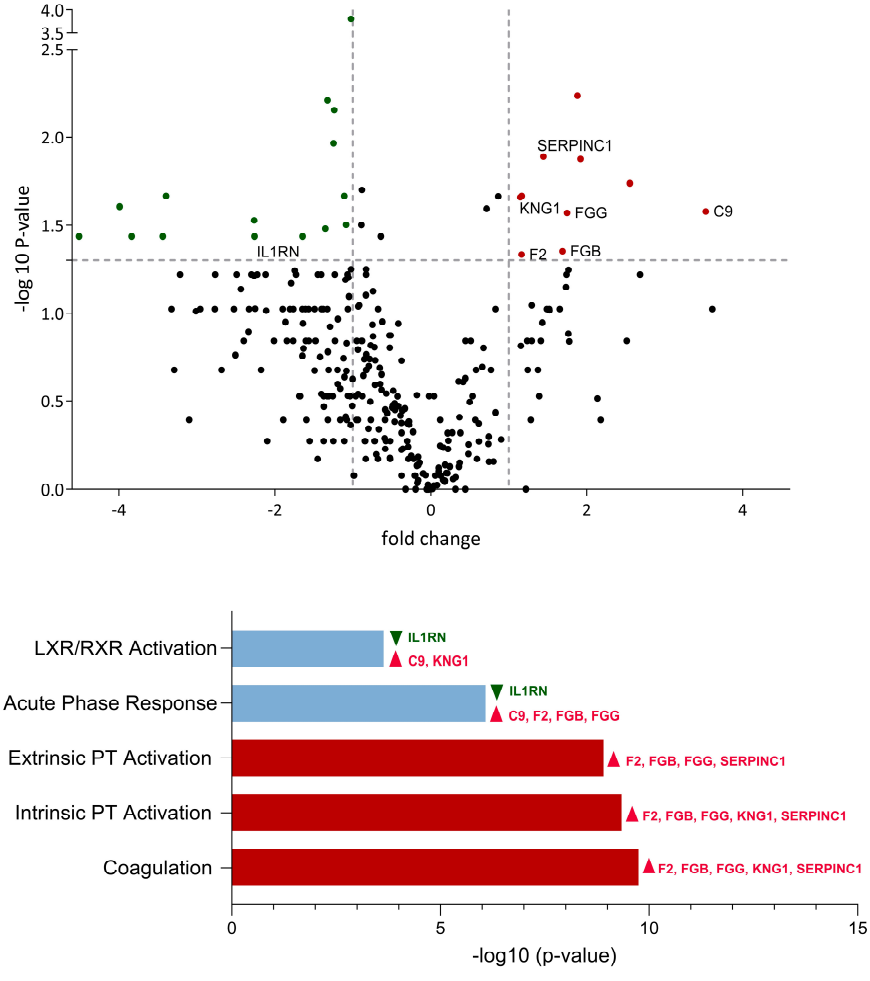

Figure 3. Comparative analysis of protein expression and associated biological pathways in bronchoalveolar lavage of *M. bovis*-infected calves vs. controls. Upregulated proteins and pathways are illustrated in red, whereas downregulated proteins and pathways are represented in green. Pathways exhibiting both upregulated and downregulated proteins are indicated in light blue. The proteins involved in the top-five significantly affected pathways are named with their gene name. All illustrated proteins are listed in the supplementary information (Supplement S1).

To investigate the effect of IDV during co-infection with *M. bovis*, we compared the proteome of IDV+*M. bovis*-infected calves and *M. bovis*-infected calves. A total of 58 differentially expressed proteins were detected, of which 17 were downregulated and 41 were upregulated by the addition of IDV (Figure 4). Despite being able to identify thirty-seven of the upregulated proteins, the software was not able to find any of these proteins among the top-five pathways. By performing a manual analysis of each of the upregulated proteins, six proteins (GPI, CD177, OSTF1, S100A11, CTSD, and GSTP1) can be associated with neutrophil degranulation. A total of thirteen downregulated proteins were identified

in the five most significantly expressed pathways. The coagulation pathway was observed to be the most significantly affected pathway, with a −log 10 (*p*-value) of 12, similar to *M. bovis*-infected calves when compared to controls. However, whereas this pathway was upregulated by *M. bovis* during a single infection, it appeared downregulated by IDV during co-infection. The acute-phase response signaling pathway contained the highest number of detected proteins, with a total of nine proteins: A2M, AMBP, C4A/C4B, FGA, FGB, FGG, FN1, and PLG. Plasminogen was particularly differentially expressed, with a *p*-value of 0.004, and, similar to FGA, AMBP, and FETUB, was differentially expressed only in the comparison between IDV+*M. bovis*-infected calves vs. *M. bovis*-infected calves (Table 1).

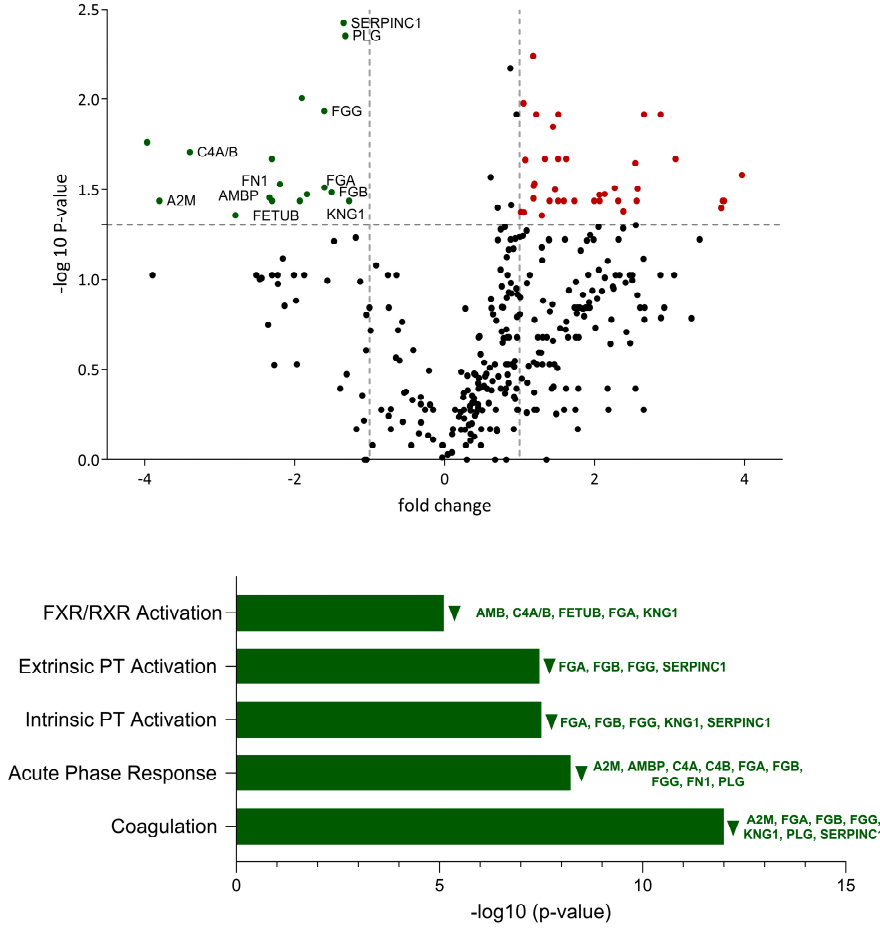

Figure 4. Comparative analysis of protein expression and associated biological pathways in bronchoalveolar lavage of IDV+*M. bovis*-infected calves vs. *M. bovis*-infected calves. Downregulated proteins and pathways are represented in green. The proteins involved in the top-five significantly affected pathways are named with their gene name. All illustrated proteins are listed in the supplementary information (Supplement S1).

Table 1. Proteins that were differentially expressed only by the addition of IDV to the *M. bovis* infection.

Protein	Description	Fold Change	*p*-Value	Biological Process
FGA	Fibrinogen alpha chain	−1.6	0.03	acute-phase response
PLG	Plasminogen	−1.3	0.004	blood coagulation
AMBP	Alpha-1-microglobulin	−2.29	0.03	negative regulation of immune response
FETUB	Fetuin B	−2.3	0.03	response to systemic inflammation

To assess the role of *M. bovis* as a co-pathogen at 2 dpi, we compared the proteome of IDV+*M. bovis*-infected calves with that of IDV-infected calves. Only eight proteins were differentially identified in the analysis, of which four were downregulated (mucin, LPO, cathepsin G, bnbd9, and PIGR) and three were upregulated (APOA1, C5, and AOC3) (Figure 5). None of these proteins was identified when comparing *M. bovis*-infected calves with non-infected calves. As mentioned above, due to the low number of proteins identified, it was not possible to identify canonical pathways.

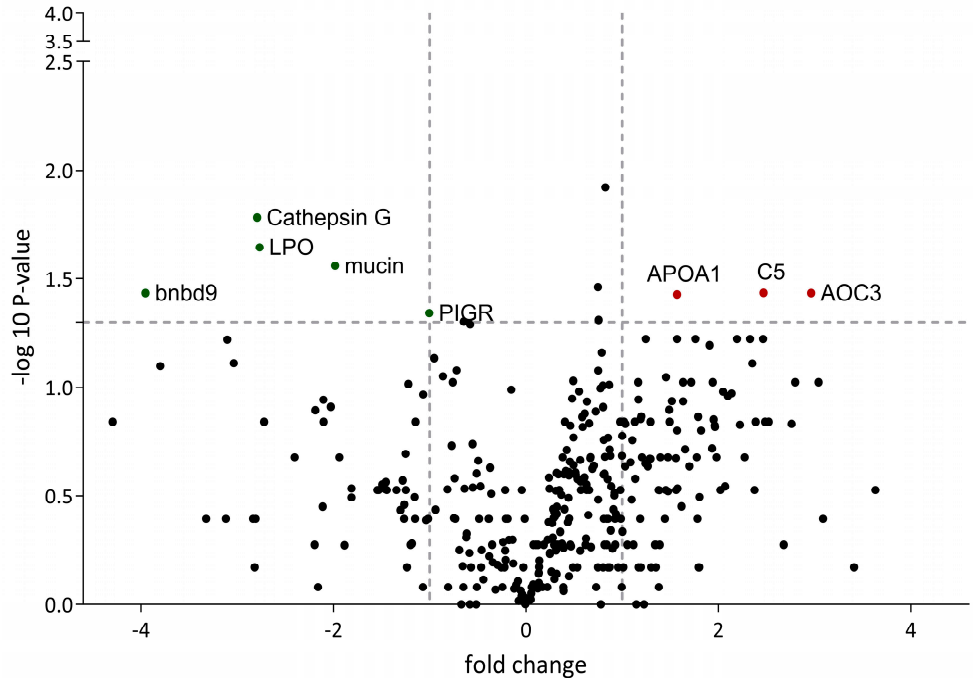

Figure 5. Comparative analysis of protein expression in bronchoalveolar lavage of IDV+*M. bovis*-infected calves vs. IDV-infected calves. Upregulated proteins are illustrated in red, whereas downregulated proteins are represented in green. All illustrated proteins are listed in the supplementary information (Supplement S1).

3.2. Co-Infection with IDV and M. bovis Induced a Higher Oxylipid Concentration in BAL Samples Than Either Single Infection at Day 7 Post-Infection

To investigate the processes of inflammation and resolution in more detail, a mass spectrometry (LC-MS/MS) analysis of lipid intermediates and mediators was performed in BAL samples obtained from all calves at 2, 7 and 14 dpi. Based on their polyunsaturated fatty acid (PUFA) precursors, 23 lipids were associated with 3 main oxylipid pathways.

The arachidonic acid (ARA) pathway consisted of PGE2, 6K-PGF1A, TXB2, 5-HETE, LTB4, 15-HETE, LXA4, LXB4, and 12-HETE. The docosahexaenoic acid (DHA) pathway included 14-HDOHE, 7(R)-MAR1, MAR2, 17-HDOHE, PDX, PD1, RVD1, RVD2, RVD5, RVD3, and RVD4. Furthermore, the eicosapentaenoic acid (EPA) pathway involved 18-HEPE, RVE1, and RVE2.

At 2 dpi, no significant difference in the activation of any of the oxylipid pathways was detected between the calves in the different groups, but the controls had the lowest overall oxylipid concentration (Figure 6).

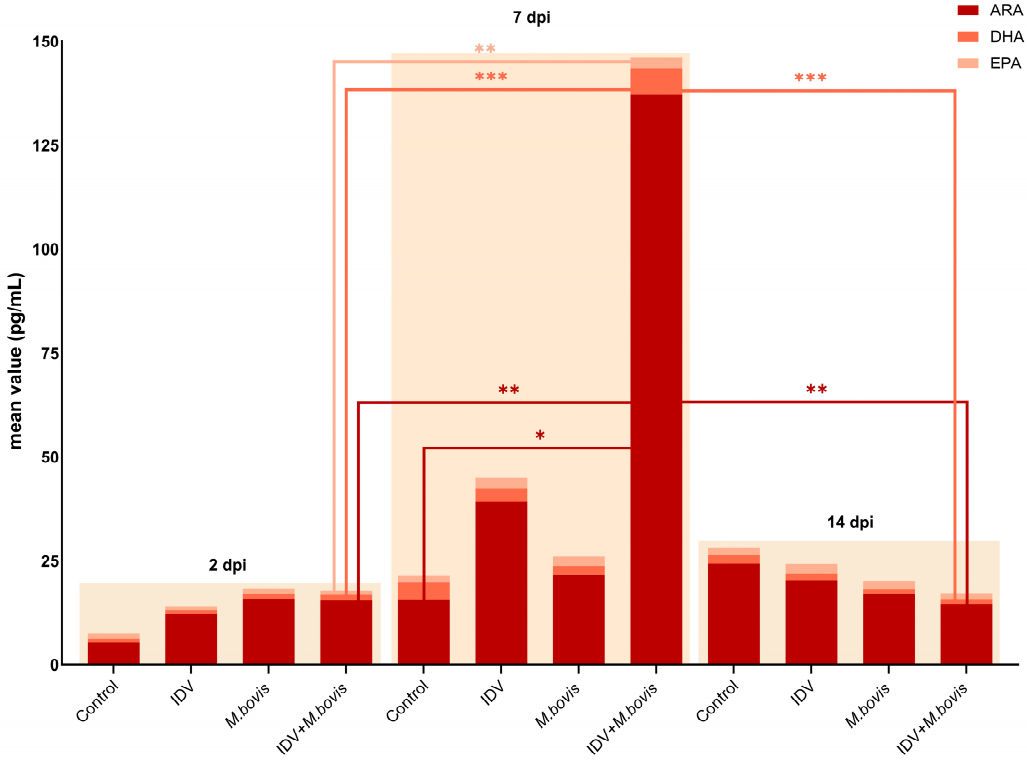

Figure 6. Dynamic changes in lipid oxidation in bronchoalveolar lavage from calves infected with IDV, *M. bovis* or IDV+*M. bovis*, or from uninfected controls, at 2, 7 and 14 days post-infection (dpi). ARA: Arachidonic Acid, DHA: Docosahexaenoic Acid, EPA: Eicosapentanoic Acid. Significance levels are indicated as follows: * (p-value < 0.05), ** (p-value < 0.01), and *** (p-value < 0.001).

At 7 dpi, a high expression of the ARA pathway was detected in the IDV+*M. bovis*-infected calves, followed by IDV-infected calves, *M. bovis*-infected calves, and controls. The increase in lipid expression within the ARA pathway between 2 and 7 dpi was significant only in the IDV+*M. bovis*-infected calves ($p < 0.01$). Between day 2 and 7, the average concentration of the different oxylipids in this pathway increased 8.8-fold in IDV+*M. bovis*-infected calves, 3.2-fold in IDV-infected calves, 1.4-fold in *M. bovis*-infected calves and 2.9-fold in the controls. Notably, among the controls, the increase was mainly attributed to one animal (No. 9238). Despite that, neither IDV nor *M. bovis* was detected in the respiratory tract of this calf, and despite having a normal body temperature and no microscopic lesions in all investigated organs, calf 9238 showed mild clinical signs from 1 dpi, such as

decreased appetite, a cough, and abnormal respiratory rate. The removal of calf 9238 from the statistical analysis did not change the final results of any of the assessments throughout the study. This only affected the mean values within the control group and consequently the visualization of the graphs due to changed standard deviations.

Whereas the expression of ARA lipids at 7 dpi was significantly higher in IDV+*M. bovis*-infected calves than in controls (Figure 6), no significant difference was observed between IDV+*M. bovis*-infected and single-infected calves. Nevertheless, a strong statistical difference was observed in the expression of ARA, DHA, and EPA oxylipids at 7 dpi compared to both 2 dpi (ARA, DHA, and EPA) and 14 dpi (ARA and DHA) in double-infected calves (Figure 6).

A non-significant reduction in the concentration of oxylipids was additionally observed in the other infected calves at 14 dpi compared to 7 dpi. The amount of ARA lipids was 9.39-, 1.93-, and 1.2-fold lower at 14 dpi than at 7 dpi for the IDV+*M. bovis*-, IDV-, and *M. bovis*-infected calves, respectively.

In terms of individual lipids, a high production of ARA-oxylipids, including PGE2, 6K-PGF1A, TXB2, 5-HETE, LTB4, 15-HETE, and 12-HETE, and the DHA lipid 14-HDOHE, was observed in all calves throughout the experiment (Figure 7). Most of the individual lipids detected in the infected calves peaked at 7 dpi.

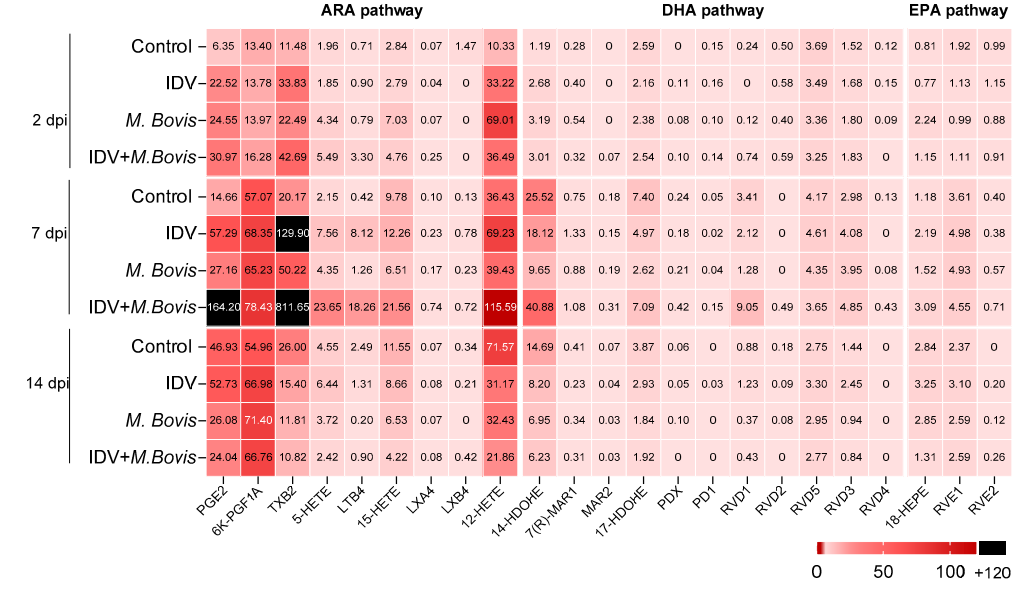

Figure 7. Heatmap displaying the mean concentration (pg/mL) of each of the 23 oxylipids analyzed.

Among the oxylipids analyzed, concentrations of PGE2, TXB2, and LTB4 were statistically different between IDV+*M. bovis*-infected calves and controls at 7 dpi (Figure 8). At 7 dpi, TXB2 was the most abundant lipid in both IDV-infected and IDV+*M. bovis*-infected calves, making it the most abundant oxylipid throughout the study.

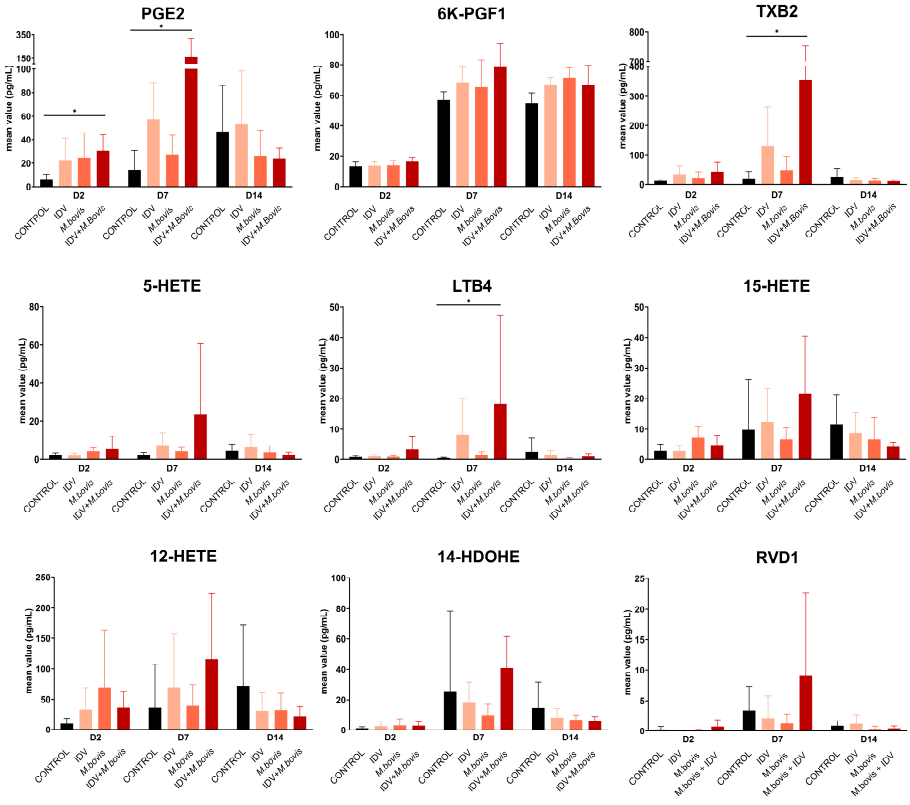

Figure 8. Oxylipids in bronchoalveolar lavage from calves infected with IDV, *M. bovis* or IDV+*M. bovis*, or from uninfected controls, at 2, 7 and 14 days post-infection. The bars denote mean value ± standard deviations. The asterisk indicates significant changes (p-value < 0.05).

4. Discussion

This study comprehensively analyzed the bronchoalveolar proteome and lipidome in calves simultaneously infected with IDV, *M. bovis*, or both.

The proteomic data indicated that IDV downregulates acute-phase response signaling and counteracts *M. bovis*-induced coagulation system activation, which are two key mechanisms in the innate immune response. In parallel, high concentrations of oxylipids that derived from the ARA pathway were detected at 7 dpi, particularly in calves infected with both pathogens.

Proteomic analyses were performed at 2 dpi, providing information on the early host response to infection. Of note, IDV, *M. bovis*, or the two pathogens in combination induced significant changes in the expression of several proteins closely associated with innate immunity as early as 2 dpi. In particular, proteins such as A2M, F2, FGA, FGG, FGB, KNG1, PLG, and SERPINC1 were found to be differentially expressed. These proteins are involved in coagulation and the acute-phase response, explaining the recurrent presence of these pathways in the enrichment analyses. While the coagulation pathway was significantly upregulated in response to *M. bovis* infection, as demonstrated by comparing *M. bovis*-infected calves with uninfected controls, the addition of IDV to induce co-infection (IDV+*M.*

bovis) resulted in an opposite effect, with the downregulation and counteraction of this response compared to *M. bovis* infection alone.

In the context of IDV vs. controls, the coagulation pathway was not identified among the top-five most significantly differentially regulated pathways; however, A2M and SERPINC1, which are proteins involved in the coagulation pathway, were downregulated with a significant −log10 *p*-value of 1.35 and 1.69, respectively (Supplementary Material). The proteins associated with the coagulation pathway (F2, FGB, FGG, KNG1, and SERPINC1) in the *M. bovis*-infected calves are predominantly associated with fibrin formation. The formation of fibrin limits infections by supporting immune cells such as neutrophils and macrophages, which are critical for pathogen clearance [23]. However, a study of *Mycoplasma pneumoniae* in children found elevated fibrinogen levels in the most severely affected children, indicating a correlation between coagulation and disease severity. The authors suggested that the excessive coagulation might be related to a massive interleukin secretion causing vascular damage [24]. Our results indicate that *M. bovis* infection induced rapid fibrin formation in the lungs of calves, as a host response. In contrast, the addition of IDV to *M. bovis* resulted in a downregulation of coagulation-associated proteins. Interestingly, with the exception of F2, proteins upregulated by *M. bovis* alone were downregulated by the addition of IDV as a co-infection. This would suggest that IDV reduces the fibrin formation triggered by *M. bovis* early in infection, delaying *M. bovis* clearance and allowing *M. bovis* replication and dissemination. Moreover, although the effect of *M. bovis* as a co-pathogen was not associated with any pathway, the observed downregulation of cathepsin G and LPO was probably related to neutrophil degranulation, which would support the idea that the combination of both pathogens delays the host response. This observation is consistent with the results of the in vivo experiment performed by Lion et al. [13].

Furthermore, A2M, PLG, and FGG were also downregulated in the IDV+*M. bovis* infection compared to *M. bovis* alone. Notably, PLG was additionally downregulated in IDV-infected calves compared to uninfected controls; however, this was with a *p*-value of 0.06 (Supplementary Material). In Influenza A virus infection, the conversion of plasminogen to plasmin activates the viral haemagglutinin and promotes the replication of the virus. Since plasminogen additionally induces cell infiltration and cytokine production, plasminogen-deficient mice show reduced lung inflammation and damage during Influenza A infection [25]. The observed reduction in PLG associated with IDV, in contrast to what has been described for Influenza A, may be one of the reasons for the low virulence in cattle and apparent non-pathogenicity in humans compared to IAV.

The acute-phase response pathway showed differential activity between infected calves. In particular, there was a downregulation of the acute-phase response when IDV was involved, in contrast to the upregulation observed in *M. bovis* single-infected calves. As previously highlighted, several coagulation proteins also contribute to the acute-phase response. However, with IDV, additional proteins (AGT, APOA2, C5, FN1, HRG, ITIH2, SERPIND1, AMBP, and C4A/B) were identified as downregulated, emphasizing the role of this pathway. The acute-phase response is responsible for the early initiation of several defense mechanisms against infectious diseases, such as inflammation, activation of immune cells, or fever [26]. Particularly, C4A/B was significantly downregulated in both IDV single- and co-infected calves, compared to uninfected controls and *M. bovis* single-infected calves, respectively. Moreover, C4, a key protein in the acute-phase response, plays an important role in both the classical and lectin complement pathways. Downregulation of C4 can lead to impaired processes such as opsonization, clearance of immune complexes, inflammatory responses, and cell recruitment, increasing susceptibility to secondary infections [27]. In parallel with the counteracting effect described for the fibrin-formation process, IDV seems to reduce the activation of basic innate immune processes. The reduced concentrations of C4 could be a key mechanism, facilitating the replication of *M. bovis*.

These results are also in line with a study by Gaudino et al., who used precision-cut lung slices as an organotypic lung model to demonstrate how IDV impairs the innate immune response induced by *M. bovis*. This impairment was characterized by a decrease

in the expression of pro-inflammatory cytokines and chemokines, such as IL-8, IL1β, and IL-17 [18].

Lipid mediator profiling of infected calves suggested the generation of several key oxylipids, produced through catalyzation by COX-1, COX-2, and LOX, across the different infection protocols. The highest total oxylipid expression was observed at 7 dpi in double-infected calves and was characterized by oxylipids that derived from arachidonic acid. Although the high ARA lipid concentrations at 7 dpi coincided with the peak of clinical signs on day 8.6 ± 1.3 in the IDV+*M. bovis*-infected calves at the group level, the individual calves with the highest oxylipid concentrations in BAL samples did not have the most pronounced clinical signs (data not shown) [13]. It should be noted that the lipidomic profile may vary depending on the viral strain studied. For example, Tam et al. demonstrated different lipid expression patterns between high and low pathogenic strains of Influenza A virus infection in mice [28]. This highlights the need to study a wider range of IDV strains in the future.

At 2 dpi, the detection of PGE2 was significantly higher in the IDV+*M. bovis*-infected calves than in controls (p-value < 0.05). Prostaglandin E2 plays a dual role in inflammation. On one hand, it is characterized by its vasodilatory properties and ability to attract macrophages to the site of inflammation [29,30]. On the other hand, it induces a switch to the resolution of inflammation by reducing the infiltration of neutrophils, promoting the production of anti-inflammatory cytokines such as interleukin-10, and inducing both apoptosis and the efferocytosis of neutrophils [31–33].

In contrast to 2 dpi, which was mainly characterized by PGE2 responses, additional lipids such as TXB and LTB4 were produced in higher amounts, particularly in the IDV+*M. bovis*-infected calves, at 7 dpi. The activation of COX-1 (TXB), COX-2 (PGE2), and LOX-5 (LTB4) in doubly infected calves on day 7 compared to uninfected controls suggests that the use of selective inhibition drugs of a single pathway may not entirely suppress inflammation. Thromboxane is involved in platelet aggregation and has vasoconstrictive properties, whereas LTB4 has a pro-inflammatory role in neutrophil recruitment, which influences immune defense mechanisms against viral infections [34,35]. A daily administration of LTB4 to IAV-infected mice enhanced the reduction of lung viral loads through the upregulation of antimicrobial peptides, compared to mice treated with a placebo [36].

Although no significant difference was found in RVD1 at 7 dpi, some of the IDV+*M. Bovis*-infected calves showed a high concentration of this pro-resolving and anti-inflammatory lipid, suggesting that a process of healing and tissue repair was carried out. In agreement with this, a significant decrease in oxylipids was observed on day 14, suggesting a significant reduction in the inflammatory process.

To fully evaluate the potential of IDV, different pathogen combinations need to be tested, including different infection orders and intervals, as well as pathogen loads. Simultaneous co-infection is a relevant starting point because calves from different herds are often grouped at markets or stocker farms, resulting in rapid and simultaneous exposure to different pathogens.

The proteomic database for cattle likely does not have the same quality and completeness as that for humans or other commonly studied model organisms. However, by comparing infected and uninfected calves, the differential protein expression provides relevant information. By including lung tissue in future investigations, additional information will be collected. The use of BAL samples presents certain challenges, including the risk of dilution effects. However, the possibility of sampling the same individual repeatedly over time, without the need to sacrifice the animal or perform surgery, is a great advantage. Bronchoalveolar lavage also provides a more representative view of the lung's immune state than analyzing a small sample of tissue, which may over- or under-represent the inflammatory state dependinsg on the area sampled.

In conclusion, this study investigated the BAL proteome and lipidome affected by IDV and *M. bovis* infection in cattle. Our observations suggest that during infection with IDV, certain pathways of the innate immune response, particularly those related to the

acute phase and coagulation, are initially suppressed. This early suppression appears to counteract the activation of the host response to *M. bovis* and consequently the clearance of this pathogen during co-infection. In contrast to single infections, co-infections with IDV and *M. bovis* acted synergistically and induced detectable changes in lipid mediators in BAL samples, which were mainly generated by the COX-1, COX-2, and LOX-5-oxidation of arachidonic acid. Although the peak of these responses matched the peak of clinical signs, there was no association between oxylipid concentrations and disease severity in individual calves. Further studies are required to improve our understanding of the role of the identified proteins and oxylipids during Influenza D detection. Further studies with different IDV strains are needed to validate the current findings and to contribute to a more comprehensive understanding of the role of IDV in the pathogenesis of BRD.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v16030361/s1, Supplement S1: Spreadsheet detailing proteins expressed in (A) IDV vs. control (B) IDV+*M. bovis* vs. *M. bovis* (C) *M. bovis* vs. control (D) IDV+*M. bovis* vs. IDV (E) Full name of lipids.

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References

1. Fulton, R.W. Viruses in Bovine Respiratory Disease in North America: Knowledge Advances Using Genomic Testing. *Vet. Clin. N. Am. Food Anim. Pract.* **2020**, *36*, 321–332. [CrossRef]
2. Mitra, N.; Cernicchiaro, N.; Torres, S.; Li, F.; Hause, B.M. Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus. *J. Gen. Virol.* **2016**, *97*, 1771–1784. [CrossRef]
3. Ng, T.F.; Kondov, N.O.; Deng, X.; Van Eenennaam, A.; Neibergs, H.L.; Delwart, E. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. *J. Virol.* **2015**, *89*, 5340–5349. [CrossRef]
4. Ferguson, L.; Eckard, L.; Epperson, W.B.; Long, L.P.; Smith, D.; Huston, C.; Genova, S.; Webby, R.; Wan, X.F. Influenza D virus infection in Mississippi beef cattle. *Virology* **2015**, *486*, 28–34. [CrossRef]
5. Alvarez, I.J.; Fort, M.; Pasucci, J.; Moreno, F.; Gimenez, H.; Naslund, K.; Hagglund, S.; Zohari, S.; Valarcher, J.F. Seroprevalence of influenza D virus in bulls in Argentina. *J. Vet. Diagn. Investig.* **2020**, *32*, 585–588. [CrossRef]
6. Oliva, J.; Eichenbaum, A.; Belin, J.; Gaudino, M.; Guillotin, J.; Alzieu, J.P.; Nicollet, P.; Brugidou, R.; Gueneau, E.; Michel, E.; et al. Serological Evidence of Influenza D Virus Circulation Among Cattle and Small Ruminants in France. *Viruses* **2019**, *11*, 516. [CrossRef]

7. Rosignoli, C.; Faccini, S.; Merenda, M.; Chiapponi, C.; De Mattia, A.; Bufalo, G.; Garbarino, C.; Baioni, L.; Bolzoni, L.; Nigrelli, A.; et al. Influenza D virus infection in cattle in Italy. *Large Anim. Rev.* **2017**, *23*, 123–128.
8. Snoeck, C.J.; Oliva, J.; Pauly, M.; Losch, S.; Wildschutz, F.; Muller, C.P.; Hubschen, J.M.; Ducatez, M.F. Influenza D Virus Circulation in Cattle and Swine, Luxembourg, 2012–2016. *Emerg. Infect. Dis.* **2018**, *24*, 1388–1389. [CrossRef]
9. O'Donovan, T.; Donohoe, L.; Ducatez, M.F.; Meyer, G.; Ryan, E. Seroprevalence of influenza D virus in selected sample groups of Irish cattle, sheep and pigs. *Ir. Vet. J.* **2019**, *72*, 11. [CrossRef]
10. Horimoto, T.; Hiono, T.; Mekata, H.; Odagiri, T.; Lei, Z.; Kobayashi, T.; Norimine, J.; Inoshima, Y.; Hikono, H.; Murakami, K.; et al. Nationwide Distribution of Bovine Influenza D Virus Infection in Japan. *PLoS ONE* **2016**, *11*, e0163828. [CrossRef]
11. Salem, E.; Cook, E.A.J.; Lbacha, H.A.; Oliva, J.; Awoume, F.; Aplogan, G.L.; Hymann, E.C.; Muloi, D.; Deem, S.L.; Alali, S.; et al. Serologic Evidence for Influenza C and D Virus among Ruminants and Camelids, Africa, 1991–2015. *Emerg. Infect. Dis.* **2017**, *23*, 1556–1559. [CrossRef]
12. Zhai, S.L.; Zhang, H.; Chen, S.N.; Zhou, X.; Lin, T.; Liu, R.X.; Lv, D.H.; Wen, X.H.; Wei, W.K.; Wang, D.; et al. Influenza D Virus in Animal Species in Guangdong Province, Southern China. *Emerg. Infect. Dis.* **2017**, *23*, 1392–1396. [CrossRef]
13. Lion, A.; Secula, A.; Rancon, C.; Boulesteix, O.; Pinard, A.; Deslis, A.; Hagglund, S.; Salem, E.; Cassard, H.; Naslund, K.; et al. Enhanced Pathogenesis Caused by Influenza D Virus and Mycoplasma bovis Coinfection in Calves: A Disease Severity Linked with Overexpression of IFN-gamma as a Key Player of the Enhanced Innate Immune Response in Lungs. *Microbiol. Spectr.* **2021**, *9*, e01690-21. [CrossRef]
14. Salem, E.; Hagglund, S.; Cassard, H.; Corre, T.; Naslund, K.; Foret, C.; Gauthier, D.; Pinard, A.; Delverdier, M.; Zohari, S.; et al. Pathogenesis, Host Innate Immune Response, and Aerosol Transmission of Influenza D Virus in Cattle. *J. Virol.* **2019**, *93*, e01853-18. [CrossRef]
15. Skelton, R.M.; Shepardson, K.M.; Hatton, A.; Wilson, P.T.; Sreenivasan, C.; Yu, J.; Wang, D.; Huber, V.C.; Rynda-Apple, A. Contribution of Host Immune Responses Against Influenza D Virus Infection Toward Secondary Bacterial Infection in a Mouse Model. *Viruses* **2019**, *11*, 994. [CrossRef]
16. Robinson, E.; Schulein, C.; Jacobson, B.T.; Jones, K.; Sago, J.; Huber, V.; Jutila, M.; Bimczok, D.; Rynda-Apple, A. Pathophysiology of Influenza D Virus Infection in Specific-Pathogen-Free Lambs with or without Prior Mycoplasma ovipneumoniae Exposure. *Viruses* **2022**, *14*, 1422. [CrossRef]
17. Zhang, X.; Outlaw, C.; Olivier, A.K.; Woolums, A.; Epperson, W.; Wan, X.F. Pathogenesis of co-infections of influenza D virus and Mannheimia haemolytica in cattle. *Vet. Microbiol.* **2019**, *231*, 246–253. [CrossRef]
18. Gaudino, M.; Lion, A.; Sagne, E.; Nagamine, B.; Oliva, J.; Terrier, O.; Errazuriz-Cerda, E.; Scribe, A.; Sikht, F.Z.; Simon, E.; et al. The Activation of the RIG-I/MDA5 Signaling Pathway upon Influenza D Virus Infection Impairs the Pulmonary Proinflammatory Response Triggered by Mycoplasma bovis Superinfection. *J. Virol.* **2023**, *97*, e0142322. [CrossRef]
19. Jean Beltran, P.M.; Federspiel, J.D.; Sheng, X.; Cristea, I.M. Proteomics and integrative omic approaches for understanding host-pathogen interactions and infectious diseases. *Mol. Syst. Biol.* **2017**, *13*, 922. [CrossRef]
20. Hagglund, S.; Blodorn, K.; Naslund, K.; Vargmar, K.; Lind, S.B.; Mi, J.; Arainga, M.; Riffault, S.; Taylor, G.; Pringle, J.; et al. Proteome analysis of bronchoalveolar lavage from calves infected with bovine respiratory syncytial virus-Insights in pathogenesis and perspectives for new treatments. *PLoS ONE* **2017**, *12*, e0186594. [CrossRef]
21. Aguilan, J.T.; Kulej, K.; Sidoli, S. Guide for protein fold change and p-value calculation for non-experts in proteomics. *Mol. Omics* **2020**, *16*, 573–582. [CrossRef]
22. Le Faouder, P.; Baillif, V.; Spreadbury, I.; Motta, J.P.; Rousset, P.; Chene, G.; Guigne, C.; Terce, F.; Vanner, S.; Vergnolle, N.; et al. LC-MS/MS method for rapid and concomitant quantification of pro-inflammatory and pro-resolving polyunsaturated fatty acid metabolites. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2013**, *932*, 123–133. [CrossRef]
23. Weisel, J.W.; Litvinov, R.I. Fibrin Formation, Structure and Properties. *Subcell Biochem.* **2017**, *82*, 405–456. [CrossRef]
24. Li, Y.T.; Zhang, J.; Wang, M.Z.; Ma, Y.M.; Zhi, K.; Dai, F.L.; Li, S.J. Changes in coagulation markers in children with Mycoplasma pneumoniae pneumonia and their predictive value for Mycoplasma severity. *Ital. J. Pediatr.* **2023**, *49*, 143. [CrossRef]
25. Berri, F.; Rimmelzwaan, G.F.; Hanss, M.; Albina, E.; Foucault-Grunenwald, M.L.; Le, V.B.; Vogelzang-van Trierum, S.E.; Gil, P.; Camerer, E.; Martinez, D.; et al. Plasminogen controls inflammation and pathogenesis of influenza virus infections via fibrinolysis. *PLoS Pathog.* **2013**, *9*, e1003229. [CrossRef]
26. Monach, P.A. Complement. *Arthritis Rheumatol.* **2024**, *76*, 1–8. [CrossRef]
27. Wang, H.; Liu, M. Complement C4, Infections, and Autoimmune Diseases. *Front. Immunol.* **2021**, *12*, 694928. [CrossRef]
28. Tam, V.C.; Quehenberger, O.; Oshansky, C.M.; Suen, R.; Armando, A.M.; Treuting, P.M.; Thomas, P.G.; Dennis, E.A.; Aderem, A. Lipidomic profiling of influenza infection identifies mediators that induce and resolve inflammation. *Cell* **2013**, *154*, 213–227. [CrossRef]
29. Funk, C.D. Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science* **2001**, *294*, 1871–1875. [CrossRef]
30. Nakayama, T.; Mutsuga, N.; Yao, L.; Tosato, G. Prostaglandin E2 promotes degranulation-independent release of MCP-1 from mast cells. *J. Leukoc. Biol.* **2006**, *79*, 95–104. [CrossRef]
31. Honda, A.; Sugimoto, Y.; Namba, T.; Watabe, A.; Irie, A.; Negishi, M.; Narumiya, S.; Ichikawa, A. Cloning and expression of a cDNA for mouse prostaglandin E receptor EP2 subtype. *J. Biol. Chem.* **1993**, *268*, 7759–7762. [CrossRef]
32. Levy, B.D.; Clish, C.B.; Schmidt, B.; Gronert, K.; Serhan, C.N. Lipid mediator class switching during acute inflammation: Signals in resolution. *Nat. Immunol.* **2001**, *2*, 612–619. [CrossRef]

33. Bystrom, J.; Evans, I.; Newson, J.; Stables, M.; Toor, I.; van Rooijen, N.; Crawford, M.; Colville-Nash, P.; Farrow, S.; Gilroy, D.W. Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. *Blood* **2008**, *112*, 4117–4127. [CrossRef]
34. Borgeat, P.; Samuelsson, B. Transformation of arachidonic acid by rabbit polymorphonuclear leukocytes. Formation of a novel dihydroxyeicosatetraenoic acid. *J. Biol. Chem.* **1979**, *254*, 2643–2646. [CrossRef]
35. Le Bel, M.; Brunet, A.; Gosselin, J. Leukotriene B4, an endogenous stimulator of the innate immune response against pathogens. *J. Innate Immun.* **2014**, *6*, 159–168. [CrossRef]
36. Gaudreault, E.; Gosselin, J. Leukotriene B4 induces release of antimicrobial peptides in lungs of virally infected mice. *J. Immunol.* **2008**, *180*, 6211–6221. [CrossRef] [PubMed]

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This thesis explores the role of influenza D virus (IDV) in bovine respiratory disease (BRD), with a focus on the detection in Sweden and interactions with other respiratory pathogens. Bovine respiratory disease is a complex disease with a major impact on cattle health and productivity, and IDV is increasingly recognized as a potential co-pathogen worldwide. To better understand the role of IDV, the work combined epidemiological surveys, genetic characterization, and in vitro and in vivo models of coinfection with key respiratory pathogens. Analyses of immune responses revealed that IDV modulates innate immune pathways, particularly during coinfections. Altogether, the findings suggest a modulatory role for IDV in BRD pathogenesis and highlight the need for ongoing surveillance.

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