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Mme MARIA GAUDINO

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Directeur(s) de Thèse :

MME MARIETTE DUCATEZ

M. GILLES MEYER

Rapporteurs :

M. FENG LI, University of Kentucky

MME SABINE RIFFAULT, INRAE JOUY-EN-JOSAS

Membre(s) du jury :

M. JEAN-FRANÇOIS VALARCHER, UPPSALA UNIVERSITET SUEDE, Président

M. GILLES MEYER, ECOLE NATIONALE VETERINAIRE DE TOULOUSE, Membre

MME CHANTAL SNOEK, LUXEMBOURG INSTITUTE OF SC. & TECH, Membre

MME MARIETTE DUCATEZ, ECOLE NATIONALE VETERINAIRE DE TOULOUSE, Membre

MME SARA HÄGGLUND, UPPSALA UNIVERSITET SUEDE, Membre

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Abbreviations

7-AAD: 7-aminoactinomycin D

BAstV: Bovine Astrovirus

BAT2: bovine alveolar type 2

BCoV: Bovine Coronavirus

BoHV-1: Bovine Herpesvirus type 1

BPIV-3: Bovine Parainfluenza type 3

BRD: Bovine Respiratory Disease

BRSV: Bovine Respiratory Syncytial Virus

BVDV: Bovine Viral Diarrhea Virus

BWA: Burrows-Wheeler Aligner

CCL5: C-C Motif Chemokine Ligand 5

CFU: Colony forming unit

CXCL10: C-X-C motif chemokine ligand 10

DAPI: 4',6-diamidino-2-phenylindole

ELISA: Enzyme-Linked Immunosorbent Assay

FBS: Fetal Bovine Serum

GFP: Green Fluorescent Protein

HA: Hemagglutinin

HEF: Hemagglutinin-Esterase-Fusion

HEK: Human Embryonic Kidney

HI: Hemagglutination Inhibition

hRT18g: human Rectal Tumor 18g

IAV: Influenza A virus

IBV: Influenza B virus

ICV: Influenza C virus

ICTV: International Committee on Taxonomy of Viruses

IDV: Influenza D virus

IFN: Interferon

IL: Interleukin

iNOS: inducible nitric oxide synthase

ISG15: Interferon-stimulated gene 15

LDH: lactate dehydrogenase

MHC-II: major histocompatibility complex class 2

MN: Microneutralization

MyD88: Myeloid differentiation primary response 88

NA: Neuraminidase

NF-kB: Nuclear Factor Kappa-light-chain-enhancer of activated B cells

NGS: Next Generation Sequencing

NLPR3: NOD-like receptor family, pyrin domain containing 3

NOD2: nucleotide-binding oligomerization domain 2

ORF: Open Reading Frame

Pam3CSK4: Pam3CysSerLys4

PB1: Polymerase basic 1

PI: Propidium Iodide

PCR: Polymerase chain reaction

PFA: Paraformaldehyde

Poly(I:C): Polyinosinic-polycytidylic acid

RLR: RIG-I-like receptor

RNA: Ribonucleic Acid

RNP: Ribonucleoprotein

RT-qPCR: Quantitative reverse transcription PCR

SEM: Standard Error of Mean

SPF: Specifi-pathogen free

ST: Swine testis

TCID50: 50% of Tissue Culture Infectious Dose

TLR: Tool-like receptor

tMRCA: time to the Most Recent Common Ancestor

TRAF6: TNF Receptor Associated Factor 6

TRIF: TIR-domain-containing adapter-inducing interferon-β

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Introduction

Co-infecting pathogens within the Bovine Respiratory Disease (BRD) complex

Bovine Respiratory Disease (BRD) complex is a general term for a range of respiratory disorders that can affect the lower respiratory tract of cattle. BRD is the second most devastating disease that touches the beef industry, after neonatal calf diarrhea (1), and it leads to poor animal health and economic losses (2). Several pneumonia predisposing factors have been described in cattle, such as stress, herd management, poor air quality and the presence of respiratory pathogens (3,4). Indeed, BRD is recognized as a polymicrobial disease (5) and during respiratory outbreaks multiple pathogens, especially viruses and bacteria, are isolated from animals with clinical signs (6–8). In cattle, synergistic effects of viral and bacterial co-infections have been described in literature and the simultaneous presence of multiple pathogens is often associated with an exacerbation of the clinical outcome of the respiratory pathology (5). Primary viral infections that modulate the immune response and that facilitate subsequent bacterial superinfections are frequently described and their role in BRD onset is also supported by experimental evidence (9,10). To date, the viruses most frequently associated with BRD include bovine parainfluenza type 3 (BPIV-3), bovine herpesvirus type 1 (BoHV-1), bovine respiratory syncytial virus (BRSV), and bovine viral diarrhea virus (BVDV) (11). On the other hand, the most important bacterial pathogens causing pneumonia in cattle are *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni* and *Mycoplasma bovis* (12).

Based on current scientific literature, we can classify these respiratory pathogens in four different categories depending on their tropism and their known role in BRD:

i) Pathogens with a known respiratory tropism

This group includes pathogens that are isolated from animals with respiratory signs and, upon experimental infection *in vivo*, can cause respiratory signs of different intensity in challenged animals, therefore fulfilling all Koch's postulates. We can classify within this group BRSV (13–

16), BoHV-1 (17–19), and to a lesser extent BPIV-3 (20,21), IDV (22–24), Bovine adenoviruses (25–28) and *M. bovis* (29–31) .

ii) Bacterial commensal in other anatomical districts that can become opportunistic in the lower respiratory tract, inducing pneumonia

This second group is based on the current hypothesis that some bacteria that are commensal of the upper respiratory tract of healthy cattle can colonize the lower respiratory tract following the exposure of a triggering factor and can subsequently become opportunistic, inducing pneumonia. The list includes the bacteria belonging to the *Pasteurellaceae* family (taxonomic class of Gammaproteobateria), such as *Pasteurella multocida*, *Mannhemia haemolytica* and *Histophilus somni*. The pathogens belonging to this category do not fulfil necessarily all Koch's postulates, as upon experimental inoculation *in vivo*, the induction of pneumonia is not systematically observed in all calf models, suggesting that other factors intervene in their complex pathogenesis. For *P. multocida*, in different experiments the inoculation of the A3 serotype could induce clinical signs and lung lesions in calves (32–34) but also in buffalos with the A1 serotype (35). On the contrary, in other studies the lesions and the overall pathology seemed milder (36). Similarly, experimental infections with *M. haemolytica* alone often failed to induce severe bronchopneumonia (9,21,37,38), but in some studies experimentally infected animals developed severe clinical illness and reached end-point limits during the study (39,40). Such differences among studies could be possibly due to intrinsic characteristics of the animals (immune status, age and breed) but also intrinsic differences in the bacterial strains that are not known yet, as the reported studies used the same serotype, similar doses of pathogens and they were performed on animals of a similar age.

The classification of certain respiratory bacterial pathogens within this group has however certain limitations, as pneumonia onset within a feedlot can be also caused by the circulation of virulent bacterial strains and not solely the exposure to stress factors that trigger lung colonization by commensals. As a matter of fact, differences in the pathogenicity of different bacterial strains were described for *M. haemolytica* (41) but also for *P. multocida* (42). This suggests that BRD onset may be caused by both the circulation of virulent bacterial strains but

also the exposure to stress factors that can compromise the host immunity and hence allow the LRT colonization by commensals that inhabit the URT.

iii) Pathogens with non-exclusive respiratory tropism but with known consequences on respiratory pathology after infection

The pathogens belonging to this category can be isolated from animals with BRD, however upon experimental challenge their primary tropism is not exclusively respiratory. For instance, BVDV induces a wide spectrum of pathologies such as lesions of mucosal (especially intestinal) and lymphoid tissues that can result in acute diarrhea, thrombocytopenia and respiratory signs (43,44). Likewise, BCoV has long been defined as virus with a double pneumoenteric tropism. Upon challenge its primary tropism is intestinal, however in some studies it could also induce pneumonic lesions in cattle (45–48). In the field BCoV is often isolated during respiratory outbreaks (6,49) but its etiological role in BRD remains questioned today (50).

iv) Pathogens detected in the respiratory tract in sick animals but with unknown role on BRD

The last category includes viruses that were detected in the respiratory tract of animals with respiratory signs but so far attempts of isolation have failed/or their inoculation did not induce any clinical signs. Over the past decade, the Next Generation Sequencing (NGS) technology has seen a substantial evolution, with an increase in precision, throughput and accessibility (51). This has led to a critical improvement in pathogen's discovery (52), by facilitating the detection of novel pathogens that could escape the traditional detection methods (specific PCRs, serology or culture). In the BRD context, the NGS technique has allowed to improve the study of the respiratory microbiome, as well as metagenomic investigation of the presence of pathogenic viruses and bacteria in the upper and the lower respiratory tract of cattle. In a few recent studies, the metagenomic approach revealed the presence of different viruses in the respiratory tract that were known to have a different tropism. One example is represented by Bovine Astroviruses (BAstV), which are enteric viruses that are known etiological agents for

calf diarrhea (53,54). In different studies, BAstV were detected by NGS in the upper and lower respiratory tract of calves with bronchopneumonia (55–58). However, BAstV were never been isolated so far, therefore preventing the investigation of their possible respiratory tropism upon challenge. Another example are Bovine Rhinitis Virus Type A and B (BRAV and BRBV), which upon inoculation in naïf calves failed to induce any clinical signs (59), suggesting therefore a limited role in BRD onset. A summary of the classification of different pathogens within different categories (based on their putative role in BRD onset) is available in Table 1.

Table 1: Summary of the different pathogens involved in BRD and their putative role in its onset.

Category	Pathogens	References
Pathogens with known respiratory tropism	BRSV	(13–16)
	BoHV-1	(17–19)
	BPIV-3	(20,21)
	IDV	(22–24)
	M. bovis	(29,31)
	Bovine Adenovirus	(25–28)
Bacterial commensals in other anatomical districts that can become opportunistic in the lower respiratory tract, inducing pneumonia	*Mannheimia haemolytica*	(60–62)
	Histophilus somni	(63,64)
	Pasteurella multocida	(33,65)
Pathogens with non-exclusive respiratory tropism but with known consequences on respiratory pathology after the infection	BVDV	(66–68)
	BCoV	(45–48)

Pathogens detected in respiratory tract in sick animals but with unknown role on BRD	Bovine Astrovirus	(55,57,58)
	Influenza C virus	(57,69,70)
	Bovine rhinitis virus	(55–57)
	Ungulate bocaparvovirus 1	(55–57)

BRD co-infections and the novel IDV: State of the art and aim of the work

Respiratory outbreaks in cattle have a multifactorial origin often involving several pathogens (7,8,71) and many of the microbial associations in respiratory co-infections are now considered to negatively impact the clinical outcome of the infection. These respiratory co-infections, are frequently associated with an increase in severity of disease, gross lung lesions, and in some cases a decrease in the survival rate (19,72,73). As reported in humans (74,75), primary viral infections can aggravate the respiratory illness caused by bacterial superinfections in cattle. Studies in young cattle have shown a predisposing role for Bovine ParaInfluenza Type 3 (BPIV-3) (20), Bovine Herpesvirus Type 1 (BoHV-1) (18,19,37,73), and Bovine Respiratory Syncytial Virus (BRSV) (72,76,77) in facilitating secondary bacterial infections. In these studies, primary viral infections were followed a few days later by an inoculation with *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, or *Mycoplasma bovis*, which are all important bacterial pathogens involved in the BRD complex (78–80).

Although some respiratory viruses have been known for several years and their pathogenesis is well characterized, recently discovered viruses were described in cattle affected by respiratory illness and could now be included in the list of BRD pathogens. It is the case of the novel Influenza D virus (IDV), which was first isolated in 2011 from swine with Influenza-like illness in the United States (81). IDV circulation was documented in humans (82–84) and several animal species (85–89), but cattle is considered its primary host. In the last few years, IDV detection in clinical samples has been positively associated with respiratory disease in cattle through metagenomic approaches (55,56). This was further confirmed by experimental infections with IDV that revealed viral replication in both the upper and lower respiratory tract with moderate respiratory signs onset in calves (22,23). At necropsy, infected animals displayed subacute broncho-interstitial pneumonia with neutrophil infiltration in bronchial lumens and neutrophilic and macrophagic alveolitis (23). Transcriptomic and proteomic analyses on broncho-alveolar lavages of infected animals revealed an up-regulation of the pathogen recognition receptors (especially of cytosolic receptors NOD2 and RIG-I, but also Toll-like receptors TLR3, TLR7, TLR9), pro-inflammatory cytokines, and chemokines, consistent with the inflammatory lesions observed in lungs at necropsy (23,24). If clinical signs induced

by IDV infection were moderate, experimental co-infection with IDV and *M. bovis* resulted in enhanced bacterial colonization of the lower respiratory tract leading to exacerbated clinical manifestations associated with severe microscopic and macroscopic lung lesions (24). Although experimental evidence suggests a potential IDV impact on cattle respiratory health, the mechanisms underlying the possible increase in susceptibility to bacterial superinfections remain partially unknown for IDV.

There is still a lack of data about IDV actual circulation and about its possible role in BRD onset. In the present work we therefore investigated IDV prevalence in cattle herds in France and other European countries and we studied its evolution in the last decade, since its first discovery on the continent. In addition, we studied IDV role in bacterial superinfections in experimental *ex vivo* models.

The main scientific questions that will be investigated in this work will be the following:

i) What is the experimental evidence about the impact of co-infections on BRD?

Before experimentally studying the impact of IDV on co-infections, an extensive literature research about experimental *in vivo* and *in vitro* studies was done in order to review the existing data about the predisposing role of respiratory viruses on bacterial superinfections in cattle. In addition, data about mixed viral and mixed bacterial infections was also collected.

ii) What is IDV prevalence in cattle herds?

IDV was recently discovered and cattle seems its primary host. IDV infection was described for the first time in Europe in France in 2012 and the last positive samples were collected in 2014 (90), however no data about IDV circulation in the following years is available for this country. We first reviewed the available IDV serology results in literature and compared their prevalence between cattle and swine and between different European countries. We then collected new cohorts of samples (in a South Western France region) for molecular screening to investigate IDV circulation in this geographic region in veal calves' farms. In addition, IDV circulation was also investigated in a cohort of 883 nasal swabs collected in Québec, Canada.

iii) How did IDV evolve since its discovery?

Two major clades of IDV (named D/OK and D/660) have been described on different continents so far. In addition, divergent clades have been described but have been limited to

specific geographic areas (i.e. Japan, California). In order to better understand IDV evolution throughout the years and its introduction on the European continent, phylogenetic analyses were carried out on all available IDV sequences and on newly generated sequences from this work. The tMRCA of different clades and the evolutionary rate of IDV HEF were estimated by BEAST analysis.

iv) What is the impact of the novel respiratory IDV on BRD in cattle?

If clinical signs induced by IDV infection alone *in vivo* are moderate (22,23), experimental co-infection with IDV and *M. bovis* resulted in enhanced bacterial colonization of the lower respiratory tract leading to exacerbated clinical manifestations associated with severe microscopic and macroscopic lung lesions (24). Although experimental evidence suggests a potential IDV impact on cattle respiratory health, the mechanisms underlying the possible increase in susceptibility to bacterial superinfections remain partially unknown for IDV. We therefore carried out experimental co-infections with IDV and *M. bovis* on *ex vivo* organotypic model of bovine lung (PCLS) in order to decipher the mechanisms underlying IDV and *M. bovis* synergistic interplay upon airway colonization.

To resume, to better understand the role of new pathogens that were never considered before as BRD triggers, using Influenza D virus as case study, we will use two different approaches:

1) Investigation of the prevalence, distribution and evolution of IDV in cattle farms
2) Investigation of the impact of IDV in BRD in experimental conditions in co-infection with bacteria

Chapter 1: The Bovine Respiratory Disease (BRD) complex and the role of co-infections in its pathogenesis

1.1 Review article: Understanding the mechanisms of viral and bacterial co-infections in bovine respiratory disease: a comprehensive review on experimental evidence

PhD candidate's contribution:

The candidate conceptualized the work, collected the data on relevant literature, drafted the article and generated the figures.

Summary of the review

Bovine respiratory disease (BRD) is one of the most important diseases impacting the global cattle industry, resulting in significant economic loss. Commonly referred to as shipping fever, BRD is especially concerning for young calves during transport when they are most susceptible to developing disease. Despite years of extensive study, managing BRD remains challenging as its aetiology involves complex interactions between pathogens, environmental and host factors. While at the beginning of the 20th century, scientists believed that BRD was only caused by bacterial infections ("bovine pasteurellosis"), we now know that viruses play a key role in BRD induction. Mixtures of pathogenic bacteria and viruses are frequently isolated from respiratory secretions of animals with respiratory illness. The increased diagnostic screening data has changed our understanding of pathogens contributing to BRD development. In this review, we aim to comprehensively examine experimental evidence from all existing studies performed to understand coinfections between respiratory pathogens in cattle. Despite the fact that pneumonia has not always been successfully reproduced by in vivo calf modelling, several studies attempted to investigate the clinical significance of interactions between different pathogens. The most studied model of pneumonia induction has been reproduced by a primary viral infection followed by a secondary bacterial superinfection, with strong evidence suggesting this could potentially be one of the most common scenarios during BRD onset. Different in vitro studies indicated that viral priming may increase bacterial adherence and colonization of the respiratory tract, suggesting a possible mechanism underpinning

bronchopneumonia onset in cattle. In addition, a few in vivo studies on viral coinfections and bacterial coinfections demonstrated that a primary viral infection could also increase the pathogenicity of a secondary viral infection and, similarly, dual infections with two bacterial pathogens could increase the severity of BRD lesions. Therefore, different scenarios of pathogen dynamics could be hypothesized for BRD onset which are not limited to a primary viral infection followed by a secondary bacterial superinfection.

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VETERINARY RESEARCH

REVIEW Open Access

Understanding the mechanisms of viral and bacterial coinfections in bovine respiratory disease: a comprehensive literature review of experimental evidence

Maria Gaudino, Brandy Nagamine, Mariette F. Ducatez* and Gilles Meyer*

Abstract

Bovine respiratory disease (BRD) is one of the most important diseases impacting the global cattle industry, resulting in significant economic loss. Commonly referred to as shipping fever, BRD is especially concerning for young calves during transport when they are most susceptible to developing disease. Despite years of extensive study, managing BRD remains challenging as its aetiology involves complex interactions between pathogens, environmental and host factors. While at the beginning of the twentieth century, scientists believed that BRD was only caused by bacterial infections ("bovine pasteurellosis"), we now know that viruses play a key role in BRD induction. Mixtures of pathogenic bacteria and viruses are frequently isolated from respiratory secretions of animals with respiratory illness. The increased diagnostic screening data has changed our understanding of pathogens contributing to BRD development. In this review, we aim to comprehensively examine experimental evidence from all existing studies performed to understand coinfections between respiratory pathogens in cattle. Despite the fact that pneumonia has not always been successfully reproduced by in vivo calf modelling, several studies attempted to investigate the clinical significance of interactions between different pathogens. The most studied model of pneumonia induction has been reproduced by a primary viral infection followed by a secondary bacterial superinfection, with strong evidence suggesting this could potentially be one of the most common scenarios during BRD onset. Different in vitro studies indicated that viral priming may increase bacterial adherence and colonization of the respiratory tract, suggesting a possible mechanism underpinning bronchopneumonia onset in cattle. In addition, a few in vivo studies on viral coinfections and bacterial coinfections demonstrated that a primary viral infection could also increase the pathogenicity of a secondary viral infection and, similarly, dual infections with two bacterial pathogens could increase the severity of BRD lesions. Therefore, different scenarios of pathogen dynamics could be hypothesized for BRD onset which are not limited to a primary viral infection followed by a secondary bacterial superinfection.

Keywords: Bovine respiratory disease, respiratory viruses, respiratory bacteria, coinfections, cattle, bacterial superinfection, in vitro, experimental infections, influenza D virus

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*Correspondence: mariette.ducatez@envt.fr; gilles.meyer@envt.fr
IHAP, Université de Toulouse, INRAE, ENVT, Toulouse, France

1 Bovine respiratory disease: the prelude of a respiratory outbreak

Bovine respiratory disease (BRD) is a general term for a range of respiratory disorders that can affect the lower respiratory tract in cattle. BRD is the second most common disease impacting the global beef industry, after neonatal calf diarrhoea [1], being a particular burden in young cattle and pre-weaned calves. Economic loss due to treatment costs, reduced performance (i.e. loss of weight or absence of weight gain, lighter carcass at slaughter or reduced milk production in dairy farms) and animal death can be substantial for producers [2]. Moreover, the high consumption of antibiotics to treat BRD causes concern over the emergence of antimicrobial resistance in cattle and also in humans, indirectly via the food chain, water, air, and manured and sludge-fertilized soils [3], thus threatening both animal and human health.

Early BRD manifestations include general signs, such as lack of appetite, self-isolation, depression and fever. These signs can evolve to more severe respiratory signs including nasal and eye discharge, salivation, rapid breathing, dyspnoea and prominent coughing [4]. BRD is known to be a multifactorial syndrome, triggered by a combination of environmental factors and infectious agents. Among environmental factors, events such as transportation and handling (i.e. for dehorning) are the most important stressful experiences for animals, as well as weaning or changes of feed [5, 6]. Cattle transportation alone is an important trigger in BRD, causing an increase in mortality during respiratory outbreaks, especially when following secondary bacterial infection [7]. Other environmental factors include the combination of insufficient ventilation, wet and dirty bedding, dust exposure and overcrowding, which can increase the possibility of pathogens transmission [8]. Also, the general microbial pressure in the environment due to lack of good hygiene practice can increase the risk of infections. Elements such as good colostrum quality and management, normal level of essential nutrients and adequate rest (especially after shipping) are essential for calves to maintain a normal immune function in response to challenging pathogens [9], as well as minimum stress exposure (i.e. good care when handling and using low stress techniques). Biosecurity measures (i.e. isolating new or sick animals and avoiding housing animals of mixed ages together) can also significantly decrease the risk of pneumonia outbreaks in cattle herds [9]. Lastly, routine feedlot vaccination can reduce the likelihood of primary viral infection, significantly reducing mortality [10]. In this review we will focus on the principal infectious agents involved in BRD and how the interactions between these pathogens impact pathogenesis.

2 Most common infectious agents involved in BRD: from the twentieth century up to now

At the beginning of the twentieth century, BRD was believed to be solely caused by bacterial infections and thus referred to as "bovine pasteurellosis" or, as reported in the first descriptions of the disease in late nineteenth century, as "haemorrhagic septicaemia" [11]. Around the 30 s', scientists started to observe that beside *Pasteurella spp.* infection, other factors played a role in the disease development [12]. Animals experimentally inoculated with bacteria alone failed to reproduce the typical pneumonia signs [11, 13]. In addition, these bacteria could be cultured from apparently healthy animals after they were stressed such as during shipping (for this reason BRD was often referred as "shipping fever" during the last century) but also overcrowding, weaning and weather variations [14, 15]. In the 50 s', the theory of viral causation gained support in North America, when bovine herpesvirus-1 (BoHV-1), the etiological agent of infectious bovine

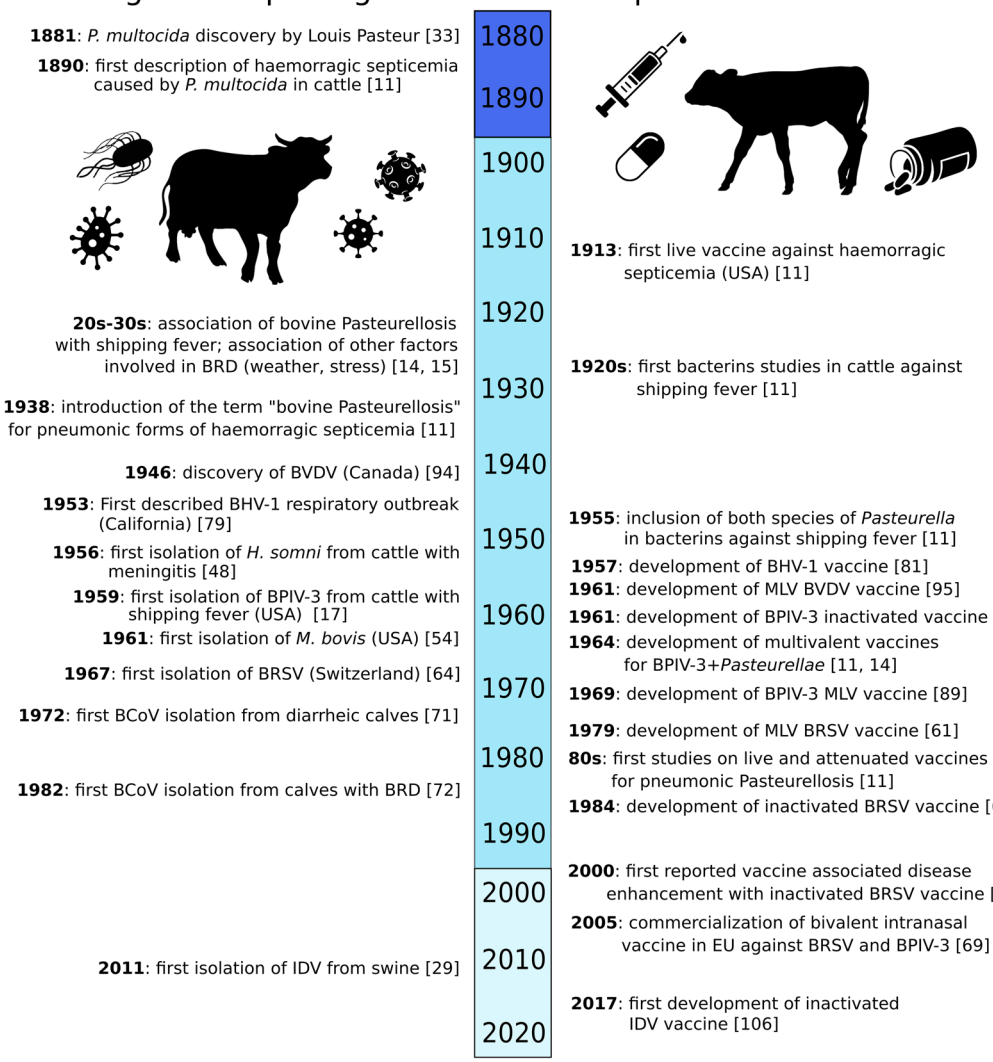

Figure 1 Timeline showing examples on the history of BRD pathogens discovery and adopted countermeasures throughout the years. An emphasis on vaccine countermeasures taken in Europe was given.

rhinotracheitis (IBR) [16], and bovine parainfluenza virus type 3 (BPIV-3), known as myxovirus parainfluenza 3 at that time, were isolated from cattle with shipping fever [12, 17]. During experimental infection, BPIV-3 mimicked natural pneumonia [18] with bacterial superinfections often accentuating the clinical signs and lesions in animals (Figures 1, 2).

BRD is now globally recognized as a polymicrobic disease, with bacterial coinfections known to affect the morbidity and mortality during viral respiratory infections [19]. Although the majority of pneumonia outbreaks are predominantly caused by bacteria and viruses, some fungi belonging to *Aspergillus* spp. genus [20] and parasites, commonly known as "lungworms" [21], can also trigger respiratory disease. Bacteria are generally isolated at higher prevalence in cattle with respiratory signs and because of this, antibiotic treatment is often the first choice made by veterinarian practitioners to avoid a rapid progression to severe BRD [22]. The most common bacteria isolated from cattle with respiratory signs belong to the *Pasteurellaceae* family, the most prevalent being *Pasteurella multocida*, *Mannhemia haemolytica* and *Histophilus somni* [23]. These three pathogens are also commensals of the upper respiratory tract (nasopharynx and tonsils) in healthy calves but can subsequently become opportunistic when host defences are compromised, leading to colonization of the lower respiratory tract [24]. Another class of bacteria that plays an

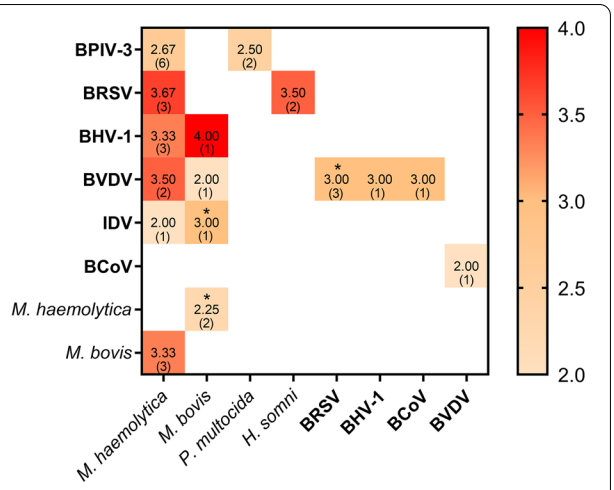

Figure 2 Heat map showing the impact of sequential coinfections on respiratory pathology in cattle on in vivo experiments. On the y-axis, the virus used for the primary viral infection is represented. On the x-axis, the pathogen used for the secondary superinfection is listed. The severity of coinfections on in vivo studies (compared to single pathogens) was given a score from 1 to 4 (colour code for the score is given in function of the increase in clinical signs, light orange to dark orange). The description of the scoring system that we used to describe the impact of coinfection in vivo is available as Additional file 1. Cell values represent the mean between the scores given to different in vivo studies performed with the same pathogens. The value in parentheses represents the number of trials carried out for each couple of pathogens that were used to calculate the mean score. White cells indicate an absence of in vivo studies for that specific couple of pathogens. *: the two pathogens were simultaneously inoculated in some studies.

important role in BRD belongs to the *Mycoplasmataceae* family, specifically the *Mycoplasma* spp. genus. Among these, *Mycoplasma bovis* is one of the most widespread, leading to the highest morbidity [25]. *Mycoplasma dispar* and *Mycoplasma bovirhinis* can be isolated from sick cattle as well [26, 27]. On the other hand, viruses also play an important role in BRD. Some viruses have been well known BRD agents for years and their pathogenesis is well characterized, whereas others have less clear roles. This list of viruses includes bovine respiratory syncytial virus (BRSV), bovine coronavirus (BCoV), bovine herpesvirus 1 (BoHV-1), BPIV-3 and bovine viral diarrhea virus (BVDV) [28]. Also, thanks to the advent of new generation of sequencing technologies (next generation sequencing (NGS)) new viruses have been discovered and could now be part of the official list of BRD pathogens, i.e. influenza D virus (IDV) [29–31]. Some viruses are thought to be more benign with an incidental finding during coinfection, but others such as BRSV can have a major pathogenic potential and can be the only etiological agent responsible for a respiratory outbreak in cattle herds, especially during the winter season [32].

To better understand the dynamic interactions between the various cattle respiratory pathogens, we will discuss the most common BRD-associated pathogens in the following paragraphs. Treatment options and preventive measures (i.e. vaccines) will also be covered for each pathogen.

2.1 *Pasteurella multocida*

Pasteurella multocida is a Gram-negative bacterium that can infect a wide range of mammals and domestic birds. It was first discovered by Louis Pasteur around 1881 during the investigation of the etiological agent of fowl cholera [33]. Since the same bacteria could produce disease in different animal species, in 1939, scientists proposed to classify all these bacterial strains under the same genus and species, thereafter named *Pasteurella multocida* [34]. It is currently classified into five capsular groups (named from A to E) and 16 somatic serotypes (1 to 16). In cattle, *P. multocida* A:3 is the most common serotype isolated from animals displaying BRD and its pathogenicity has been confirmed in experimental studies [35]. In addition, serogroups B, E and F can be pathogenic in this species [36]. *P. multocida* infection in cattle can cause different types of bronchopneumonia, ranging from subacute to chronic fibrinopurulent but also fibrinous and fibro-necrotizing, which can be accompanied by a variable amount of intra-alveolar haemorrhage with moderate to severe neutrophils and macrophages infiltration in bronchi and bronchioles [37]. Vaccines to prevent *P. multocida* infection consist of bacterins (killed bacteria) [38] and the only available treatments are antibiotics, despite rising antibiotic resistance, as recently reported [39].

2.2 *Mannheimia haemolytica*

M. haemolytica is another important Gram-negative bacterium involved in calf pneumonia. It was previously known as "*Pasteurella haemolytica*" but a revisitation of the *Pasteurellaceae* classification based on genetic similarity suggested its removal from the *Pasteurella* genus and thus the creation of a new genus named *Mannheimia* [40]. Hence, in this review, some scientific studies from before 1999 still contain the ancient nomenclature "*Pasteurella haemolytica*". Currently, *M. haemolytica* is classified based on 12 capsular serotypes (named A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16 and A17) [41]. Serotypes associated with respiratory disease in cattle are prevalently A1 and A6 [42]. Infected animals can first display general clinical signs such as fever along with loss of appetite and weight loss but also respiratory signs such as cough, nasal discharge and respiratory distress. The

principal cause of death is acute fibrinous pleuropneumonia due to the obstruction of bronchioles and alveoli with fibrinous exudate [43]. Necropsy commonly reveals fibrinosuppurative pneumonia, necrotizing inflammatory response and alveolar damage and necrosis due to neutrophil and macrophage infiltration in the lung and fibrin deposition in the alveoli [41]. Vaccines containing *M. haemolytica* leucotoxin, its main virulent factor [44], are currently available. However, there is still a lack of data in the scientific literature to reinforce the full efficacy of this preventive measure [45]. Intranasal probiotic administration of *Lactobacillus* strain in order to prevent *M. haemolytica* colonization of the upper respiratory tract has been evaluated in a clinical trial and could represent a future possibility for the prevention of cattle pneumonia [46].

2.3 *Histophilus somni*

H. somni is a Gram-negative bacterium that mainly affects cattle but can occasionally also infect small ruminants [47]. Unlike *P. multocida* and *M. haemolytica*, the circulating strains of *H. somni* are not currently classified into specific serotypes and no comprehensive nomenclature is available to date. It was first isolated in 1956 from cattle with meningitis [48]. Animals of all ages can be affected but recently, it was shown that weaned calves seem to be at higher risk of infection [49]. Although *H. somni* is considered, like the other mentioned *Pasteurellaceae*, a commensal bacterium of the nasal tract, different strains have also been isolated from urogenital secretions, which can be responsible for venereal spread [50]. When the bacterium colonizes lungs and gains access to the blood stream, it can cause systemic disease that is not limited to the respiratory tract. *H. somni* infection can thus also cause encephalitis, myocarditis and sudden death due to acute septicaemia [51]. Post-mortem findings in the lungs include bronchopneumonia and fibrinous pleurisy [52]. Diagnosis based on gross lesions is accompanied by bacterial culture and molecular testing. Treatment options include large-spectrum antibiotics such as florfenicol but, similarly to *M. haemolytica*, bacterins are currently available as preventive measure, although they have failed to demonstrate effective protection in vaccinated animals [53].

2.4 *Mycoplasma bovis*

M. bovis is a particular type of bacteria that greatly differs from those we previously described. Its represents one of the most challenging bacterial BRD pathogens. First isolated in 1961 [54], *M. bovis* causes pneumonia outbreaks in calves and young cattle but also mastitis in dairy cows, as well as otitis and abortion [55]. Like all the other members of the *Mycoplasmataceae* family, it is the smallest known bacteria. It lacks a cell wall, making it naturally resistant to several classes of antibiotics [56]. Clinical signs of infected animals can include fever, depression, nasal discharge, shallow breathing and cough. Post-mortem findings include bronchopneumonia with characteristic caseous necrotic lesions and also fibrinosuppurative bronchopneumonia [57]. Once introduced to a farm (i.e. through contaminated animals), eradication is difficult due to its strong environmental resistance [58] and widespread herd dissemination through direct contact [57]. Being a persistent intracellular bacterium lacking a cell wall reduces the choice for antibiotic treatment, representing another obstacle for its elimination. In addition, other major challenges include high antigenic variability of surface glycoproteins and the ability to evade host immune system [59]. Treatment efficacy is questionable with treated animals relapsing after a few weeks, in part due to increased antibiotic resistance over time [60]. A few vaccines are currently commercialized in North America, consisting of bacterins which offer limited protection [59].

2.5 Bovine respiratory syncytial virus

BRSV (also known as bovine orthopneumovirus) is one of the most important viral pathogens involved in BRD. It is a single-stranded RNA virus belonging to the *Pneumoviridae* family (order *Mononegavirales*) [61]. Although it is similar to the human respiratory syncytial virus (around 40% of nucleotide identity) [62], BRSV has only been diagnosed in cattle as well as wild and domesticated small ruminants [63] and it is not considered a zoonotic pathogen. The first report of BRSV infection in cattle dates from 1967 in Geneva, Switzerland [64], after which it spread to other countries. There are currently ten circulating lineages [65], as based on genotyping of a small immunogenic region in the glycoprotein G which is important for antibody recognition. The biological significance of the antigenic variation in this region might thus be relevant for vaccine efficacy [32]. BRSV has the highest pathogenic potential among all circulating viruses in cattle with clinical signs ranging from mild-moderate to subclinical. Less frequently, BRSV infection can progress to respiratory acute distress syndrome including fever, depression, decreased food intake, and dyspnoea with open-mouth breathing that can exacerbate during late stage infection [66]. In some cases, up to 80% of morbidity is reported, with mortality reaching up to 20% [67]. Emphysematous and haemorrhagic lung lesions, as well as necrotizing bronchiolitis and interstitial pneumonia, especially in the cranial lobes, are characteristics of BRSV infection at necropsy [66]. The infection can also produce the typical multi-nucleated syncytial cells formed by the fusion of several cells caused by the fusion protein F.

Several vaccines are available on the market as a prophylactic measure against BRSV infection [68, 69].

2.6 Bovine coronavirus

BCoV is a single-stranded RNA virus belonging to the *Coronaviridae* family (*Coronavirinae* subfamily, order *Nidovirales*), and is classified within the Betacoronavirus 1 subgroup (Embecovirus) [70]. It was first isolated in 1972 from diarrheic calves [71] and in 1982 from BRD calves [72]. Endemic in cattle worldwide, it is known for its pneumo-enteric tropism, causing both enteric disease (especially calf diarrhoea) and pneumonia outbreaks [73]. After experimental BCoV inoculation, colostrum-deprived calves develop cough, nasal discharge, respiratory distress and diarrhoea [74]. Treatment for the enteric disease associated with BCoV infection is largely limited to supportive care (i.e. rehydration, electrolyte administration, and the use of nonsteroidal anti-inflammatory drugs [75, 76]). Several vaccines against the enteric form are currently available [77]. Vaccines protecting against BCoV respiratory-associated disease are still missing.

2.7 Bovine herpesvirus type 1

BoHV-1 is a DNA virus belonging to the *Herpesviridae* family (subfamily *Alphaherpesvirinae*, order *Herpesvirales*) and the known etiological agent for infectious bovine rhinotracheitis (IBR) [78]. It is believed to have been first isolated from German cattle with venereal disease in the nineteenth century and later associated with respiratory disease during a 1954 outbreak in California [79–81]. BoHV is divided into two circulating subtypes, BoHV-1.1 and BoHV-1.2 [82], which are both characterized by acute inflammation of the upper respiratory tract but can also sporadically cause abortion in cattle, as well as conjunctivitis, vaginitis and enteritis [83]. In particular, respiratory signs associated with BoHV-1 infection include mucopurulent nasal discharge (sometimes accompanied by ulcers in mouth and nose), conjunctivitis, coughing, sneezing, and difficult breathing [84]. BoHV-1 in cattle is characterized by lifelong latent infection with sporadic viral reactivation and shedding when immune defences are compromised (i.e. following a stressful event such as shipping) [85]. Commercially available vaccines are broadly used in various European countries to prevent BoHV-1 associated syndrome leading to progressive eradication of the disease as part of a monitoring program for control maintenance and eradication [86].

2.8 Bovine parainfluenza type 3

BPIV-3 is a single-stranded RNA virus belonging to the *Paramyxoviridae* family (genus *Respirovirus*, order *Mononegavirales*) [87]. It was first isolated in 1959 from cattle with shipping fever and named "myxovirus shipping fever 4" (SF-4) [17, 88, 89]. BPIV-3 is now endemic, with three circulating genetic groups worldwide, named A, B, and C [90]. Infection with BPIV-3 usually leads to mild respiratory signs, such as fever, dry cough, nasal and ocular discharge, increased respiratory rate and dyspnoea [91, 92]. Infection of the upper respiratory tract can also lead to a transient immunosuppression, creating an opportunity for secondary bacterial superinfections [87], a component of calf enzootic pneumonia. Several vaccines are commercially available, often in association with BRSV [68].

2.9 Bovine viral diarrhea virus

BVDV is a single-stranded RNA virus belonging to the *Flaviviridae* family [93]. It is a member of the genus *Pestivirus*, first discovered in North America during the 40 s' and later isolated in 1957 [94, 95]. Two different Pestivirus species are currently in circulation, Pestivirus A (formerly known as BVDV-1) and Pestivirus B (formerly known as BVDV-2) [93]. Infection with BVDV often manifests as respiratory and gastrointestinal disease, the latter being associated with diarrhoea and mucosal disease (when a cytopathic strain is involved), especially during persistent infections [96, 97]. BVDV induces lesions of mucosal (especially intestinal) and lymphoid tissues that can result in acute diarrhoea, thrombocytopenia and respiratory signs [98, 99]. Its main role during BRD is immunosuppressive, paving the way for subsequent superinfections by other viral or bacterial respiratory pathogens. Vaccine prophylaxis via maternally derived antibodies has been shown effective at protecting cows and newborn calves but efforts are still to be made to eradicate the disease [100].

2.10 Influenza D virus

Influenza D virus (IDV) is a single-stranded RNA virus belonging to the *Orthomyxoviridae* family (genus *Deltainfluenzavirus*, order *Articulavirales*). Like Influenza C (ICV), it has a segmented genome consisting of seven genomic segments, unlike Influenza A and B viruses (IAV and IBV) that harbour eight segments [29]. IDV was discovered in 2011, making it the most novel bovine respiratory pathogens to date [29]. Unlike the other genera of the *Orthomyxoviridae* family, IDV is most prevalently found in cattle, which is considered its primary host [101]. To a lesser extent, IDV can also infect small ruminants, swine and feral swine, camelids, horses and hedgehogs [102]. Several lines of evidence suggest that IDV can be zoonotic but to what extent is currently being investigated [101].

Different circulating IDV genotypes have been characterized through sequence analysis of the hemagglutinin

esterase-fusion (HEF) segment, the most prevalent being "D/OK" and "D/660" with divergent lineages present in Japan, Canada and the United States of America [8, 103, 104]. IDV also seems to undergo genetic reassortments among its different lineages which is a common feature of influenza viruses [8]. Pathogenic differences amongst the different circulating strains remain questionable as IDV can be isolated from both sick and healthy animals and is often found alongside other pathogens in cattle with BRD signs [8]. Calves experimentally infected with IDV display mild to moderate signs of repeated spontaneous coughing, abdominal dyspnoea with increased respiratory rates, and abnormal lung sounds [105]. Upon necropsy, the lung tissue reveals subacute bronchointerstitial pneumonia with neutrophils in bronchial lumens, neutrophilic and macrophagic alveolitis, as well as microscopic alveolar lesions [105]. A vaccine that confers partial protection in cattle was developed in a research study but has not been commercialized [106].

2.11 Other influenza viruses

The role of other influenza viruses in BRD still remains unclear to date. Natural infections of IAV virus in cattle have been reported, as well as few studies showing low seroprevalence of IAV infection in this species [107]. In addition, experimental challenges showed that cattle can develop moderate to severe clinical signs and seroconversion following IAV infection [107]. Despite all these pieces of evidence, cattle is not considered a host for IAV, unlike swine and avian species. Several reports described ICV detection in samples from sick cattle [108–110], suggesting its circulation in cattle population, similarly to IDV. However, studies of experimental infections in cattle are currently missing in literature and convincing proof of its pathogenicity and role in BRD in cattle are still to be provided.

3 Prevalence of coinfections in cattle herds: an interplay between viruses and bacteria

RT-qPCR commercial kits and decreased NGS costs have made the detection of multiple respiratory pathogens from clinical samples simpler and cost effective. Today, BRD is recognized as a polymicrobial disease with numerous studies acknowledging the high frequency of coinfections. 50.73% of nasal swabs taken over a four-year period from Canadian cattle ($n=883$) showing respiratory signs were positive for at least two respiratory pathogens [8], supporting a 2018 study, that detected at least two pathogens in 41% of the nasal swabs ($n=23$) collected from steers during a respiratory outbreak in Brazil [111]. Bronchoalveolar lavages collected in Denmark from 46 healthy calves and 46 sick calves tested for respiratory pathogens revealed similar coinfecting pathogenic abundance. However, *H. somni* was the only pathogen that was positively associated to cattle with BRD [112]. In another study, lungs from Irish cattle with BRD were submitted for post-mortem examinations and dual infections were detected in 58% of lungs, with a high prevalence especially for *M. haemolytica* and *H. somni* coinfection [49]. The authors reported that *P. multocida* was the pathogen identified alone with the greatest frequency and the most frequently detected virus/bacteria coinfections were *P. multocida*/BPIV-3, *H. somni*/BPIV-3, or *H. somni*/BRSV. Studies using metagenomics approaches on respiratory samples also confirmed that presence of multiple pathogens is more associated with illness than mono-infections. In a first study, the virome found in nasal swabs of 50 young dairy cattle with BRD was compared to 50 location-matched healthy control animals [30]. Viruses were detected in 68% and 16% of sick animals and healthy control animals, respectively. In addition, 38% of sick animals (versus 8% of controls) were infected with multiple respiratory viruses. Similar results were reported in another case–control study [110]. However, in another study that used a similar metagenomic approach, the authors failed in finding differences in terms of viral presence between sick and healthy animals in nasal swabs from feedlot cattle [31].

4 Impact of coinfections on respiratory pathology in cattle: what is the experimental evidence?

4.1 Viral and bacterial coinfections: the importance of primary viral infections preluding secondary bacterial superinfection

The occurrence of a primary viral infection followed by a secondary bacterial superinfection is the most common and well documented coinfection model of respiratory syndrome complex applied to cattle, swine [113], and humans [114]. Over the past 60 years, several studies have investigated the clinical ramifications of different bacterial and viral pathogenic interactions. The majority of the studies describes in vivo challenges during which young calves were inoculated with a viral pathogen followed by a bacterial superinfection a few days later. Most of the bacterial strains used belonged to the *Pasteurellaceae* family (*M. haemolytica*, *P. multocida* or *H. somni*), the classical etiological agents causing pneumonia in cattle. In two studies, *M. bovis* was concomitantly or subsequently inoculated after a viral strain. In this section, we comprehensively review the underlying mechanisms leading to enhanced pathogenicity during mixed respiratory infections in cattle. Table 1 summarizes the in vivo studies that were performed in calves to study the viral/bacterial respiratory coinfections. The description of the scoring system used to describe the impact of coinfection in vivo is available as Additional file 1.

Table 1 In vivo studies from the scientific literature performed on young calves to assess the impact of virus/bacteria coinfection on BRD

Reference	Primary viral challenge (route of infection and dose/animal)	Time between exposure to the two pathogens	Secondary bacterial challenge (route of infection and dose/animal)	Main results of the clinical trials	Impact of the coinfection on BRD (score 1 to 4)	Study limitations
Collier et al. [115]	BoHV-1	3 days	*M. haemolytica*	Coinfected group: longer duration of illness	3	
Hamdy et al. [15]	BPIV-3	6 h	*P. multocida*/*M. haemolytica*	BPIV-3 group: no respiratory disease, transient leukopenia Coinfected group: severe respiratory disease and pneumonic lesions	3	The animals were stressed, which could be a confounding factor
Saunders et al. [92] Trial 7	BPIV-3 (Intratracheal, 5 mL of 10^6 $TCID_{50}$/mL)	30 days	*P. multocida*/*M. haemolytica* (Intratracheal, 10 mL of 10^9 CFU/mL)	BPIV-3 group: slight febrile response and leukopenia, nasal discharge and cough Exposure to *M. haemolytica* and *P. multocida* one month later did not provoke illness	2	Small number of animals (2), lack of mono-infected controls
Saunders et al. [92] Trial 8	BPIV-3 (Intratracheal, 5 mL of 10^6 $TCID_{50}$/mL)	Simultaneous	*P. multocida*/*M. haemolytica* (Intratracheal, 10 mL of 10^9 CFU/mL)	BPIV-3 group: nasal discharge until day 3 Coinfected group: 40 °C fever and increased nasal discharge until day 11	3	Small number of animals (2), lack of non-infected controls
Baldwin et al. [116]	BPIV-3		*M. haemolytica*	Coinfected group: more severe respiratory symptoms upon subsequent exposure to *M. haemolytica*	3	
Collier et al. [117]	BoHV-1 (Intratracheal)	30 days	*M. haemolytica* (Aerosol)	Coinfected group: bronchopneumonia leading to the death of one calf	4	
Jericho et al. [118]	BoHV-1 (Aerosol, 10^6 to 10^{10} $TCID_{50}$/mL)	3 to 4 days	*M. haemolytica* (Aerosol, 5.5×10^5–1.8×10^{10} CFU/mL)	Coinfected group: signs of bronchopneumonia ~ 4 days after virus exposure *M. haemolytica* group: no clinical signs	3	Viral and bacterial shedding were determined only after exposure; unclear number of animal/group

Table 1 (continued)

Reference	Primary viral challenge (route of infection and dose/animal)	Time between exposure to the two pathogens	Secondary bacterial challenge (route of infection and dose/animal)	Main results of the clinical trials	Impact of the coinfection on BRD (score 1 to 4)	Study limitations
*Al-Darraji et al. [120, 124, 165, 166]	BRSV (Transtracheal, 20 mL of 2.9×10^4 PFU/mL)	3 and 5 days	*M. haemolytica* biotype A serotype 1 (Transtracheal, 5 mL of 3×10^7 CFU/mL)	*M. haemolytica* group: reduced physical activity BRSV group: inactivity, fever Coinfected group: inactivity, dry and intermittent cough, fever, increased respiratory rate, dyspnoea, anorexia, signs more pronounced in 5 day delayed group, loss of condition until the end of the experiment	4	
Yates et al. [119]	BoHV-1 (Aerosol, 10^7 PFU)	4, 10, 20 and 30 days	*M. haemolytica* biotype A serotype 1 (Aerosol, 10^6 PFU)	Coinfected group: higher fever, lung and pharyngeal lesions more severe in animals with a 4-day delay	3	Lack of control groups (non-infected, mono-infected)
Carrière et al. [120]	BPIV-3 (Aerosol, 100 mL of 5×10^4 $TCID_{50}$)	4 and 7 days	*M. haemolytica* biotype A serotype 1 (Aerosol, 100 mL of 10^{12} CFU)	All groups (BPIV-3, *M. haemolytica* and coinfected): no difference in terms of lung lesions, increase in rectal temperature and respiratory rate	2	
*Trigo et al. [124]	BRSV (Aerosol)	0, 3 and 6 days	*M. haemolytica* (Intranasal)	Virus or bacteria alone groups: mild clinical response BRSV + *M. haemolytica* superinfected group: increased pulmonary lesions; mono-infected groups: no observed lesions Coinfected group: higher rectal temperature compared to *M. haemolytica* group	3	
Potgieter et al. [121]	BVDV (Endobronchial inoculation)	5 days	*M. haemolytica* (Endobronchial inoculation)	BVDV group: fever, nasal discharge, cough *M. haemolytica* group: mild signs Coinfected group: severe fibrinopurulent bronchopneumonia and pleuritis	3	

Table 1 (continued)

Reference	Primary viral challenge (route of infection and dose/animal)	Time between exposure to the two pathogens	Secondary bacterial challenge (route of infection and dose/animal)	Main results of the clinical trials	Impact of the coinfection on BRD (score 1 to 4)	Study limitations
Potgieter et al. [129]	BRSV (Endobronchial inoculation, 10^8 $TCID_{50}$)	8 days	*H. somni* (Endobronchial inoculation, 10^7-10^9CFU)	BRSV group: no signs *H. somni* group: mild signs (fever, occasional cough and depression) Coinfected group 10^9 CFU: severe clinical signs (mortality, diffused pneumonic lesions); 10^7 CFU: mild clinical signs, less extended lung lesions	4	
*Sharma et al. [125]	BRSV (Intranasal)	6 days	*M. haemolytica* biotype A serotype 1 (Intranasal and intratracheal, 5 mL of 9×10^7 CFU/mL)	Coinfected group: increased disease score, higher fever and higher mortality than both mono-infected groups	4	BRSV dose is not reported
Gånheim et al. [122]	BVDV (Intranasal, 2 mL of 10^6 $TCID_{50}$/mL)	5 days	*M. haemolytica* (Intranasal, 10 mL of 5×10^7 CFU/mL)	Mono-infected groups: a few calves had fever and depression Coinfected group: all animals had fever and mild to severe depression, one calf did not recover, slower bacterial clearance, duration of elevated APPs lasted longer in coinfected group than BVDV but similar in *Mannheimia* group	4	
Gershwin et al. [128]	BRSV (Aerosol, 5 mL of 10^6 $TCID_{50}$)	6 days	*H. somni* (Intratracheal, (10 mL of 10^8 CFU)	BRSV group: no lung lesions *H. somni* group: limited lung lesions Coinfected group: higher magnitude and duration of clinical signs, isolation of *H. somni* in lungs, extended lung lesions	3	Pathogen replication profile in animals is missing
Prysliak et al. [126]	BVDV (Intranasal aerosol[7], 4 mL of 10 PFU/mL)	4 days	*M. bovis* (Intratracheal, 4 mL of 1.5×10^{10} CFU)	BVDV group: no lung lesions nor clinical signs, rectal temperature spike at 8 dpi Coinfected group: no lung lesions nor clinical signs	2	

Table 1 (continued)

Reference	Primary viral challenge (route of infection and dose/animal)	Time between exposure to the two pathogens	Secondary bacterial challenge (route of infection and dose/animal)	Main results of the clinical trials	Impact of the coinfection on BRD (score 1 to 4)	Study limitations
Prysliak et al. [126]	BoHV-1 (Intranasal aerosol, 4 mL of 10^5 $TCID_{50}$/mL)	4 days	*M. bovis* (Intratracheal, 4 mL of 1.5×10^{10} CFU)	Coinfected group: higher weight loss and rectal temperature, higher rate of *M. bovis* isolation from blood of coinfected animals, more extensive lung lesions and shorter survival terms	4	Lack of BoHV-1 mono-infected group
Zhang et al. [130]	IDV (Intranasal, 10 mL of 10^7 $TCID_{50}$/mL)	5 days	*M. haemolytica* biotype A serotype 1 (Intratracheal, 30 mL of 10^9 CFU)	Coinfected group: decreased clinical score compared to *Mannheimia* group, retarded viral shedding compared to IDV group; *Mannheimia* group: slightly increased lung lesions	2	
Lion et al. [131]	IDV (Nebulization, 10 mL of IDV 10^7 $TCID_{50}$/mL)	Simultaneous	*M. bovis* (Nebulization, 10^{10} CFU)	All infected groups: clinical signs present Coinfected group: earlier appearance and increased severity of clinical signs, gross lung lesions at 6 dpi	3	

*These studies were performed on a lamb model.

The first mixed infection studies were from 1960 to 1983, the majority being in vivo challenges using BPIV-3 or BoHV-1, the two viruses first associated with BRD, for the primary infection followed by inoculation with *M. haemolytica* [15, 92, 115–120]. In Jericho et al., two- to five-month-old calves exposed to aerosolized BoHV-1 then to *M. haemolytica* developed pneumonia when the delay between the viral and the bacterial infection was > 4 days. Calves infected solely with *M. haemolytica* did not develop severe pneumonia, underlining the importance of a viral pre-infection for the development of severe respiratory disease [118]. In Yates et al., six- to eight-month-old calves were exposed to BoHV-1 before being subsequently infected with *M. haemolytica* four to thirty days later. Although fibrinous pneumonia and pleuritis occurred in all four groups, animals exposed to the virus and bacteria four days apart had the most extensive and severe pathologic findings including foci of necrosis and/or focal areas of mucopurulent exudate on mucosal surfaces of the upper respiratory tract, with the pharyngeal tonsillar surfaces being most severely affected. Moreover, fibrinous pneumonia in coinfected calves resulted in the persistence of the viral antigen in the respiratory tract despite the resolution of the necrotic virus-induced lesions [119]. In contrast, a study by Carrière et al., did not observe any synergy in calves coinfected with the same pathogens, noting only mild lung lesions in all infected groups [120]. Similar findings were published by Saunders et al., where calves infected with BPIV-3 followed by different *Pasteurellaceae* species did not display increased respiratory disease severity, except increased nasal discharge [92].

Other experiments noted enhanced clinical signs when animals were pre-exposed to BVDV or BRSV before *M. haemolytica* or *H. somni* bacteria [121–126]. In Potgieter et al., two groups of six-month-old calves were inoculated at day 0 with either BVDV or *M. haemolytica* while a third coinfected group was inoculated first with BVDV and the subsequent bacterial pathogen 5 days later [121]. The authors reported pneumonic lesions reaching 2 to 15% of the total lung volume in the BVDV and *M. haemolytica* groups while the coinfected group developed severe fibrinopurulent bronchopneumonia and pleuritis comprising 40% to 75% of the total lung volume. In Gånheim et al., nine- to eighteen-month-old calves inoculated with either BVDV or *M. haemolytica* or coinfected with BVDV at day 0 and *M. haemolytica* 5 days later all had increased body temperature and depression, but the coinfected group had the most severe clinical signs with some animals not able to fully recover post-experimentation. The authors reported that both mono- and coinfected groups had similar magnitudes of acute phase proteins (AAPs) responses, particularly fibrinogen, haptoglobin and serum amyloid A, but the duration of elevated APPs expression was significantly longer in the BVDV/*M. haemolytica* group than in the BVDV group, reflecting the duration of clinical signs [122].

The first in vivo report of BRSV experimental infection in combination with *Pasteurellaceae* strains was actually performed in four-week-old lambs mono- or coinfected with BRSV or *M. haemolytica* at the same time. Pneumonic lesions were more frequent, extensive, and severe in coinfected lambs than in lambs inoculated with either agent alone. The authors postulated that BRSV compromised the lungs through the formation of lesions, promoting *M. haemolytica* establishment and subsequently, more severe pneumonic lesions than it could produce alone [127]. In the same animal model, similar findings were reported by Trigo et al. [124]. Later, in Gershwin et al., 9-month-old calves inoculated with a virulent strain of BRSV and *H. somni* 6 days later demonstrated significant mean clinical score differences compared to the groups infected with a single pathogen alone. Necropsy revealed severe bilateral consolidation in the anterior ventral lung lobes only in the coinfected group [128]. These results are in accordance to a similar coinfection study where calves pre-infected with BRSV and *H. somni* eight days later showed significantly more severe clinical signs and pneumonic lesions than animals inoculated with one pathogen alone [129].

In Prysliak et al., the pathogenicity of *M. bovis* was studied in six- to eight-month-old calves pre-exposed to BVDV or BoHV-1. Animals challenged with BoHV-1 prior to *M. bovis* inoculation 4 days later displayed weight loss, increased body temperature, and significantly shorter survival. At necropsy, the lungs of the BoHV-1/*M. bovis* group had extensive areas of bronchopneumonia, consolidation, and multifocal white nodules containing caseous material, whereas those from the *M. bovis* group displayed small consolidations without white nodules. No body weight loss was recorded for the BVDV/*M. bovis* group and there were no typical *M. bovis* pneumonia lesions found at necropsy [126].

As IDV was recently discovered to be a cattle pathogen, researchers started to investigate its possible role in BRD onset, assessing if IDV infection could worsen respiratory signs when co-inoculated with other pathogens in a manner similar to the viruses mentioned above. Four- to six-month-old calves infected with IDV at day 0 and *M. haemolytica* at day 5 had similar overall clinical scores as calves infected with IDV alone, while calves only infected with *M. haemolytica* had more severe gross lung lesions compared to the negative control group. *M. haemolytica* severe bronchopneumonia signs could not be reproduced in the coinfected calves suggesting that IDV and *M. haemolytica* coinfection does not alter the

respiratory pathology of calves [130]. In another study, six-week-old calves were infected with either IDV, *M. bovis*, or IDV and *M. bovis* together [131]. Although the *M. bovis* group did not present bronchopneumonia and caseonecrotic lesions typical of *M. bovis* infection, the authors reported that the coinfected group had a shorter time span of presented clinical signs and significantly increased clinical score, as well as increased severity of trachea and lung macroscopic and microscopic lesions. Starting at 2 days post-infection, upregulated IFNγ levels were found in bronchoalveolar lavages from the coinfected group, reflecting increased leukocyte recruitment in the airway lumen. The authors also noted that *M. bovis* colonization of the lower respiratory tract was aided by the viral infection.

4.2 In vitro approaches to further elucidate viral and bacterial coinfection pathogenicity mechanisms

Several studies attempt to explain the mechanisms underlying the enhanced pathology often observed during coinfection, mostly through in vitro approaches. One of the most well studied mechanisms of bacterial superinfection is the enhancement of bacterial adherence resulting from prior viral infection. In Sudaryatma et al., trachea, bronchus and lung primary cell lines were infected with BRSV before *P. multocida* [132]. The authors noticed that *P. multocida* adherence was greatly increased in pre-infected cells derived from the lower respiratory tract compared to cells that were not previously exposed to BRSV, together with an up-regulation of IL-6 mRNA expression. The same authors later reported an increased accumulation of the platelet-activating factor receptor (PAFR) in vitro and also demonstrated that *P. multocida* adherence depended on PAFR expression [133]. This work highlights a possible mechanism of bacterial superinfection caused by *P. multocida* following BRSV infection, that is often observed in field conditions [8]. In another recent work, the same authors observed an increase in *P. multocida* adherence following BCoV infection, noticing an increase in intercellular adhesion molecule-1 (ICAM-1) and PAFR, thus highlighting that the same mechanism could be shared among other BRD viruses [134]. In Agnes et al., infections with BRSV and superinfections with *H. somni* were carried out in BAT2 alveolar type 2 cell model [135]. The coinfection resulted in enhanced cytotoxicity for alveolar epithelial cells, increased transmigration of *H. somni* across the alveolar cell barrier, and matrix metalloproteinases MMP1 and MMP3 increased expression and activity. This could explain the observed results in their previous in vivo experiment, where they showed that *H. somni* and BRSV act synergistically in vivo to cause more severe bovine respiratory disease than either agent alone [128]. The same authors also reported, that BAT2 cell treatment with *H. somni* infected supernatants up-regulated antiviral genes and dramatically reduced a subsequent BRSV replication, showing once again that the timing of each pathogen infection is an important factor for the overall impact on pathology [136]. Finally, in McGill et al., the authors observed that in peripheral blood mononuclear cells (PBMC), coinfection with BRSV and *M. haemolytica* exacerbated IL-17 production, which plays a critical role in neutrophil recruitment and inflammation, a characteristic trait of *M. haemolytica* severe pasteurellosis in calves [137].

4.3 Viral coinfections: a less explored model of increased pathogenesis in BRD

The "viral infection followed by bacterial superinfection" model seems to be the most frequent and best described dynamic in cattle herds. There is currently very little information about viral superinfections in BRD. After an exhaustive literature search, we found three in vivo studies investigating the impact of a primary viral infection followed by a second viral infection [138–140]. BVDV was used in the three studies as the primary viral infection, likely due to its immunosuppressive nature [141]. We also identified two other studies investigating the impact of simultaneous BRSV and BVDV coinfection [142, 143]. All in vivo viral/viral respiratory coinfection calf studies are summarized in Table 2.

In Pollreisz et al., nine- to twelve-month-old calves simultaneously infected with BRSV and BVDV developed more severe clinical signs, including fever and diarrhoea, and lung lesions than their mono-infected counterparts. In addition, coinfected calves had a longer duration of viral shedding in nasal secretions and higher infectious titres compared to the groups infected with BRSV or BVDV alone [142]. An in vitro study performed on alveolar macrophages demonstrated that concomitant infection with BRSV and BVDV suppressed alveolar macrophage functionality [144], potentially explaining the increased lung lesions observed in Pollreisz et al. [142]. In contrast, Elvander et al. reported no change in clinical signs in three-month-old calves concurrently infected with BVDV and BRSV [138].

In Risalde et al., eight-month-old calves pre-inoculated with a non-cytopathic BVDV strain followed by BoHV-1 inoculation twelve days later had more intense clinical signs and lesions, correlating with greater TNFα secretion and reduced IL-10 production than animals inoculated with BoHV-1 alone. Delayed IFNγ production and low IL-12 levels were also observed in coinfected animals [145]. In a following paper, the same authors

Table 2 In vivo studies from the scientific literature performed on young calves to assess viral coinfections impact on BRD

Reference	Primary viral challenge (route of infection and dose/animal)	Time between exposure to the two pathogens	Secondary viral challenge (route of infection and dose/animal)	Main results of the clinical trials	Impact of the coinfection on BRD (score 1 to 4)	Study limitations
Pollreisz et al. [142]	BVDV-1 (Intranasal and intratracheal, 5 mL of 2×10^8 $TCID_{50}$)	Simultaneous, 1 day and 2 days	BRSV (Intranasal and intratracheal, 5 mL of 10^6 $TCID_{50}$/mL)	BVDV group: mild signs BRSV group: serous nasal discharge, rapid and shallow respiration and depression Coinfected group: excessive serous or mucopurulent nasal discharge, rapid breathing, diarrhoea, severe depression, one calf had to be euthanized	4	
Brodersen et al. [143]	BVDV	Simultaneous	BRSV	Coinfected group: increased clinical signs, higher viral shedding and increased lung lesions than infection with either virus alone	3	
Elvander et al. [138]	BVDV (non-cytopathogenic) (Intratracheal, 10^5 $TCID_{50}$/mL)	Simultaneous	BRSV (Intratracheal, BRSV group: 10 mL of 10^4 $TCID_{50}$/mL, coinfected group: 10 mL of BRSV 10^5 $TCID_{50}$/mL)	No increase in clinical signs in coinfected group	2	Lack of BVDV group; different BRSV dose in mono-infected and coinfected groups
Risalde et al. [145]	BVDV-1 (non-cytopathogenic) (Intranasal, 1 mL/nostril of 10^5 $TCID_{50}$/mL)	12 days	BoHV-1.1 (Intranasal,1 mL/nostril of BoHV-1.1 $\times$ 10^7 $TCID_{50}$/mL)	Appearance of clinical signs in all groups but increase in severity in coinfected group; increase in pro-inflammatory cytokines and APPs in coinfected group (IL-1β) and more severe inflammatory lesions	3	Lack of BVDV group
Ridpath et al. [140]	BVDV-2a (Intranasal aerosol, 4 mL of 10^6 $TCID_{50}$/mL)	3, 6 and 9 days	BCoV (Intranasal aerosol)	BCoV group: pyrexia but no gross lesions Coinfected group: higher fever, lung lesions present in all infected groups but more pronounced in 6-day delay group; peripheral blood lymphocytes count returned to baseline in 6-day delay group but not in 9-day delay group	3	BCoV dose is not reported

Table 2 (continued)

Reference	Primary viral challenge (route of infection and dose/animal)	Time between exposure to the two pathogens	Secondary viral challenge (route of infection and dose/animal)	Main results of the clinical trials	Impact of the coinfection on BRD (score 1 to 4)	Study limitations
Ridpath et al. [140]	BCoV (Intranasal aerosol)	3 days	BVDV-2a (Intranasal aerosol, 4 mL of 10^6 $TCID_{50}$/mL)	BCoV group: pyrexia but no gross lesions Coinfected group: pyrexia and lung lesions in some coinfected calves consisting in pale, firm foci randomly scattered throughout the lungs but particularly obvious in the ventral caudal lobes	2	BCoV dose is not reported

described important lung vascular alterations produced by fibrin microthrombi and platelet aggregations within the blood vessels that were earlier and more severe in the BVDV and BoHV-1 coinfected group, suggesting that coinfection facilitates a procoagulant environment modulated by inflammatory mediators such as significantly decreased iNOS expression released by pulmonary macrophages [146]. In two subsequent studies, the same authors reported that coinfected animals displayed inhibited CD8 + and CD4 + T lymphocyte responses against BoHV-1, suggesting that BVDV pre-infection could impair local cell-mediated immunity to secondary respiratory pathogens [139] and provoke thymic lesions that temporarily downregulate Foxp3 lymphocytes and TGFβ expression and medullary CD8 + T cells development [147].

In Ridpath et al., BVDV and BCoV dual infection studies in vivo were performed using different sequences and delays of superinfection to assess pathogenicity. Calves inoculated with BVDV followed by BCoV 6 days later displayed more pronounced clinical signs and lung lesions compared to 3 days of delay, demonstrating that the timing of the secondary infection along with the pathogen itself plays an important role in coinfection pathogenesis [140]. In the same study, calves were also inoculated with BCoV followed by BVDV 3 days later but clinical signs and lung lesions were not as pronounced as in animals pre-infected with BVDV followed by BCoV challenge, questioning the role of BCoV as BRD initiator.

The in vivo studies described above support the notion that BVDV pre-infection aggravates the respiratory pathology induced by other viruses in a manner similar to bacterial superinfections, as previously discussed above. What the field lacks is data concerning other viral coinfections involved in BRD. For example, BRSV, also known to modulate host immune responses [148], could play a similar role, despite the absence of experimental in vivo evidence during respiratory coinfections. The small number of available studies on viral superinfections limits our understanding of the role of viruses in priming the immune system before causing a subsequent viral superinfection.

4.4 Bacterial coinfections: can bacteria initiate BRD without the presence of primary triggers?

Contrary to viral/bacterial coinfections, bacterial coinfection models have been rarely explored in BRD studies. Multiple bacterial respiratory pathogens are often simultaneously detected from sick animals [8]. Despite this, respiratory bacteria interactions remain unclear. Some are part of the normal microbial flora of the upper respiratory tract of healthy animals (notably *Pasteurellaceae* family members) but are also often isolated from animals with respiratory signs [8, 49]. Different experimental in vivo infections with single bacterial challenge have been carried out throughout the years in calf models [35, 52, 149]. However, reproducing classical bronchopneumonia signs has been highly variable. Inoculation of the A3 serotype of *P. multocida* in calves induced clinical signs and lung lesions [35, 150, 151] whereas buffalo are susceptible to the A1 serotype [37]. In contrast, other studies reported milder lesions and overall pathology [152]. Animals experimentally infected with *M. haemolytica* alone either fail to develop bronchopneumonia [118, 120, 121, 130], or manifest severe clinical illness and reach end-point limits during the study [149, 153]. Such confounding study differences could be due to intrinsic characteristics of the animals (immune status, age and breed) as well as differences in the bacterial strains that are not yet known and therefore, could not be accounted for the highly controlled experiments.

We retrieved two in vivo studies from the literature investigating the impact of dual bacterial infection in calves. In Houghton and Gorlay, calves simultaneously inoculated with *M. bovis* and *M. haemolytica* were more severely affected than animals inoculated with only one pathogen. Vast differences were seen during necropsy with coinfected animals displaying 34 to 55% of lung consolidation compared to only 1–6% for calves from the *M. bovis* group and 0–1% for calves from the *M. haemolytica* group [154]. Subsequently, the same authors performed different dual bacterial challenges in gnotobiotic calves [155]. Calves were first inoculated with *M. bovis* followed by *M. haemolytica* one or two days later. Two animals also received a *M. haemolytica* strain that was cultured for 18 h previously to the challenge and two others received a strain that was cultured for 6 h. No clinical signs were reported for the calf infected with only *M. haemolytica*, whereas calves that were inoculated with *M. haemolytica* 2 days later displayed severe illness and 16% of lung consolidation at necropsy. However, calves that received the second pathogen one day later were more ill compared to the group inoculated 2 days later. In addition, high lung consolidation (50–64%) was reported for this group. Two calves inoculated with *M. haemolytica* then *M. bovis* two days later only developed mild signs without pneumonia. Similar challenges were performed on conventionally reared calves, with simultaneous inoculation of *M. bovis* and *M. haemolytica*, or, inoculation by *M. bovis* first followed by *M. haemolytica* one day later. Calves in the *M. haemolytica* group did not display any lesions or illness and only a few animals in the coinfected group had fever and 6–8% of lung consolidation at necropsy. In contrast, calves first dosed with *M. bovis* followed by *M. haemolytica* one day later had severe respiratory signs, resulting in the death of one calf and high lung consolidation (28 to

Table 3 In vivo studies from the scientific literature performed on young calves to study bacterial coinfections impact on BRD

Reference	Primary bacterial challenge (route of infection and dose/animal)	Time between exposure to the two pathogens	Secondary bacterial challenge (route of infection and dose/animal)	Main results of the clinical trials	Impact of the coinfection on BRD (score 1 to 4)	Study limitations
Houghton and Gorlay, [154]	*M. bovis* (Intranasal)	Simultaneous	*M. haemolytica* (Intranasal)	Dually inoculated animals were more severely affected than animals inoculated with one pathogen *M. bovis* group: 1–6% lung consolidation *M. haemolytica* group: 0–1% lung consolidation Coinfected animals displayed 34 to 55% of lung consolidation	3	
Gourlay et al. [155] (gnotobiotic calves)	*M. bovis* (Intranasal and intratracheal, 6×10^8 CFU)	1 day and 2 days	*M. haemolytica* (Intranasal and intratracheal, 7×10^8 CFU cultured for 6 h or for 18 h before inoculation)	*Mannheimia* group: no illness or gross lesions Coinfected group: severe clinical illness and 16% of lung consolidation at necropsy with 2-days delay, severe illness and high area of lung consolidation in dually infected calves (50–64%) inoculated with 1-day delay between pathogens, increased lesions when using 6 instead of 18 h culture of *Mannheimia*	3	Lack of *M. bovis* group
Gourlay et al. [155] (gnotobiotic calves)	*M. haemolytica* (Intranasal and intratracheal, 7×10^8 CFU cultured for 6 h or for 18 h before inoculation)	2 days	*M. bovis* (Intranasal and intratracheal, 6×10^8 CFU)	No significant increase in pneumonia in coinfected animals, mild signs in all groups	2	Lack of *M. bovis* group
Gourlay et al. [155] (Conventionally reared calves)	*M. bovis* (Intranasal and intratracheal, 6×10^8 CFU)	Simultaneous	*M. haemolytica* (Intranasal and intratracheal, 7×10^8 CFU cultured for 6 h or for 18 h before inoculation)	*Mannheimia* group: no illness and no gross lung lesions Coinfected group: only few calves displayed fever and clinical signs and 6–8% of lung consolidation at necropsy, the remaining calves displayed no clinical signs	2.5	

Table 3 (continued)

Reference	Primary bacterial challenge (route of infection and dose/animal)	Time between exposure to the two pathogens	Secondary bacterial challenge (route of infection and dose/animal)	Main results of the clinical trials	Impact of the coinfection on BRD (score 1 to 4)	Study limitations
Gourlay et al. [155] (Conventionally reared calves)	*M. bovis* (Intranasal and intratracheal, 6×10^8 CFU)	1 day	*M. haemolytica* (Intranasal and intratracheal, 7×10^8 CFU cultured for 6 h or for 18 h before inoculation)	*M. bovis* group: moderate clinical signs and reduced extent of lung consolidation (27–40%) Coinfected group: severe clinical signs resulting in the death of one calf and higher extent of lung consolidation (28 to 60%)	4	

60%). The *M. bovis* group showed moderate clinical signs and less lung consolidation (27–40%). These data underline the relationship between coinfection and the development of severe pneumonia [155]. This is in agreement with another study, where the death of two gnotobiotic calves was reported 24 h followed simultaneous inoculation with *M. bovis* and *M. haemolytica* [156]. Table 3 summarizes the calf in vivo studies on bacterial respiratory coinfections.

4.5 Bacterial coinfection studies: synergy or antagonism?

Currently, few in vitro studies investigating the interactions among different bacteria exist. In Corbeil et al., different bacterial strains (*P. multocida*, *M. haemolytica* and *H. somni*) isolated from bovine microbial flora were grown together to examine whether they would inhibit or enhance their growth [157]. The authors discovered that the majority of microbial strains could enhance the growth of the tested pathogens, especially those from the *Micrococcus*, *Corynebacterium* and *Staphylococcus* genera, whereas a discrete number of isolates did not affect their growth. In contrast, only some *Bacillus* genus strains could inhibit *Pasteurellaceae* growth. In Bavananthasivam et al., the authors tested growth competition between *P. multocida* and *M. haemolytica* and found that each showed similar growth when cultured together but upon physical separation by a membrane, *M. haemolytica* growth was inhibited by a contact-proximity mechanism [158], similar to what was already observed for *Bibersteinia trehalosi* in sheep pneumonia [159], hypothesizing that the inhibition occurred though similar molecular mechanisms. Inhibition of *M. haemolytica* by probiotic bacteria was also demonstrated in vitro [160]. Since previous studies reported that *P. multocida* can be isolated from the lower respiratory tract from calves experimentally infected with *H. somni* [161] but also during natural cases of BRD [49], the co-existence of *H. somni* and *P. multocida* in polymicrobial film was investigated in vitro and in vivo [162]. In the in vitro model, both pathogens were shown to co-exist and to contribute to biofilm formation. Two eight-week-old calves were then intratracheally challenged with 10^9 CFU of *H. somni* so that lung tissues could be analysed for polymicrobial formation. Both pathogens were detected by PCR in the lungs, supporting the hypothesis that *H. somni* and *P. multocida* can cohabit in polymicrobial films in vivo. In another study, the carriage of *H. somni*, *P. multocida* and *M. haemolytica* was assessed by qPCR from nasal swabs collected from healthy beef calves ($n=60$) during a 75-day study [163]. Co-carriage of two or three bacterial species was detected in 47 animals but *P. multocida* remained the most prevalent during the entire study, either as co-carriage with *H. somni* occurring the most frequently followed by *M. haemolytica* and lastly with *H. somni*. Taking all the experimental evidence into consideration, we cannot conclude whether a synergistic or antagonistic effect is present among different *Pasteurellae* bacterial strains. Further studies are needed to investigate the interactions among these pathogens in the context of BRD.

5 Discussion

In this review, we consolidated experimental evidence describing coinfection mechanisms potentiating pneumonia aetiology in cattle. The most studied mechanism of BRD onset in calves is the primary viral infection followed by a secondary bacterial superinfection model, with evidence suggesting it to be one of the most common scenarios triggering BRD. Several in vivo experiments showed that a primary viral infection impacts *M. haemolytica* superinfection. The viruses that seem to enhance secondary bacterial infection the most include BRSV, BVDV and BoHV-1 with mean scores higher than 3. BPIV-3 received a mean score of 2.67, also indicating a close association. Despite this, no solid conclusions can be drawn due to the very limited number of undertaken studies. In addition, two of the BRSV studies were performed in lambs, not in calves. A few studies using *P. multocida*, *H. somni* and *M. bovis* as bacterial secondary infection could be retrieved, with the highest impact score for BoHV-1 followed by *M. bovis* (mean score of 4) and BRSV followed by *H. somni* (mean score of 3.5). Multiple in vitro studies showed that viral priming increased bacterial adherence and colonization of the respiratory tract, suggesting a possible mechanism underlying the onset of bronchopneumonia in cattle. This could explain why viruses and bacteria are often co-detected in the respiratory tract of field animals with BRD signs. A limited number of viral coinfection studies ($n=4$) was also retrieved, showing that a primary viral infection increases the pathogenicity of a secondary viral infection. Despite this, only the role of BVDV has been explored throughout the years for viral coinfections. The mechanisms utilized by other viral pathogens such as BCoV and IDV remain unclear. One of the most important questions concerning the dynamics of bacterial derived respiratory infection is whether contagious spread between animals stems from bacterial replication in the lungs or whether said bacteria is already present in the nasopharynx, accessing the lower respiratory tract when immune responses are impaired from a primary trigger (the secondary bacterial superinfection model).

A few studies have attempted to adress this question. Young bulls ($n=112$) arriving at a fattening facility were divided into different pens and observed for 40 days. Nasal swabs and transtracheal aspirations were collected

to detect *M. haemolytica* and to study the clonal diversity between the upper and lower respiratory tracts. During the BRD outbreaks that occurred at the facility, *M. haemolytica* was frequently isolated from sick animals with 75 bulls testing positive during the study. Among these, *M. haemolytica* was cultured from transtracheal aspirates from 23 asymptomatic bulls. Pulse field gel electrophoresis (PFGE) analysis revealed a moderate agreement in clone diversity within nasal swabs and transtracheal aspirates within the same animals but high within-pen diversity, indicating that the disease was due to predisposing triggers enabling the bacteria to overcome the animal immune system and the normal flora. Despite this, the authors observed horizontal gene transfers from bulls in the nearest pen as well [24]. High genetic diversity within the same feedlot was also observed in other studies for *M. haemolytica* [164] and for *P. multocida* [165]. These results suggest that BRD episodes associated with these pathogens are probably due to predisposing factors overcoming the normal flora than the spread of a contagious clone among animals within a herd. Young pre-weaned calves recently arrived to feeder farms are exposed to high stress levels, likely the most important trigger to BRD aetiology.

A separate evaluation should be made for *M. bovis*, as this pathogen is not part of the commensal flora of healthy animals. In experimental conditions, a primary *M. bovis* challenge followed by *M. haemolytica* one day later increased the severity of illness compared to calves singly challenged or simultaneously challenged with both pathogens [155]. The conditions of the experimental infection do not represent the reality of animals in the field within a herd (the pathogens are challenged intratracheally with a high infectious dose), however these data suggest that *M. bovis* could potentially initiate BRD development.

Different in vitro studies tried to elucidate bacterial pathogenic interactions; however, mechanisms of synergy or antagonism among the studied bacterial strains remain unknown as there are too few studies, leaving a gap in knowledge about the polymicrobial aetiology of BRD.

In this study, we developed a scoring system to evaluate the impact of coinfection on overall cattle BRD pathology. This scoring system is meant to generalize the effects of specific pathogen pairs during coinfection with the caveat that there are major limits obfuscating the true impact, including poorly described control groups in certain studies and differences in induced respiratory pathology upon challenge of the same pathogen among all the studies. For example, inoculation with *M. haemolytica* induced BRD in some studies but not others, making it difficult to compare the true impact of *M. haemolytica* during coinfection. High heterogeneity across studies leads to additional difficulties when comparing results as parameters considerably change from one study to another, notably the infection route or pathogen dose, the assays used to confirm infection and seroconversion, and the age and breed of the animals. In addition, in vivo studies assessing the impact of coinfections among respiratory pathogens in cattle are limited, as are the number of animals used per study. One way to control for error is by using specific-pathogen free (SPF) calves, negating confounding effects associated with animals previously exposed to different pathogens and immunologically primed to combat infection, potentially resulting in altered pathological changes upon challenge.

Few studies ($n=7$) have attempted to study coinfections using alternative models to animal testing. The onset of new in vitro, ex vivo or in-vivo-like models in recent years could represent a valid replacement for primary studies before confirmation in animals. In particular, primary cell cultures, tissue cultures, organ slices and organoids provide a good start to change, both addressing the 3 R's principle (Reduction, Replacement and Refinement) and expanding the global scientific field (Figure 1).

Over the course of the 20th and the twenty-first century, the impact of different pathogens on BRD has changed. On one side, the development of prophylactic measures has helped control some infectious diseases in cattle, as notably shown by the eradication program for IBR and BVDV [86]. On the other hand, new emerging pathogens continue to appear, probably due to intensified cattle farming from the twentieth century like the appearance of high-density animal feedlots. New pathogens potentially involved in BRD that were not considered before (i.e. Influenza D virus) can be quickly discovered through NGS [166], potentially leading the way for an early risk assessment surveillance program in which cattle herds are monitored for emerging pathogens in order to prevent their circulation. New techniques like NGS facilitate studies on respiratory pathogenic interactions with the surrounding normal bacterial species as well as the mechanisms underlying the pathogenesis of respiratory disease in cattle. During surveillance, longitudinal studies could also be conducted to observe the dynamics of respiratory outbreaks caused by mixed infections, providing insight about the timing of pathogen introduction during BRD development (Figure 2).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13567-022-01086-1.

Additional file 1. Description of the scoring system criteria to evaluate the impact of coinfections on BRD.

Authors' contributions

MG conceptualized the work, collected the data on relevant literature, drafted the article and generated the figures. BN proofread English and revised the manuscript. MD and GM contributed to the conception and the critical revision of the article and funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

1.2 The cattle respiratory microbiota "in sickness and in health"

Although throughout the years the field of the microbiome has rapidly evolved, there is still a lack of current consensus on a commonly agreed definition of this term (91). Here, we will therefore attempt to give a simple definition and to describe the composition of the cattle "respiratory microbiota", based on current knowledge. The respiratory microbiota can be defined as the commensal microbes that are associated to the respiratory tract mucosae of healthy individuals (92). In particular, the microbiota of the upper respiratory tract includes the commensal microbes colonizing the nasopharynx tract, whereas the microbiota of the lower respiratory tract includes the commensal microbes colonizing the lungs (93). The majority of studies are concentrated solely on the bacterial component, however the respiratory microbiota includes a wider range of microorganisms beyond bacteria, such as viruses, fungi, archaea and eukaryotic parasites (94,95). The respiratory tract of healthy cattle is inhabited by 5 of the existing 55 bacterial phyla, which include: Firmicutes, Proteobacteria, Tenericutes, Actinobacteria, and Bacteroidetes (96,97). The disruption of the normal microbiota that results in an imbalance of the microbial communities in healthy individuals is defined as "dysbiosis" (98). In humans, several studies confirmed the association of microbiome imbalance with the development of different diseases, including allergies and asthma in children (99) but also pneumonia development in elderly (100). Similarly to humans, some studies described alterations in nasopharyngeal microbiota in BRD-affected calves compared to healthy animals (101,102). Other studies however failed to demonstrate a change in overtime composition of the microbiota in animals developing BRD (103).

The newborn calves acquire the bacterial communities in their airways by natural transfer via the birth canal during delivery (104). The bacterial abundance in the upper respiratory tract increases then progressively during the first month of age (105). In a recent study, the composition of the microbiota of the upper respiratory tract was found to be highly similar to the composition of the maternal vaginal microbiota (106). Interestingly, *Mannheimia* was the most abundant genus in the vaginal microbiota of cows whose calves developed pneumonia and/or otitis compared to the microbiota of cows whose calves did not develop disease. This suggests that the maternal microbiota could play a crucial role in defining the future health of newborn calves (106). Similar findings were described in humans, where the maternal gut

microbiome was shown to impact the offspring's risk of asthma and allergic disease (107). In particular, it has been hypothesized that the maternal carriage during pregnancy of *Prevotella copri* is associated to decreased IgE-mediated food allergy in the offspring (107).

The respiratory microbiota is not static and it is subjected to constant changes throughout the life of the individuals, especially following changes within the herd, such transport and arrival to new feedlots (97,108,109). In healthy adults, the nasopharynx is suggested to be the primary source of pathogenic bacteria that cause BRD, as in a recent study the nasopharyngeal microbiota was most similar to the lung bacterial microbiota, serving therefore potentially as the primary source of bacteria to the lung (110). Changes in the respiratory microbial composition were also observed between healthy individuals and animals with BRD during longitudinal studies. In a study that enrolled 174 dairy calves, deep nasal swabs were collected at 3, 14, 28 and 35 days of life to assess the colonizing microbiome of the upper respiratory tract and to study changes in its composition in calves with pneumonia and/or otitis (105). The calves were divided in four categories based on their health status: healthy calves, calves diagnosed with pneumonia, otitis or both diseases. The authors observed that the total bacterial load in newborn calves was higher for animals that developed pneumonia than for healthy animals. In addition, the relative abundance of the genera *Mannheimia*, *Mycoplasma* and *Moraxella* was significantly higher in sick versus healthy animals (105). These results support the hypothesis that *Mannheimia* and *Mycoplasma* are two dominant genera associated with otitis and pneumonia, but also that the neonatal total bacterial load is a significant risk factor in BRD onset. Interestingly, similar findings were observed in children, where early *Moraxella* colonization of the URT was associated with younger age of first virus respiratory infection (111). This indicates that the respiratory microbiome composition, and particularly the carriage of specific bacterial species, is a predisposing factor in the development of respiratory disease that is conserved between different species (112).

Therefore, limiting the colonization of opportunistic pathogens could be a possible strategy in order to reduce the prevalence of BRD in cattle (96). Similar results were described in a study where trans-tracheal aspirates and nasal swabs were obtained from steers (113). The bacterial metacommunity of steers with bronchopneumonia had a particular abundance of *P. multocida*, *M. haemolytica* and *M. bovis*. On the other hand, in healthy steers the metacommunity was enriched with *Mycoplasma dispar*, *Lactococcus lactis* and *Lactobacillus*

casei both in trachea and nasal swabs, suggesting that the presence of these bacterial genera could have a protective effect against opportunistic colonization. This is in agreement with a previous study, where cattle that had a higher abundance of *Lactobacillaceae* in nasopharynx at feedlot entry were less likely to develop BRD within the first 60 days on feedlot (114). The capacity of bacterial strains isolated from nasal normal flora to inhibit the growth of bacterial pathogens such as *P. multocida* and *M. haemolytica* was already tested *in vitro* (115) and could represent a possible mechanism for the observed results in the studies described above. These discoveries open up new important paths for control strategies of BRD in cattle herds, such as the possible application of probiotics, based on *Lactobacillus* and *Lactococcus* strains (116,117), to prevent the colonization with opportunistic pathogens (96).

1.3 Discussion

In this first part of the manuscript we described that the most studied mechanism of BRD onset in cattle is the primary viral infection followed by a secondary bacterial superinfection model, with evidence suggesting it to be one of the most common scenarios triggering BRD. Several *in vivo* experiments indeed suggested that a primary viral infection impacts a secondary bacterial superinfection, which was shown in particular for BRSV, BVDV, BPIV-3 and BoHV-1. Yet, a very limited number of studies were conducted and very limited data about the fundamental mechanisms were provided. Multiple *in vitro* studies proved that viral priming increased bacterial adherence and colonization of the respiratory tract, suggesting a possible mechanism underlying the onset of bronchopneumonia in cattle. This could explain why viruses and bacteria are often co-detected in the respiratory tract of field animals displaying BRD signs. A limited number of viral coinfection studies (n=4) were also retrieved, showing that a primary viral infection increases the pathogenicity of a secondary viral infection, and the mechanisms were well described for BVDV (118,119). The mechanisms exploited by other viral pathogens such as BCoV and IDV remain however still unclear.

The susceptibility to BRD is known to be multifactorial, influenced by a complex interaction between pathogens and the host immune response. In addition, stress is also an important factor in BRD outcome. Stress factors in calves can include transport, weaning, veterinary procedures, crowding and dietary changes. There is however conflicting evidence in the literature of stress impact on the alteration of serum stress markers (i.e. cortisol levels) and how these can influence the co-infection pathogenesis within BRD (120). In cattle, prenatal stress exposure was linked to an imprinting of the immune system in the offspring by inducing an impairment of the cellular immune status of the pre-weaned calf (121). For instance, maternal heat stress during late gestation was shown to inhibit the immune response of the offspring by modifying T and B cell functions (122). In addition, other findings reported a reduction in lymphocyte percentage and downregulation of blood immune markers, such as TLR2 and TNF-α, in the first several weeks after birth of calves born to cows exposed to heat stress during late gestation (123).

In young animals, weaning and transportation were demonstrated to contribute to BRD severity. This was shown in experimental infection, where calves co-infected with BHV-1 and

M. haemolytica exposed to stress (weaning and maternal separation) showed increased innate immune responses (IFN-γ secretion, acute-phase inflammatory response, CD14 expression, and TNF-α production) and mortality compared to the co-infected group without stress exposure (124). In another study, transport alone significantly increased BHV-1 shedding and IFN-γ production in the URT (125).

In adult animals, it has been proposed that stress has a negative impact on the immune system via the cell mediated and humoral immune responses: the activation of the hypothalamic-pituitary-adrenal axis, and therefore the increase of peripheral levels of glucocorticoids, suppresses the synthesis and release of various cytokines (notably IL-4, IL-5, IL-5, IL-12, IFN-γ and TNF-α) (126). Despite all these findings, the exact mechanisms and the molecular basis of stress effects on the immune function have not been extensively described in cattle, unlike in humans and mice relevant models (127). In addition, how the history of infections for an individual will impact his response to further infections via trained immunity pathways and adaptive memory in young calves within the BRD context and respiratory co-infections has still to be determined.

Another important questions concerning the dynamics of bacterial derived respiratory infection is whether contagious spread between animals stems from bacterial replication in the lungs or whether the bacteria is already present in the nasopharynx, accessing the lower respiratory tract when immune responses are impaired from a primary trigger (the secondary bacterial superinfection model). A few studies have attempted to answer this question. Young bulls (n=112) arriving at a fattening facility were divided into different pens and observed for 40 days. Nasal swabs and transtracheal aspirations were collected to detect *M. haemolytica* and study the clonal diversity between the upper and lower respiratory tract. During the BRD outbreaks that occurred at the facility, *M. haemolytica* was frequently isolated from sick animals with 75 bulls testing positive during the study. Among these, *M. haemolytica* was cultured from transtracheal aspirates from 23 asymptomatic bulls. Pulse field gel electrophoresis (PFGE) analysis revealed a moderate agreement in clone diversity within nasal swabs and transtracheal aspirates within the same animals but high within-pen diversity, indicating that the disease was due to predisposing triggers enabling the bacteria to overcome the animal's immune system and the normal flora. However, the authors observed horizontal gene transfers from bulls in the nearest pen as well. High genetic diversity within the same

feedlot was also observed for *M. haemolytica* (128) and for *P. multocida* (129). These results suggest that BRD episodes associated with these pathogens are probably due to the presence of predisposing factors and the overcoming of the normal flora, rather than the spread of a contagious clone among animals within a herd. However, in the field bacterial pneumonia onset is probably due to both the circulation of virulent bacterial strains and the overcome of host immunity due to the exposure of stress triggers.

A separate evaluation should be made for *M. bovis*, as this pathogen is not part of the commensal flora of healthy animals. In experimental conditions, a primary *M. bovis* challenge followed by *M. haemolytica* one day later increased the severity of illness compared to calves singly challenged or simultaneously challenged with both pathogens (130). The conditions of the experimental infection do not represent the reality of animals in the field within a herd (the pathogens were inoculated intratracheally with a high infectious dose), however these data suggest that *M. bovis* could have a potential role as initiator for BRD development. Different *in vitro* studies tried to elucidate bacterial/bacterial interactions; however, mechanisms of synergy or antagonism among the studied bacterial strains remain unknown as there is still an insufficient number of studies, leaving a gap in knowledge on the polymicrobial aetiology of BRD.

The high heterogeneity across studies leads to additional difficulties in comparing the results, as several important parameters considerably change from one study to another, notably the infection route or pathogen dose, the techniques used to analyse the pathogen's replication and the seroconversion of animals, as well as the age and breed of the challenged animals. In addition, *in vivo* studies assessing the impact of co-infections among respiratory pathogens in cattle are limited, as are the number of animals used per study. One way to control for error would be to use specific-pathogen free (SPF) calves, negating confounding effects associated with animals previously exposed to different pathogens and immunologically primed to combat infection, potentially resulting in altered pathological changes upon challenge.

A few studies have attempted to study co-infections using alternative models to animal testing. The increasing use of new *in vitro*, *ex vivo* or *in-vivo*-like models in recent years could represent a valid replacement for primary studies before confirmation of only the most interesting studies in animals. In particular, primary cell cultures, tissue cultures, organ slices

and organoids provide a good start to change, both addressing the 3 R's principle (Reduction, Replacement and Refinement) and expanding the global scientific field.

Over the course of the 20th and 21st centuries, the impact of different pathogens on BRD has changed. On one side, the development of prophylactic measures has helped control some infectious diseases in cattle, as notably shown by the eradication programs for IBR and BVDV (131). On the other hand, new pathogens continue to emerge, probably due to intensified cattle farming from the 20th century like the appearance of high-density animal feedlots. New pathogens potentially involved in BRD that were not considered before (i.e. Influenza D virus) can be quickly discovered through NGS (52), potentially leading the way for an early surveillance program in which cattle herds are monitored for emerging pathogens in order to prevent their circulation. These new technologies can facilitate studies on respiratory pathogenic interactions with the surrounding normal bacterial species as well as the mechanisms underlying the pathogenesis of respiratory disease in cattle. During surveillance, longitudinal studies could also be conducted to observe the dynamics of respiratory outbreaks caused by mixed infections, providing insight about the timing of pathogen introduction during BRD development.

Chapter 2: New putative viral agents involved in BRD: the case of Influenza D virus

2.1 Discovery of IDV: where it all started

IDV was isolated for the first time in April 2011 from nasal swabs of swine (Oklahoma, United States) exhibiting influenza-like symptoms (81). The samples were negative for influenza A virus, therefore an isolation on cell culture using the ST (swine testis) cell line was done. At three-day post-infection, cytopathic effects similar to those induced by influenza viruses were observed and by electron microscopy study the virus seemed to display morphological characteristics of an *Orthomyxoviridae* member. Enzymatic assays showed a weak neuraminidase activity but a detectable O-acetylesterase activity, suggesting a likely belonging to the genus influenza C, which was however then disproved by RT-qPCR. The whole viral genome was therefore sequenced by using the Ion Torrent technology and a first BlastP search of the ORFs identified the human ICV as the most similar existing virus, leading to its provisional designation as C/swine/Oklahoma/1334/2011 (C/OK). Similarly to ICV, the novel C/OK genome was composed of seven segments (Fig. 1), unlike IAV and IBV whose genomes include eight genomic segments. The identity of PB1 segment (which is considered as the most conserved segment among influenza viruses) was only of 69-72% when compared to the PB1 protein of Influenza C virus, and only of 39-41% with Influenza A and B virus, suggesting that the novel isolated virus was likely to belong to a new influenza genus. In addition, the new virus hemagglutinin esterase-fusion (HEF) protein shared 53% identity with ICV HEF. The HEF protein allows ICV to bind to the sialic acid receptors and to destroy them through the acetylesterase activity with a subsequent membrane fusion. On the contrary, for IAV and IBV this activity is achieved through two separate proteins which work in cooperation, the hemagglutinin (HA) and the neuraminidase (NA). The non-coding regions of the 3' and 5' terminals for each genomic segment, which are highly conserved within the same influenza genus and allow reassortment events between different clades, displayed only one single nucleotide polymorphism at 5' end and another polymorphism at the first position of the 3' end between ICV and the novel C/OK. However, in a following study reassortments between human ICV and the novel C/OK were not observed *in vitro* (132).

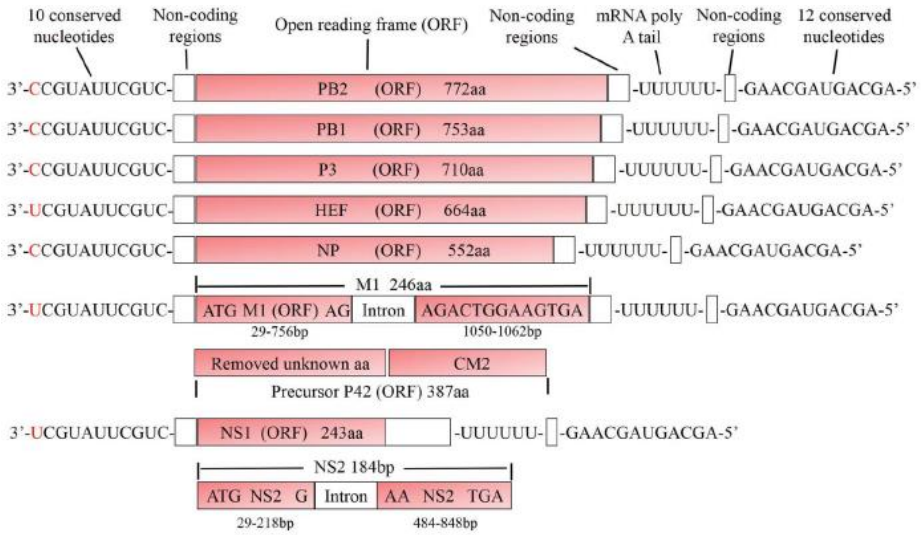

Figure 1: Structure of the 7 Influenza D virus genome segments. Source: (Su S et al., 2017)

In addition, the analysis of viral transcriptome by NGS revealed internal introns and different splicing strategies for the matrix and non-structural protein segments (Fig. 2), underlining the genomic differences with human ICV (132). In the same study, agar gel immunodiffusion assay was carried out in order to assess C/OK cross-reactivity with IAV, IBV and ICV antisera but no recognition of the C/OK antigens was detected. In cell culture, C/OK had a broader tropism than human ICV and could replicate at 33°C and at 37°C, unlike ICV which replicates only at 33°C. In experimental infection, ferrets and swine were intranasally challenged with C/OK. In both species, C/OK was able to replicate in the upper respiratory tract and to transmit to naive animals by direct contact. In addition, all challenged animals seroconverted within three weeks post-infection. However, no clinical signs or typical influenza histopathological changes were observed at necropsy (81).

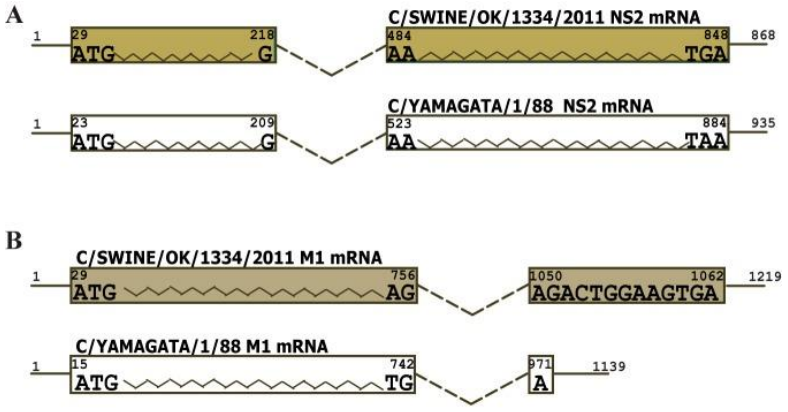

Figure 2: Splicing strategies of C/OK virus for the NS segment (A) and M segment (B). Source: (Hause BM et al., 2014)

As a consequence of these discoveries, in 2014 the authors submitted the proposal to the International Committee on Taxonomy of Viruses (ICTV) for the insertion of a new genus within the influenza virus family. The proposal was justified by the following reasons, which resume the assertions listed in the introduction above: C/OK virus is distantly related to ICV, but also to IAV and IBV, it failed to reassort with ICV in *in vitro* co-infections, it did not cross-react with the other Influenza genera antisera in agar gel immunodiffusion, it shows polymorphisms in highly conserved non-coding regions but also different splicing mechanisms for at least two viral segments. In addition, C/OK was detected in cattle, which is rarely infected by IAV, IBV or ICV and it was not prevalent in human population. The new genus is currently known as *Deltainfluenzavirus influenza* (ICTV Taxonomy), extending therefore the *Orthomyxoviridae* to currently nine genera.

2.2 IDV entry receptor and life cycle

The first step of IDV viral cycle begins with the viral binding to sialic acids on the host cell. In particular, IDV HEF uses for entry the human 9-O-acetylated N-acetylneuraminic acid (Neu5,9Ac2) and non-human N-glycolylneuraminic acid (Neu5Gc9Ac) (133,134). IDV HEF is a dimer transmembrane protein and each monomer is made of three domains (membrane fusion, esterase and binding) (135). The receptor binding site is located on the top of HEF1

globular head (Fig.) (81,133). The esterase domain of HEF harbors the receptor-destroying enzyme activity. To catalyse virus entry by membrane fusion HEF is proteolytically processed into the HEF1 (the largest among the two) and HEF2 (which is membrane-bound). HEF contains a monobasic cleavage site, a single arginine residue at the C-terminus of HEF1 (R439). HEF2 C-terminus contains two widely spaced basic residues (RTLTPATR) and the HEF2 N-terminus is identical to human ICV (IFGIDDLI) (135). The monobasic cleavage indicates that the replication of the virus is limited to the site of the infection, similarly to ICV and unlike polybasic cleavage sites of highly pathogenic IAV, which are recognized by a wide range of proteases and therefore give rise to systemic infections. However, the exact enzymes that activate IDV HEF are unknown.

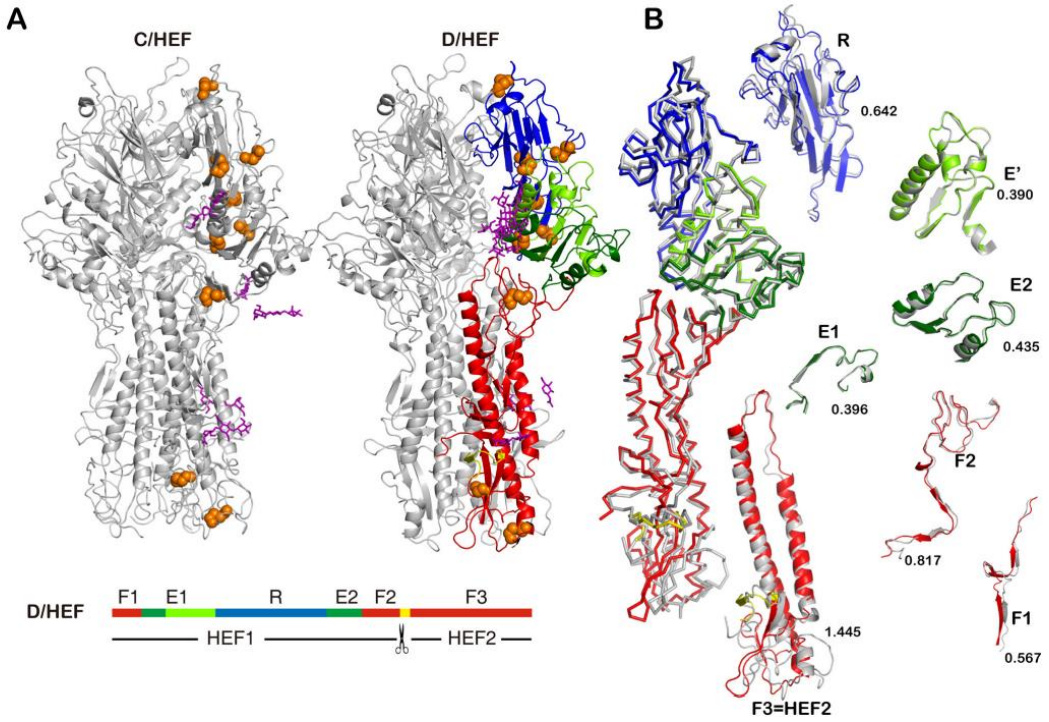

Figure 3: Overall crystal structure of IDV HEF protein (Source: Song et al., 2016)

After cleavage, the subunits display partially exposed fusion peptide that does not insert into cavity (133). The exact mechanisms of the endocytosis process have not been demonstrated for IDV, however it is hypothesized that IDV likely exploits a low-pH-dependent endocytosis route to enter the cell and fuse with the endosomal membrane to initiate infection, which is

a common pathway for all influenza viruses (136). The membrane M2 protein functions as ion channel which acidifies the endosome and allows the fusion of viral membranes and facilitates therefore the viral disassembly and the release of the viral RNA segments that are packaged as vRNPs (137), as described for other influenza viruses (138). Once disassembled, the vRNPs migrate in the nucleus by interacting with importins-α. In particular, for IDV nucleoprotein the carboxy-terminal tail interacts with importin-α7 and mediates nuclear transport (139). Data about IDV transcription and polymerase activity are missing in literature. Based on the knowledge on influenza viruses' replication cycle, once in the nucleus the RNA-dependent RNA polymerase (Rdrp) complex (which consists of PB2, PB1 and P3), binds to each RNA segment to initiate the replication and the transcription of viral RNAs (135,140). The negative-sense templates are therefore transcribed by the Rdrp complex into positive-sense mRNAs that will be translated into viral proteins within the cytoplasm, using the host's ribosomal machinery (140). The NS2 protein then mediates the nuclear export of newly formed vRNPs. For IDV, three nuclear export signals mediated by the CRM1 pathway were identified (141). Interestingly, despite the presence of only seven genomic segments, the majority (>70%) of IDV new particles are packaged with eight RNPs and not seven (142). The release of new viral particles from infected cells is then once again mediated by HEF protein through its esterase activity (140,143).

2.3 Understanding IDV host range: from surveillance to experimental infection

To date, IDV was detected in several countries on almost all continents. Since serious epidemiological studies have only been carried out in a few countries, conclusions about its actual prevalence worldwide are difficult to draw.

So far, IDV or anti-IDV antibodies have been detected worldwide (Figure 4) and in multiple hosts such as cattle, swine and feral swine, sheep and goats, horses and camelids contrasting with the host range of other influenza viruses. Although IDV was first isolated from a swine displaying influenza-like illness symptoms (81), epidemiological studies revealed a higher prevalence in cattle compared to other species, suggesting bovine as the primary host of IDV.

Cattle were never suspected of being a host for influenza viruses before (144), but recent studies suggested otherwise (70,86). Altogether, these data advocate for an underestimation of the role of cattle as a host for influenza viruses. IDV was also detected in bioaerosols in a poultry farm in Malaysia in 2018 (145), raising new questions about an even wider host range. Increasing evidence suggests IDV spillover into the human population (82,83,146–148) with yet unknown consequences for public health.

2.3.1 Cattle

Cattle is currently considered IDV primary host, due to the high prevalence in this species. Its first documented detection in cattle was in the United States, where a serological study conducted on a cohort of sera collected from 1977 to 2010 revealed the first positive sera in 2003 (149). In the same years, cattle in Nebraska already displayed high seroprevalence (93-94%) (150). Since then, molecular screening and serologic surveys described its detection in this species worldwide, including the American continent (Canada, United States, Mexico, Argentina, Brazil) (56,150–152), Africa (Morocco, Benin, Togo, Côte d'Ivoire, Uganda) (86,153), Europe (Italy, France, Switzerland, Luxembourg, Denmark, Ireland, the UK) (87,154–160) and Asia (Turkey, China, Japan) (161–164). The serologic prevalence in cattle is generally high and ranges from 31.0-48% to 95.6% (165). The notion of IDV as cattle pathogen was strengthened by different *in vivo* experimental infections, where IDV could successfully replicate in both the upper and lower respiratory tract and cause mild respiratory signs, as well as microscopic lesions in lung tissue (22,23). Aerosol transmission among animals was also demonstrated (23).

2.3.2 Swine

If IDV is widespread in cattle, its diffusion in swine population seems very limited. IDV positive swine nasal swabs and sera were detected in USA, Italy, Ireland, Luxembourg, France and China (81,157,161,166,167), with an overall prevalence ranging from 0.6-1.6% to 11.7%. In addition, virological surveillance in pigs in Northern Vietnam failed to detect the presence of the virus (168). In experimental infection no clinical signs were recorded in challenged animals,

however IDV could replicate in the upper respiratory tract and could be transmitted by direct contact to donor animals (81,169).

2.3.3 Camelids

Camelids were never described in literature as hosts for influenza viruses. Interestingly, a cohort of 293 camel sera collected in 2015 in Kenya was tested positive for IDV with high prevalence (99%). However, a cross-reactivity with ICV was also highlighted, indicating that anti-ICV and anti-IDV antibodies could not be discriminated in this species (86). In a following study another cohort of camels from Ethiopia was tested by HI assay and tested positive for IDV using three different antigens but negative for ICV, indicating there an absence of ICV-IDV cross-reactivity (170). However, in the two studies two different ICV antigens were used, namely C/Victoria/1/2011 in the first study and C/Ann Arbor/1/1950 in the second one. These studies suggest that although cross-reactivity between ICV and IDV is possible, camelids are likely susceptible to IDV infection. Molecular surveillance in camelids should be carried out in order to support the serologic data.

2.3.4 Horses

A very limited number of studies is present in literature that describe IDV infection in equids. Serology studies done by HI and MN assays suggested its circulation in 141 farms of the Midwest United States, however cross-reactivity between ICV and IDV was also highlighted, similarly to camelids (88). In experimental infection, horses did not develop any clinical signs but they showed seroconversion 13 days' post-infection. In addition, IDV could replicate in the upper respiratory tract, suggesting a possible transmission among horses, which remains to be fully investigated (171).

2.3.5 Small ruminants

IDV infection was described in small ruminants by serology assays on different continents. In the United States, small cohorts of sheep (n=85) and goat (n=64) sera collected from 2001 to

2007 in different States tested negative for IDV. However, in cohorts collected in 2014 the seroprevalence increased for both species, with 15.3% (17/111) of sheep farms positive for IDV and 25.9% (7/27) of goat farms (89). In France, a serosurvey was conducted on 181 sheep and 430 goat sera collected from 45 and 13 farms, respectively. IDV seroprevalence ranged from 0 to 5.5% in sheep and from 1.3 to 5.8% in goats, indicating that despite the serologic evidence for IDV positivity, its circulation in these two species seems very limited in the country (87). Similarly, in West Africa a seroprevalence of 4.1% and 3.7% was observed in sheep and goats in Côte d'Ivoire and of 2 and 4.4% in Togo (153). Finally, in the province of Guangdong in China IDV was molecularly detected in 27 nasal swabs collected from 80 different goats. Although molecular evidence for IDV presence in China was provided, serosurveys to understand its actual prevalence in small ruminants in this geographic area are still missing. In experimental infection in SPF lambs, challenged animals did not display any clinical signs following IDV infection and histological evaluation showed no evidence of microscopic lesions or immune cell recruitment associated to inflammation. The virus however replicated, as observed by the analysis of viral shedding in nasal swabs, and the lambs seroconverted at 14 days post-challenge (172).

2.3.6 Wild fauna

To date, IDV circulation was principally investigated in domestic animals. However, a few studies described its detection also in wild fauna. In the United States, feral swine displayed high seroprevalence unlike domestic swine. In a first cohort of 256 sera collected in 2012-2013, 19.1% tested positive. In a second cohort (n=96) collected during 2010-2013, the seropositivity was even higher, with an overall prevalence of 42.7%, indicating that the virus could actively circulate in this population. In addition, in the same study, an experimental infection was carried out to study IDV replication and transmission among feral swine and the results indicated that challenged animals could shed and could transmit IDV to contact animals and both challenged and contact animals seroconverted 21 days post-infection (173). A recent study described white-tailed deer exposure to IDV in North America by serology assay on 264 sera collected from 2011 to 2017. 13 samples tested positive for IDV (4.9%), suggesting a limited presence in this population in this timeframe. Cohorts of wild *Cervidae* also highlighted

a limited spread to roe deers in Belgium and Germany (165). IDV spread was also suggested for hedgehogs and lamas, however the results have to be confirmed by additional serological testing and molecular assays (165). Finally, in Namibia IDV was molecularly detected in a black wildebeest (*Connochaetes gnou*) and in two giraffes (*Giraffa camelopardalis*). However, due to low genetic material HEF sequence could only be obtained for the wildebeest and not for the giraffes (174).

2.3.7 Humans

IDV presence has been highlighted in humans by serologic assay and molecular detection in urban environment in different studies. In a first study conducted in Florida, HI and MN assays were carried out on 46 sera collected in 2011 and 2012. Among these, 35 serum samples came from people with occupational exposure to cattle and 11 were from non-cattle-exposed adults. In this study, a seroprevalence of 91% was detected via HI assay, and 97% by MN assay among individuals working with cattle. On the other hand, among non-cattle-exposed individuals, seropositivity determined via MN assay was lower (18%) (84). Anti-IDV antibodies presence was also highlighted by HI and MN assays in a cohort of randomly selected human sera (n=1281) from two different geographic Italian regions from 2005 to 2017. Interestingly, a few sera were already positive in 2005 (5.1%) with a constant increase in time (9.8% in 2007, 24.1% in 2010, 39.0% in 2013, 42.0% in 2014) and a decrease starting from 2015 (21.8% in 2015 and 7.9% in 2017) (82). Anti-IDV antibodies were also reported in four veterinarian sera collected in 2004 (4.9% 4/82) in Italy (83). In addition to serology assays, IDV was detected in urban environments such as a hospital emergency room bioaerosol (148), in an airport bioaerosol (146), but also in human samples such as a nasal swab of a farmer working on a pig farm in Malaysia (147). However, in other studies different cohorts of samples tested negative for IDV. In a cohort of 316 serum samples collected in the Greater Vancouver area of British Columbia in Canada, or in Connecticut during the 2007–2008 and 2008–2009 influenza seasons, only 1.3% of sera tested positive for IDV. In another study, 741 adult sera collected during 2008-2009 and 2012-2013 influenza seasons revealed 1% of anti-IDV antibodies; however, after ICV pre-absorption the specific anti-IDV antibodies were no longer detectable (149). In addition, in a study conducted in Scotland 3300 respiratory samples from children

and adults collected from 2006 to 2008 were tested in pools of 10 by RT-qPCR for all four influenza genera. IDV was not detected in any sample, whereas ICV was present in 6 samples (0.2%), while frequencies of 3.3% and 0.9% were reported for IAV and IBV. However, possible RNA degradation due to prolonged storage reported from authors, as well as the dilution factor of the RNA testing in pools of 10, could be responsible for the observed results (175). The current knowledge on IDV host by serology and molecular assays is summarized in Table 2.

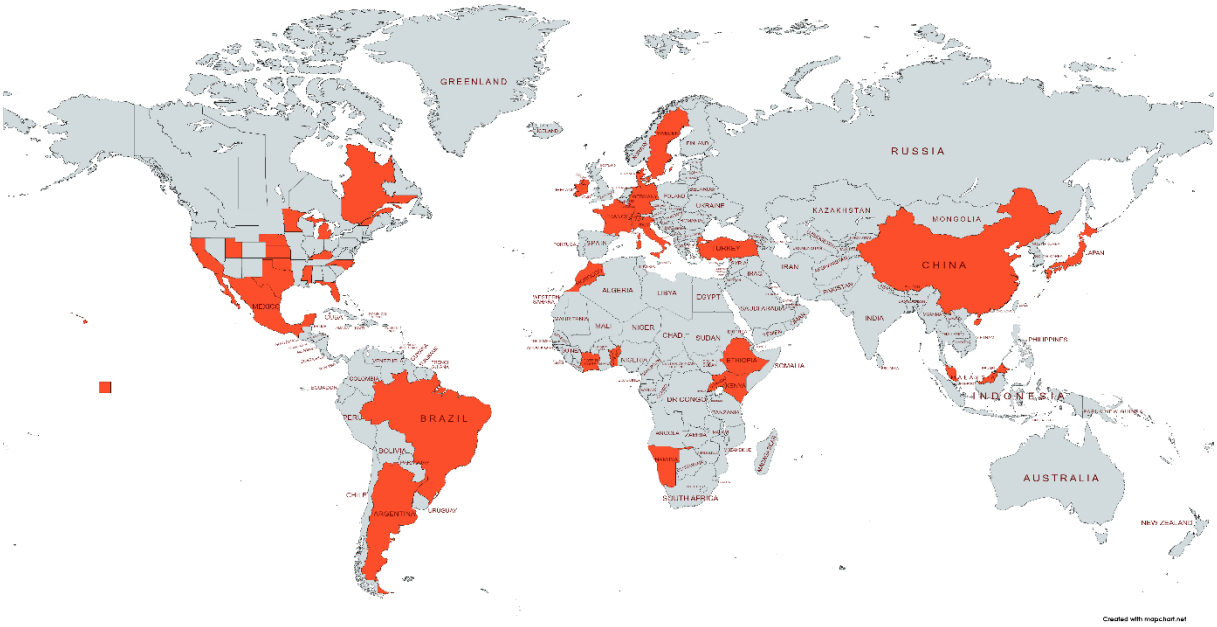

Figure 4. Countries where IDV or IDV-antibodies were detected by molecular or serological assay so far.

Table 2: Summary on the current knowledge on IDV host range by molecular and serological assays.

Host	Positive by molecular assay	Positive by serology assay	Countries of detection	Reference
Cattle	Yes	Yes	USA, Mexico, Brazil, Argentina, Morocco,	(6,86,150–154,157,15

Domestic animals				Benin, Togo, Côte d'Ivoire, Uganda, Namibia, Italy, France, Ireland, Switzerland, Denmark, UK, Luxembourg, Turkey, China, Japan	8,160–162,176)
	Swine	Yes	Yes	USA, Italy, Ireland, France, China	(81,161,166,167,177)
	Camelids	No	Yes	Benin, Togo, Côte d'Ivoire, Morocco, Uganda	(86,153)
	Horses	No	Yes	USA	(88)
	Small ruminants	Yes	Yes	USA, France, China, Côte d'Ivoire, Togo, Uganda	(86,87,89,153,161)
Wild fauna	*Feral swine*	No	Yes	USA	(173)
	White-tailed deer	No	Yes	USA	(178)
	Giraffe	Yes	No	Namibia	(174)
	Black wildebeest	Yes		Namibia	(174)
Humans		No	Yes	Italy, USA	(81–84)

2.4 Phylogeny and genetic diversity of circulating IDV

Two major circulating IDV clades, designated as D/OK and D/660, have been described in North America, Europe and China based on HEF diversity. In addition, multiple reassortment events between these two clades were detected (6,85,155). In Europe, one genetically divergent clade was described in France in 2012 and Ireland in 2014, represented by D/bovine/France/2986/2012 and D/bovine/Ireland/007780/2014 strains (90,160). Divergent local clades are also present in other countries on other continents, such as in Japan, California, Brazil and Namibia (152,162,163,174,179). The studies that we conducted on IDV prevalence, genetic diversity and evolution of circulating IDV in Europe and Canada are available in the paragraphs 2.5, 2.6 and 2.7 of the current Chapter.

2.5 IDV ecology and origins

In 2002, the definition of natural reservoir of a given pathogen was proposed as “one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population”, with the target population defined as “the population or species in which the pathogen causes disease” (180). As discussed in the previous paragraph, cattle is the only known species in which IDV could induce disease so far; it could therefore be considered a target population. IDV has probably low pathogenicity in cattle, it is highly prevalent and it is transmissible among individuals. Therefore, cattle could also be a potential candidate for a natural reservoir (181). However, molecular and serological data suggest that IDV has reached cattle population in relatively recent times.

Based on the few epidemiological data available in different species, two hypotheses could be drawn on IDV ecology: i) IDV could have been recently transmitted from an unknown host to cattle population. Cattle, being highly receptive for the virus, currently supports its maintenance in the environment. IDV could transmit from cattle to less receptive species like domestic animals and humans, but also occasionally to wild fauna, potentially explaining the

low IDV prevalence in these hosts. ii) IDV has an unknown natural reservoir that is still responsible for its maintenance in the environment and several spillovers from this unknown reservoir are regularly made to domestic animals and wild fauna.

Based on epidemiological data, the first hypothesis seems more likely. However, cattle alone could not have contributed to IDV global spread in a short period of time. A second accidental host could have helped its spread. Humans could have participated in IDV diffusion in the last decade and several evidence already pointed towards a possible IDV spillover to humans. Other hosts that were never considered so far are also migratory birds, which have already a known role in intercontinental transmission of avian influenza (182). However, birds are not suspected of being receptive for IDV and the infection in these species was never been investigated so far. Thus, the complete lack of data on IDV epidemiology in wild birds do not allow to substantiate this hypothesis.

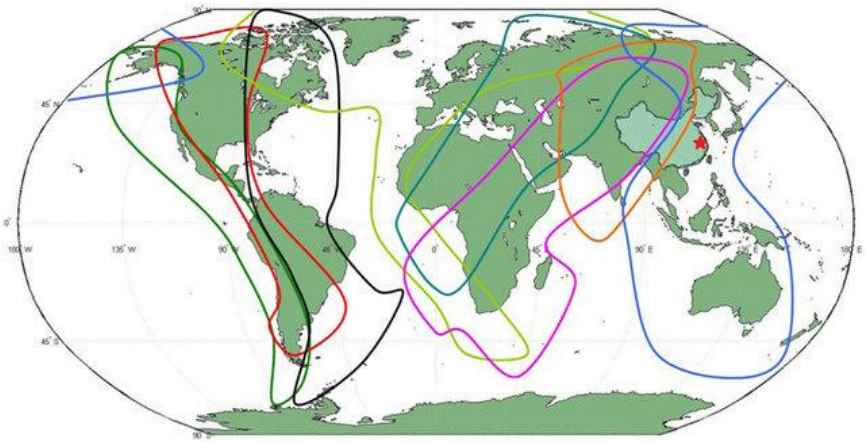

Figure 5: Migration routes of migrant birds in the world. Source: *(182)*

Since IDV discovery in 2011, a lot of questions about IDV origins remain unanswered. The first description of IDV infection dates to the early years of the 21st century in the United States, where in a cohort of cattle sera collected from 1977 to 2010 the first positive sera were from 2003 (149). In addition, more than 80% of sera were positive for IDV in a cohort of 293 samples collected from 2003 to 2004 in Nebraska farms, indicating that IDV was already largely circulating in cattle in that timeframe. Our evolutionary studies indicate a high mutation rate and multiple reassortment events of circulating IDV in cattle, suggesting a recent introduction

of the virus in bovine and therefore a lack of adaptation in this population. Unfortunately, due to the lack of high molecular detection rate in other species, it is still not possible to compare the mutation rate of IDV in cattle to the one of other animals.

Different scenarios about IDV origin could be hypothesized: i) IDV could have differentiated from bovine or human ICV throughout the years and the absence of ancient sequences of IDV may bias the tMRCA analyses, which was estimated as 1500 years ago (135). A recent change in IDV antigenicity and genetic diversity could be responsible for an increase in detection starting from 2003 by HI assay when testing sera with recent IDV antigens or false negatives by RT-qPCR assays that were designed based on recent sequences. ii) IDV could alternatively have differentiated from human ICV a long time ago, circulated silently in another host until recent spillover to domestic animals. iii) IDV could also have originated from another influenza-like virus, such as the recently discovered influenza viruses in amphibians (183) in another host until recent spillover to humans and domestic animals.

More serology and molecular studies with samples collected before 2003 could help understand the dynamics of IDV spread to humans and domestic animals. In particular, the retrieval of IDV sequences from 2000 to 2010 would be essential to reconstruct IDV origins, as the oldest sequence available for IDV was retrieved from a sample collected in Kansas in 2010. The main limit of serology approach, unlike molecular assays, is also the uncertainty of the specificity of antibodies, especially when using HI assay. A cross-reaction between ICV and IDV has been highlighted for humans (149), camelids (86) and horses (88), arising therefore the question of the true IDV seroprevalence observed in humans. However, the frequency of anti-ICV antibodies is generally high in the adult population (184). An increase in IDV seroprevalence as suggested by a study in the general Italian adult population is therefore not likely attributable to an increase in anti-ICV antibodies (82).

2.6 Review article: Emerging Influenza D virus infection in European livestock as determined in serology studies: Are we underestimating its spread over the continent?

PhD candidate's contribution:

The candidate collected the data on relevant literature (serology studies and livestock trade data), drafted the article and generated the figures.

Summary of the review

Influenza D virus (IDV) is a novel orthomyxovirus that was first isolated in 2011 in the United States from a swine exhibiting influenza-like disease. To date, its detection is extended to all continents and in a broad host range: IDV is circulating in cattle, swine, feral swine, camelids, small ruminants and horses. Evidence also suggests a possible species jump to humans, underlining the issue of zoonotic potential. In Europe, serological investigations in cattle have partially allowed the understanding of the virus diffusion in different countries such as Italy, France, Luxembourg and Ireland. The infection is widespread in cattle but limited in other investigated species, consolidating the assumption of cattle as IDV primary host. We hypothesize that commercial livestock trade could play a role in the observed differences in IDV seroprevalence among these areas. Indeed, the overall level of exposure in cattle and swine in destination countries (e.g. Italy) is higher than in origin countries (e.g. France), leading to the hypothesis of a viral shedding following the transportation of young cattle abroad and thus contributing to larger diffusion in countries of destination. IDV large geographic circulation in cattle from Northern to more Southern European countries also supports the hypothesis of a viral spread through livestock trade. This review summarizes available data on IDV seroprevalence in Europe collected so far and integrates unpublished data from IDV European surveillance network of the last decade. In addition, the possible role of livestock trade and biosecurity measures in this pathogen's spread is discussed.

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REVIEW ARTICLE

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Emerging Influenza D virus infection in European livestock as determined in serology studies: Are we underestimating its spread over the continent?

Maria Gaudino[1] | Ana Moreno[2] | Chantal J. Snoeck[3] | Siamak Zohari[4] | Claude Saegerman[5] | Tom O'Donovan[6] | Eoin Ryan[6] | Irene Zanni[7] | Emanuela Foni[7] | Aurelie Sausy[3] | Judith M. Hübschen[3] | Gilles Meyer[1] | Chiara Chiapponi[7] | Mariette F. Ducatez[1]

[1]IHAP, Université de Toulouse, INRAE, ENVT, Toulouse, France

[2]Istituto Zooprofilattico Sperimentale Della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Brescia, Italy

[3]Clinical and Applied Virology Group, Department of Infection and Immunity, Luxembourg Institute of Health, Esch-sur-Alzette, Luxembourg

[4]National Veterinary Institute, Uppsala, Sweden

[5]Fundamental and Applied Research for Animals and Health (FARAH) Center, University of Liège, Liège, Belgium

[6]Central Veterinary Research Laboratory, Celbridge, Co. Kildare, Celbridge, Ireland

[7]Istituto Zooprofilattico Sperimentale Della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Parma, Italy

Correspondence
Maria Gaudino, IHAP, Université de Toulouse, INRAE, ENVT, Toulouse, France.
Email: m.ducatez@envt.fr

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Abstract

Influenza D virus (IDV) is a novel orthomyxovirus that was first isolated in 2011 in the United States from a swine exhibiting influenza-like disease. To date, its detection is extended to all continents and in a broad host range: IDV is circulating in cattle, swine, feral swine, camelids, small ruminants and horses. Evidence also suggests a possible species jump to humans, underlining the issue of zoonotic potential. In Europe, serological investigations in cattle have partially allowed the understanding of the virus diffusion in different countries such as Italy, France, Luxembourg and Ireland. The infection is widespread in cattle but limited in other investigated species, consolidating the assumption of cattle as IDV primary host. We hypothesize that commercial livestock trade could play a role in the observed differences in IDV seroprevalence among these areas. Indeed, the overall level of exposure in cattle and swine in destination countries (e.g. Italy) is higher than in origin countries (e.g. France), leading to the hypothesis of a viral shedding following the transportation of young cattle abroad and thus contributing to larger diffusion at countries of destination. IDV large geographic circulation in cattle from Northern to more Southern European countries also supports the hypothesis of a viral spread through livestock trade. This review summarizes available data on IDV seroprevalence in Europe collected so far and integrates unpublished data from IDV European surveillance framework of the last decade. In addition, the possible role of livestock trade and biosecurity measures in this pathogen's spread is discussed.

KEYWORDS

cattle, epidemiology, influenza D virus, livestock trade, seroprevalence, small ruminants, swine, zoonosis

Gaudino, Moreno, Snoeck, Chiapponi and Ducatez equally contributed to the study.

Gaudino, Moreno, Snoeck should be considered as co-first authors.

Chiapponi and Ducatez should be considered as co-last authors.

1 | INTRODUCTION

Influenza D virus (IDV) was isolated for the first time in 2011 in the United States from a swine exhibiting influenza-like syndrome (Hause et al., 2013). It shared 50% of genetic identity with human Influenza C virus (ICV), leading to its provisional designation as C/swine/Oklahoma/1334/2011. In addition, this novel virus showed no reassortment with the other genera of Influenza viruses (Influenza A and B viruses, IAV and IBV, or ICV) (Hause et al., 2014). As a consequence, the International Committee on Taxonomy of Viruses (ICTV) was decided in 2016 to create a new genus in the influenza virus family. The new genus is currently known as influenza D virus, extending therefore the *Orthomyxoviridae* from six to seven genera. Based on their similarity in terms of genetic and morphological structures, some authors suggested the possibility of IDV derivation from ICV (Su, Xinliang, Li, Kerlin, & Veit, 2017). Indeed, the nucleotide identity between ICV and IDV is 50% for the Hemagglutinin Esterase-Fusion segment (HEF), the most variable segment among Influenza viruses, and 70% for PB1 (Polymerase basic protein 1), the most conserved segment (Hause et al., 2013). Similarities between ICV and IDV include the genomic composition of seven segments, and both only have one major surface glycoprotein that fulfils the functions of receptor recognition and binding, its destruction and the fusion between the virions and the host cell membranes. On the contrary, IAV and IBV are composed of eight genomic segments and these functions are accomplished by two different proteins, the hemagglutinin (HA), which binds to the host cell receptors and mediates the membrane fusion, and neuraminidase (NA), which allows for receptor destroying and new viral particles release (Asha & Kumar, 2019).

So far, two major circulating IDV lineages have been described in North America and Europe, often designated as D/OK and D/660. Reassortment events between these two lineages were also revealed (Chiapponi et al., 2019; Collin et al., 2015). In Europe, a third genetically divergent lineage was described in France in 2012 and Ireland in 2014 (designated as D/bovine/France/2986/2012 and D/bovine/Ireland/007780/2014, respectively). Though, further IDV sequences are needed to assess whether another different lineage is circulating in Europe. In addition, other genetically divergent lineages are present in Japan and they have not been reported on other continents to date (Murakami et al., 2016, 2020)(Hayakawa, Masuko, Takehana, & Suzuki, 2020).

So far, IDV seems to have a broad host range and has been described almost on all continents, showing an intercontinental transmission. Despite its first isolation from swine, cattle is currently considered as IDV principal host. Indeed, various studies report a high prevalence of IDV in this species (Luo et al., 2017; O'Donovan, Donohoe, Ducatez, Meyer, & Ryan, 2019; Oliva et al., 2019; Rosignoli et al., 2017) whereas historically cattle had never been considered a potential reservoir of influenza A viruses (Sreenivasan, Thomas, Kaushik, Wang, & Li, 2019). Currently, the list of susceptible species include cattle, swine (Foni et al., 2017; Gorin et al., 2019), small ruminants (O'Donovan et al., 2019; Oliva et al., 2019; Quast et al., 2015), camelids (Murakami et al., 2019; Salem et al., 2017), feral swine (Ferguson et al., 2018) and horses (Nedland et al., 2018). The emergence of the novel IDV in pigs initially raised public health concerns, as swine is a well-known host of other zoonotic Influenza viruses. However, whether IDV could be a threat to human is still unclear. Studies in the ferret model, where IDV replicates efficiently (Hause et al., 2013), as well as IDV receptors characterization (Song et al., 2016), suggest that humans may be susceptible. Furthermore, IDV replicates well in a human airway epithelium model (Holwerda et al., 2019) and its genetic material has been detected in a bioaerosol sample collected at an airport (Bailey, Choi, Zemke, Yondon, & Gray, 2018), in a hospital emergency room (Choi et al., 2018), as well as in a nasal swab of a farmer working on a pig farm in Malaysia (Borkenhagen et al., 2018). Serologic surveys conducted in persons with occupational contact with cattle in Florida (White, Ma, McDaniel, Gray, & Lednicky, 2016) and in the general population in Italy (Trombetta et al., 2019) suggested a zoonotic potential. In contrast, a prevalence of only 1.3% of anti-IDV antibodies was initially observed in a Canadian elderly cohort (Hause et al., 2013) and cross-reactivity between anti-ICV and anti-IDV antibodies was highlighted in human and camelids, suggesting that further controls and optimizations should be carried out in the serology assays before conclusions can be drawn on IDV seropositivity in these species (Eckard, 2016; Salem et al., 2017). Wide epidemiological investigations are still lacking to assess a risk level for humans, and they could provide additional insights about the real IDV zoonotic potential.

Epidemiological investigations suggest cattle to be IDV primary host and, so far, the virus has been detected both in healthy and diseased animals. Nevertheless, studies conducted through metagenomic approaches suggested its implication in Bovine Respiratory Disease Complex (BRDC) (Mitra, Cernicchiaro, Torres, Li, & Hause, 2016; Ng et al., 2015; Zhang et al., 2019), one of the most concerning health issues in cattle industry that has multifactorial aetiology and causes major economic losses. Experimental infections showed mild to moderate clinical signs in cattle, as well as direct contact and aerosol transmission among animals (Ferguson et al., 2016; Salem et al., 2019). The real implication of IDV on BRDC severity in the field is still not clear, and further studies would be needed to demonstrate its role.

In Europe, IDV was first reported in cattle in France in 2012 (Ducatez, Pelletier, & Meyer, 2015) and was then detected in surrounding countries Italy (Chiapponi et al., 2016) and Luxembourg (Snoeck et al., 2018), but also in Ireland (Flynn et al., 2018) and the UK (Dane et al., 2019). On this continent, as in other parts of the world, the livestock trade across national borders each year is of great importance. Livestock trade essentially includes import and export of live animals to neighbouring countries for production (fattening), breeding and slaughtering. This sector substantially contributes to the European economy, representing almost half of the total agricultural activity (Eurostat). In a 'One Health' context, livestock health is a major link in the global health chain. Animal-based product consumption has been a fast-growing component of food industry in the last decades, particularly in some developing countries in

Asia and South America but concerning also industrialized countries. A continuous surveillance on emerging livestock pathogens is thus required in order to ensure animal well-being but also to prevent health-related challenges in a more complex setting of animal-to-human pathogen transmission prevention.

The aim of this review was to summarize IDV infection spread in the European continent in different animal species. The review focuses on serological data obtained during the last ten years of surveillance and includes unpublished data coming from the consortium for European surveillance of this novel virus. In addition, the role of livestock trade in IDV transmission between different countries is discussed.

2 | IDV SEROPREVALENCE IN EUROPEAN LIVESTOCK: A WIDESPREAD INFECTION IN CATTLE WITH LIMITED DIFFUSION IN SWINE AND SMALL RUMINANTS

IDV seroprevalence in different species (which will be detailed in the following paragraphs) was mainly assessed by HI assay (Hemagglutination Inhibition). In all cases, a threshold of positivity was set at antibody titres ≥ 1:20. ELISA test (Enzyme-linked immunosorbent assay) was also used. A summary of technical details and results for each study is presented in Table 1 (Cattle), Table 2 (Swine) and Table 3 (Small ruminants).

2.1 | Cattle

In France, a serosurvey was carried out on bovine sera (n = 3,703) collected from 2014 to 2018 in 5 French regions (Oliva et al., 2019). Sera were tested by HI assay (with 1% solution of horse red blood cells). All animals were older than 1-year of age, excluding interference with maternally derived antibodies. The overall resulting seroprevalence was 47.2% but results varied depending on the geographical region (with seroprevalence ranging from 31.0% to 70.0%). In Italy, the overall reported IDV seroprevalence in cattle was higher than in France. Cohorts of bovine sera coming from both active (n = 420) (Rosignoli et al., 2017) and both active/passive surveillance (n = 315) (Moreno et al., 2019) were tested for anti-IDV antibodies by using HI assay (0.5% solution of turkey red blood cells) and solid-phase competitive ELISA (Moreno et al., 2019). Overall resulting seroprevalence was 92.4% and 74%, respectively. In addition, an observational cohort study conducted on 914 cattle samples collected in 2016–2018 showed a seroprevalence of 69%. In Luxembourg, high IDV seroprevalence (80.2%–82.5%) was found in cattle sera (n = 450 and n = 108) collected in 2016 (Snoeck et al., 2018) and 2019, respectively. Authors reported no difference between IDV seroprevalence in dairy and meat production cattle. Similar seroprevalence rates were found when testing the same 2016 cohort by HI (80.2%) or solid-phase competitive ELISA (81.8%). Finally, in 2017 in Ireland (O'Donovan et al., 2019) sera were collected from slaughterhouses across the country (n = 1,219) and screened for anti-IDV antibodies. An additional cohort of sera collected in 2016 and 2017 for diagnostic purposes to screen for antibodies to bovine respiratory disease (BRD) pathogens was also tested for IDV antibodies (n = 1,183). A high difference was found in terms of seroprevalence between the two cohorts, with 94.6% and 64.9% for active and passive surveillance, respectively. Relevant differences in overall IDV seroprevalence in cattle were also found in Italy based on the type of surveillance (active or passive), suggesting that it could be a relevant factor that should be taken into consideration to assess future sampling plans. Available serological results in cattle in Europe are summarized in Table 1.

2.2 | Swine

A different scenario emerged from serological studies conducted on serum samples collected in swine farms. A serosurvey across France was conducted on 2090 sera collected from 102 different farms between 2012 and 2018 (Gorin et al., 2019). Herds were mostly located in Brittany region, known to have the highest pig density in France. Samples were also collected in Nouvelle Aquitaine, Occitanie, Hauts-de-France, Normandie, Pays de la Loire and Corsica regions. While anti-IDV antibodies in cattle were found in most of these regions (Oliva et al., 2019), positive swine sera were found only from Brittany and Corsica regions. The overall IDV seroprevalence was 1.6% (represented by 31 positive samples on a total of 2090 tested sera). In these two regions, seroprevalence varied from 3.3% to 73.3% in Brittany and 7.1%–16.7% in Corsica. In Brittany, two herds with high within-herd seroprevalence (73.3% and 3.3%, where samples were collected in 2014 and 2015, respectively) were re-tested in 2017 to assess virus persistence but they then exhibited 13.3% and 3.3%. In Italy, cohorts from 2009 to 2018 coming from active and passive surveillance were screened for anti-IDV antibodies. All herds originated from the Po Valley (Northern Italy), one of the most intensive pig farming areas in Europe. Overall IDV seroprevalence ranged from 0.6% to 11.7%, depending on the year of sampling (Foni et al., 2017). IDV monitoring was also conducted on wild boars from the Alpine and Northern Apennine areas. A total of 1,350 samples collected in 2018 and 2019 was tested with a low prevalence (1.92%). Details of sera tested for Italian cohorts for each year are available in Table 2. In Luxembourg, the first cohort from 2012 (n = 258) was found seronegative, and then, a second cohort (n = 287) including sera collected at slaughter in 2014–2015 harboured 5.9% seroprevalence (Snoeck et al., 2018). In Ireland, a seroprevalence of 5.8% was found in swine (n = 377) (O'Donovan et al., 2019). Results from serological studies in pigs are summarized in Table 2.

2.3 | Small ruminants

So far, limited serological investigations have been performed on small ruminants. In France, sheep and goat sera were tested

TABLE 1 Overview over available serological results in cattle in France, Italy, Luxembourg and Ireland

Year of sampling	Country	Region/Department	Type of Surveillance	Nr. herds	Nr. collected sera	Nr. of positive sera	% of IDV positive sera	Screening method	References
2014–2018	FR	Occitanie	Active[a]	31	1,409	248	48.2	HI assay (1% HRBCs)	Oliva et al., (2019)
2014–2015	FR	Nord, Hauts-de-France	Active[a]	6	477	112	31.0	HI assay (1% HRBCs)	Oliva et al., (2019)
2015	FR	Vendée, Pays de la Loire	Active[a]	8	480	308	70.0	HI assay (1% HRBCs)	Oliva et al., (2019)
2017–2018	FR	Côte d'Or, Bourgogne Franche-Comté	Active[a]	20	480	158	39.6	HI assay (1% HRBCs)	Oliva et al., (2019)
2016	FR	Bretagne	Active[a]	27	480	168	45.2	HI assay (1% HRBCs)	Oliva et al., (2019)
2013	IT	Northern Italy, po valley	Active[a]	35	945	903	95.6	HI assay (0.5% TRBCs)	Unpublished data
2015	IT	Mantua, Lombardy	Active[a]	42	420	398	92.4	HI assay (0.5% TRBCs)	Rosignoli et al., (2017)
2016–2018	IT	Northern Italy (Po Valley)	Passive[a]	44	914	634	69.0	HI assay (0.5% TRBCs)	Unpublished data
2016–2017	IT	Northern Italy (Po Valley)	Both[a]	31	315	233	74.0	Competitive ELISA	Moreno et al., (2019)
2016–2018	IT	Northern Italy (Po Valley)	Active[b]	29	556	493	88.6	HI assay (0.5% TRBCs)	Unpublished data
2016	LU	Whole country	Active[a]	44	450	361	80.2	HI assay (1% HRBCs)	Snoeck et al.. (2018)
2019	LU	Whole country	Active[a]	64	1,108	914	82.5	Competitive ELISA	Unpublished data
2017	IE	Whole country	Active[a]		1,219	1,153	94.6	HI assay (0.75% TRBCs)	O'Donovan et al., (2019)
2017	IE	Whole country	Passive[a]		1,183	768	64.9	HI assay (0.75% TRBCs)	O'Donovan et al., (2019)

Abbreviations: ELISA, Enzyme-linked immunosorbent assay; FR, France; IT, Italy; LU, Luxembourg; IE, Ireland; HI, Hemagglutination Inhibition; HRBCs, Horse Red Blood Cells; TRBCs, Turkey Red Blood cells.

[a]While surveillance for IDV was carried out from animals with/without respiratory clinical signs, none of the sera was collected specifically for IDV seroprevalence studies (rather co-products from infectious bovine rhinitis or swine influenza surveillance programs mainly): surveillance stands for observational study here.

[b]Sera collected specifically for an IDV seroprevalence study.

TABLE 2 Overview over available serological results in swine in France, Italy, Luxembourg and Ireland

Year of sampling	Country	Region/ Department	Type of surveillance	Nr. herds	Nr. collected sera	Screening method	Nr. of positive sera	% of IDV positive sera	References
2012–2018	FR	Bretagne-Corse	Active[a]	102	2090	HI assay (0.5% CRBCs)	31	1.6	Gorin et al. (2020)
2009	IT	Northern Italy (Po Valley)	Passive[a]	25	502	HI assay (0.5% TRBCs)	3	0.6	Foni et al., (2017)
2013	IT	Northern Italy (Po Valley)	Both[a]	11	333	HI assay (0.5% TRBCs)	10	3	Unpublished data
2015	IT	Northern Italy (Po Valley)	Active[a]	143	3,106	HI assay (0.5% TRBCs)	364	11.7	Foni et al., (2017)
2017–2018	IT	Northern Italy (Po Valley)	Active[b]	13	173	HI assay (0.5% TRBCs)	5	2.8	Unpublished data
2012	LU	Whole country	Active[a]	27	258	HI assay (1% HRBCs)	0	0	Snoeck et al., (2018)
2014–2015	LU	Whole country	Active[a]	29	287	HI assay (1% HRBCs)	17	5.9	Snoeck et al., (2018)
2015	IE	Whole country	Passive[a]		377	HI assay (0.75% TRBCs)	65	5.8	O'Donovan et al., (2019)

Abbreviations: CRBCs, Chicken Red Blood Cells; FR, France; HI, Hemagglutination Inhibition; HRBCs, Horse Red Blood Cells; IE, Ireland; IT, Italy; LU, Luxembourg; TRBCs, Turkey Red Blood Cells.

[a]While surveillance for IDV was carried out from animals with/without respiratory clinical signs, none of the sera was collected specifically for IDV seroprevalence studies (rather co-products from infectious bovine rhinitis or swine influenza surveillance programs mainly): surveillance stands for observational study here.

[b]Sera collected specifically for an IDV seroprevalence study.

TABLE 3 Overview over available serological results in small ruminants (ovine and caprine species) in France, Italy, and Ireland

Year of sampling	Country	Region/ Department	Type of surveillance[a]	Nr. of herds	Nr. collected sera and species	Screening method	Nr. of positive sera	% of IDV positive sera	References
2016	FR	Bretagne	Active	4	164 (sheep)	HI assay (1% HRBCs)	0	0	Oliva et al., (2019)
2016	FR	Bretagne	Active	10	104 (goat)	HI assay (1% HRBCs)	6	5.8	Oliva et al., (2019)
2014–2015	FR	Hauts-de-France	Active	7	306 (sheep)	HI assay (1% HRBCs)	16	5.5	Oliva et al., (2019)
2015	FR	Hauts-de-France	Active	1	80 (goat)	HI assay (1% HRBCs)	1	1.3	Oliva et al., (2019)
2014–2018	FR	Occitanie	Active	34	960 (sheep)	HI assay (1% HRBCs)	3	0.4	Oliva et al., (2019)
2014–2018	FR	Occitanie	Active	10	441 (goat)	HI assay (1% HRBCs)	12	2.9	Oliva et al., (2019)
2016–2017	IT	Northern Italy (Po Valley)	Active	7	506 (sheep)	HI assay (0.5% TRBCs)	32	6.3	Unpublished data
2016–2017	IT	Northern Italy (Po Valley)	Active	4	188 (goat)	HI assay (0.5% TRBCs)	6	3.1	Unpublished data
2016–2017	IE	Whole Country	Passive		288 (sheep)	HI assay (0.75% TRBCs)	12	4.5	O'Donovan et al., 2019

Abbreviations: FR, France; HI, Hemagglutination Inhibition; HRBCs, Horse Red Blood Cells; IE, Ireland; IT, Italy; TRBCs, Turkey Red Blood Cells.

[a]While surveillance for IDV was carried out from animals with/without respiratory clinical signs, none of the sera was collected specifically for IDV seroprevalence studies (rather co-products from infectious bovine rhinitis or swine influenza surveillance programs mainly): surveillance stands for observational study here.

within the same framework as IDV serosurveillance in cattle (Oliva et al., 2019). In Brittany, no evidence of past exposure was found in sheep sera cohorts (n = 164), whereas in goats (n = 104) 5.8% of samples tested positive. In Hauts-de-France, 5.5% (n = 306) and 1.3% (n = 80) of sheep and goats were seropositive, respectively. In Occitanie, the overall seroprevalence was 0.4% (n = 960) for sheep and 2.9% (n = 441) for goats. The authors reported a significant difference between IDV seroprevalence in cattle and small ruminants

in these regions. Similar results in seroprevalence were found in Italy in sheep (n = 506) and goats (n = 188) cohorts of sera collected in 2016–2017, with 6.3% and 3.1% of tested sera IDV seropositive, respectively (unpublished data). A very low prevalence of 0.98% was observed when wild ungulates (n = 204) collected under the Italian wildlife monitoring program were tested (unpublished data). Finally, in Ireland a seroprevalence of 4.5% (n = 288) was reported in sheep (O'Donovan et al., 2019). Results from serological studies in small ruminants are summarized in Table 3.

Taken together, high IDV seroprevalence in cattle suggested the potential role of the species as primary host of this emerging virus, while available data on pigs and small ruminants suggest that its circulation is limited in these species. Overall the median IDV seroprevalence was significantly higher in cattle than in swine and small ruminants (Kruskal–Wallis equality-of-populations rank test; chi-squared = 24 with 2 *df* and p-value = .0001) but they are not significant between swine and small ruminants (Kruskal–Wallis equality-of-populations rank test; chi-squared = 0.009 with 1 *df* and p-value = .92). High IDV seroprevalence in European cattle is consistent with the findings generated in other continents: in the United States the overall IDV seroprevalence in cattle was 77.5% nationally, ranging from 47.7% to 84.6% depending on the region (Silveira et al., 2019), whereas in South America 73% of tested farms had at least one positive animal (Alvarez et al., 2020). The infection seems less extended in cattle in African countries (Salem et al., 2017) (Fusade-Boyer et al., 2020) than in Europe or America. This could be possibly due to a lower density of animals in cattle industry, as cattle density was found to be a major risk factor for IDV infection occurrence (Fusade-Boyer et al., 2020). Although some studies highlighted IDV circulation in Asian countries by using molecular tools (Murakami et al., 2016; Zhai et al., 2017), little data on IDV seroprevalence in cattle are available for this continent at the moment. In Japan, a recent study highlighted IDV seroprevalence ranging from 45% to 71% in sera collected in Hokkaido prefecture from 2009 to 2018 (Hayakawa et al., 2020), underlining the virus circulation on the island since at least 10 years.

3 | OVERVIEW ON LIVESTOCK TRADE BETWEEN DIFFERENT COUNTRIES IN EUROPE

The European Union has a substantial livestock population: in 2018, Europe counted 148 million heads of pigs, 87 of cattle, 98 of sheep and goats (Eurostat 2018). The most important cattle producer is France, reaching 19 million heads of animals in 2018 (Eurostat), followed by Germany (12 million heads). A considerable number of animals is then exported to neighbouring countries, mostly for production but also for slaughtering and for breeding. In 2018, 3,073,082 cattle heads were traded among EU countries for production, 654,938 heads for slaughtering and 607,226 for breeding. The most important movements of cattle for production took place from France to Italy (almost one million heads), followed by Germany to the Netherlands (531,597 heads), France to Spain (420,774 heads) and Belgium to

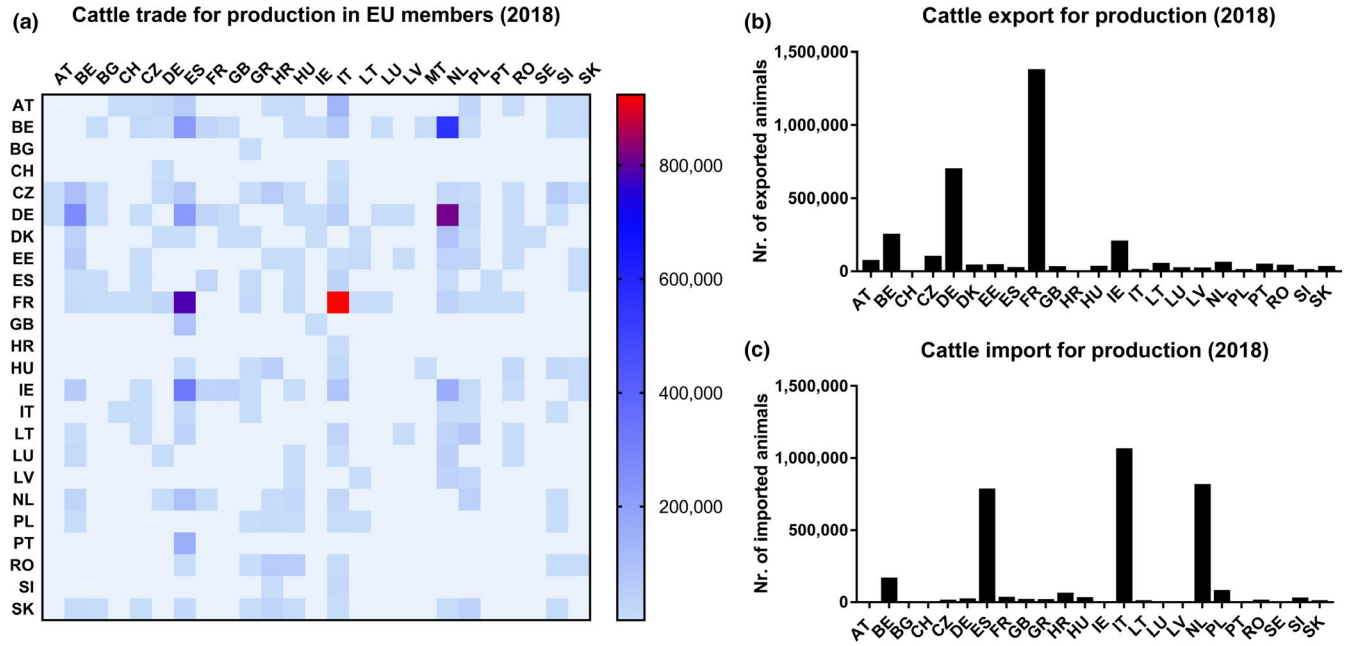

FIGURE 1 Cattle trade in the European Union (2018) (a) Trade matrix showing commercial exchanges of cattle for production in 2018 among EU member countries (source: ec.europa.eu). On the y-axis, the cattle origin country is represented, and on the x-axis, the destination country is showed. The figure legend on the right hand side of the matrix represents the number of exchanged animals. Country names were expressed with two letters of the official ISO code for European Union countries. (b) Histogram showing the number of exported cattle for production in 2018 in different EU member states. (c) Histogram showing the number of imported cattle for production in 2018 in different EU member states

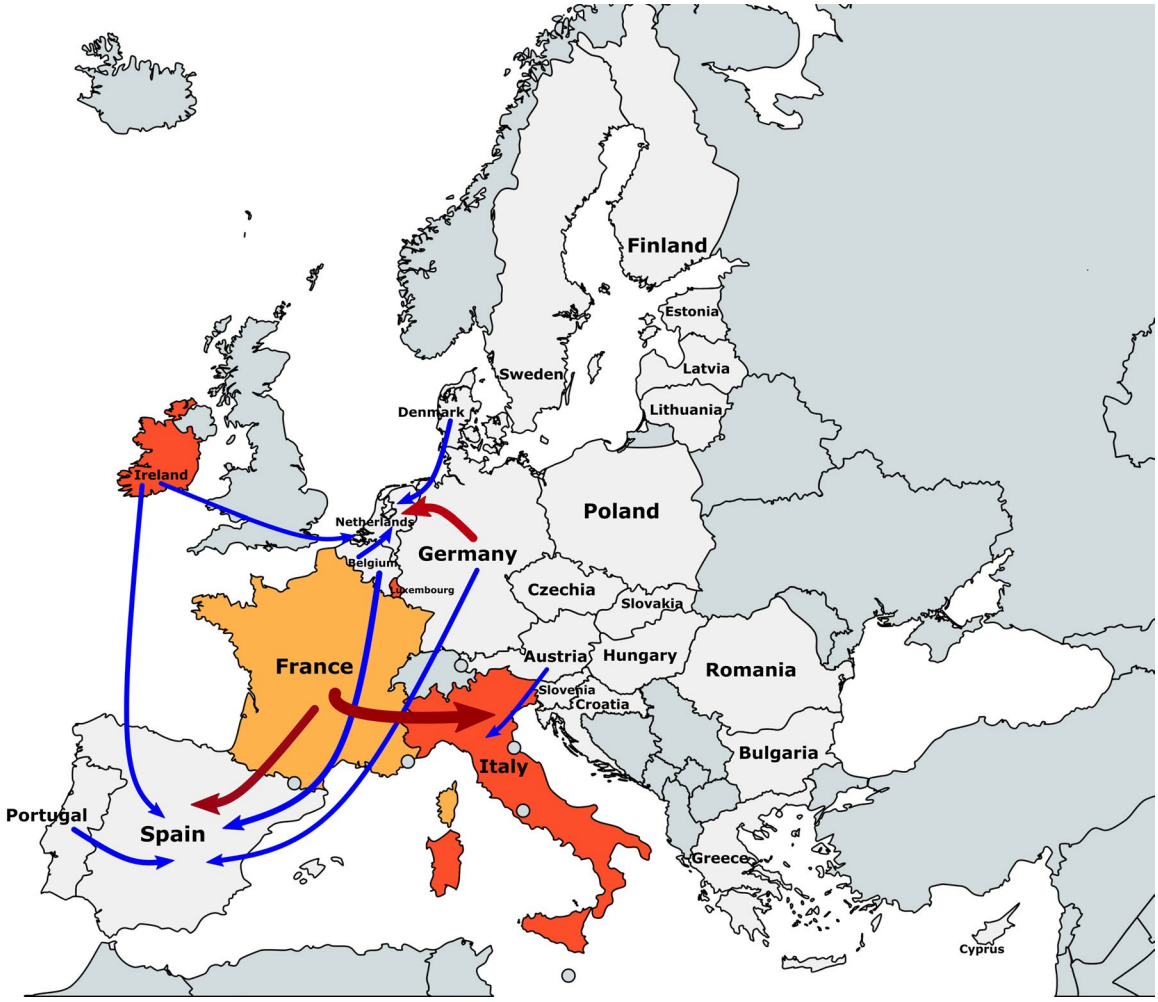

FIGURE 2 Map of Europe representing cattle commercial trades for production among different countries in 2018 (source: ec.europa.eu). The map only shows cattle that has been transported for production. Countries in colour represent areas where IDV seroprevalence has been investigated. Dark red arrows represent cattle movements that include more than 500,000 heads (France-to-Italy direction was highlighted with a bigger arrow, indicating the biggest trade above all in Europe). Blue arrows indicate cattle movements with more than 30,000 heads per year (for simplicity, only trades including more than 30 thousand heads are shown)

the Netherlands (153,508 heads). This makes Italy, Netherlands and Spain the three most important cattle importers in Europe and France and Germany the leading countries for export. A different situation is observed in export for slaughtering: the Netherlands is the leading country for export, Austria and Belgium for import. Cattle trade between different EU countries is summarized in a trade matrix in Figure 1 and on a geographic map in Figure 2.

With regard to swine production, Spain and Germany are leading countries for pig farming, reaching a population of 30,804,102 and 26,445,400 heads in 2018, respectively. In Europe, the total number of traded pigs has greater importance than cattle: in 2018, 8,388,712 heads were traded for slaughter, 24,279,371 were traded for production and 752,501 for breeding. Among pigs traded for production, the vast majority is exported abroad by Denmark, with more than 14 million heads per year, followed by Netherlands (7 million per year). European countries importing most swine are Germany (almost 11 million heads per year) and Poland (7 million heads per year) (Eurostat). Swine trade between different EU countries is summarized in a trade matrix in Figure 3.

Among small ruminants, sheep occupy a much more important place on the market of traded animals than goats. Sheep are mostly traded in Europe for slaughtering, with a total of 2,442,066 heads in 2018 (mostly from France to Spain, UK to Ireland and Hungary to Italy). Also 932,946 heads were traded for fattening (mostly from Spain to Portugal and from Romania to Greece and Hungary). Trade for sheep breeding concerned only 48,104 heads overall. Finally, 25,330 goats were traded for slaughtering, 8,409 for fattening and 4,840 for breeding (Eurostat).

4 | DISCUSSION

Livestock trade is of great economic importance, allowing animal-origin products offer at affordable price for the final consumer,

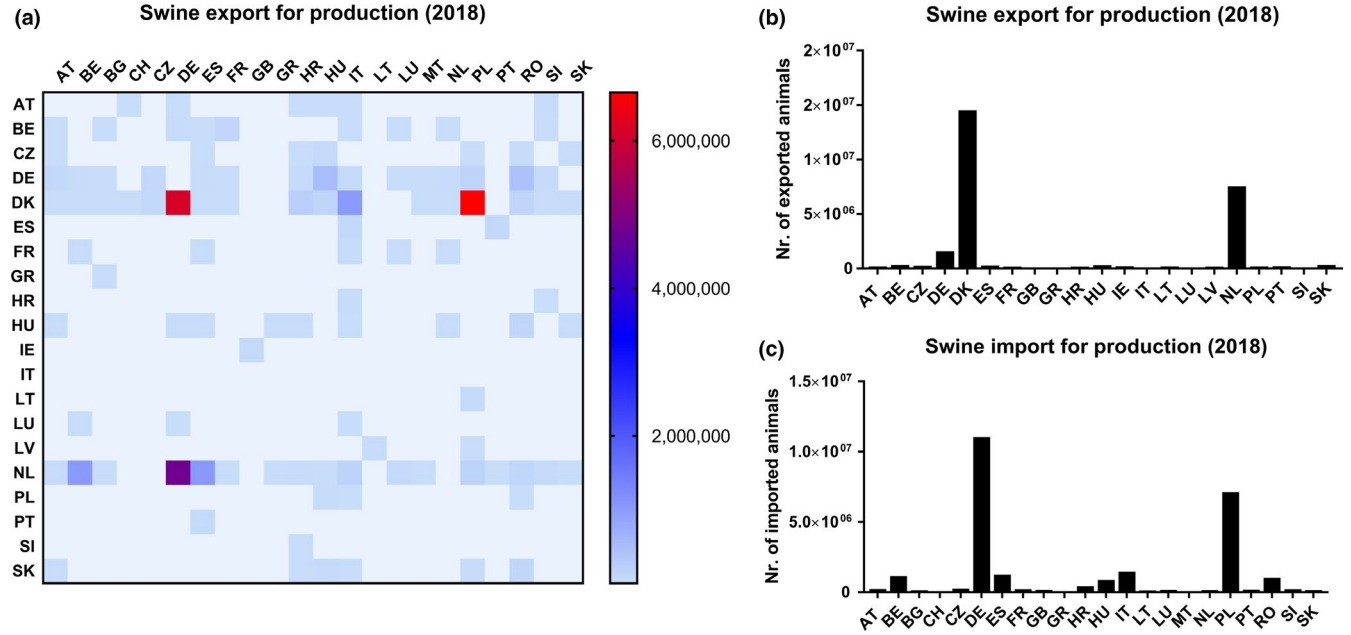

FIGURE 3 Swine trade in the European Union (2018) (a) Trade matrix showing commercial exchanges of swine for production in 2018 among EU member countries (source: ec.europa.eu). On the y-axis, the cattle origin country is represented, and on the x-axis, the destination country is showed. The figure legend on the right hand side of the matrix represents the number of exchanged animals. Country names were expressed with two letters of the official ISO code for European Union countries. (b) Histogram showing the number of exported swine heads for production in 2018 in different EU member states. (c) Histogram showing the number of imported swine heads for production in 2018 in different EU member states

as well as a substantial contribution to the local economy and development. Nevertheless, live animal transport can also lead to health issues that are often only noticed at the destination country. Transport is a very stressful event for animals, with a clear impact on cattle health and production and has a well-documented role in BRDC onset (Buckham Sporer, Weber, Burton, Earley, & Crowe, 2008; Van Engen & Coetzee, 2018). Transportation can cause immunosuppression in young calves, allowing for the colonization by opportunistic pathogens and sometimes causing severe disease (Earley, Buckham Sporer, & Gupta, 2017). Pathogen shedding following transportation has been demonstrated to increase, not only for bacteria such as *Mannhemia haemolytica*, *Mycoplasma bovis* and *Pasteurella multocida* but also for viruses such as Bovine Coronavirus (BCoV) and Bovine Respiratory Syncytial Virus (BRSV) (Cirone et al., 2019). Most importantly, in a study conducted in Mississippi, young calves were sampled before and after admission in herd facility for anti-IDV antibodies and viral RNA detection (Ferguson et al., 2015). Results showed that IDV infection could occur after arrival in the conditioning yard, as some calves tested negative before the arrival by RT-PCR but were positive 1 week later. In addition, the same study showed that almost all neonatal calves were able to acquire anti-IDV antibodies through colostrum after birth but the antibody titres seemed to decrease with age, as at 6–8 months only 3.7%–11.5% of the same calves were IDV seropositive. Seropositivity increased then at 1-year age, suggesting that calves mostly encounter IDV between 6 months to 1 year of age. In Europe, this often corresponds to the period where calves are transported abroad for fattening but also slaughtering, strengthening the hypothesis that trading of young calves in a period of immunologic weakness could contribute to pathogen shedding in the herd of arrival.

In this context, biosecurity is an important measure to prevent livestock pest and disease introduction in farms. In European regulations, biosecurity is defined in the 'Animal Health Law' and other legislation aimed at minimizing animal disease contained in Regulation (EU) 2016/429. On a practical level, some of the recommended practices include isolation for at least 4 weeks for all purchased animals arriving at a farm but also regular equipment sanitation, correct storage of food and water and, when applicable, preventive measures such as vaccination. There are different individuals that play a role in biosecurity implementation, including not only government authorities and legislators but above all farmers and veterinarians. It is often assumed that farmers have the necessary resources and knowledge to minimize the risk of disease introduction. In a survey conducted on dairy cattle farmers in Ireland, most of the interviewees declared that biosecurity is important. Still, half of them also declared a lack of necessary knowledge that would help them in improving their biosecurity measures (Sayers et al., 2013). In addition, a lack of trust of farmers towards governing authorities was shown, arising the belief that biosecurity is primarily a government responsibility, and leading to inobservance of recommended good practices (Higgins, Bryant, Hernandez-Jover, Rast, & McShane, 2018).

As IDV is an emerging pathogen, its veterinary monitoring is still partial. Its novelty and the possible absence of clinical manifestation in infected cattle impair early pathogen detection without specific molecular tools and active surveillance. Although IDV does not cause

concerns for cattle farming to date despite its implication in BRDC, there is a need for a more rigorous surveillance and implementation of biosecurity measures. In particular, observance of recommended practices such as quarantine for purchased animals and testing on the arriving lots is once more advised (Damiaans et al., 2020), as a survey showed that only half of the interviewed farmers apply the quarantine practice and only 7% test animals after purchase (Sayers et al., 2013). Among interviewed farmers answering 'no' to the post-purchase testing, 21% of them thought it was of 'no benefit', 20% declared 'not to know what to test for', 45% were never advised to do so and 13% complained about the cost of testing.

Interestingly, the overall IDV prevalence was found to be lower in countries that mainly export cattle (e.g. France, with a seroprevalence ranging from 33% to 64% depending on the region) than in countries that mainly import cattle from abroad, from instance Italy (from 65% to 95%). This suggests that cattle may come in contact with IDV during or just after transportation and that viral shedding mainly occurs after transportation in the destination countries, contributing to larger diffusion than in origin countries. The role of inter-herd livestock exchanges in disease spread is already known, being of particular concern for airborne transmission pathogens (Pandit, Hoch, Ezanno, Beaudeau, & Vergu, 2016). The assumption of IDV spread though livestock trade is also strengthened by the large diffusion in cattle across all Europe, from Northern to more Southern. The high movement of cattle from France to Italy could have contributed to IDV spread in this country. IDV introduction in Ireland and Luxembourg could have occurred through the import of infected cattle from France or other European countries. Considering the large number of traded animals every year, we speculate that IDV is probably present also outside the four territories surveyed, as already suggested previously for countries bordering Luxembourg given frequent cross-border grazing and trade (Snoeck et al., 2018). A surveillance network extended to other EU members would provide more information about the real spread of this emerging pathogen, in particular in countries importing cattle from areas where IDV is already known to circulate. For instance, IDV surveillance could be useful in leading countries for cattle import in Europe, such as Spain and the Netherlands, where a similar (or even higher) seroprevalence than the origin country could be hypothesized. In addition, a longitudinal study with monitoring of IDV in calves traded from origin country to arrival country would provide additional insight about the real shedding of this pathogen during transport. IDV surveillance implementation is justified by its zoonotic potential and its possible implication in BRDC aggravation.

5 | CONCLUSION

Influenza D virus infection in cattle has spread across different countries in Europe. Surveillance in countries where IDV presence has not been investigated is required in order to understand the real spread of the virus. IDV role in BRDC onset, especially after stress transport experience, is still not clear to date and further analysis could help in determining its actual implication in diseased cattle. We hypothesize the role of livestock trade in the observed differences of IDV seroprevalence among European countries where data are available. In addition to surveillance, implementation of biosecurity measures are once more emphasized (Damiaans et al., 2020), especially at arrival of young cattle in a facility, in order to limit the geographical spread of this emerging respiratory pathogen with zoonotic potential.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this is a review article.

DATA AVAILABILITY STATEMENT

For the most part, data sharing is not applicable to this article as little new data were created or analysed in this study. The unpublished data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Ana Moreno https://orcid.org/0000-0002-8497-9708
Chantal J. Snoeck https://orcid.org/0000-0002-0000-1850
Mariette F. Ducatez https://orcid.org/0000-0001-9632-5499

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2.7 Research article: Influenza D virus in respiratory disease in Canadian, province of Québec, cattle: Relative importance and evidence of new reassortment between different clades

PhD candidate's contribution:

The candidate carried out IDV full-genome sequencing and phylogenetic analyses and participated in drafting the manuscript.

Summary of the research article:

Background: Influenza D virus (IDV), a segmented single-stranded negative-sense ribonucleic acid (RNA) virus, belongs to the new Delta influenza virus genus of the *Orthomyxoviridae* family. Cattle were proposed as the natural reservoir of IDV in which infection was associated with mild-to-moderate respiratory clinical signs (i.e. cough, nasal discharge and dyspnoea). **Methods and principal findings:** In order to investigate the role of IDV in bovine respiratory disease, during the period 2017–2020, 883 nasal or naso-pharyngeal swabs from Canadian cattle with respiratory signs (cough and/or dyspnoea) were tested by (RT-)qPCR for IDV and other major bovine viral (bovine herpesvirus 1, bovine viral diarrhoea virus, bovine respiratory syncytial virus, bovine parainfluenza virus 3 and bovine coronavirus) and bacterial (*Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis*) respiratory pathogens. In addition, whole genome sequencing and phylogenetic analyses were carried out on five IDV-positive samples. The prevalence of IDV RT-qPCR (with cut-off: Cq < 38) at animal level was estimated at 5.32% (95% confidence interval: 3.94–7.02). Positive result of IDV was significantly associated with (RT-)qPCR-positive results for bovine respiratory syncytial virus and *Mycoplasma bovis*. While phylogenetic analyses indicate that most IDV segments belonged to clade D/660, reassortment between clades D/660 and D/OK were evidenced in four samples collected in 2018–2020.

Conclusions and significance: Relative importance of influenza D virus and associated pathogens in bovine respiratory disease of Canadian dairy cattle was established. Whole-genome sequencing demonstrated evidence of reassortment between clades D/660 and D/OK. Both these new pieces of information claim for more surveillance of IDV in cattle production worldwide.

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ORIGINAL ARTICLE

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Influenza D virus in respiratory disease in Canadian, province of Québec, cattle: Relative importance and evidence of new reassortment between different clades

Claude Saegerman[1,*] | Maria Gaudino[2,*] | Christian Savard[3] | André Broes[3] | Olivier Ariel[3] | Gilles Meyer[2] | Mariette F. Ducatez[2]

[1]Fundamental and Applied Research for Animal and Health (FARAH) Center, University of Liège, Liège, Belgium

[2]INRAE UMR 1225 IHAP-ENVT, Toulouse, France

[3]Biovet Inc., Saint-Hyacinthe, Québec, Canada

Correspondence
Claude Saegerman, Fundamental and Applied Research for Animal and Health (FARAH) Center, University of Liège, B-4000 Liège, Belgium.
Email: claude.saegerman@uliege.be; mariette.ducatez@envt.fr

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Abstract

Background: Influenza D virus (IDV), a segmented single-stranded negative-sense ribonucleic acid (RNA) virus, belongs to the new Delta influenza virus genus of the *Orthomyxoviridae* family. Cattle were proposed as the natural reservoir of IDV in which infection was associated with mild-to-moderate respiratory clinical signs (i.e. cough, nasal discharge and dyspnoea).

Methods and principal findings: In order to investigate the role of IDV in bovine respiratory disease, during the period 2017–2020, 883 nasal or naso-pharyngeal swabs from Canadian cattle with respiratory signs (cough and/or dyspnoea) were tested by (RT-)qPCR for IDV and other major bovine viral (bovine herpesvirus 1, bovine viral diarrhoea virus, bovine respiratory syncytial virus, bovine parainfluenza virus 3 and bovine coronavirus) and bacterial (*Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis*) respiratory pathogens. In addition, whole-genome sequencing and phylogenetic analyses were carried out on five IDV-positive samples. The prevalence of IDV RT-qPCR (with cut-off: Cq < 38) at animal level was estimated at 5.32% (95% confidence interval: 3.94–7.02). Positive result of IDV was significantly associated with (RT-)qPCR-positive results for bovine respiratory syncytial virus and *Mycoplasma bovis*. While phylogenetic analyses indicate that most segments belonged to clade D/660, reassortment between clades D/660 and D/OK were evidenced in four samples collected in 2018–2020.

Conclusions and significance: Relative importance of influenza D virus and associated pathogens in bovine respiratory disease of Canadian dairy cattle was established. Whole-genome sequencing demonstrated evidence of reassortment between clades D/660 and D/OK. Both these new pieces of information claim for more surveillance of IDV in cattle production worldwide.

1 | INTRODUCTION

Influenza D virus (IDV), a segmented single-stranded negative-sense ribonucleic acid (RNA) virus, belongs to the new Delta influenza virus genus of the *Orthomyxoviridae* family (https://talk.ictvonline.org/ictv-reports/ictv_online_report/). The virus was discovered in animals showing respiratory signs; initially in 2011, in pig from the United States of America (Hause et al., 2013) and further, in cattle and pigs from Europe and Asia (e.g. Chiapponi et al., 2016; Dane et al., 2019; Ducatez et al., 2015; Murakami et al., 2016; Snoeck

*Claude Saegerman, Maria Gaudino contributed equally to the work.

et al., 2018; Zhai et al., 2017). The list of susceptible hosts growing since the discovery of the virus as IDV-specific antibodies were demonstrated in domestic, feral and wild swine, cattle, small ruminants, horses and camelids (e.g. Dane et al., 2019; Ferguson et al., 2015, 2018; Flynn et al., 2018; Gaudino et al., 2020; Gorin et al., 2019; Murakami et al., 2019; Nedland et al., 2018; O'Donovan et al., 2019; Oliva et al., 2019; Salem et al., 2017; Silveira et al., 2019; Snoeck et al., 2018). A review paper proposed the cattle as reservoir and amplification host with periodic spill over to other hosts listed before (Liu et al., 2020). In addition, a recent study identified IDV genome in Asian poultry farm aerosol. However, other animal species were also present in the same farm (Bailey et al., 2020) and previous serological study indicated no evidence of infection in chicken and turkeys (Quast et al., 2015).

In cattle, IDV infection is associated with mild-to-moderate respiratory clinical signs (e.g. Ferguson et al., 2016; Salem et al., 2019), but the virus also likely plays a role in the bovine respiratory disease complex, so-called BRDC (e.g. Mitra et al., 2016; Ng et al., 2015; Zhang, Hill, Godson, Ngeleka, et al., 2020; Zhang, Outlaw, et al., 2019). The BRDC is one of the most concerning health issues in worldwide cattle industry, being a primary cause of major economic loss in both dairy and beef production. Its aetiology is a combination of multiple factors (Taylor et al., 2010) such as breeding selection, environmental conditions, herd management, transportation and infectious agents of both bacterial and viral origins (though parasites and opportunistic fungi can also be responsible of pneumonia in cattle) (Lekeux, 1995). In the field, BRDC can be triggered by a transient immunosuppression, which can be caused by a stressful event for the animal and/or a primary infection with an encountered pathogen. A bacterial superinfection may then follow, frequently caused by bacteria that are commensal of the bovine respiratory tract. Members of the *Pasteurellaceae* family such as *Mannheimia haemolytica* (*M. haemolytica*), *Pasteurella multocida* (*P. multocida*) and *Histophilus somni* (*H. somni*) have been described as superinfecting agents (Hodgins et al., 2002). In addition, *M. bovis* (class of Mollicutes, family *Mycoplasmataceae*) is another important bacterial pathogen with recognized role in BRDC aetiology, being frequently isolated from diseased cattle (Caswell & Archambault, 2007). Some of the early-stage clinical signs in cattle include hyperthermia, depression, self-isolation, lack of appetite and nasal discharge (McGuirk, 2008). If left untreated, respiratory signs can rapidly progress to cough, fever and rapid or difficulty breathing, sometimes resulting in fatal outcomes. To date, the list of viral agents known to participate in BRDC includes bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus-3 (BPiV-3), bovine adenovirus, bovine viral diarrhoea virus (BVDV), bovine herpesvirus 1 (BoHV-1) and bovine coronavirus (BCoV) (Grissett et al., 2015). Some of these viruses can successfully cause severe disease alone (e.g. BRSV) (Antonis et al., 2010; Odeón et al., 1999), whereas some others are less pathogenic (i.e. BPiV-3) and fatal cases due to infection by itself are rare. In addition, evaluation of attributable lesions is usually complicated by the involvement of multiple pathogens (Ellis, 2010). So far, IDV pathogenesis has been investigated in an experimental in vivo model, and the virus was shown to cause mild-to-moderate respiratory signs with successful replication and lesions in both, the URT and LRT (Ferguson et al., 2016; Salem et al., 2019). Little data are currently available about the possible role of IDV in BRDC onset. In some case–control studies, using a metagenomics approach, IDV has been detected both in healthy and diseased animals but with significantly higher association in animals affected by respiratory disease (Mitra et al., 2016; Ng et al., 2015; Zhang, Hill, et al., 2019) supporting this initial hypothesis. Despite this, no experimental data are to date available to confirm a real effect of IDV on this complex infectious disease.

In addition, studies suggested that the IDV may infect human (Borkenhagen et al., 2018; Hause et al., 2013; Trombetta et al., 2019), especially when they are exposed to cattle (White et al., 2016). However, despite the presence of pieces of evidence, its zoonotic potential is still matter of debate and strong evidence are still lacking to establish a real species jump (Kumari & Kumar, 2019; Trombetta et al., 2019).

Since its discovery, IDV has evolved into two main clades (depending on the species where it was isolated (D/swine/Oklahoma/1334/2011 (D/OK) and D/bovine/Oklahoma/660/2013 (D/660)) (Collin et al., 2015). In addition, two new Japan clades have been described in cattle (D/Yama2016 and D/Yama2019) (Murakami et al., 2020). In Japan, IDV continues to evolve in cattle (Hayakawa et al., 2020). Indeed, some clades co-circulate in cattle and pig facilitating reassortment (between clades) with possible impact on pathogenicity of the reassortant. Similar evolutionary patterns have been frequently detected with influenza A viruses in birds and pigs where numerous reassortment events have been described (reviewed in Steel & Lowen, 2014). However, reassortment events between influenza genera cannot occur (Gao et al., 2019; Hause et al., 2014).

To date, IDV circulation has only been described in Western Canada and data about its presence in the eastern part of the country are still lacking. In addition, no current genetic characterization of IDV circulating in Canada is available. In order to investigate the role of IDV in the BRDC, a large number of cattle exhibit respiratory signs were sampled and systematically tested by (RT-)qPCR to detect the genome of the main common viral and bacterial respiratory pathogens. In addition, whole-genome sequencing and phylogenetic analyses were carried out on some IDV-positive samples in order to characterize the strains currently circulating in cattle of this Canadian region.

2 | MATERIALS AND METHODS

2.1 | Samples

Samples (n = 883 animals) were collected from January 2017 to August 2020 in cattle herds from the province of Québec in the eastern part of Canada. In this province, around 5,000 dairy cattle farms are registered (on average, each farm contains 70 dairy

cows, which produce 600,000 litres of milk by year). Animals stay on farm during the year. Samples consisted in nasal or nasopharyngeal swabs from cattle with respiratory disorders (i.e. cough and/or dyspnoea). Most of them were born and raised in dairy farms in the province of Québec. Breed (mostly Holstein) and age (months to years) of tested animals were not specified in most of the cases.

2.2 | Climate

With an average temperature of 19.9°C, July is the hottest month of the year in Québec. January is the coldest month of the year with an average temperature of −11.1°C. February is the driest month of the year with 71mm of rainfall in average. In July, the rainfall is the heaviest of the year with an average of 117 mm (https://fr.climate-data.org/amerique-du-nord/canada/Québec/Québec-663/).

2.3 | Samples preparation

First 500 μl of PBS is added to the swabs, which are then vortexed vigorously. Nucleic acids were extracted from 200 μl suspension using a nucleic acids purification kit (MagMAX™ Pathogen RNA/DNA Kit, Thermo Fisher) on automated KingFisher™ Flex Purification System (Thermo Fisher) according to manufacturer's instructions and eluted with 90 μl of nuclease-free water.

2.4 | Polymerase chain reaction testing for respiratory pathogens

Samples were examined for the major bovine viral (BoHV-1, BRSV, BPiV-3 and BCoV) and bacterial (*M. haemolytica*, *P. multocida*, *H. somni* and *M. bovis*) respiratory pathogens using Bovichek® MRB bacteria qPCR and Bovichek® MRB virus qPCR kits (Biovet, Saint-Hyacinthe, QC, Canada). Testing was performed according to manufacturer's instructions. Samples were examined for IDV using primers and probe previously described (Hause et al., 2013). Samples were also examined for presence of BVDV using a commercial detection kit (EXOone BVDV-BDV, ONEmix detection kit, Exopol, Zaragoza, Spain). Results were interpreted as 'positive' when the Cq value was ≤38.

2.5 | Whole-genome sequencing

Whole genome of five IDV-positive samples was amplified by RT-PCR with QIAGEN One-Step RT-PCR Kit (Qiagen) by using primers described in (Ducatez et al., 2015). PCR products were sequenced with Sanger technology (Eurofins, GATC), and obtained sequences were manually curated with BioEdit v7.1. Multiple sequence alignments were generated using ClustalW algorithm (http://www.clustal.org). Genomic characterization and evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). After determining the best DNA model to use for each alignment, maximum likelihood phylogenetic trees were constructed for all seven IDV segments. For statistical support, 500 bootstrap replicates were used for the analysis.

2.6 | Statistical analysis

2.6.1 | Basic statistics

Different statistics were used depending on the objective followed. To estimate the 95% confidence interval (CI) of the prevalence of pathogens, an exact binomial distribution was used (Petrie & Watson, 2013). For the comparison of frequency between the identification of pathogens alone or in association with other selected pathogen(s), Pearson correlation coefficient test was used (Petrie & Watson, 2013). The correlation between selected pathogens was assessed using binary Jaccard similarity coefficient (Chung et al., 2019). All analyses were performed using Stata SE 14.2 (StataCorp). The limit of significance was .05.

2.6.2 | Binary logistic regression

A univariate followed by a multivariate binary logistic regression using backward stepwise approach was used to check the relation between the IDV status of cattle (confirmed or unconfirmed cases) and other selected viruses and bacteria (Petrie & Watson, 2013). First, the multivariate binary logistic regression included all explanatory variables with a p-value ≤ .2 as assessed in the univariate binary logistic regression. Secondly, to assess the collinearity, a backward elimination of variables was performed (Preux et al., 2005). In this stepwise approach, the non-significant variables (p-value > .05) were removed starting from the less significant (highest p-value). At each step, a likelihood-ratio test comparing the two nested models allowed for the comparison of the simplified with the more complex model. The final model was selected when the likelihood-ratio test highlighted a significant difference between the more complex and the simplified model (p-value < .05). Goodness of fit was assessed using the Hosmer–Lemeshow goodness-of-fit test (Petrie & Watson, 2013). All analyses were performed using Stata SE 14.2 (StataCorp). The limit of significance was .05.

2.6.3 | Overall pondered score

An overall pondered score (OPS) by cattle was defined using significant variable identified by the binary multivariate logistic regression and pondered by its respective odds ratio (see formula presented in the Results section).

2.6.4 | Receiver operating characteristic curve

A ROC curve (probability curve) was plotted with true-positive results (Y-Axis) against the false-positive results (X-Axis). The AUC-ROC is the performance measurement for the classification of the OPS at various thresholds settings. The higher the AUC-ROC, the better the OPS is able to distinguish between confirmed and unconfirmed IDV cases (i.e. measurement of the separation of the two sub-populations). In addition, Youden's index 'J' is frequently used in conjunction with the ROC curve analysis to estimate the best cut-off (Petrie & Watson, 2013), with:

$$\text{Youden}'\text{s index} = \text{sensitivity} + \text{specificity} - 1 \quad (1)$$

The value of AUC-ROC ranges from 0 to 1 (inclusive). A zero value is observed when a diagnostic test gives the same proportion of positive results for groups confirmed or unconfirmed IDV cases. A value of 1 indicates that there are no false positives or false negatives, that is that the test is perfect.

2.6.5 | Classification and regression tree analysis

A classification tree analysis (CTA) was conducted on the data set. The dependent variable was IDV status (confirmed versus unconfirmed cases by RT-qPCR). The independent variables were other pathogen(s) detection. A CTA is a non-linear and non-parametric model that is fitted by binary recursive partitioning of multidimensional covariate space (Breiman et al., 1984; Saegerman et al., 2011; Saegerman et al., 2004; Speybroeck et al., 2004). Using Salford Predictive Modeler (SPM) 8.3.2. (Minitab LLC) (Steinberg & Colla, 1997), the analysis successively splits the data set into increasingly homogeneous subsets. The Gini index was used as the splitting method, and 10-fold cross-validation was used to test the predictive capacity of the obtained trees (Breiman et al., 1984). CTA performs cross-validation by growing maximal trees on subsets of data, then calculating error rates based on unused portions of the data set. To accomplish this, CTA divides the data set into 10 randomly selected and roughly equal parts, with each 'part' containing a similar distribution of the data from the populations of interest (i.e. IDV confirmed versus unconfirmed cases). The CTA then uses the first nine parts of the data, constructs the largest possible tree and uses the remaining 1/10 of the data to obtain initial estimates of the error rate of the selected subtree. The process is repeated using different combinations of the remaining nine subsets of data and a different 1/10 data subset to test the resulting tree. This process is repeated until each 1/10 subset of the data has been used to test a tree that has been grown using a 9/10 data subset. The results of the 10-mini tests are then combined to calculate error rates for trees of each possible size. These error rates are applied to prune the tree grown using the entire data set. The consequence of this complex process is a set of reliable estimates of the independent predictive accuracy of the tree. For each node in a CTA, the 'primary splitter' is the variable that best splits the node, maximizing the purity of the resulting nodes.

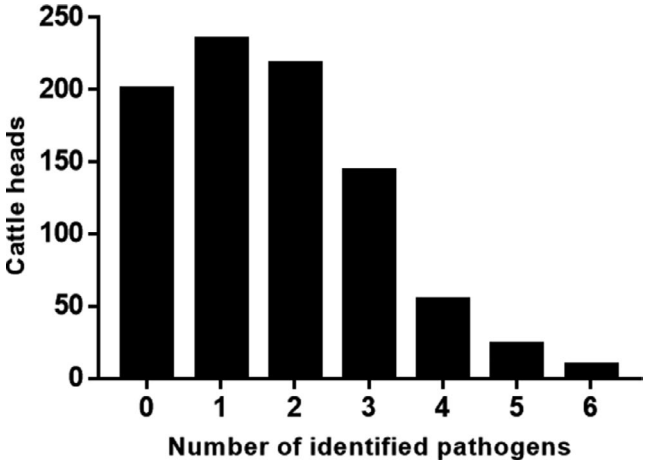


FIGURE 1 Number of pathogens detected per cattle head showing respiratory signs ($N = 883$)

3 | RESULTS

3.1 | Prevalence of selected pathogens in cattle showing respiratory signs

The number of cattle showing respiratory signs in function of the number of pathogens identified was depicted in Figure 1. Two hundred cattle heads (23%) were negative for all the tested respiratory pathogens despite respiratory signs. For 453 cattle (51%), one or two pathogens were identified, and for 230 cattle, more than two pathogens (up to six) were identified (26%).

The prevalence of each pathogen identified is presented in Table 1. Influenza D virus was identified in 47 samples, and similar prevalence was found for BPiV-3 and BoHV-1. BCoV was assessed as the most prevalent virus in the studied cohort, and bacteria from the *Pasteurellaceae* family showed the highest importance among all detected respiratory pathogens. In particular, *P. multocida* was present in more than 50% of tested samples. The pathogen with the lowest prevalence was BVDV, for which only 12 samples were RT-qPCR positive.

The frequencies between the identification of pathogens alone or in association with other selected pathogen(s) are highly correlated (Pearson correlation coefficient = .92; p-value = .0002). In order to assess the relative contribution of each pathogen in the respiratory disorders, we created a ratio between the frequency of presence of the pathogen alone and the frequency of the pathogen associated to other pathogens (Table 2). In mono-infections, the most frequently detected pathogens were *P. multocida*, *M. haemolytica*, BCoV and *H. somni*, in decreasing order. In co-infections, the most frequently detected pathogen was *M. bovis*, followed by IDV and BPiV-3. Our results showed that IDV is found with higher frequency in co-infections than alone, similarly to other viruses such as BPiV-3 and BRSV. Relative contribution of BVDV could not be assessed for co-infections as it was only detected in combination with other pathogens in all samples.

TABLE 1 Prevalence of selected pathogens in cattle showing respiratory signs ($N = 833$)

Pathogens	Prevalence (one or more selected pathogens)			Prevalence (pathogen alone)		
	Np	%	95% CI	Np	%	95% CI
Viruses						
BCoV	231	25.2	23.3–19.2	27	3.1	2.0–4.4
BRSV	123	13.9	11.7–16.4	13	1.5	0.8–2.5
IDV	47	5.3	3.9–7.0	3	0.3	0.07–1.0
BPiV–3	47	5.3	3.9–7.0	3	0.3	0.07–1.0
BoHV–1	45	5.1	3.7–6.8	7	0.8	0.3–1.6
BVDV	12	1.4	0.7–2.4	0	0	0–0.4(¶)
Bacteria						
P. multocida	450	51.0	47.6–54.3	88	10.0	8.1–12.1
M. haemolytica	265	30.0	27.0–33.2	45	5.1	3.7–6.8
H. somni	211	23.9	21.1–26.8	26	2.9	1.9–4.3
M. bovis	207	23.4	20.7–26.4	12	1.4	0.7–2.4

Note: Np, Number of positive animals; CI, confidence interval; BCoV, bovine coronavirus; BRSV, bovine respiratory syncytial virus; IDV, influenza D virus; BPiV-3, bovine parainfluenza virus 3; BoHV-1, bovine herpesvirus 1; BVDV, bovine viral diarrhoea virus; (¶) one-sided, 97.5% confidence interval.

TABLE 2 Relative contribution of each pathogen in bovine respiratory disease complex (presented in decreasing and increasing orders for pathogens in mono- and co-infections, respectively)

Pathogen	Relative contribution		Ratio	
	Mono-infection [A]	Co-infection [B]	[A]/[B]	[B]/[A]
P. multocida	88	362	0,24	4.11
M. haemolytica	45	220	0,2	4.89
BoHV–1	7	38	0,18	5.43
H. somni	26	185	0,14	7.12
BCoV	27	204	0,13	7.56
BRSV	13	110	0,12	8.46
BPiV–3	3	44	0,07	14.67
IDV	3	44	0,07	14,67
M. bovis	12	195	0,06	16.25
BVDV	0	12	0	-

Note: Code of colour for the coefficients in function of the increasing importance of the binary similarity (green to red). Bo-CoV, bovine coronavirus; BRSV, bovine respiratory syncytial virus; IDV, influenza D virus; BPiV-3, bovine parainfluenza virus 3; BoHV-1, Bovine herpesvirus 1; BVDV, bovine viral diarrhoea virus.

3.2 | Correlation between selected pathogens in respiratory disease

In order to visualize the possible correlation between selected pathogens associated with BRDC of Canadian dairy cattle, a matrix of binary Jaccard similarity coefficients was calculated (Figure 2). The six most important correlation (>.2) of selected pathogens found were, in decreasing order, *P. multocida* and *M. bovis*, *P. multocida* and *M. haemolytica*, *H. somni* and *M. bovis*, *P. multocida* and BoCV, *M. bovis* and BoCV, and *H. somni* and *P. multocida*.

3.3 | IDV is more associated with BRSV and *M. bovis*

In order to identify associated selected pathogens with IDV detection, binary univariate and multivariate logistic regression analyses were performed. First variables with *p*-value less than .2 in the univariate analysis (BRSV, BVDV, *M. bovis*, *M. haemolytica* and *P. multocida*) were introduced in a binary multivariate logistic regression using a backward stepwise approach. Three pathogens were retained but only the two first were significant: BRSV with an odd ratio of 2.16 (95% CI: 1.07–4.35; *p*-value = .03), *M. bovis* with an odd ratio of 2.63 (95% CI: 1.42–4.88; *p*-value = .002) and *P. multocida* with an odd ratio of 1.44 (95% CI: 0.75–2.74; *p*-value = 0.27). The Hosmer–Lemeshow goodness-of-fit test showed that the model adequately fits the data (Hosmer–Lemeshow Chi2 (4 *df*) = 1.63 with *p*-value = .80).

A sensitivity analysis was added considering the positivity threshold at Cq <35 in (RT-) qPCR and led to the same result. Finally, using a classification tree analysis (CTA), the previous result was confirmed (Figure 3). In addition, BVDV was shown to play a role in association with IDV in a few number of cases. The sensitivity and specificity of the CTA reached 59.6% (95% CI: 44.3–73.6) and 67.9% (95% CI: 64.7–71.1), respectively.

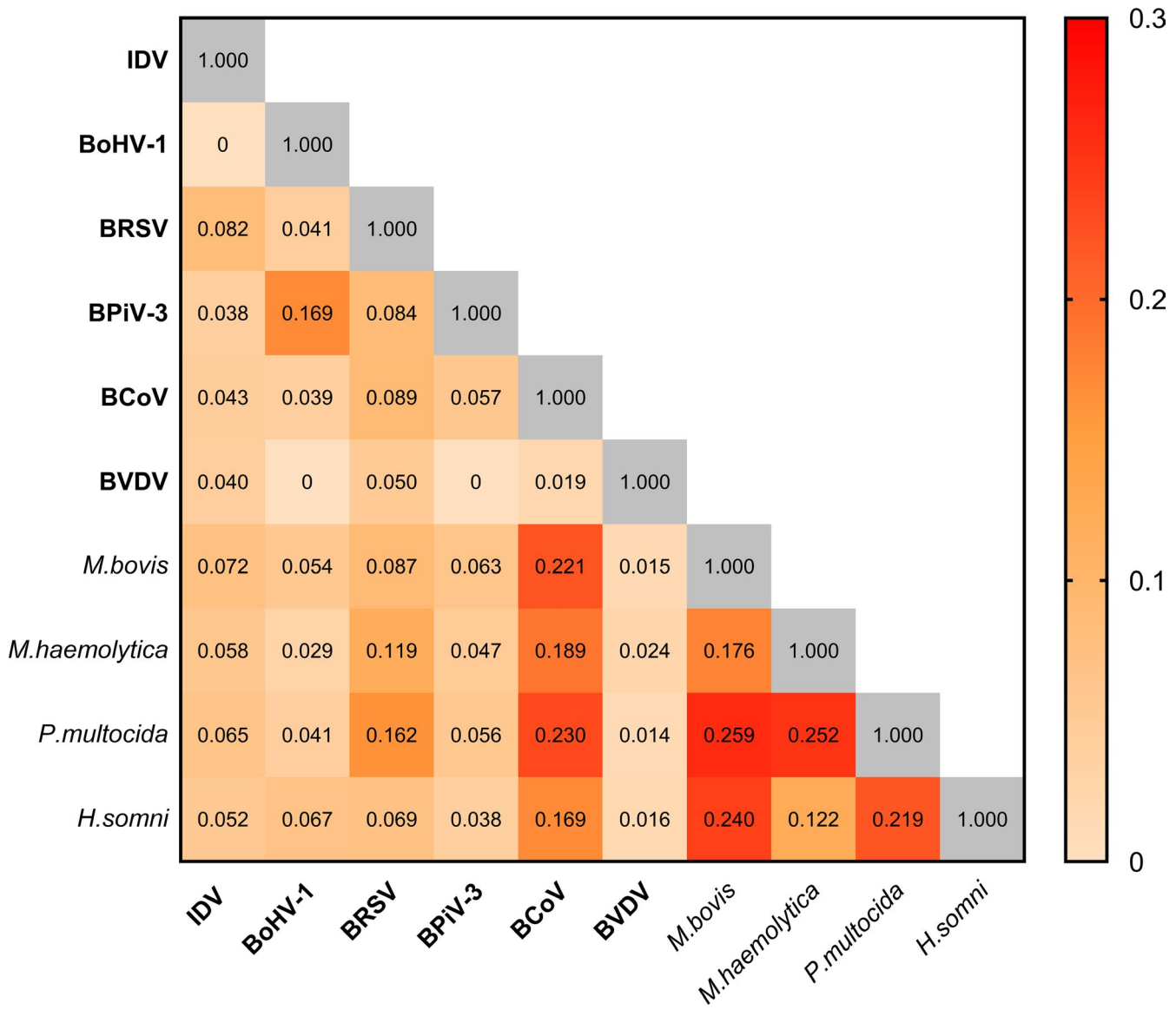


FIGURE 2 Matrix of binary Jaccard similarity coefficients between influenza D virus and selected pathogens in respiratory disease of Canadian dairy cattle ($N = 883$). Colour code for the coefficients in function of the increasing importance of the binary similarity: light orange to dark orange. Viruses: BoHV-1, bovine herpesvirus 1; BVDV, bovine viral diarrhoea virus; BRSV, bovine respiratory syncytial virus; BPiV-3, bovine parainfluenza virus 3; BCoV, bovine coronavirus

3.4 | Overall pondered score and area under the receiver operating characteristic curve

Using the output of the binary multivariate logistic regression, a pondered score was created and used to perform a receiver operating characteristic (ROC) curve (Figure 4). This score considers the significant associated pathogens with IDV detection. Ponderation of each significant associated pathogen takes into account each odds ratio obtained.

The overall pondered score (OPS) by cattle was defined using the following formula:

$$\text{OPS} = [(\text{Presence of BRSV} = 1) * (\text{OR}_{\text{BRSV}})] + [(\text{Presence of Mycoplasma bovis} = 1) * (\text{OR}_{\textit{Mycoplasma bovis}})]$$

With OPS, overall pondered score; OR, odds ratio presented in the previous section.

The area under the ROC curve was 0.67 with standard error of 0.05 and a 95% CI between 0.57 and 0.78, suggesting a contribution of BRSV and/or *M. bovis* in the detection of IDV. Using the Youden index (i.e. 0.20), the best cut-off to discriminate the two sub-groups (positive and negative IDV RT-qPCR) was OPS = 2.

3.5 | Molecular characterization of IDV

Four whole genomes and one partial genome of IDV sampled in cattle in Québec province were obtained, and sequences were deposited in GenBank and designated as described in Table 3.

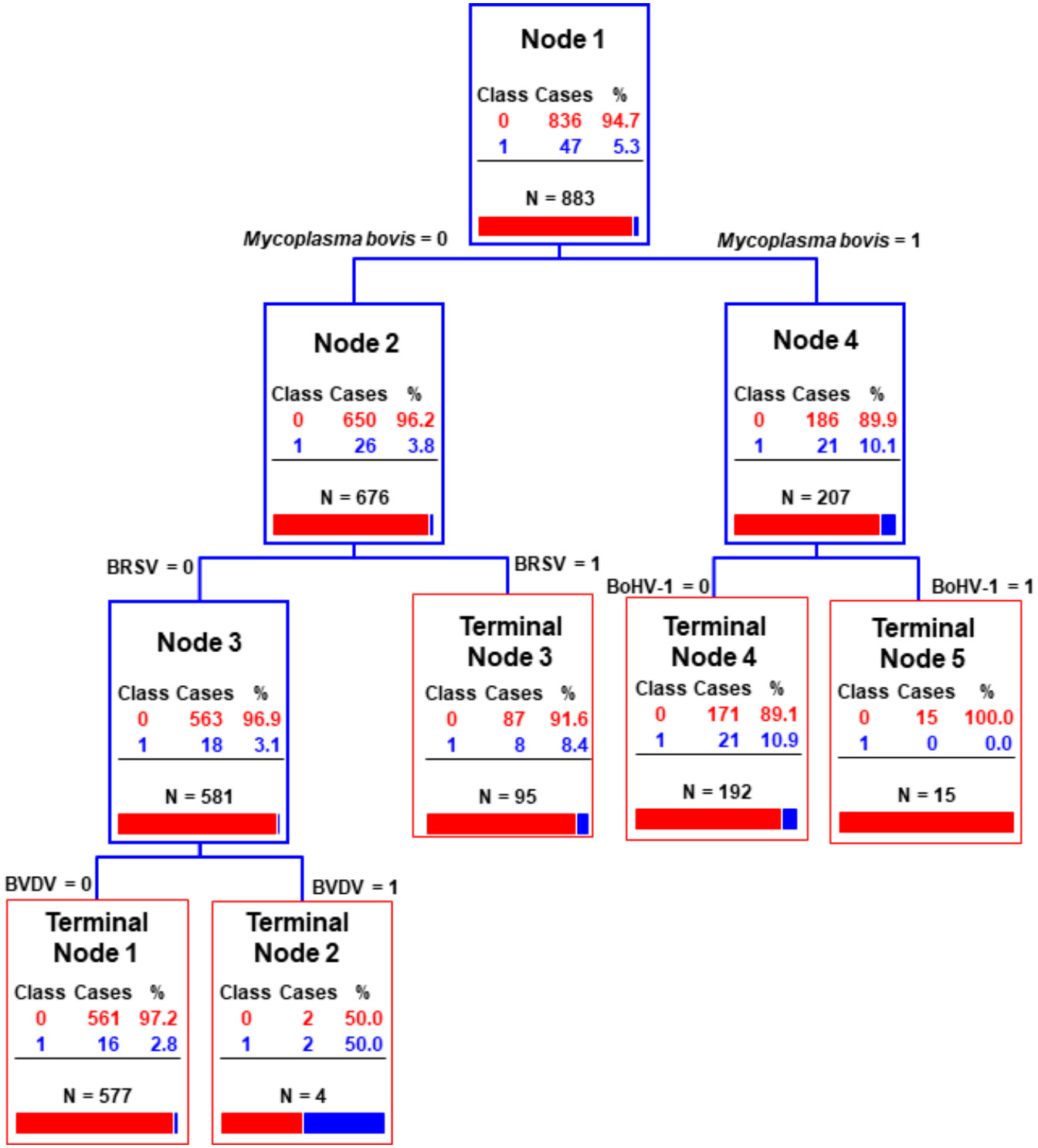


FIGURE 3 Associated selected pathogens with influenza D virus detection using classification tree analysis ($N = 883$). Legend: Class 0 (red), IDV negative qPCR; Class 1 (blue), IDV-positive qPCR; BRSV, bovine respiratory syncytial virus; BVDV, bovine viral diarrhoea virus; BoHV-1, bovine herpesvirus 1

Phylogenetic analyses were performed on all IDV seven segments for each specimen. Our phylogenetic analyses revealed, as already reported in literature, PB1 as the most conserved gene in Influenza D virus, being the only segment that prevents a genetic discrimination between the two major circulating clades (D/OK and D/660). While most segments belonged to clade D/660 (Figure 5), evidence of reassortment between clades D/660 and D/OK were observed in three samples collected in 2019 and 2020 for polymerase P3 segment (Figure 5). In addition, the fifth segment coding for the nucleoprotein seemed to diverge from D/660 clade in samples D/bovine/ Québec/5E-H/2018 and D/bovine/ Québec/1 M-H/2019 (Figure 5). The NP gene segment of these

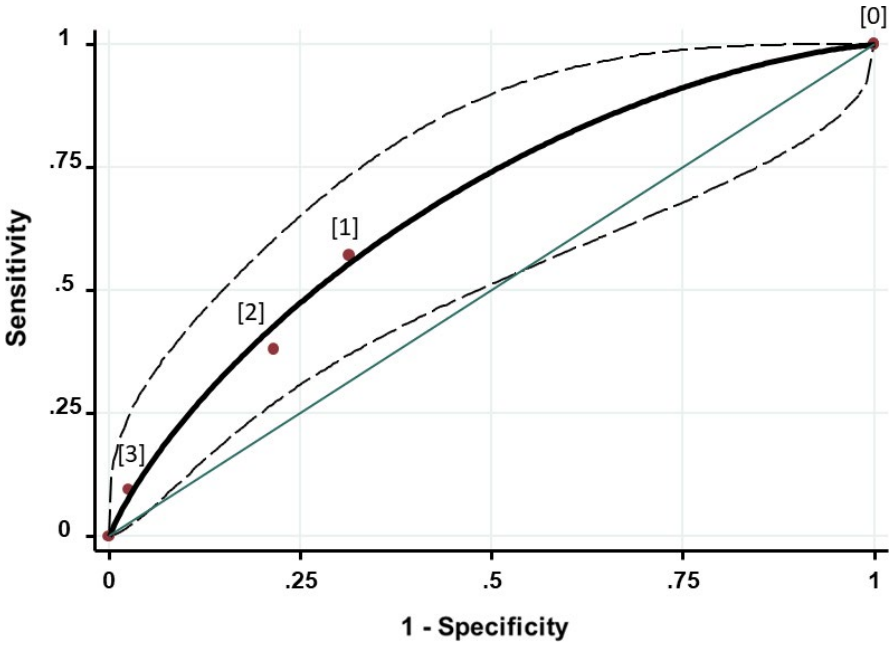


FIGURE 4 Receiver operating characteristic curve of the overall pondered score of IDV. Points are observed values; the solid curve in black and its 95% confidence interval (broken curves in black) was fitted according to a binormal distribution. Area under curve = 0.67 (95% CI: 0.57–0.78) with standard error =0.05. [0], [1], [2], [3], values of the overall pondered score; with [2], the best cut-off point

TABLE 3 Molecular characterization of cattle samples positive for IDV ($N = 5$)

Virus name	Date of sampling	Region of sampling	GenBank Accession Nos.
*D/bovine/Québec/3E-H/2018	November 2018	Estrie	MT246280–85
D/bovine/Québec/5E-H/2018	December 2018	Estrie	MT246266–72
D/bovine/Québec/1 M-H/2019	April 2019	Mauricie	MT246273–79
D/bovine/Québec/3 M-B/2020	January 2020	Québec	MT246286–92
D/bovine/Québec/4Q-J20/2020	January 2020	Montérégie	MT246293–99

*Indicates that partial genome was obtained.

two 2018–2019 Québec strains closely clustered with D/swine/Kentucky/17TOSU1262/2017 in a branch sharing a common ancestor with D/OK-like viruses. They, however, had a closer genetic identity with D/660-like NP sequences. Their classification in a given cluster is therefore not possible at this stage and would require more surveillance and IDV genome sequence data (Figure 5). No information about NP segments is available for fifth sample D/bovine/ Québec/3E-H/2018 (NP could not be amplified by RT-PCR). In addition, HEF was found to be divergent from D/660 clade in one sample collected in January 2020, suggesting a possible new divergent circulating clade.

4 | DISCUSSION

Since its first detection in 2011 (Hause et al., 2013), IDV was qualified as an emerging issue by Emerging Risks Exchange Network of the European Food Security Authority (EFSA, 2016). This novel IDV was shown to infect farm animals including swine and cattle and to efficiently replicate and transmit in ferrets (*Mustela putorius furo*), the animal model of choice for transmission of influenza A virus to humans (Chiapponi et al., 2020).

In this study, we identified the presence of IDV nucleic acids in a large cohort of dairy cattle (both young and adult) showing respiratory clinical signs ($N = 883$), from eastern Canada, in province of Québec, between 2017 and 2020. In addition, we investigated its association with other selected respiratory pathogens of both bacterial and viral origins. IDV is currently circulating in the studied region with an overall prevalence of 5.3% from cattle with respiratory clinical signs, a rate similar to BPiV-3 and BoHV-1. Another study in North America described swabs testing positive by RT-qPCR for IDV, in 16 of 55 (29.1%) respiratory sick calves and 2 of 82 (2.4%) healthy calves (Ferguson et al., 2015). More recently, Zhang et al. (2020a) showed that 53 out of 232 samples collected in western Canada (prevalence of 22.8%) were positive to IDV. The difference in prevalence rate with these studies may be attributed to the age and origin of animals. In addition, the relative contribution of IDV in mono- and co-infections was estimated using a ratio between samples only positive for IDV and samples positive for both IDV and other respiratory pathogen(s). Similarly to other viruses, such as BPiV-3, BRSV and BCoV, the prevalence of IDV was higher in co-infections than in mono-infections, suggesting that the level of clinical severity of infection might be related to some specific association of pathogens. Despite recent field study evidenced that IDV viral

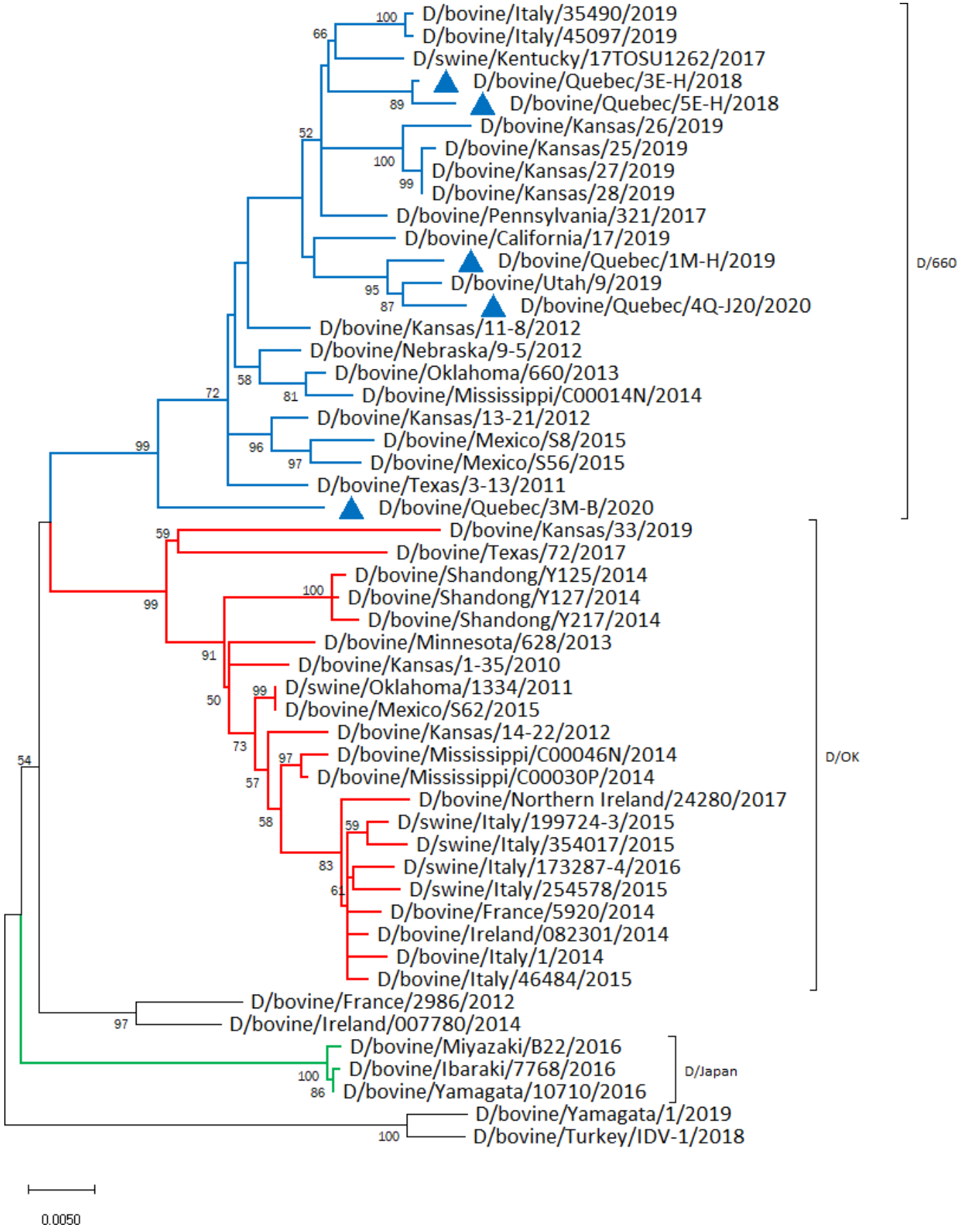


FIGURE 5 Phylogenetic trees obtained for each of the seven influenza D virus (IDV) segments Legend: Triangles depict the study IDV specimens. Maximum likelihood phylogenetic trees were constructed for all seven IDV segments (HEF [a], PB2 [b], PB1 [c], P3 [d], NP [e], P42 [f] and NS [g]) using 500 bootstrap replicates. Clade D/660, D/OK, D/Japan viruses were depicted with blue, red and green branches, respectively. Québec sequences are indicated with triangle shaped symbols

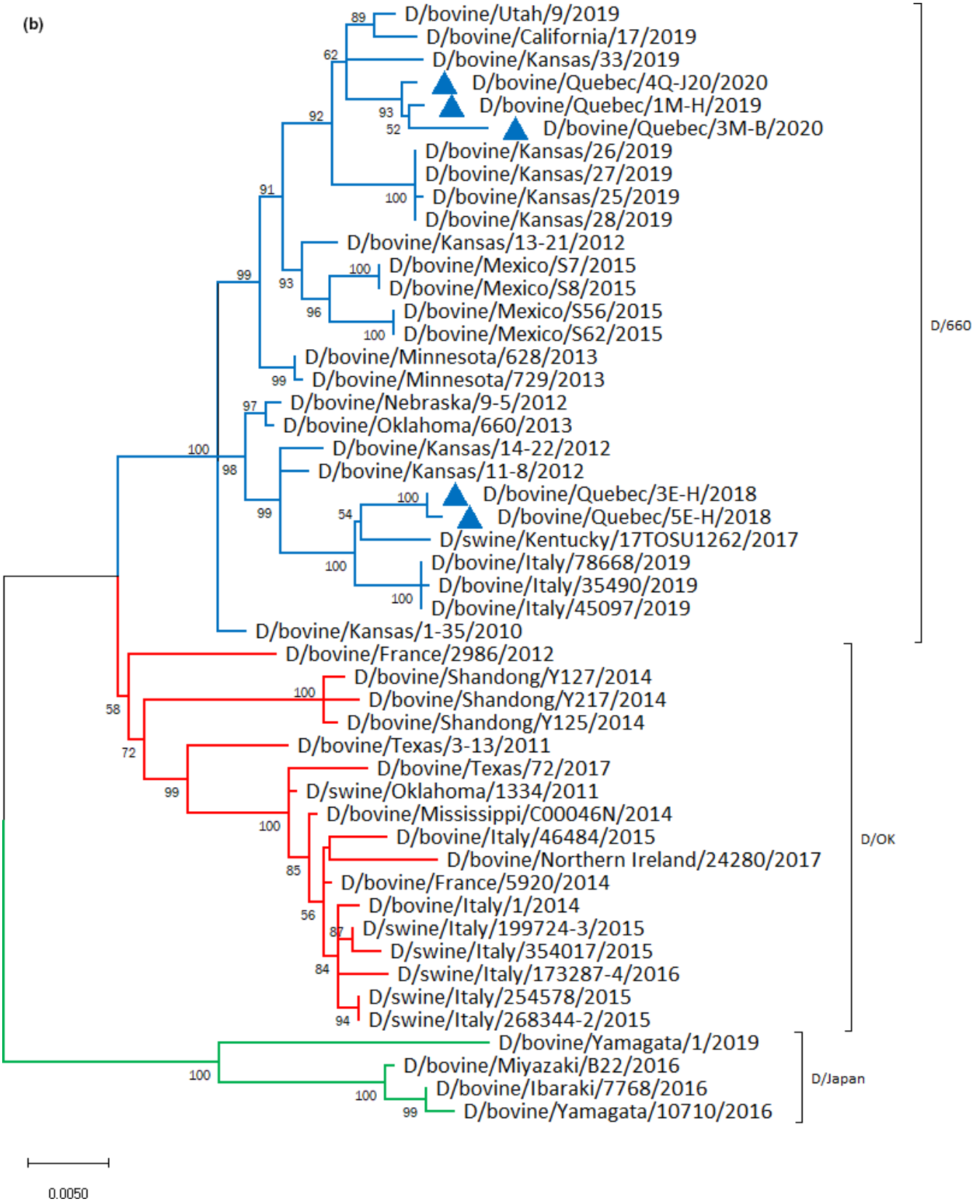
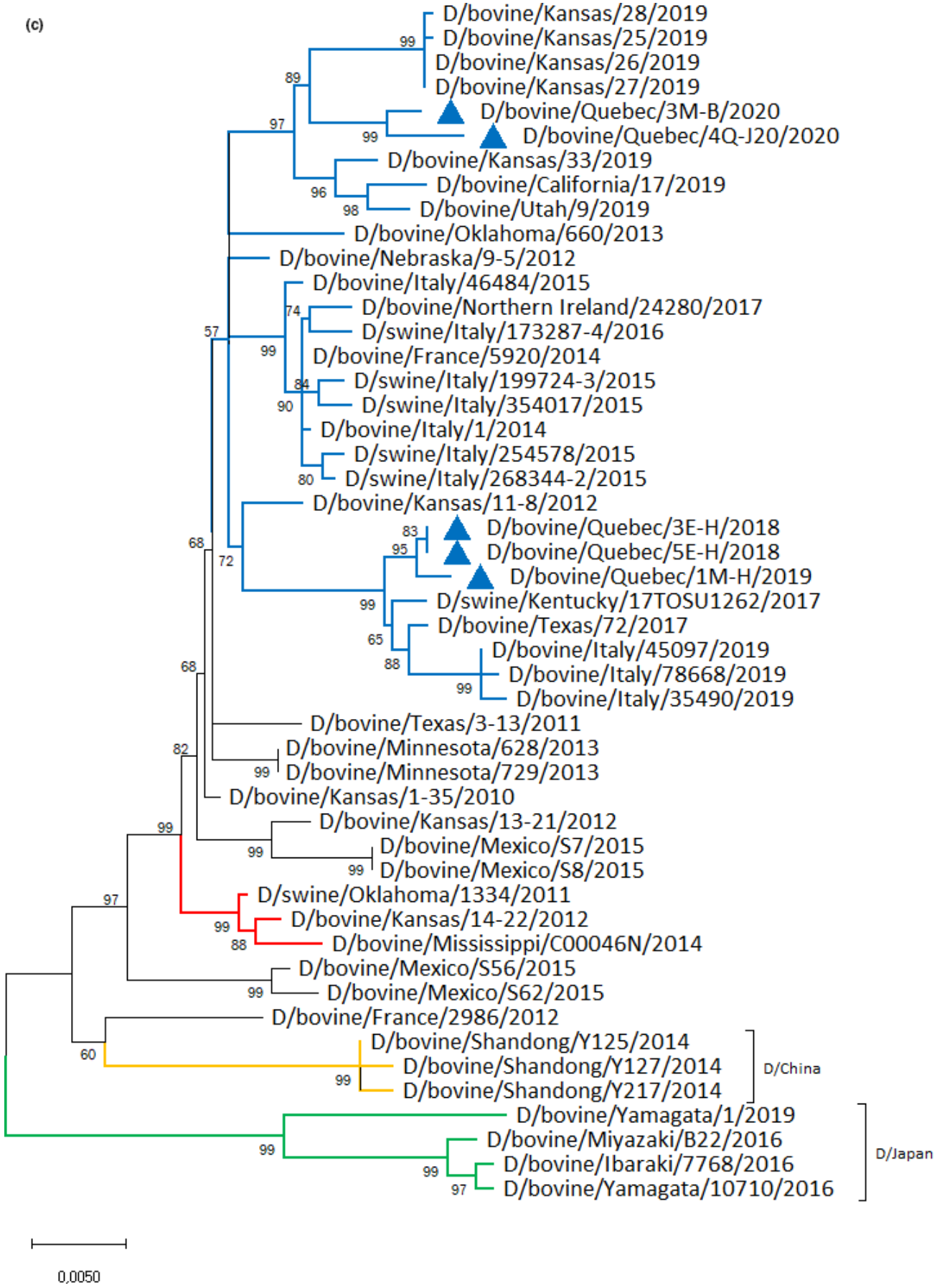


FIGURE 2 Continued

load in cattle correlates with BRDC (Nissly et al., 2020), more studies are needed to confirm this hypothesis. In addition, the strategy of IDV to interact with airway epithelium should be more studied since other viruses of the BRDC follow different strategies for this interaction (BPiV-3 preferentially targets the apical membrane with ciliated cells, BoHV-1 mainly targets basal cells, while BRSV neither



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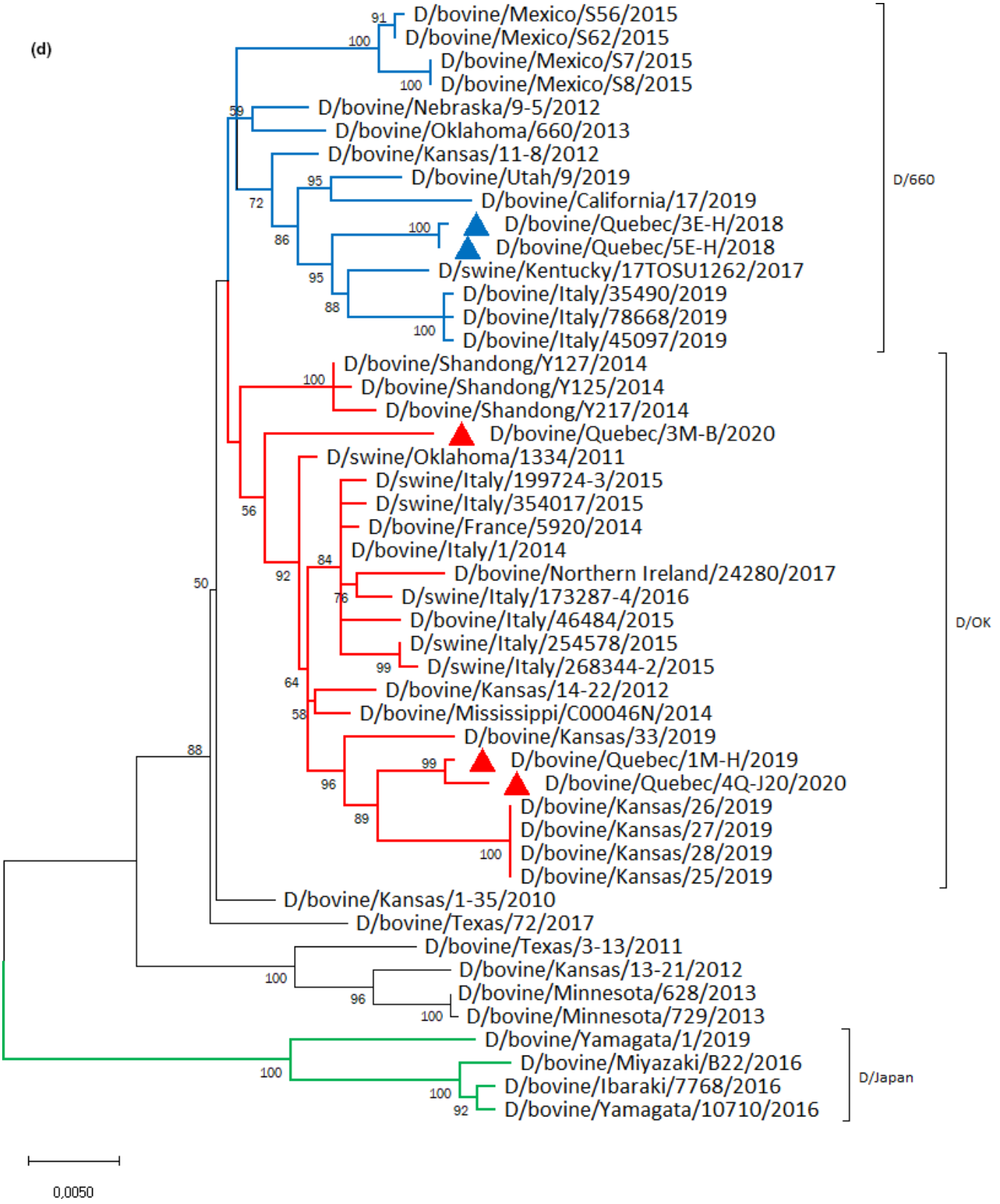

FIGURE 2 Continued

targets differentiated epithelial cells nor basal cells but well sub-epithelial cells) (Goris et al., 2009; Kirchhoff et al., 2014). On the contrary, most bacteria of the *Pasteurellaceae* family seemed to play a bigger role in mono- than in co-infections, suggesting their major pathogenicity in the bovine respiratory disease complex (BRDC) context. As the upper respiratory tract alone was sampled (no broncho-alveolar lavages were available), it is, however, difficult to fully link presence of pathogen and disease. In contrast, *M. bovis* presented a

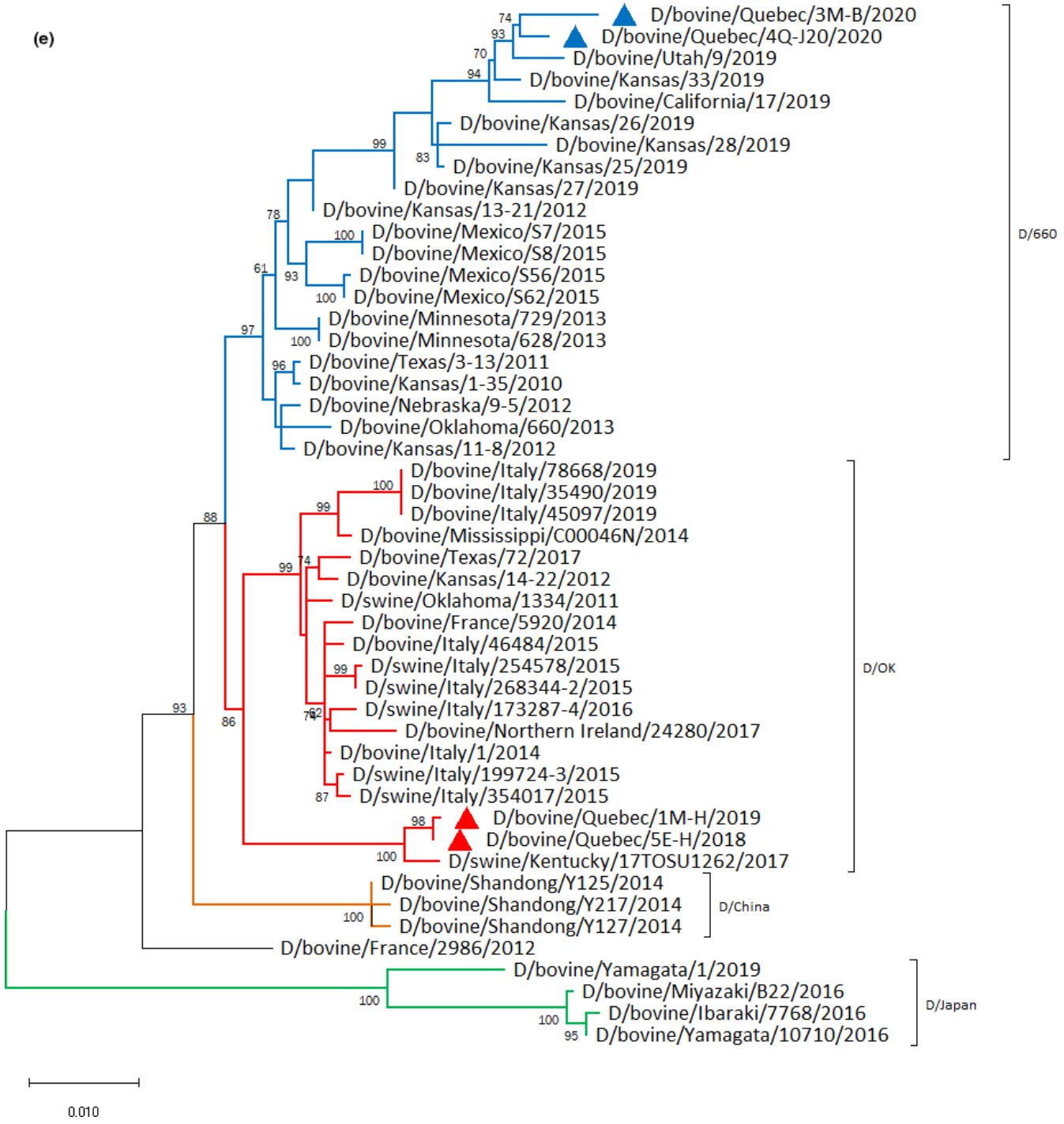

FIGURE 2 Continued

different trend from the other bacteria, having the highest contribution in co-infections among all other pathogens included in the molecular screening. In addition, our analyses revealed that the two strongest associations with IDV were BRSV and *M. bovis*. Indeed, IDV higher prevalence in co-infections suggests an involvement in BRDC. However, a bias in the present study is the absence of a control group (healthy controls). Samples from asymptomatic animals are much more difficult to obtain from the field and were therefore not included. A negative control cohort would however be essential in a future study to confirm the results obtained here. In addition, IDV low pathogenicity in absence of co-infecting pathogens ($n = 3$ samples positive for IDV alone versus $n = 44$ samples positive for IDV and other pathogens) suggests a similar role of this novel virus as of other 'minor' respiratory viruses (i.e. BPiV-3 and BVDV). Another limitation of the study was the fact that the study focused on the most important pathogens involved in BRDC but not all. Recently, no relationship was found between BRDC development in Western Canada and the number of viruses detected by metagenomics, or

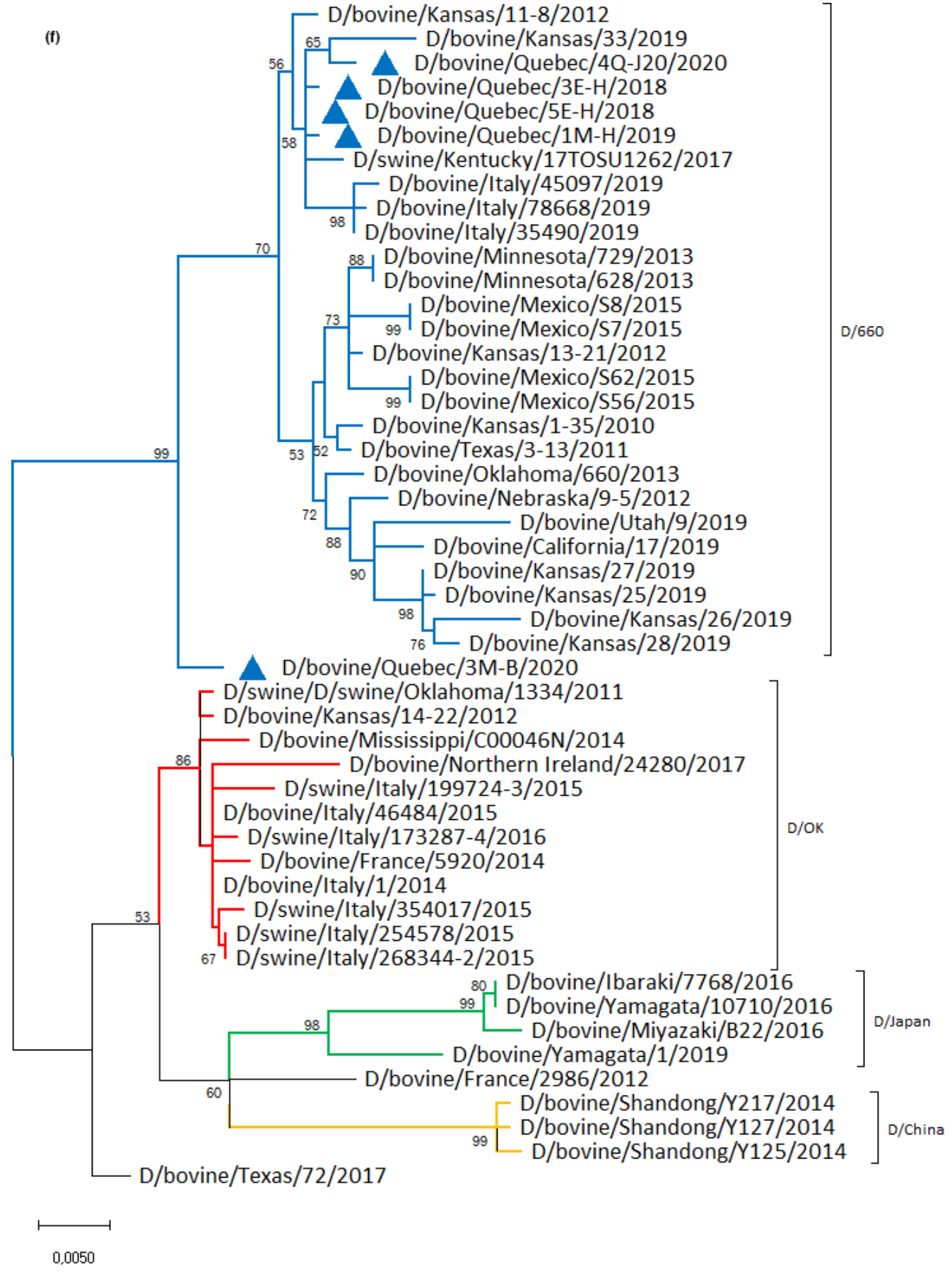

FIGURE 2 Continued

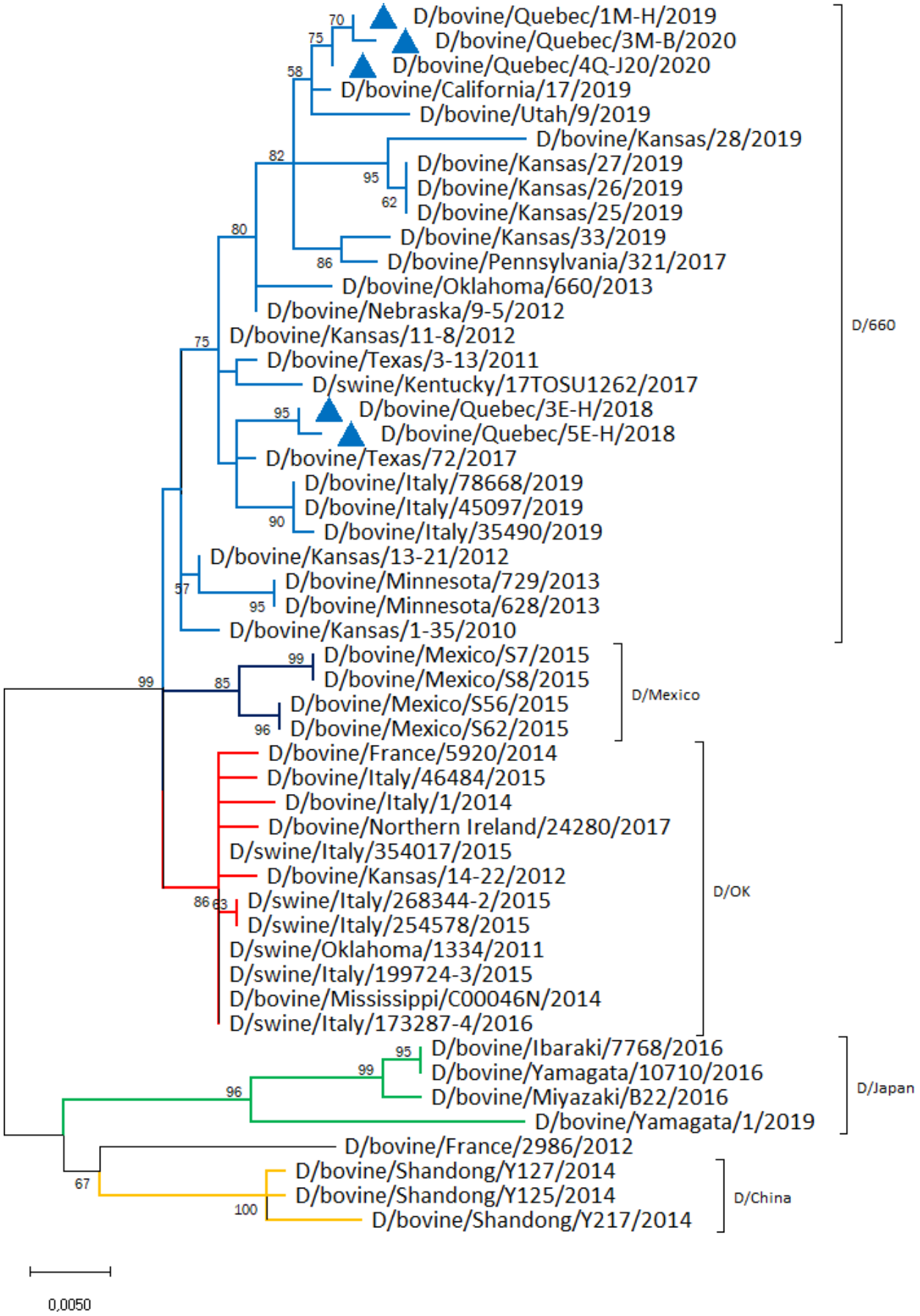

FIGURE 2 Continued

the presence of any specific individual virus or combination of viruses (Zhang, Hill, Godson, Ngeleka, et al., 2020). However, another study evidenced that IDV viral load in cattle correlates with BRDC (Nissly et al., 2020).

It is so far unknown whether IDV genetic characteristics might predict and impact its propensity to be found associated with other respiratory pathogens. Genotyping IDV so far at least allows for understanding viral evolution, geographical origin and spread. Five complete IDV genomes were therefore obtained from positive Québec samples (with low Cq values in RT-qPCR), and phylogenetic analyses were performed on all seven segments. Our results showed that IDV segments belong mostly to D/660 clade. However, reassortment events with D/OK clade were also evidenced. The P3 segment seemed to have encountered reassortment in 2019 and 2020 samples, and a D/OK-like NP segment was evidenced in two samples collected in the same timeframe. In addition, HEF was found to be divergent from D/660 clade in one sample collected in January 2020, suggesting a possible new divergent circulating clade. High IDV genetic diversity with reassortment events was also evidenced in the USA and in Europe (e.g. Chiapponi et al., 2019; Collin et al., 2015). On the European continent, a high overall prevalence of IDV circulation in cattle could be explained by the extended commercial livestock trade between different countries, which could increase as a consequence, the dynamic genetic evolution of circulating viruses (Chiapponi et al., 2020; Gaudino et al., 2020). Livestock trade is also of great importance between Canada and USA (international trade statistics, available at the following URL address: https://www.trademap.org/tradestat/Index.aspx), where both high IDV prevalence and multiple reassorting genotypes in different States were already evidenced. Influenza D virus genetic diversity in Québec could therefore be due to the trade between the countries. Reassortment events evidenced also suggest a high viral load in the field (in this study, average and standard deviation of Cq values for IDV detection were 28.17 and 6.26, respectively—data not shown). Despite the overall low IDV viroprevalence in our cohort (5.32%), a serological survey could provide additional insight into its real spread in Canadian cattle (exposure assessment).

As IDV seems to be involved both in BRDC (Nissly et al., 2020) and be frequently detected in cattle farms, the question of mitigation measures should be raised. Approaches to control the BRDC include breed selection, prevention (vaccination programme, biosecurity, diminution of stress during transport of animals) and therapy (antimicrobial drugs, inflammation modulators) (Lekeux, 1995). More specifically, for some of the respiratory viruses (in particular for BPiV-3, BVDV, BoHV-1 and BRSV) (Chamorro & Palomares, 2020), control measures are already available in the field in order to reduce infection rate and clinical signs. Vaccine administration as BRDC onset prevention is advised, especially with multivalent vaccines due to the complex aetiology of this disease. In Québec, most dairy cows are routinely vaccinated against BoHV-1, BPiV-3, BVDV and BRSV in order to protect themselves and also supply passive immunity to their calves through the colostrum. In some herds, female calves are vaccinated intranasally with modified live vaccines against BoHV-1, BPiV-3, BRSV, *M. haemolytica* and *P. multocida* during their first week of life (Sébastien Buczinski, personal communication). Replacement heifers are regularly vaccinated around 6 months of age against the same viral and bacterial agents. Whether IDV is a trigger for disease development or whether its viral shedding increases following BRDC onset is still unclear. This gap in knowledge is actually not only true for IDV but for influenza and other viruses in general as illustrated by the limited literature in the field (Nickbakhsh et al., 2019; Schultz-Cherry, 2015). Overall seroprevalence of IDV was found to be higher in cattle in countries that mostly import than export, supporting the hypothesis of its shedding after a stressful event such as transportation (Gaudino et al., 2020). However, both hypotheses still have to be tested thanks to experimental studies. Regarding the study and in relation with BRDC, we recommend starting experimental co-infection with combination between IDV and either/both BRSV and *M. bovis*. As suggested by some authors and in order to compare studies and progress in the understanding of the complex interactions between microorganisms regarding BRDC, authors should clearly summarize their co-infection/superinfection experimental setup (i.e. strains used, doses of pathogens, inoculation route, delays between infections, environmental and management conditions, genetic of the host animal and their sanitary status, assays used) (e.g. Saade et al., 2020). In addition, trained immunity (innate immune memory, which influences the type and magnitude of the immune response developed against subsequent infections) is accompanied by epigenetic changes and most often associated with modifications in cellular metabolism (reviewed by Kumar, Stecher, et al., 2018; Netea et al., 2016). Indeed, a look at potential epigenetic changes and cellular metabolism modifications would be of high interest in further respiratory co-infections (Kumar, Stecher, et al., 2018; Saade et al., 2020). In addition, genome-wide transcriptomics and proteomics, coupled with small interfering RNA are proposed to identify key molecules implicated in innate and adaptive pathogen interference (Kumar, Stecher, et al., 2018).

Preventive measures against IDV could be useful to reduce its spread in cattle herds and subsequently to better manage BRDC. A prototype experimental vaccine against IDV has been developed and showed partial protection in calves (Hause et al., 2017) but no commercial vaccine is yet available. Finally, taken into account of the AUC-ROC, 67% of the IDV result prediction might be explained by the OPS rendering marge of progress for the discovery of other associated pathogens and risk factors (e.g. environmental and management factors).

5 | CONCLUSION

IDV circulation in Eastern Canada in the province of Québec was demonstrated. Relative importance of influenza D virus and particular associated pathogens in BRDC of Canadian dairy cattle was assessed. Whole-genome sequencing demonstrated evidence of reassortment between clades D/660 and D/OK. In addition, HEF segment was divergent from D/660 clade in one recent sample

collected in 2020, suggesting a possible new divergent circulating clade. These new pieces of information claim for more surveillance of IDV in cattle production as well as preventive measures that could limit its spread.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

Due to the nature of the study and the low risk posed to participants, formal approval from an Ethics Committee was not a requirement at the time of the study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Claude Saegerman https://orcid.org/0000-0001-9087-7436

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2.8 Research article: Evolutionary and temporal dynamics of emerging Influenza D virus in Europe (2009-2022)

PhD candidate's contribution:

The candidate carried out the experiments (French samples collection and molecular screening, virus isolation, full-genome Sanger sequencing), curated IDV sequences data set, carried out the phylogenetic and evolutionary analyses, drafted the article and generated the figures.

Summary of the research article:

Influenza D virus (IDV) is an emerging influenza virus that was isolated for the first time in 2011 in the United States from swine with respiratory illness. Since then, IDV has been detected worldwide in different animal species and it was also reported in humans. Molecular epidemiological studies revealed the circulation of two major clades, named D/OK and D/660. Additional divergent clades have been described but have been limited to specific geographic areas (i.e. Japan, California). In Europe, IDV was detected for the first time in France in 2012 and subsequently also in Italy, Luxembourg, Ireland, UK, Switzerland and Denmark. To understand the time of introduction and the evolutionary dynamics of IDV on the continent, molecular screening of bovine and swine clinical samples was carried out in different European countries and phylogenetic analyses were performed on all available and newly generated sequences. Until recently, D/OK was the only clade detected in this area. Starting from 2019, an increase of D/660 clade detections was observed, accompanied by an increase in the overall viral genetic diversity and genetic reassortments. The time to the most recent common ancestor (tMRCA) of all existing IDV sequences was estimated as 1995 – 16 years before its discovery, indicating that the virus could have started its global spread in this timeframe. Despite D/OK and D/660 clades having a similar mean tMRCA (2007), the mean tMRCA for European D/OK sequences was estimated as January 2013 compared to July 2014 for European D/660 sequences. This indicated that the two clades were likely introduced on the European continent at different time points, as confirmed by virological screening findings. The mean nucleotide substitution rate of the Hemagglutinin-Esterase-Fusion (HEF)

glycoprotein segment was estimated as 1.403 x 10^{-3} substitutions/site/year, which is significantly higher than the one of the HEF of human Influenza C virus ($p<0.0001$). IDV genetic drift, the introduction of new clades on the continent and multiple reassortment patterns shape the increasing viral diversity observed in the last years. Its elevated substitution rate, diffusion in various animal species and the growing evidence pointing towards zoonotic potential justify continuous surveillance of this emerging Influenza virus.

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Research Article

Evolutionary and temporal dynamics of emerging influenza D virus in Europe (2009–22)

Maria Gaudino,[1] Chiara Chiapponi,[2] Ana Moreno,[2] Siamak Zohari,[4] Tom O'Donovan,[5] Emma Quinless,[5] Aurélie Sausy,[3] Justine Oliva,[1] Elias Salem,[1] Maxime Fusade-Boyer,[1] Gilles Meyer,[1] Judith M. Hübschen,[3] Claude Saegerman,[6,‡] Mariette F. Ducatez,[1,*,†,§] and Chantal J. Snoeck[3,*,†,**]

[1]IHAP, Université de Toulouse, INRAE, ENVT, Toulouse 31076, France, [2]Department of Virology, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna 'Bruno Ubertini', Brescia 25124, Italy, [3]Clinical and Applied Virology Group, Department of Infection and Immunity, Luxembourg Institute of Health, Esch-sur-Alzette L-4354, Luxembourg, [4]Department of microbiology, National Veterinary Institute, Uppsala SE-751 89, Sweden, [5]Central Veterinary Research Laboratory, Celbridge, Co. Kildare W23 X3PH, Ireland and [6]Fundamental and Applied Research for Animals and Health (FARAH) Center, University of Liège, Liège 4000, Belgium

[†]These authors contributed equally.

[‡]https://orcid.org/0000-0001-9087-7436

[§]https://orcid.org/0000-0001-9632-5499

[**]https://orcid.org/0000-0002-0000-1850

*Corresponding authors: E-mail: chantal.snoeck@lih.lu; mariette.ducatez@envt.fr

Abstract

Influenza D virus (IDV) is an emerging influenza virus that was isolated for the first time in 2011 in the USA from swine with respiratory illness. Since then, IDV has been detected worldwide in different animal species, and it was also reported in humans. Molecular epidemiological studies revealed the circulation of two major clades, named D/OK and D/660. Additional divergent clades have been described but have been limited to specific geographic areas (i.e. Japan and California). In Europe, IDV was detected for the first time in France in 2012 and subsequently also in Italy, Luxembourg, Ireland, the UK, Switzerland, and Denmark. To understand the time of introduction and the evolutionary dynamics of IDV on the continent, molecular screening of bovine and swine clinical samples was carried out in different European countries, and phylogenetic analyses were performed on all available and newly generated sequences. Until recently, D/OK was the only clade detected in this area. Starting from 2019, an increase in D/660 clade detections was observed, accompanied by an increase in the overall viral genetic diversity and genetic reassortments. The time to the most recent common ancestor (tMRCA) of all existing IDV sequences was estimated as 1995—16 years before its discovery, indicating that the virus could have started its global spread in this time frame. Despite the D/OK and D/660 clades having a similar mean tMRCA (2007), the mean tMRCA for European D/OK sequences was estimated as January 2013 compared to July 2014 for European D/660 sequences. This indicated that the two clades were likely introduced on the European continent at different time points, as confirmed by virological screening findings. The mean nucleotide substitution rate of the hemagglutinin-esterase-fusion (HEF) glycoprotein segment was estimated as 1.403×10^{-3} substitutions/site/year, which is significantly higher than the one of the HEF of human influenza C virus ($P < 0.0001$). IDV genetic drift, the introduction of new clades on the continent, and multiple reassortment patterns shape the increasing viral diversity observed in the last years. Its elevated substitution rate, diffusion in various animal species, and the growing evidence pointing towards zoonotic potential justify continuous surveillance of this emerging influenza virus.

Key words: cattle; epidemiology; influenza D virus; viroprevalence; swine; zoonosis; virus evolution; molecular clock.

Introduction

Influenza D virus (IDV) was discovered in 2011 (Hause et al. 2013) and classified within the *Orthomyxoviridae* family in 2016 under the *Deltainfluenzavirus* genus. This family includes three other genera of flu viruses: *Alphainfluenzavirus* (comprising influenza A viruses, IAVs), *Betainfluenzavirus* (comprising influenza B viruses, IBVs), and *Gammainfluenzavirus* (comprising influenza C viruses, ICVs). All influenza A, B, and C viruses infect humans and different animal species and represent a group of viruses with a complex ecology. Indeed, IAV can infect birds and mammals, such as swine (Chauhan and Gordon 2022), horses (Sack et al. 2019), dogs (Borland et al. 2020), marine mammals (Webster et al. 1981; Fereidouni et al. 2014), and bats (Tong et al. 2012; Tong et al. 2013). Importantly, it is also responsible for annual influenza epidemics in humans, together with IBV, and pandemics with high fatality in the past (Kilbourne 2006). IBV and ICV mainly infect humans, but IBV was also detected in seals (Osterhaus et al. 2000) and ICV in pigs (Kimura et al. 1997) and cattle (Zhang et al. 2018; Nissly et al. 2020). The segmented genome of *Orthomyxoviridae* viruses enables them to undergo genetic reassortment when two viruses of the same genus infect the same cell (Lowen 2018; Trifkovic et al. 2021). The exchange of intact genes is a frequent evolutionary

mechanism for influenza viruses, giving rise to chimeric genomes that can result in an increase in viral fitness and cross-species transmission, as reported for the H1N1 IAV pandemic in 2009 (Smith et al. 2009). ICV is the most genetically similar virus to IDV, sharing approximately 50 per cent overall amino acid identity (Hause et al. 2013). ICV and IDV genomes are both organized into seven genomic segments. However, transmission electron microscopic tomography revealed that ICV and IDV virions tend to package eight ribonucleoprotein complexes (Nakatsu et al. 2018), similar to IAV and IBV. Both ICV and IDV only possess one surface glycoprotein named hemagglutinin-esterase-fusion (HEF), which is responsible for viral receptor recognition and binding to the host cell. The genomic segment coding for HEF is the most variable and is, therefore, the most frequently used in phylogenetic analyses for molecular typing of different strains.

So far, IDV or anti-IDV antibodies have been detected worldwide and in multiple hosts such as cattle (Chiapponi et al. 2016; Murakami et al. 2016; Luo et al. 2017; Oliva et al. 2019), swine (Foni et al. 2017; Zhai et al. 2017) and feral swine (Ferguson et al. 2018), sheep and goats (Quast et al. 2015; Oliva et al. 2019), horses (Nedland et al. 2018), camelids (Salem et al. 2017; Murakami et al. 2019), and hedgehogs (Oliva 2019), contrasting with the host range of other influenza viruses. IDV was first isolated from a swine displaying influenza-like illness symptoms (Hause et al. 2013); however, epidemiological studies revealed a higher prevalence in cattle compared to other species (Oliva et al. 2019; Gaudino et al. 2021), suggesting bovine as the primary virus host. Cattle were never suspected of being a host for influenza viruses (Sreenivasan et al. 2019), but recent studies on ICV and IDV prevalence in cattle suggested otherwise (Zhang et al. 2018; Nissly et al. 2020). Altogether, these data advocate for an underestimation of the role of cattle as a host for influenza viruses. IDV was also detected in bioaerosols in a poultry farm in Malaysia in 2018 (Bailey et al. 2020), raising new questions about an even wider host range. Increasing evidence suggests IDV spillover into the human population (White et al. 2016; Bailey et al. 2018; Borkenhagen et al. 2018; Choi et al. 2018; Trombetta et al. 2019) with yet unknown consequences for public health.

IDV origin remains unknown, and evolutionary analyses estimated the most recent common ancestor (tMRCA) of both ICV and IDV about 1,304–1,539 years ago (Sheng et al. 2014; Su et al. 2017). So far, two major circulating IDV clades (designated as D/OK and D/660) have been described in North America and Europe based on HEF diversity, and multiple reassortment events between these two clades were also detected (Collin et al. 2015; Chiapponi et al. 2019; Saegerman et al. 2022). In Europe, one genetically divergent clade was described in France in 2012 and Ireland in 2014, represented by D/bovine/France/2986/2012 and D/bovine/Ireland/007780/2014 strains (Ducatez 2015; Flynn et al. 2018). Divergent local clades are also present in other countries on other continents, such as in Japan and California (Murakami et al. 2016; Murakami et al. 2020; Huang et al. 2021). In addition to Italy, Ireland, and France, IDV or anti-IDV antibodies were also detected in Luxembourg (Snoeck et al. 2018), the UK (Dane et al. 2019), Switzerland (Studer et al. 2021), Sweden (retrospective serological survey of IDV among cattle in Sweden—Zohari, pers. comm.), and Denmark (Goecke et al. 2022). The clade D/OK was detected during the last decade of virological surveillance in Europe (2012–9) (Ducatez 2015; Chiapponi et al. 2016; Foni et al. 2017; Flynn et al. 2018). The D/660 clade was only recently detected in Italy for the first time in 2019 (Chiapponi et al. 2019), suggesting a more recent introduction of this latter clade. The current extent of IDV infection spread on the continent, and its genetic diversity, is still poorly understood. In addition, the limited number of IDV sequences available so far has prevented the scientific community from the development of an official genotyping system for clade assignment of different IDV sequences. To better understand IDV prevalence in Europe, surveillance through molecular screening of bovine and swine clinical samples was carried out in several European countries in the last decade. New cohorts were tested for the presence of IDV in this study. Genetic population analyses and phylogenetic reconstruction based on published and newly generated sequences were carried out to assess the evolutionary dynamics of the novel pathogen on the continent and to estimate the date of emergence of the main lineages in Europe.

Materials and methods

IDV molecular screening

In France, RNA extraction was performed on 140 µl of the clinical sample with the QIAamp viral RNA minikit (Qiagen), following the manufacturer protocol, and stored at –80°C. IDV screening in clinical samples was performed by Quantitative reverse transcription PCR (RT-qPCR) using primers (0.8 µM of final concentration) and hydrolysis probe (0.2 µM of final concentration) as described in Hause et al. (2013) using the QuantiNova probe RT-PCR kit (Qiagen, Germany). The RT-qPCR reactions were carried out on a LightCycler ninety-six real-time PCR system (Roche, Switzerland) with the following cycling conditions: 45°C for 30 min, 95°C for 15 min, followed by forty cycles at 95°C for 5 s, and 60°C for 30 s. In Italy, IDV molecular screening was carried out as described in Faccini et al. (2017). In Luxembourg, RNA extraction was performed with the QIAamp viral RNA minikit (Qiagen). The presence of IDV in clinical samples was tested by real-time RT-PCRs by using the primers (0.4 µM of final concentration) and probe (0.15 µM of final concentration) as described in Hause et al. (2013) using the QuantiTect probe RT-PCR kit (Qiagen). Cycling conditions were as follows: 50°C for 30 min, 95°C for 15 min, followed by forty-five cycles at 95°C for 15 s, and 60°C for 40 s.

Virus isolation

In France, attempts of virus isolation were made for samples with the lowest *Cq* values on 70–80 per cent confluent human rectal tumour 18G (HRT-18G) (ATCC CRL-11663) cells and swine testis cells (ATCC CRL-1746) in twenty-four-well plates at 37°C and with 5 per cent of CO_2. For viral isolation, two passages with 5 days of incubation per passage were performed in Dulbecco's modified Eagle's medium (Dutscher, France) in the presence of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin (1 µg/ml; Thermo Fisher Scientific, MA), amphotericin B (2.5 µg/ml; Sigma-Aldrich), BM-cyclin (15 µg/ml; Sigma-Aldrich), ciprofloxacin (10 µg/ml; Sigma-Aldrich, MO), and 1 per cent of penicillin-streptomycin (10,000 U/10 mg/ml, Pan Biotech, France). In Luxembourg, virus isolation was attempted on swine testis cells in 25-cm^2 flasks and six-well plates with or without TPCK trypsin, and no isolate was recovered after two blind passages. Sequencing was thus performed directly on the original material. In Italy, samples positive by real-time RT-PCR were tested for virus isolation in HRT-18G, without trypsin added to the medium, as previously described (Foni et al. 2017). Viral isolation was attempted by three passages with 5 days of incubation per passage, and viral growth of IDV was confirmed by hemagglutination test and by IDV sandwich virological enzyme-linked immunosorbent assay (ELISA) performed as described in Moreno et al. (2019).

IDV complete genome sequencing

In France, seven French isolates were amplified using primers as described in Ducatez (2015) by a one-step RT-PCR kit (Qiagen). Amplicons were purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) and sequenced with Sanger technology (Eurofins GATC). Obtained contigs were aligned with BioEdit v7.1 using ClustalW, and an additional fifteen IDV were sequenced using Illumina HiSeq sequencer. Contigs were assembled with D/bovine/France/5920/2014 as reference using Burrows-Wheeler Alignment tool v.0.7.12-r1039 implemented on Galaxy workbench (Giardine et al. 2005). In Italy, isolates (if available) or clinical samples were sequenced by the next-generation sequencing technique on an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, USA) as previously described (Chiapponi et al. 2016; Chiapponi et al. 2019). Contigs from Italian strains were assembled using CLC Genomic Workbench v. 11 (Qiagen, Hilden, Germany) with D/swine/Oklahoma/1334/2011 as assembly reference. The strain from Luxembourg was amplified by overlapping nested RT-PCRs using one-step RT-PCR kit (first round; Qiagen), Platinum® Taq DNA Polymerase (nested; Life Technologies, Merelbeke, Belgium), and a combination of previously published (Ducatez 2015) and newly designed primers (sequences available upon request; detailed PCR conditions in Snoeck et al. (2013)). After purification, amplicons were sequenced in both directions on an ABI 3130 Avant capillary sequencer (Applied Biosystems) as previously described (Snoeck et al. 2013). Contigs of the IDV strain from Luxembourg were assembled using SeqScape v2.5 (Applied Biosystems) and D/swine/Italy/199724-3/2015 or D/swine/Italy/254578/2015 (for PB2 gene only) as references. The sequences generated in this study were deposited in GenBank and comprised thirty-five IDV sequences in cattle in France, Italy, and Luxembourg collected over years 2018–22, including twenty whole genomes (see Supplementary Table S1 for details).

Data set curation, phylogenetic reconstruction, and reassortment detection

All publicly available IDV full-length coding sequences were downloaded from the National Center for Biotechnology Information GenBank. Partial sequences or sequences derived from synthetic constructs were excluded from the analysis. Sequences were aligned with Clustal Omega (Sievers et al. 2011) on European Molecular Biology Laboratory (EMBL)-EBI search and sequence analysis tools (Madeira et al. 2019). The alignment for *HEF* comprised 142 sequences from years 2010 to 2022, and the number of sequences in the analysis for the other segments was 104 covering years 2010–20. In particular, the data set contained 133 bovine and 9 swine sequences derived from North America, China, and Europe. Phylogenetic trees were calculated for each genome segment with the maximum likelihood (ML) method using the nucleotide substitution model with the lowest Bayesian information criterion scores, as identified for each alignment in MEGA-X v10.1.7 software (Kumar et al. 2018). Tree robustness was assessed by 1,000 bootstrap replicates. The mean distance between different genetic groups was also carried out in MEGA-X v10.1.7 software, using the maximum composite likelihood method with gamma distribution rate (shape parameter = 1). Codon positions included were 1st + 2nd + 3rd + Noncoding. Reassortment events between the two main clades were inferred based on phylogenetic incongruence between tree topologies. Within-clade reassortments were searched using the Genetic Algorithm of Recombination Detection method (Kosakovsky Pond et al. 2006) in the Datamonkey server of HyPhy v2 (Kosakovsky Pond, Frost, and Muse 2005; Delport et al. 2010).

Clade assignment for IDV sequences

As no official system to classify IDV strains into different clades is available to date, we adopted the following criteria to assign IDV strains to a clade using a method similar to those used for the classification of IAVs (Smith and Donis 2014) or Newcastle disease virus (Dimitrov et al. 2019):

1. Despite the presence of multiple strains that originated from reassortment events, we classified IDV strains based on the complete coding sequence of the *HEF* segment (1,992 nucleotides) due to its variability and biological function.
2. The analyses were carried out based on a data set containing sequences from all the different existing clades.
3. The division into different clades was done based on tree topology inferred by the ML method, and the tree topology of the clades was then confirmed by Bayesian Evolutionary Analysis Sampling Tree (BEAST) analysis.
4. A bootstrap value at the defining nodes of monophyletic groups of a minimum of 70 per cent was considered.
5. A monophyletic group was considered an IDV clade when its mean genetic distance to other clades was higher than 2.5 per cent. The mean genetic distance was computed using the maximum composite likelihood method with gamma distribution rate.
6. A genetic group was considered a sub-clade when nested within a major clade of IDV *HEF*, but the mean genetic distance between the two was lower than 2.5 per cent.

Using this method, we classified the *HEF* sequences currently available into eight clades and two subclades.

Evolutionary rate and tMRCA estimation by Bayesian analysis

Complete coding HEF sequences ($n = 142$) were used to estimate IDV evolutionary dynamics. Nucleotide substitution rates and evolutionary time scale of divergence of IDV strains were inferred using the year of sample collection as tip-calibrations in the lognormal relaxed molecular clock method under the Bayesian Markov chain Monte Carlo (MCMC) framework in BEAST v1.8.1 (Suchard et al. 2018). The Hasegawa–Kishino–Yano nucleotide substitution model and gamma as site heterogeneity were specified under a constant population size coalescent model, based on previous estimates for influenza viruses. The analysis was run across two separate partitions (the first and second codon positions in partition one and the third codon positions in partition 2). The analysis was run for 10^8 generations, sampling every 10,000 generations and removing 10 per cent 'burn-in'. The distribution of priors was assessed using Tracerv1.6 (Rambaut et al. 2018). The maximum clade credibility trees with the mean tMRCA and their 95 per cent highest posterior density (HPDs) were summarized using the TreeAnnotator program included in the BEAST package and visualized in FigTree v1.4.3. A literature search was done to compare the evolutionary rate of IDV HEF glycoprotein with those of hemagglutinins (HA) of other influenza viruses. The inclusion criteria for IAV, IBV, and ICV evolutionary rate studies were the following: (1) for simplicity, analyses were carried out on influenza sequences only of human origin, (2) analyses were performed on HA segment for IAV and IBV and on HEF for ICV, (3) analyses were performed on data derived only from clinical samples (studies describing the evolutionary rate based on data produced in experimental models were excluded), and (4) evolutionary rates expressed as substitutions/site/year. The studies included in this

Table 1. Overview over IDV viroprevalence in cattle in Europe.

Country	Year of sampling	Region/ department	Type of surveillance	Type of specimen	No. of positive samples/no. of collected samples (per cent)	No. of positive herds/total no. of sampled herds (per cent)	Clade detected	References
FR	2010–4	Saône-et-Loire	Passive	NS, BAL, lung fragments	6/134 (4.5)		D/OK D/France-2012	Ducatez et al. (2015)
FR	2013–4	Occitanie	Passive	NS, BAL	4/140 (2.9)	1/23 (4.3)	D/OK	This study
FR	2018	Côte-d'Or	Passive	NS	3/96 (3.12)		D/OK	This study
FR	2018	Occitanie	Active	NS	64/145 (44.1)	3/3 (100)	D/OK	This study
FR	2018	Occitanie	Active	NS	0/182	0/13		This study
FR	2018	Normandie	Active	NS	16/51 (31.3)			This study
FR	2019	Normandie	Active	NS	0/59			This study
IT	2014–6	Po Valley	Active	NS	52/744 (7.0)		D/OK	Rosignoli et al. (2017)
IT	2014–6	Po Valley	Active	Lung fragments	6/151 (4.0)		D/OK	Rosignoli et al. (2017)
IT	2014–6	Po Valley	Active	BAL	0/22 (0)			Rosignoli et al. (2017)
IT	2014–5	Po Valley	Passive	NS	2/150 (1.3)			Chiapponi, et al. (2016)
IT	2018–9	Po Valley	Passive	NS	92/664 (13.9)		D/OK, D/660	Chiapponi, et al. (2019)
IT	2018–9	Po Valley	Passive	Lung fragments	7/250 (2.8)		D/660	Chiapponi et al. (2019)
IT	2018–9	Po Valley	Passive	BAL	0/22 (0)			Chiapponi et al. (2019)
IT	2020–2	Po Valley	Passive	NS	44/719 (6.1)	35/270 (13.5)	D/660	This study
IT	2020–2	Po Valley	Passive	Lung fragments	3/126 (2.3)	3/131 (2.2)	NA	This study
LU	2016–21		Passive	NS, lung fragments	1/25 (4.0)		D/OK	This study
IE	2014–6	Whole country	Passive	NS	18/320 (5.6)		D/OK, D/France-2012	Flynn et al. (2018)
UK	2017–8	Northern Ireland	Passive	NS/trachea, lung fragments	9/104 (8.7), 5/104 (4.8)		D/OK	Dane et al. (2019)
DK	2015		Passive	BAL			D/OK	Goecke et al. (2022)
DK	2018–20		Active	NS		12/100	D/OK (2019–20), D/660 (2020)	Goecke et al. (2022)
CH			Passive	NS	31/764 (4.1)			Studer et al. (2021)

Abbreviations: FR, France; IT, Italy; LU, Luxembourg; IE, Ireland; UK, United Kingdom; DK, Denmark; CH, Switzerland. NS, Nasal swab; BAL, Bronchoalveolar lavage.

comparison are listed in Supplementary Table S2. For statistical analysis, an unpaired parametric t-test with a false-discovery rate approach was performed on GraphPad Prism v9.3.1 (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com).

Results

Frequency of IDV infection in cattle and swine in Europe

An overview of IDV viroprevalence in cattle and swine in different European countries is available in Tables 1 and 2, respectively. These tables include newly generated results and results from previously published data. IDV is more frequently detected in cattle than swine: IDV was detected in almost every cattle cohort tested (Table 1), while it was only detected at low prevalence rates in 6/12 swine cohorts (Table 2). In cattle, IDV has been present in France since at least 2011 (Ducatez, Pelletier, and Meyer 2015). Our molecular epidemiological data show that IDV has continued to circulate since then, being detected in the 2017–8 and 2018–9 winter seasons. In particular, high IDV shedding was detected in three veal calves' farms (named A, B, and C, respectively) in February and March 2018 in the Occitanie region. While in Farms A and C, the animals displayed no to limited respiratory signs with a positivity rate ranging from 13 per cent (Farm C) to 31 per cent (Farm A), IDV was isolated during a respiratory outbreak in Farm B. Animals displayed mild to severe respiratory signs. In the second cohort of nasal swabs collected from thirteen farms in the winter season of 2018–9 in the same region, the samples tested negative for IDV presence. IDV was also detected in the Normandie region and the Côte-d'Or department (sample CO-E2256.01), where the virus presence was never described before. In Italy, IDV was detected for the first time in 2014 (Rosignoli et al. 2017) and in almost every cohort tested since then, as well as in a recent cohort from 2020 to 2022. Despite previous serological results

Table 2. Overview over IDV viroprevalence in swine in Europe.

Country	Year of sampling	Region/ department	Type of surveillance	Type of specimen	No. of positive samples/no. of collected samples (per cent)	No. of positive herds/total no. of sampled herds (per cent)	Clade detected	References
FR	2015–8	Whole country	Passive	NS	0/452 (0)	0/137 (0)		Gorin et al. (2019)
IT	2013	Po Valley	Passive	NS	0/32 (0)			Foniet al. (2017)
IT	2014	Po Valley	Passive	NS	0/22 (0)			Foni et al. (2017)
IT	2014–5	Po Valley	Passive	NS	1/150 (0.7)		D/OK	Chiapponi et al. (2016)
IT	2015–6	Po Valley	Passive	NS	14/350 (4.0)	9/448 (2)	D/OK	Foni et al. (2017)
IT	2015–6	Po Valley	Passive	Lung fragments	3/361 (0.8)	9/448 (2)	D/OK	Foni et al. (2017)
IT	2015–6	Po Valley	Passive	Oral fluids	4/134 (3.0)	9/448 (2)	D/OK	Foni et al. (2017)
IT	2017–22	Po Valley	Passive	NS, lung fragments	3/594 (0.5)	3/263 (1.1)	NA	This Study
SE	2014–5		Active	NS	0/330 (0)	0/22 (0)		This study
LU	2009		Active	NS	0/232 (0)	0/56 (0)		Snoeck et al. (2018)
LU	2014–5		Active	NS	3/427 (0.7)	2/36 (5.6)	D/OK	Snoeck et al. (2018)
LU	2018–21		Passive	Lung fragments	0/23 (0)			This study
Twelve countries	2015–7		Passive	NS, lung fragments, oral fluids, BAL	1/4033 (0.02)	1/707 (0.14)	D/OK	Henritzi et al. (2019)

Abbreviations: FR, France; IT, Italy; LU, Luxembourg; SE, Sweden; NS, Nasal swab.

indicating IDV circulation in Luxembourg cattle (Snoeck et al. 2018), IDV was not molecularly detected at high prevalence in this geographic region. In a cohort of twenty-five samples submitted for IDV diagnostic between 2016 and 2021, one was positive for IDV in 2018. The presence of IDV in swine has been investigated to a lower extent than in cattle. IDV was not molecularly detected in France so far but was detected in several swine cohorts in Italy. In Sweden, no positive samples were detected in a cohort of 330 swine samples collected in 2014–5. In Luxembourg, active surveillance previously detected IDV in swine in 2014–5, while none of the twenty-three samples screened for passive surveillance in 2018–21 were positive. Supplementary Fig. S1 shows the genetic distances between different European IDV sequences.

Genetic diversity and phylogeny of IDV in Europe

In recent years, different provisional names have been used to refer to emerging circulating lineages of IDV. In this work, we therefore adopted different criteria to assign IDV strains to specific clades based on the complete coding sequence of the HEF segment. A summary of mean genetic distance within different clades is available in Fig. 1 and Supplementary Fig. S2.

All IDV sequences obtained from 2011 to 2018 samples collected in France and Luxembourg belong to the D/OK clade, as shown in Fig. 2. Sequences belonging to the D/France-2012 clade were not detected in cohorts used in this study, suggesting a possibly minor circulating clade compared to the D/OK and D/660 clades. In Italy, ten new IDV sequences from nasal swabs collected from 2020 to 2022 were obtained. Starting in 2019, only clade D/660 sequences were detected, as previously reported (Chiapponi et al. 2019). Similarly to Italy, an increase in D/660 group detection was also observed starting in 2020 in Denmark (Goecke et al. 2022). The topology inferred by the BEAST analysis confirmed the clade assignment inferred by the phylogenetic reconstruction carried out by the ML method (Supplementary Fig. S3).

The estimated mean within-group distance of European D/OK sequences is 0.84 per cent, whereas for European D/660 is calculated as 0.59 per cent, whereas the mean within-group genetic distance of all existing D/OK sequences at a global level is 1.26 per cent and 1.46 per cent for clade D/660. Intra-farm genetic diversity analyses were carried out on IDV sequences obtained for two veal farms in France (Farms A and B), where multiple genomes were obtained from the same herd. The genetic distance within the same farm was overall limited, ranging from 0.0 per cent to 0.31 per cent. A higher genetic distance was found between the two different farms, ranging from 0.11 per cent to 0.71 per cent (Supplementary Table S3). No within-clade reassortment events were detected in IDV sequences included in the data set. However, we detected reassortments within the two main clades (D/OK and D/660) for the Italian sequences for the Nucleoprotein segment, similar to what was previously described in the same country (Chiapponi et al. 2019). Phylogenetic trees obtained from the other six IDV genomic segments are available in Supplementary Fig. S4.

IDV evolutionary rate

We used a molecular clock approach to calculate IDV evolutionary rate for the *HEF* gene. The rate of nucleotide substitution of all existing IDV sequences was estimated as an overall

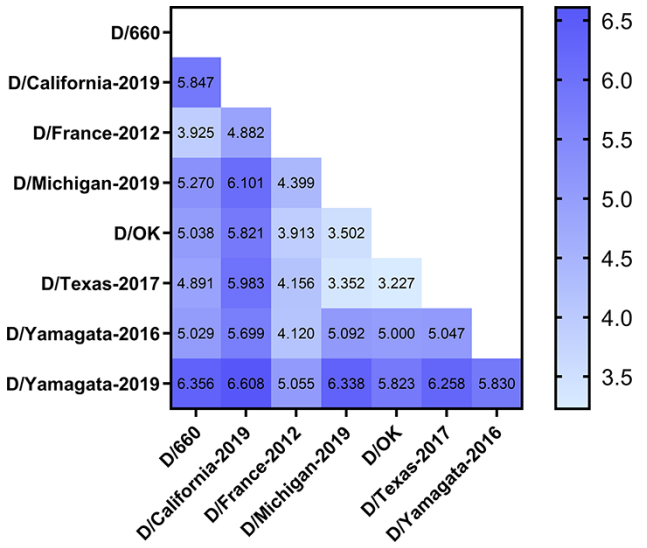

Figure 1. Matrix showing the mean distance between different IDV clades. As the mean genetic distance between D/OK and D/Shandong-2014 was ≤2.5 per cent, this latter was considered a D/OK subclade in phylogenetic analyses. Similarly, D/Quebec-2020 was considered a D/660 subclade. The mean distance between different genetic groups was calculated using the maximum composite likelihood method with gamma distribution rate (shape parameter = 1). In the matrix, the mean distance is expressed as a percentage. The colour code for the mean distance is in the function of increasing genetic distance (light to dark blue).

mean of 1.403×10^{-3} substitutions/site/year (95 per cent HPD interval: 1.156×10^{-3}–1.633×10^{-3}). As reconstructed by BEAST analysis, an increasing pattern of diversification of IDV lineages was highlighted, especially between 2013 and 2018, when the majority of sequences were retrieved (Fig. 3A). The mean substitutions/site/year was similar for all IDV clades. However, it was higher for European D/OK (0.0017) compared to European D/660 (0.0014), probably due to the greater number of sequences available for European D/OK (Table 3). We then compared the IDV HEF rates of nucleotide substitution with those of HA of other influenza viruses reported in the literature (Fig. 3B; evolutionary rates of HA segments of IAV and IBV were included as a comparison to the evolutionary rates of ICV and IDV). The nucleotide substitution rate of IDV HEF glycoprotein was significantly higher than HEF of ICV ($P < 0.0001$), and no significant differences were found with the HA of seasonal human H1N1 ($P = 0.0792$), H3N2 ($P = 0.0259$), and IBV ($P = 0.0286$), probably due to the high variability of the estimated mutation rates among the studies.

Dating the tMRCAs of IDV clades

Based on *HEF* sequences, tMRCAs were inferred for different IDV clades and European clusters of both D/OK and D/660 clades by the Bayesian method using MCMC (Fig. 4; Supplementary Fig. S5 (equivalent to *Te* 4 but with sequences colouring based on their geographic origin); Table 3). The tMRCA of all existing IDV sequences was estimated as 1995 (95 per cent HPD interval: 1989–2000)—16 years before its discovery. The tMRCA was estimated as 2007 for both clades D/660 and D/OK. However, the mean tMRCA of European D/OK sequences was estimated to be January 2013 (95 per cent HPD interval from May 2012 to July 2013) and July 2014 for European D/660 (95 per cent HPD interval from July 2012 to May 2015). In addition, the tMRCA for D/France-2012 was estimated earlier, in 1998.

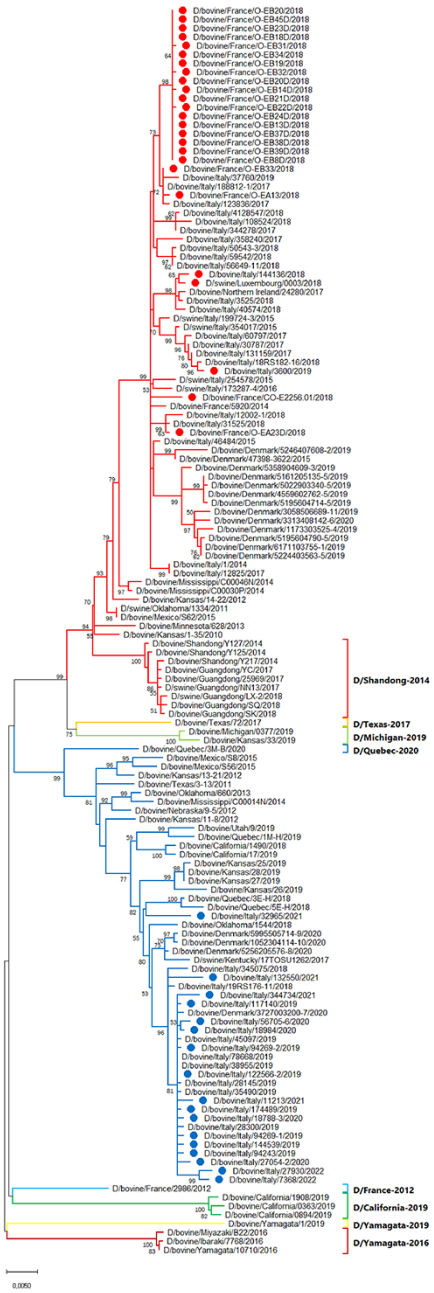

Figure 2. ML phylogenetic tree of complete HEF sequences showing the different clades and subclades of IDV. The new D/OK and D/660 European sequences generated in this work are displayed with a red and a blue dot. The tree was constructed using 1,000 bootstrap replicates. The scale bar represents the number of nucleotide substitutions/site/year.

Discussion

As the genetic diversity of IDV unfolds, the introduction of criteria for clade assignment becomes necessary. Therefore, in this work, we proposed standardized criteria that can be used to classify new strains based on their *HEF* sequence. Since IDV discovery, this emerging pathogen has been detected on almost all continents and in several animal species. Our virological screening confirmed previous serological results, where IDV circulation was described at a higher prevalence in cattle than in swine. Indeed, IDV was present in almost all bovine cohorts tested and all countries investigated in our study, whereas only a few swine samples tested

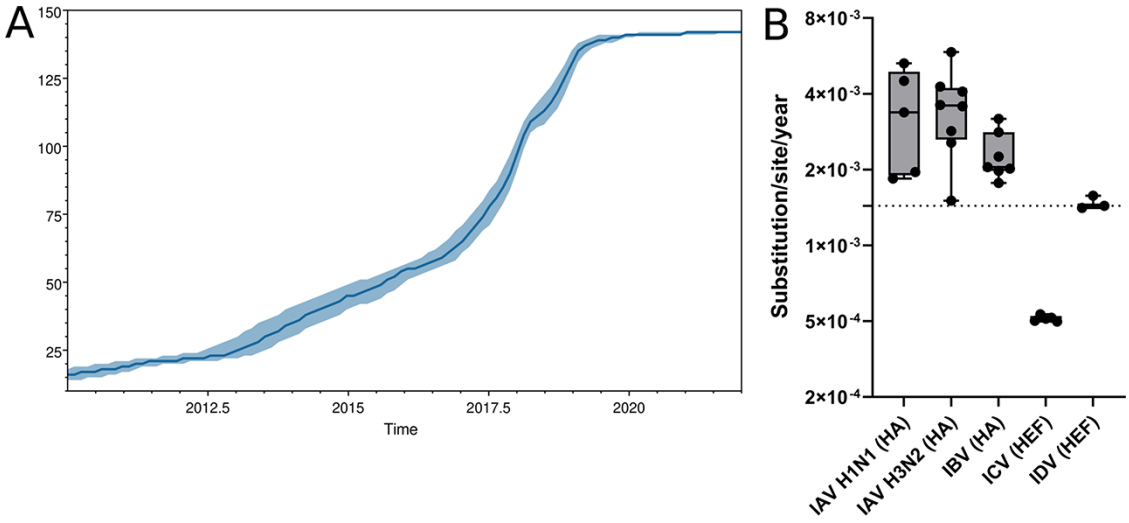

Figure 3. (A) Lineages-through-time plot as reconstructed by molecular dating analysis with BEAST and visualized in Tracer v1.7.2. The shading represents the 95 per cent HPD for the number of lineages (y-axis) distributed in time (x-axis). (B) Estimates of the nucleotide substitution rates/site/year for IDV HEF and the hemagglutinin of other influenza viruses (list of references available in Supplementary Table S2).

Table 3. tMRCA and mean evolutionary rates of the HEF gene for different IDV clades, as estimated by BEAST analysis. The estimated month is indicated after the year and the dot in the table.

Group	Mean tMRCA	95 per cent HPD	Node mean rate (s/s/y × 10^{-3})
All IDV	1995.1	1989.1-2000.7	0.0014
D/Yamagata-2019	1997.1	1993.3-2001.7	0.0014
D/Yamagata-2016	2015.4	2015.1-2016.3	0.0014
D/France-2012	1998.1	1993.3-2002.2	0.0013
D/California-2019	2018.2	2017.8-2019.3	0.0015
D/Texas-2017	2006.8	2003.9-2010.3	0.0013
D/Michigan/2019	2006.8	2003.9-2010.3	0.0013
D/OK	2007.8	2006.5-2009.6	0.0014
European D/OK	2013.1	2012.5-2013.9	0.0017
D/660	2007.8	2006.5-2009.6	0.0015
European D/660	2014.7	2013.1-2015.8	0.0014

Abbreviation: s/n/y, substitutions/site/year.

positive for IDV. While the genetic diversity of IDV in swine seems to reflect the circulating cattle strains, IDV diversity in other hosts remains elusive, and future studies might also reveal the existence of distinctly evolving clades for IAV circulating in avian, swine, or humans. Therefore, the genetic thresholds proposed as part of those criteria will need to be monitored and possibly adapted in the future as a consequence of improved coverage of viral diversity at both geographic and host levels, as well as constant viral evolution.

Our genetic analyses based on molecular screening and sequencing of recent cohorts showed an increase in IDV diversity in Europe throughout the years, resulting from a combination of genetic drift (mutations on the HEF glycoprotein), introduction of a new clade, and genetic shift (reassortment). In fact, the surveillance data collected from 2012 to 2019 only highlighted the presence of D/OK as the main circulating clade (Ducatez, Pelletier, and Meyer 2015; Chiapponi et al. 2016; Foni et al. 2017; Flynn et al. 2018). A minor clade D/France-2012 was only detected in France in 2012 (Ducatez, Pelletier, and Meyer 2015) and Ireland in 2014 (Flynn et al. 2018), but the paucity of detections is probably due to limited surveillance. The presence of the D/660 clade on the continent was observed for the first time in 2019 in Italy (Chiapponi et al. 2019) and then in Denmark in 2020 (Goecke et al. 2022). Since then, the relative frequency of D/OK versus D/660 detections shifted, and no new sequence belonging to D/OK was reported over the last 2 years in Europe. This suggests that D/660 could currently be the major circulating strain on the continent compared to the last decade. However, a systematic IDV surveillance in the majority of European countries is missing and could provide additional insight into the real genetic diversity on the continent. Reassortant viruses combining segments from the D/OK and D/660 clades were also observed for the first time in Europe and Italy in 2019. In North America, multiple reassortment patterns between different clades have been detected since 2015 (Collin et al. 2015; Saegerman et al. 2022). This apparent difference in the time frame and frequency of reassortant detection could be due to (1) longer circulation of IDV on the American continent, (2) the greater size of cattle farms, (3) higher intensity of mixing animals from different farms, and/or (4) the higher number of exchanges between distant locations in Northern America, providing more opportunities for co-circulation of genetically different viruses in a single setting.

The mechanisms suspected to drive IDV evolution are already well described for other influenza viruses (Guarnaccia et al. 2013; Barbezange et al. 2018; Kim 2018; Linster et al. 2019) and are associated with an increase in viral fitness and antibody escape (Ma et al. 2015; Rajão et al. 2015; Pulit-Penaloza et al. 2018; Gao et al. 2019). However, the consequences of reassortment on viral fitness remain unknown for IDV. Our estimated evolutionary rates for the HEF gene are similar to what was previously described (Su et al. 2017; He et al. 2021). Despite the genomic similarity between IDV and ICV, IDV seems to evolve faster than ICV. This could indicate that the novel IDV is still not fully adapted to the cattle population, from which the majority of sequences used in this study are derived. Sequences from animal species other than swine are currently missing and could provide additional insight into IDV evolution in other hosts.

To better understand the introduction dynamics of IDV in Europe, the tMRCA of European clusters was estimated through molecular clock analysis with the Bayesian method. The tMRCA of D/France-2012 was estimated as 1998, indicating that it was

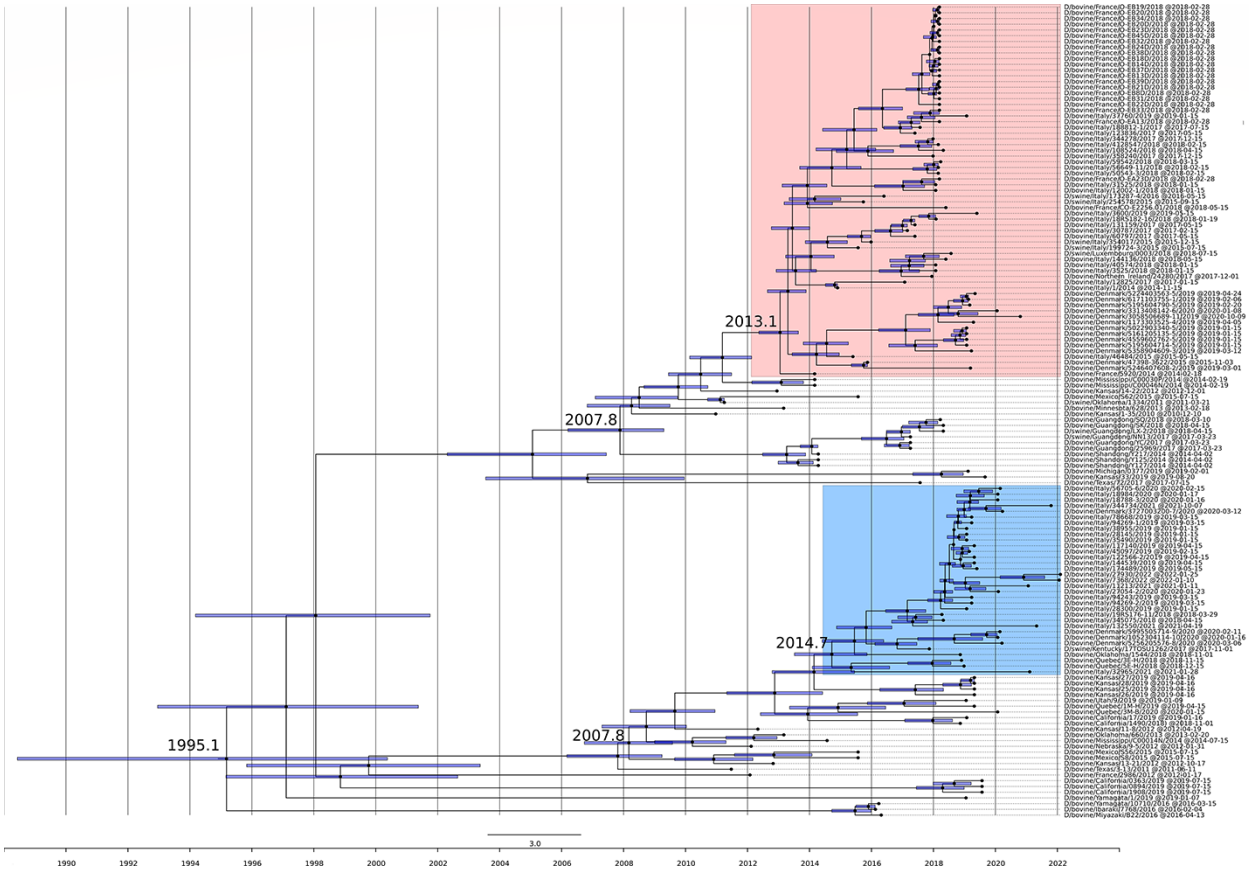

Figure 4. tMRCA of IDVs. The tree was generated using BEAST under a relaxed clock model and a constant coalescent tree prior. The nodes correspond to the mean tMRCA, and the 95 per cent HPD interval is represented with blue boxes. The D/OK European strains are highlighted in pink, whereas the D/660 Italian strains are highlighted in blue. The scale bar represents the number of nucleotide substitutions/site/year.

probably the first clade present on the continent. However, it is difficult to draw a precise phylodynamic pattern for this clade, given the lack of related sequences. The tMRCAs of the two major circulating clades were different, being estimated as January 2013 for D/OK and July 2014 for D/660 viruses. This could indicate a first introduction of D/OK in Europe followed by a more recent independent introduction of clade D/660, which is also supported by virological results. The high nucleotide identity (99.55 per cent) between IDV detected in swine in the USA in 2017 (D/swine/Kentucky/17TOSU1262/2017) (Thielen et al. 2019) and in Denmark in 2020 (D/bovine/Denmark/5256205576-8/2020) (Goecke et al. 2022) suggests that the introduction of D/660 had potentially taken place from North America. Likewise, 99.9 per cent of nucleotide identity was found between the first isolated IDV (D/swine/Oklahoma/1334/2011) in the USA and some 2014–8 D/OK European sequences. This is also supported by the fact that the sister branches of both European clades are located in America. The lack of geographic segregation in these first years of the spread of clades D/OK and D/660 suggests several recent intercontinental spillovers. However, the mode of transmission from one continent to another remains undefined. Exchanges of livestock such as cattle or swine from one continent to another are virtually absent, suggesting the implication of another unknown host. IDV was shown to bind to the epithelial surface of other domestic and wild animals (Nemanichvili et al. 2022). Additional IDV permissive species are horses, shown to seroconvert and replicate the virus (Collin et al. 2015; Sreenivasan et al. 2022), and camelids (Salem et al. 2017; Murakami et al. 2019). While exchanges of live horses between America and Europe take place (FAOstat), exchanges of camelids or horses between America and Africa, where IDV also circulates (Salem et al. 2017; Murakami et al. 2019; Sanogo et al. 2021), are not described (FAOstat). Given the extent and rapidity of IDV spread, the role of humans should also be considered. IDV was detected in urban environments such as a hospital emergency room bioaerosol (Choi et al. 2018), in an airport bioaerosol (Bailey et al. 2018), but also in human samples such as a nasal swab of a farmer working on a pig farm in Malaysia (Borkenhagen et al. 2018). Serologic evidence for IDV in humans remains of more difficult interpretation, as antibody cross-reaction with ICV was highlighted (Eckard 2016). However, the presence of anti-IDV antibodies was highlighted by hemagglutination inhibition assay and virus neutralization assay in a cohort of randomly selected human sera ($n = 1,281$) from two different geographic Italian regions from 2005 to 2017 (Trombetta et al. 2019). Interestingly, few sera were already positive in 2005 (5.1 per cent) with a constant increase in time (9.8 per cent in 2007, 24.1 per cent in 2010, 39.0 per cent in 2013, and 42.0 per cent in 2014) and a constant decrease starting from 2015 (21.8 per cent in 2015 and 7.9 per cent in 2017). The frequency of anti-ICV antibodies is generally high in the adult population (Sederdahl and Williams 2020). Therefore, an increase in the time frame as previously described is not likely attributable to an increase in anti-ICV antibodies in the general Italian adult population. In addition, anti-IDV antibodies were highlighted in four veterinarian sera collected in 2004 (4.9 per cent 4/82) in Italy (Trombetta et al. 2022), as well as at a higher prevalence in cattle-exposed workers (97 per cent) versus people

without cattle exposure (18 per cent) in Florida (White et al. 2016). While the presence of IDV in the human population has been highlighted, the precise role of humans remains yet unknown. The first description of IDV in the cattle population was assessed in 2003 in the USA by a serological study conducted on a cohort of sera collected from 1977 to 2010 (Eckard 2016). In addition, starting from 2005, an increase in IDV human seropositivity was high-lighted (Trombetta et al. 2019). It is therefore possible that humans could have been exposed as accidental hosts following IDV circulation at high prevalence in cattle (zoonotic transmission), rather than a conversely species jump in the opposite direction (anthroponotic transmission) with a consequent adaptation in cattle. However, it is also possible that IDV circulation in humans started in other geographical areas before cattle and remained undetected for several years. IDV circulation in cattle before 2003 in other geographic areas is currently unknown, and serology studies using cohorts of human and cattle sera collected from the same time frame of the putative IDV spread, as well as serology studies on other animal species that were not considered so far, could help to provide a better understanding of the role of humans in the virus origin and transmission.

Conclusion

Together, these global genomic data provide new insight into the different evolutionary dynamics exhibited by IDVs. Our molecular surveillance data confirm that the IDV is actively circulating in Europe, with an increased genetic diversity due to genetic drift, the recent introduction of the D/660 clade, and inter-clade reassortments. Extending surveillance geographically is required to understand the real prevalence of the virus in Europe and globally and obtain a better overview of its genetic diversity. Surveillance in cattle and other animal species could provide additional insight into IDV origins, evolution, and interspecies transmission. In particular, surveillance in human cohorts is warranted to assess their susceptibility to infection and their importance in IDV transmission.

Supplementary data

Supplementary data are available at *Virus Evolution Journal* online.

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Conflict of interest. The authors declare no competing interests.

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Chapter 3: Experimental evidence of IDV impact on BRD

In the previous chapters, we introduced the BRD and we described the most important pathogens involved in respiratory infections, both of viral and bacterial origins. We then focused on the recently discovered cattle pathogen IDV by describing the epidemiology and host range state of art. In the current chapter, we will focus on IDV pathogenesis in cattle and its role in BRD onset. The experimental work on the IDV impact on secondary bacterial superinfections conducted on an organotypic lung model will then be presented.

3.1 IDV pathogenesis in cattle

IDV isolation was described from both healthy and sick cattle. In addition, in sick animals IDV is often in association with other respiratory pathogens, preventing thus an understanding of the potential pathogenicity of the virus alone (6,55). IDV pathogenesis and transmission was therefore investigated in experimental infection in calves in two different studies. In a first study, three 4-month old male calves were challenged intranasally with 10 mL of 10^7 TCID50 of the strain D/bovine/C00046N/Mississippi/2014 (which belongs to the clade D/OK). In addition, three contact not infected animals were housed in the same pen as inoculated calves. Two of the three infected animals already seroconverted 6 days' post-infection and all the contact animals seroconverted starting from 9 days after the exposure to infected calves. All animals from both groups displayed viral shedding in nasal swabs. This suggests that transmission among calves by direct contact is likely in cattle farms. Daily observations for clinical signs revealed that among the directly inoculated calves one had dry cough and nasal discharge and another one was depressed and had abnormal lung sounds upon auscultation. In the contact group, calves displayed nasal and serous ocular discharge. Histological examinations of infected calves revealed tracheal inflammation characterized by multifocal areas of epithelial neutrophil infiltration and mild epithelial attenuation. However, in lung there was no evidence of pulmonary pathology (22). In another study, two-month old colostrum-deprived calves were directly inoculated by aerosol inhalation with 10^7 TCID50 of

the strain D/bovine/France/5920/2014 (also belonging to clade D/OK). In addition, a second group of contact calves that was not challenged was housed in a separate pen 3 meters apart from the infected animals. In directly inoculated animals, IDV replicated both in the upper and lower respiratory tract and the clinical signs started 4 days post-inoculation with a peak at day 8. Aerosol sentinels were IDV positive in nasal swabs 10 days after the infection of directly inoculated calves. Observed signs of respiratory disease included spontaneous coughing and slight tachypnea (35 to 40 breaths/min), repeated spontaneous coughing, and less frequently abdominal dyspnea characterized by increased respiratory rates (between 35 and 65 breaths/min) and abnormal lung sounds (wheezing), but without consequences on appetite or general state. No hyperthermia was detected in any of the infected animals, and all calves had recovered by day 12. Finally, one aerosol-sentinel calf showed mild clinical signs between days 11 and day 14. Three direct-inoculated calves were euthanized at day 8. At this time point, one calf showed major respiratory clinical signs while the other two calves were only mildly affected. Macroscopic lung lesions characterized by patchy areas of atelectasis with a deep red texture and they were restricted to the cranial right lobe and covered about 5% to 10% of the entire lung surface for two calves and less than 5% for the third one. No gross lesions were found in nasal cavities or the larynx or trachea. Microscopic lesions included typical of cases of subacute rhinitis (infiltration of the lamina propria by mononuclear cells in nasal epithelium) and subacute bronchointerstitial pneumonia with neutrophils in bronchial lumens, neutrophilic and macrophagic alveolitis, and peribronchial and septal lymphoplasmocytic infiltration in the lung (Fig. 4). No microscopic lesions were observed in respiratory tissue of aerosol-sentinel calves (23). Altogether, these two experimental infections showed that respiratory disease could be reproduced upon challenge with IDV in calves and the virus could be transmitted to contact animals, even when housed three meters apart, suggesting that IDV can be considered a cattle pathogen. No data on the pathogenicity of the other major IDV clade, named D/660, is currently available.

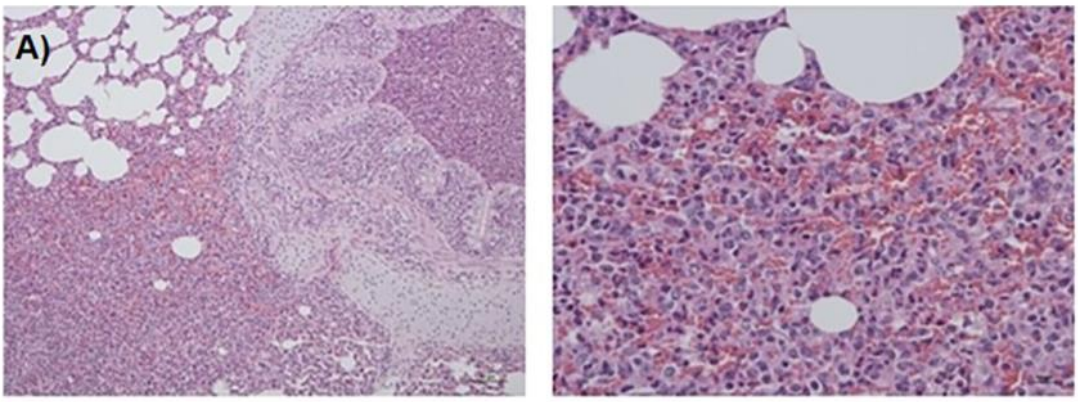

Figure 6. Lung lesions induced by IDV in calves. (A) Subacute bronchointerstitial pneumonia with neutrophils in bronchial lumens, neutrophilic and macrophagic alveolitis and peribronchial and septal lymphoplasmocytic infiltration (left; magnification, ×200) and microscopic alveolar lesions at a higher magnification (right; magnification, ×400). Source: (Salem et al., 2019)

3.2 IDV as a co-factor for BRD onset?

As IDV was recently discovered to be a cattle pathogen, researchers started to investigate its possible role in BRD onset, assessing if IDV infection could worsen respiratory signs when co-infected with other pathogens in a manner similar to the viruses BPIV-3 and BRSV. 4 to 6-month-old calves infected with IDV at day 0 and *M. haemolytica* at day 5 had similar overall clinical scores as calves infected with IDV alone, while calves only infected with *M. haemolytica* had more severe gross lung lesions compared to the negative control group. *M. haemolytica* severe bronchopneumonia signs could not be reproduced in the co-infected calves suggesting that IDV and *M. haemolytica* co-infection did not alter the respiratory pathology of calves (38). In another study, 6 week old calves were infected with either IDV, *M. bovis*, or IDV and *M. bovis* simultaneously (24). Although the *M. bovis* group did not present bronchopneumonia and caseonecrotic lesions typical of *M. bovis* infection, the authors reported that the co-infected group had a shorter time span of clinical signs and significantly increased clinical score, as well as increased severity of trachea and lung macroscopic and microscopic lesions. Compared to the clinical signs of the *M. bovis*-infected calves, those of the coinfected animals

were similar yet more severe, suggesting that IDV facilitates *M. bovis* disease. In addition, more respiratory signs of bronchopneumonia were detected in the coinfected calves than in IDV-infected calves, suggesting that each pathogen may potentiate the clinical effect of the other.

Starting at 2 days p.i., upregulated IFNγ in bronchoalveolar lavages were observed in the co-infected group, reflecting the increased leukocyte recruitment in the airway lumen. The authors also noted that *M. bovis* colonization of both the upper and lower respiratory tract was aided by the viral infection. This study shows that IDV aggravates *M. bovis*-induced pneumonia *in vivo*. However, the synergistic mechanisms and the exact immune pathways activated that potentiate the observed upregulation of IFNγ are still unknown. In the next paragraph, we will present our experimental study conducted to better understand the mechanisms exploited by IDV to aggravate secondary bacterial superinfections, mimicking therefore co-infections observed in sick animals in the field.

3.3 Research article: The activation of the RIG-I/MDA5 signalling pathway upon Influenza D virus infection impairs the pulmonary pro-inflammatory response triggered by *Mycoplasma bovis* superinfection

PhD candidate's contribution:

The candidate performed the experiments (PCLS slicing, PCLS infections with pathogens and stimulations with PRR agonists/inhibitors, FACS and LDH viability assays, RNA extractions and RT-qPCR for pathogen replication and differential gene expression, ELISA and Griess assays, immunofluorescence and confocal microscopy), carried out the statistical analyses, drafted the article and generated the figures.

Summary of the research article:

Concurrent infections with multiple pathogens are often described in cattle with respiratory illness. However, how the host-pathogen interactions influence the clinical outcome has only been partially explored in this species. Influenza D virus (IDV), was discovered in 2011. Since then, IDV has been detected worldwide in different hosts. A significant association between IDV and bacterial pathogens in sick cattle was shown in epidemiological studies, especially with *Mycoplasma bovis*. In experimental challenge, IDV aggravated *M. bovis*-induced pneumonia. However, the mechanisms through which IDV drives an increased susceptibility to bacterial superinfections remain unknown. Here, we used the organotypic lung model Precision-Cut Lung Slices to study the interplay between IDV and *M. bovis* coinfection. Our results show that a primary IDV infection promotes *M. bovis* superinfection by increasing the bacterial replication and the ultrastructural damages in lung pneumocytes. In our model, IDV impaired the innate immune response triggered by *M. bovis* by decreasing the expression of several pro-inflammatory cytokines and chemokines that are important for immune cell recruitment and the bacterial clearance. Stimulations with agonists of cytosolic helicases and TLR receptors revealed that a primary activation of RIG-I/MDA5 desensitizes the TLR2 activation, similarly to what observed with IDV infection. The cross-talk between these two Pattern Recognition Receptors leads to a nonadditive response, which alters the TLR2-mediated cascade that controls the bacterial infection. These results highlight innate immune

mechanisms that were not described for cattle so far and improve our understanding of the bovine host-microbe interactions and IDV pathogenesis.

Title: The activation of the RIG-I/MDA5 signalling pathway upon Influenza D virus infection impairs the pulmonary pro-inflammatory response triggered by *Mycoplasma bovis* superinfection

Running title: IDV promotes *M. bovis* superinfection in bovine lung

Maria Gaudino[1], Adrien Lion[1], Eveline Sagné[1], Brandy Nagamine[1], Justine Oliva[2], Olivier Terrier[2], Elisabeth Errazuriz-Cerda[3], Anaëlle Scribe[1], Fatima-Zohra Sikht[1], Elisa Simon[1], Charlotte Foret-Lucas[1], Blandine Gausserès[1], Julie Lion[1], Ana Moreno[4], Emilie Dordet-Frisoni[1], Eric Baranowski[1], Romain Volmer[1], Mariette F Ducatez[1]*, Gilles Meyer[1]*

Authors' affiliations:

[1]IHAP, Université de Toulouse, INRAE, ENVT, Toulouse, France

[2]Centre International de Recherche en Infectiologie - U1111 (Equipe VirPath) (CIRI) – Institut National de la Santé et de la Recherche Médicale : U1111, Ecole Normale Supérieure - Lyon, Université Claude Bernard Lyon 1, Centre National de la Recherche Scientifique : UMR5308 – 21 avenue Tony Garnier 69365 Lyon Cedex 07, France

[3]Centre d'Imagerie Quantitative Lyon 1, Lyon, France

[4]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Brescia, Italy

*contributed equally

*Corresponding authors: Mariette Ducatez, email: mariette.ducatez@envt.fr, Gilles Meyer, email: gilles.meyer@envt.fr

Abstract

Concurrent infections with multiple pathogens are often described in cattle with respiratory illness. However, how the host-pathogen interactions influence the clinical outcome has only been partially explored in this species. Influenza D virus (IDV), was discovered in 2011. Since then, IDV has been detected worldwide in different hosts. A significant association between IDV and bacterial pathogens in sick cattle was shown in epidemiological studies, especially with *Mycoplasma bovis*. In experimental challenge, IDV aggravated *M. bovis*-induced pneumonia. However, the mechanisms through which IDV drives an increased susceptibility to bacterial superinfections remain unknown. Here, we used the organotypic lung model Precision-Cut Lung Slices to study the interplay between IDV and *M. bovis* co-infection. Our results show that a primary IDV infection promotes *M. bovis* superinfection by increasing the bacterial replication and the ultrastructural damages in lung pneumocytes. In our model, IDV impaired the innate immune response triggered by *M. bovis* by decreasing the expression of several pro-inflammatory cytokines and chemokines that are important for immune cell recruitment and the bacterial clearance. Stimulations with agonists of cytosolic helicases and TLR receptors revealed that a primary activation of RIG-I/MDA5 desensitizes the TLR2 activation, similarly to what observed with IDV infection. The cross-talk between these two Pattern Recognition Receptors leads to a nonadditive response, which alters the TLR2-mediated cascade that controls the bacterial infection. These results highlight innate immune mechanisms that were not described for cattle so far and improve our understanding of the bovine host-microbe interactions and IDV pathogenesis.

Importance

Since the spread of the respiratory Influenza D virus (IDV) infection to the cattle population, the question about the impact of this virus on bovine respiratory disease (BRD) remains still unanswered. Animals affected by BRD are often co-infected with multiple pathogens, especially viruses and bacteria. In particular, viruses are suspected to enhance secondary bacterial superinfections. Here, we use an *ex vivo* model of lung tissue to study the effects of IDV infection on bacterial superinfections. Our results show that IDV increases the susceptibility to the respiratory pathogen *Mycoplasma bovis*. In particular, IDV seems to activate immune pathways that inhibit the innate immune response against the bacteria. This may allow *M. bovis* to increase its proliferation and to delay its clearance from lung tissue. These results suggest that IDV could have a negative impact on respiratory pathology of cattle.

Introduction

Mixed airways infections, with pathogenic bacteria and viruses often co-detected in respiratory secretions, are common in humans and animals (1). Many of these microbial associations are now considered to negatively impact the clinical outcome of infection (2–4). Cattle are no exception and respiratory outbreaks in this species have a multifactorial origin that often involves several pathogens (5–8). Co-infections are frequently associated with an increase in severity of disease and, in some cases, a decrease in the survival rate (9–14). While data illustrating the clinical importance of mixed respiratory tract infections are accumulating, the mechanisms associated with co-infections are still poorly understood (15). New respiratory viruses have been recently discovered in cattle. These include the influenza D virus (IDV), a novel *Orthomyxoviridae* isolated in 2011 from swine with influenza-like illness (16). IDV exposure was documented in humans by serologic studies (17–19) and several animal species (20–24), but cattle are considered its primary host (25). In the last few years, IDV detection in clinical samples has been positively associated with respiratory disease in cattle through metagenomic approaches (26, 27). In addition, epidemiological studies showed a significant association between IDV and *M. bovis* in sick cattle (5). Experimental *in vivo* challenge with IDV revealed viral replication in both the upper and lower respiratory tract with moderate respiratory signs onset in calves (28, 29) and subacute broncho-interstitial pneumonia with neutrophil infiltration in bronchial lumens and neutrophilic and macrophagic alveolitis at necropsy (29). Despite that clinical signs induced by IDV infection were moderate, in experimental co-infection IDV enhanced *M. bovis* colonization of the lower respiratory tract leading to exacerbated clinical manifestations (30). The mechanisms underlying the increase in susceptibility to bacterial superinfections following IDV infection remain however unknown.

The present study aimed at deciphering the mechanisms underlying IDV and *M. bovis* synergistic interplay upon airway colonization by using Precision-Cut Lung Slices (PCLS) as an organotypic lung model of infection. PCLS represents a versatile tool that has the huge benefit of meeting the 3Rs

principle (Replacement, Reduction and Refinement) to reduce the number of animals used in *in vivo* experiments. It has been extensively used for lung disease modelling (31–33), including studies with bovine pathogens (34–36). Additional advantages of this model include the preservation of the 3-dimentional structure and physiological properties of the lung tissue, the preservation of the resident cells that were present in the collected organs, as well as the possibility of doing a large number of biological replicates for different experimental conditions (37). We studied the respiratory tropism of each pathogen, their replication, the cellular ultrastructure modifications and the inflammatory and innate immune responses in mono-infections or when a primary IDV infection was followed by *M. bovis* superinfection.

Results

Viability of organotypic PCLS cultures

Bovine PCLS cultures were used to study the dynamics of IDV and *M. bovis* respiratory infections *ex vivo*. Before investigating the impact of mixed infections, we first established the viability of PCLS cultures over 7 days post-slicing by two different methods. We stained PCLS single-cell suspensions with 7-AAD (7-aminoactinomycin D), and we measured 7AAD-stained cells by flow cytometry. In parallel, we measured the cytotoxicity by quantifying the LDH (Lactate dehydrogenase) activity in PCLS supernatants.

Flow cytometry analysis of 7-AAD positive cells revealed a low percentage of apoptotic cells at 24 hours post-slicing (< 12%), with higher values at 120 hours (11 to 30%) (Fig. 1A and 1B). The LDH activity remained low up to 120 hours post-slicing (< 15%) with a decline in viability only detectable at day 7 post-slicing (10 to 30% of LDH activity) (Fig. 1C). β-tubulin staining indicated a well-preserved

alveolar structure of cultured PCLS (Figure 1D). To further characterize the viability of mock-infected PCLS cultures, necrotic cells were visualized by propidium iodide (PI) staining and confocal imaging on multiple confocal plans at 72 hours post-slicing. PI-stained cells, mainly pneumocytes and resident alveolar macrophages, were only observed on apical and basal plans (Fig. 1F), whereas cells in the inner part of the slices remained viable (Fig. 1E). Finally, the ciliary activity was daily observed under a light microscope. All mock-infected PCLS displayed ciliary motion in lung airways until the end of the experiment (7 days post-slicing). Overall these data suggest that PCLS cultures showed good cell viability for up to five days, with an increase in cell mortality on day 7. As a consequence, for co-infection experiments PCLS were cultured until maximum 5 days post-slicing.

Bovine PCLS are permissive to IDV and *M. bovis* infection and reproduce *in vivo*-like innate immune response

We then established the permissiveness of this model for each pathogen by studying their replication and the innate immune response. The replication of individual pathogens in PCLS cultures was determined by both titration and reverse transcription quantitative PCR (RT-qPCR) for IDV and bacterial numeration and qPCR for *M. bovis* (Fig. 2A and 2B). IDV and *M. bovis* displayed a 4-log10 increase in replication from 24 to 168 hours p.i. indicating that the PCLS model is permissive for both pathogens. Interestingly, infected PCLS failed to reveal enhanced cytotoxicity compared to mock conditions up to 7 days p.i. (8 days post-slicing) (Fig. S1). The PCLS innate immune response to individual infections was then assessed by RT-qPCR for a panel of cytokines at 48 hours p.i. Representive cytokines for the innate immune pathways activated upon IDV and *M. bovis* infection were selected based on transcriptomic data that were previously obtained *in vivo* (29, 30).

The innate immune response to IDV infection was characterized by the activation of type I interferon (IFN-β), Mx1 and ISG15 (Fig. 2C), as well as CXCL10. In contrast to IDV, *M. bovis* induced an up-regulation of pro-inflammatory cytokines, especially IL-1β, IL-8, CXCL10 and TNF-α (Fig. 2D), together

with the inducible nitric oxide synthase (iNOS) whose expression was associated with pneumonic lesions *in vivo* (38). In addition, IL-10 response was not observed. Overall, these data suggest that PCLS cultures allow the replication of both pathogens and the initiation of an innate immune response similar to those observed in vivo (29, 30).

PCLS infections with IDV and *M. bovis* revealed a similar distribution and tissue tropism

Immunofluorescence with confocal microscopy imaging was then carried out on PCLS to study the distribution and the tissue tropism of each pathogen. Figure S3 shows confocal images taken on mock PCLS. Z-stack imaging revealed that IDV infects predominantly bronchial epithelial cells but also the alveolar parenchyma (Fig. 3A, 3B) and Club cells (Fig. 3D), which are epithelial bronchiolar exocrine cells secreting glycosaminoglycans that protect the bronchiolar epithelium (39). Less frequently, IDV was also localized in MHC-II expressing cells (Fig. S2E, S2F) and endothelial cells (Fig. 3C), this latter cell type being a hallmark of pathogenic influenza A virus infections (40). For *M. bovis*, fluorescent foci of bacterial replication and colonies were observed particularly in bronchioles (Fig. 3E, 3F, 3H) and Club cells (Supplementary Figure S2G), but also in the alveolar parenchyma (Fig. 3G), similarly to what was observed for IDV. By confocal microscopy we could also co-localize IDV and *M. bovis* in co-infected conditions, as shown in Fig. 4. IDV and *M. bovis* infected similar cell types, namely bronchial cells (as shown in the upper Fig. 4) and alveolar pneumocyte (middle and lower Fig. 4), strengthening the notion of a similar tissue tropism.

IDV promotes *M. bovis* replication and increases the ultrastructural changes in bovine PCLS

Our results indicate that our PCLS model is permissive for IDV and *M. bovis* in mono-infection and it reproduces in vivo-like innate immune response upon infection. In addition, a similar tissue tropism

for both pathigens was highlighted by confocal microscopy studies. We then investigated the interactions between the two pathogens in co-infected conditions compared to mono-infections. In particular, to study the effects of a primary IDV infection on *M. bovis* superinfection, the PCLS were infected at 0 hours with IDV and 48 hours later with *M. bovis*. The supernatants and lung cuts were then collected at 72 and 96 hours post-IDV infection (p.i.). While no changes in IDV replication were observed between single and superinfected conditions (Fig. 5A), we observed that pre-infection with IDV caused an approximately two-fold increase in *M. bovis* replication at 96 hours p.i. (Fig. 5B).

Ultrastructural changes associated with single or co-infections were analyzed by transmission electron microscopy. Single infections with each pathogen (Fig. 6D and 6I) impacted the structure of the respiratory epithelium compared to mock conditions (Fig. 6A, 6B and 6C). Localized on the apical side of the epithelium, IDV was mainly associated with ciliated cells and pneumocytes inducing a loss of cilia, nuclear inclusions (Figure 6F), as well as cell disorganization likely caused by a loss of tight junctions (Fig. 6F). Similarly, *M. bovis* was also localized on the apical side of the epithelium and remained mainly extracellular (Fig. 6H). *M. bovis* induced more ultrastructural changes in lung cells than IDV. Damages associated to *M. bovis* infection included dense ultrastructure in the cytoplasm (Fig. 6I), cell death (apoptosis and autophagy) and mucus production (Fig. 6I).

Upon co-infections, we confirmed IDV and *M. bovis* co-localization in alveolar parenchyma, suggesting a close contact between the two pathogens (Fig. 6J, 6M). The co-infection presented damages with a composite pattern of both single infections. Co-infected PCLS presented nuclear inclusions, as shown in Figure 6K. Similar signs of both single infections were observed but with higher intensity compared to mono-infections, characterized by an increase in viro-induced ultratructures (Figure 6L) as well as phagophores (Figure S4), which are correlated with autophagy. An increase in cell death was observed in co-infected conditions compared to mono-infections (Figure 6N, 6O). Despite this, it is not known if the increased cell death can be attributed to IDV or *M. bovis* upon co-infection.

186

187 **IDV infection impairs the pro-inflammatory responses triggered by *M. bovis* superinfection**

188 We then investigated the impact of co-infection on the innate immune response compared to mono-
189 infections. The RT-qPCR analysis revealed that the pro-inflammatory response against *M. bovis* was
190 decreased in the co-infected conditions (Fig. 7A). The mRNA expression of several cytokines induced
191 by *M. bovis*, including IL-8, IL-1β and IL-17, were significantly decreased at 72 hours p.i. in co-infected
192 conditions but recovered their baseline levels of expression one day later, whereas CXCL10 and iNOS
193 remained statistically decreased also at 96 hours p.i. No effect was observed on Mx1 response
194 (Figure S5, supplementary material) in co-infected conditions, which is probably linked to no changes
195 in IDV replication (Fig. 5A). No differences were observed for IL-6 and CCL5 between mono- and co-
196 infected conditions (Figure S5, supplementary material). Interestingly, the only up-regulated cytokine
197 in co-infected conditions was IFN-γ, similarly to what we previously observed *in vivo* (30). The
198 decreased IL-1β production at 72 hours p.i. (Fig. 7B) and iNOS lower activity (Fig. 7C) were confirmed
199 by enzyme-linked immunosorbent assay (ELISA) assay and Griess assay, respectively.

200

201

202 **The NF-kB pathway contributes to the control of *M. bovis* replication**

203 Activation of the NF-kB pathway has been described for *Mycoplasma* spp. infection in different
204 species (41, 42), including bovine (43), via TLR2 binding and MyD88-dependent signaling. Our RT-
205 qPCR data on cytokine transcripts (Figure 7A) suggest that IDV may counteract this pathway, leading
206 to decreased expression of several cytokines and chemokines and increased bacterial replication. To
207 confirm the role of pro-inflammatory cytokines in the control of *M. bovis* infection, we pre-treated
208 for 6 hours the PCLS with an inhibitor of NF-kB (BAY 11-7082), followed by the inoculation of *M. bovis*
209 on treated and untreated lung slices. The decreased expression of IL-1β and IL-8 mRNAs at 48 hours

p.i. was associated with an increase in *M. bovis* replication in both lung donors at 120 hours p.i. (Fig. 8), similarly to what has been observed in superinfection (Fig. 5B). This suggests that the NF-kB pathway plays a role in the control of *M. bovis* replication in bovine lung.

RIG-I/MDA5 activation desensitizes the TRL2 signaling pathway

To investigate the mechanisms that drive a decreased expression of pro-inflammatory cytokines in the co-infected conditions, we used synthetic agonists that activate specific immune pathways. In particular, IDV is known to stimulate the RIG-I/MDA-5 cytosolic receptor, whereas *M. bovis* is known to activate the TLR2 membrane receptor upon infection (29, 43–45). As shown in Figure 9A, a primary activation of RIG-I/MDA5 induced by intracellularly-delivered poly(I:C) stimulation desensitizes the TLR2 pathway cytokines induced by Pam3CSK4 agonist added 24 hours later, suggesting a cross-talk between cytosolic helicases and TLRs. Similar results were also obtained when IDV infection was used instead of poly(I:C) stimulation (Fig. 9B) with significant decrease of IL-1β, IL-8 and iNOS mRNAs loads. IL-17 mRNA levels also decreased in presence of poly(I:C) pre-treatment, however the differences were not significant.

Discussion

Organotypic PCLS model was used in this study to better characterize IDV and *M. bovis* infections in lung and to decipher the molecular mechanisms explaining the impact of a primary IDV infection on *M. bovis* superinfection. In the present study, we focused on a primary viral infection as in literature it was described to be one of the most common triggers for bovine respiratory disease in the field (38). The use of this model could be useful in the future to investigate further conditions of co-infections. Both pathogens had a particular tropism for bronchiolar epithelial cells, which is

consistent with the respiratory signs observed in infected animals (29, 30). The co-localization of IDV and *M. bovis* in mixed infections points towards a similar tropism. A possible aggregation between these two pathogens has been suggested by several electron microscopy images, but the biological signification of these particular structures remains to be further investigated. In particular, the impact of these structures on the transmission of both pathogens simultaneously among animals could be studied more in depth.

Our RT-qPCR data revealed a down-regulation of mRNA expression of pro-inflammatory cytokines upon IDV infection, which were confirmed by ELISA assay for IL-1β and Griess test for iNOS, suggesting that this virus could negatively regulate the NF-kB pathway in the first days of infection, a mechanism that is commonly exploited by several viruses to evade the immune response (46). The impairement of pro-inflammatory cytokines and chemokines that play a key role in neutrophil recruitment may influence the response against *M. bovis* infection, thereby promoting mycoplasma growth, as we experimentally confirmed by inhibiting the NF-kB pathway. This is in agreement with what was observed for the human pathogen *Mycoplasma pneumoniae* in a mouse model, where mice unable to produce IL-1β had delayed bacterial clearance in the lungs (47). Attenuated IL-1β production was also linked with *Staphylococcus aureus* pneumonia exacerbation in another study (48). Previously described pathogenic mechanisms of human influenza A virus (IAV) in mice model include neutrophil impairement in lung, associated with increased susceptibility to *Streptococcus pneumoniae* (49) and neutrophil chemoattractants deficience that resulted in the inability to efficiently resolve *S. pneumoniae* superinfection (50). In other studies, experimental pneumococcal superinfections aggravation were associated to IFN-I presence, as shown by the increased survival of Ifnar-/- mice compared to wild-type mice (51, 52) and to decreased Th17 response (53), as shown by the impaired *S. aureus* clearance in IL-17R(-/-) mice compared to wild-type. Similarly, in our study we observed that IDV decreased IL-17 mRNA of 10-fold in superinfection. In our model, we could confirm the decreased pro-inflammatory and chemokines expression by RT-qPCR and ELISA

experiments, however the immune cell recruitment in lung, which plays a very important role in the outcome of the co-infection, cannot be studied.

Interestingly, IFN-γ was the only up-regulated cytokine in this study, similarly to what observed during *in vivo* trial with IDV and *M. bovis*, where IFN-γ up-regulation was linked to increased lung lesions in co-infected animals (30). This confirms previous studies that described the role of IFN-γ on alveolar macrophage depletion and a consequent delayed bacterial clearance (54). In addition, IFN-γ signalling was also shown to impair cell recruitment during the progression of influenza/*S. pneumoniae* co-infection (55).

IDV predisposing role to secondary bacterial superinfections was previously investigated *in vivo*. Mice were first inoculated with IDV and seven days later they were challenged with *S. aureus*. However, IDV antiviral response had a protective effect on co-infected animals by increasing the survival rate and recovery compared to the *S. aureus* group alone (56), contrary to what reported for IAV primary infection and *S. aureus* superinfection in the same model, where IAV primed and predisposed mice to secondary pneumonia (57, 58). IAV solicited host immune factors are similar to those observed in IDV infection, with an induction of ISGs and antiviral proteins. Concurrent with their antiviral effect, type I IFN production can decrease important antibacterial immune responses and neutrophil-recruiting chemokines (58), which is a similar mechanism to what we observed with IDV and *M. bovis* co-infection in this study. Therefore, the outcome of co-infections seems to be host- and pathogen-dependent, and future studies should focus on the interspecies variability to IDV immune response in co-infection.

To investigate the mechanisms that drive a decreased expression of pro-inflammatory cytokines and chemokines in co-infection in our model, we used synthetic agonists that activate specific immune pathways that mimic viral and mycoplasma infections. Our results suggest that interference between cytosolic RIG-I-like receptors (RLRs) and membrane TLR2 receptor activation takes place. In our *ex vivo* conditions, the observed down-regulation of pro-inflammatory cytokines was however transient

284 and our observations in time were limited at 72 hours post-stimulations with PRR agonists and at 96
285 hours p.i. with the replicating pathogens. The interference between TLR2 and RIG-I/MDA5 is in
286 agreement with the results observed in bone marrow-derived macrophages in mice (59). Similarly,
287 the activation of MyD88, which is pivotal for the signalling of membrane Toll-like receptors, was
288 observed to be a negative regulator for the TLR3/TRIF pathway in mice corneal epithelium (60). In
289 addition, MyD88 activation was also shown to inhibit TRIF-mediated IFN-β and RANTES by
290 suppressing IKKε-dependent IRF3 phosphorylation in macrophages (61). The interactions among
291 different PRRs could range from nonadditive to desensitizing responses, which may have a negative
292 impact when co-infecting pathogens that activate different innate pathways are present (62).
293 Altogether these results suggest that the interference between different PRRs represents a possible
294 mechanism for the increased susceptibility to respiratory disease in viral and bacterial co-infections,
295 at least for IDV and *M. bovis*. We observed that the interference between RIGI/MDA5 and TLR2 was
296 stronger with poly(I:C)/LyoVec and Pam3CSK4 treatments than with co-infections with the two
297 pathogens. This could be due to the activation of additional PRRs that have not been described for
298 IDV and *M. bovis* so far.

299 IDV matrix protein was shown to suppress RLR and NF-kB signalling in human cells HEK-293T by
300 degrading the TNF receptor-associated factor 6 (TRAF6) (63), which is known to play a pivotal role in
301 the NF-kB activation (64–66) but also in the IFN-I pathway (67). Our RT-qPCR and ELISA results in
302 bovine PCLS indicate that a counteraction of the NF-kB pathway takes place also in cattle. In addition
303 to RIG-I/MDA5 activation, the degradation of TRAF6 upon primary IDV infection could be a
304 mechanism that explains the down-regulation of the TRL2-induced cytokines via the TRAF6/NF-kB
305 pathway.

306 In our previous *in vivo* trial, animals co-infected with IDV and *M. bovis* had an increased bacterial
307 colonization in the lower respiratory tract, which was linked to an increase in the clinical scores and
308 gross lung lesions compared to the group challenged with *M. bovis* alone (30). Taken together, our *in*

309 *vivo* and *ex vivo* results suggest that IDV could have an impact on BRD in the field, especially when
310 associated with other pathogens. However, the mechanisms of pathogenesis of IDV in co-infection
311 may be specific to the bacteria, as suggested by the absence of enhanced disease when calves were
312 co-infected with IDV and *M. haemolytica* (44).

313 Overall, these findings deepen the knowledge of respiratory co-infections' pathogenesis and increase
314 the understanding of the molecular mechanisms of mixed airways infections.

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316

317 **Materials and methods**

318 *Lung donors*

319 Animal tissues were obtained in accordance to the French regulation on animal experimentation. 3
320 to 6 week old male calves were purchased from the dairy educational farm of the Engineering School
321 for Agriculture of Purpan (INP Toulouse, France) and euthanasia was carried out by intravenous
322 injection of pentobarbital sodium (Dolethal, Vetoquinol) followed by complete exsanguination.
323 Animals displaying respiratory illness or gross lung lesions at necropsy were excluded from the
324 experiments. For each animal, nasal swabs, lung fragments and serum were collected to assess the
325 pre-exposure to bovine respiratory pathogens. RNA was isolated from nasal swabs and lung
326 fragments using Qiamp Viral RNA Mini Kit (Qiagen) and the presence of *M. haemolytica*, *P.*
327 *multocida*, *M. bovis*, *H. somni*, Bovine Coronavirus (BCoV), IDV, BRSV, and BPIV-3 was assessed by
328 real-time PCR using the commercial kit Bio-T respiratory qPCR kits (BioSellal). Serology to detect the
329 presence of anti-IDV antibodies was performed using a hemagglutination inhibition (HI) assay with
330 0.5% chicken red blood cells derived from SPF animals (PFIE, INRAE Centre Val de Loire, Nouzilly,
331 France) and 45 minutes incubation at +4°C (22). Anti-*M. bovis* antibodies were searched using the

commercial ELISA kit ID Screen® Mycoplasma bovis Indirect (IDVet). All the lung donors that were positive for one of the above-mentioned pathogens were excluded from the analyses.

Precision-Cut Lung slicing and organotypic culture

The lungs were collected post-mortem and they were washed twice with phosphate-buffered saline (PBS) supplemented with 2% of penicillin-streptomycin (PS) (10,000 U/10 mg/mL, Pan Biotech). The PCLS were obtained from cranial and accessorial lobes. A gelation medium was prepared by melting 2% of low melting point agarose (Thermo Fischer) in RPMI 1640 medium (Thermo Fischer). The gelation medium was cooled and maintained at 42°C and then inflated with a cannula at the bifurcation of the principal bronchi. The lungs were incubated on ice for 30 minutes to allow agarose polymerisation. Biopsy punches of 8 mm in diameter were obtained and sliced using a Krumdieck MD6000 tissue slicer (Alabama Research&Development) in sterile conditions. PCLS of approximately 100µm thickness were obtained. After slicing, the PCLS were placed in P24-well plates containing RPMI medium supplemented with 10% FBS and 1% PS. Three washing steps with 30 minutes of incubation at 37°C with 5% CO_2 were carried out using RPMI medium supplemented with 10% FBS and 1% PS. The PCLS were then incubated at 37°C with 5% CO_2 overnight before carrying out the experiments. Two additional washing steps with RPMI medium were done before the infections. After the washing steps, the medium was then replaced with RPMI supplemented with amphotericin B (2.5 µg/mL; Sigma-Aldrich) and ampicillin (0.3 mg/mL; Sigma-Aldrich).

Viruses, bacteria and infection of PCLS

Influenza D virus isolate D/bovine/France/5920/2014 (29) was propagated on specific-pathogen-free embryonated chicken eggs (PFIE, INRAE Centre Val de Loire, Nouzilly, France). IDV was titrated by TCID50 assay using swine testis cells (CRL-1746, ATCC) that were maintained in culture using

Dulbecco's minimum essential medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and propagated at 37°C with 5% CO_2. The cells were seeded in in P96-well plates at a density of 10,000 cells per well and the following day the culture medium was removed, the cells were washed once with PBS and the medium was replaced with DMEM supplemented with 2% PS. Virus stocks were 10-fold diluted in the infection medium and were then inoculated on swine testis cells that were incubated for five days at 37°C with 5% CO_2. The virus titers were revealed by HA assay using 0.5% solution of chicken red blood cells derived from SPF animals (PFIE, INRAE Centre Val de Loire, Nouzilly, France) and 45 minutes incubation at +4°C.The titers were determined using the Reed-Muench method. *M. bovis* strain RM16 (45) was grown in SP4 medium at 37°C and the titer was determined by 10-fold dilutions numeration on SP4 agar plates after five days of incubation at 37°C. The PCLS were washed twice with RPMI medium before the infections. Then, the medium was replaced with RPMI supplemented with amphotericin B (2.5 µg/mL; Sigma-Aldrich) and ampicillin (0.3 mg/mL; Sigma-Aldrich). For replication studies, 10^3 TCID50 IDV and/or 10^3 CFU *M. bovis* were used (MOI=0.001). For innate immunity studies, 10^6 TCID50 IDV and/or 10^6 CFU *M. bovis* were used (MOI=1). Mock allantoic fluid was inoculated in non-infected and *M. bovis* conditions. To study the effect of a primary IDV infection on *M. bovis* superinfection, the PCLS were infected at 0 hours with 10^6 TCID50 IDV and, 48 hours later, 10^6 CFU of *M. bovis* were inoculated in superinfected conditions. The supernatants and lung tissues were then collected at 72 and 96 hours p.i.

Flow cytometry

The viability of mock lung tissues was assessed by flow cytometry. The PCLS and their supernatants were briefly spinned, the supernatant were discharged and a solution of 0.25 mg/mL of Liberase™ TL Research Grade (Roche) was added to obtain single-cell suspensions. The PCLS were then incubated at 37°C for one hour and the tissues were dissociated by gentle pipetting. After a washing step using cold PBS and brief centrifugation at 300 x *g* for 5 minutes, the dissociated cells were stained with 7-

amino-actinomycin D (Biolegend) at a final concentration of 5 µg/mL. A final resuspension was done in a final volume of 100 µL of cold PBS supplemented with 2% of FBS and 50 µL of samples were read immediately after on a MACSQuant Analyzer (Miltenyi Biotec). Flow cytometry results were analysed using FlowJo v10.7.1 (Tree Star) software.

LDH activity measure

To assess the viability of infected and mock PCLS, 50µL of supernatants (infected with 10^3 TCID50 IDV and/or 10^3 CFU *M. bovis*) were collected at different time points and were tested using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific), following the manufacturer's instructions. Positive controls for LDH release were created by incubating PCLS with a lysis buffer provided by the kit for 1 hour at 37°C ("Maximum LDH activity"). The optical density of positive controls and PCLS supernatants was read at 490 nm and 680 nm (background) using a CLARIOStar Plus plate reader (BMG LabTech). The 680 nm absorbance value from the 490 nm absorbance before calculating the % of cytotoxicity, using thr formula [(LDH at 490 nm) - (LDH at 680 nm)] for each sample ("PCLS sample LDH activity"). The % of cytotoxicity was then calculated using the formula: [(PCLS sample LDH activity)/(Maximum LDH activity)] x 100.

RIG-I/MDA-5 and TRL2 stimulation and NF-kB inhibition

Polyriboinosinic:polyribocytidylic acid (polyI:C) (LMW) / LyoVec™ and Pam3Cys-Ser-(Lys)4 (Pam3CSK4) (Invivogen) were selected as agonists to activate specific immune pathways on PCLS. Poly(I:C) (LMW) / LyoVec™ is a synthetic dsRNA polymer that is complexed with a transfection reagent and it is sensed by cytosolic helicases retinoic acid-inducible gene I and melanoma differentiation-associated gene 5 (RIG-I/MDA-5) in a specific manner (68) and are known essential signalling pathways upon influenza viruses infection (69). Experimental infection in calves with IDV

405 also suggests the activation of this pathway (29). Pam3CSK4 is a synthetic triacylated lipopeptide that
406 is a potent activator of NF-kB pathway upon binding of TLR2/TLR1 receptor and a MyD88-dependent
407 activation, as described during *Mycoplasma* spp. infection in different species (41–43). To inhibit NF-
408 kB pathway, BAY 11-7082 (Invivogen) was used. BAY 11-7082 was described to inhibit the
409 phosphorylation of IκB-α (which is essential for the release of NF-κB from the cytosolic IκB-α/ NF-κB
410 complex) (70) but it was also suggested to inhibit the inflammasome responses indirectly by
411 preventing the nuclear translocation of NF-κB at the priming step but also to directly inhibitory
412 functions on the NLRP3 inflammasome by blocking the sensor's ATPase activity (71). For stimulations,
413 the PCLS were treated at 0 hour with poly(I:C) at a final concentration of 500 ng/mL, followed by
414 stimulation with Pam3CSK4 at a final concentration of 100 ng/mL 24 hours later. To inhibit the NF-kB
415 pathway, the PCLS were pre-treated with BAY 11-7082 at a final concentration of 100 ng/mL,
416 followed by *M. bovis* infection 6 hours later.

417

418 *RNA extraction and RT-qPCR*

419 For pathogen replication, 170µL of PCLS supernatant was used for RNA extraction using the kit
420 NucleoMag Pathogen (Macherey-Nagel) on a KingFisher Flex ™ purification system (Thermo Fisher
421 Scientific). For purification of total RNA from lung tissues, the PCLS were placed in lysis buffer
422 (provided by the RNA purification kit) in Precellys lysing kit tubes (Bertin Technologies) and tissues
423 were homogenized using a Precellys24 system (Bertin Technologies). Samples were centrifuged at
424 6,000 g for 5 minutes and 300µL of lysed supernatant was used to extract the total RNA using the
425 NucleoMag RNA kit (Macherey-Nagel) on the same KingFischer Flex system described above.
426 Pathogens were quantified in the supernatant by RT-qPCR (16). *M. bovis* was quantified using the
427 QuantiNova probe PCR kit (Qiagen) and primers and probe described in (72). RT-qPCR analysis for
428 immune response studies was carried out with RT-qPCR by using iTaq Universal SYBR green one-step
429 kit (Bio-Rad) using the amplification protocol of the manufacturer. For the relative quantification, we

used three previously validated housekeeping genes (HPRT, YWHA7 and GAPDH) to normalize the amount of the target gene (30). The calibration formula 2−ΔΔCT was used to quantify the relative expression of targeted genes. ΔΔCT represents the following: ΔCT (sample) ([CTcytokine gene – CT geometric mean of the three housekeeping genes] of infected lung cuts) − ΔCT (calibrator) ([CTcytokine gene – CT geometric mean of the three housekeeping genes] of mock lung cuts. For each time point and for each condition, values from infected PCLS were normalized to the mock PCLS gene expression and final results were expressed as fold changes relative to the number of copies of mRNAs in infected PCLS compared to the mock ones. All the qPCR experiments were performed on a LightCycler 96 real-time PCR system (Roche) and the results were analysed using the LightCycler® 96 Software v1.1.01320 (Roche). The list of primers used for cytokine amplification in this study is available in Table S1.

Measurement of IL-1β secretion by enzyme-linked immunosorbent assay (ELISA)

To confirm the RT-qPCR results, IL-1β was quantified in mock and infected supernatants using the IL-1 beta Bovine Uncoated ELISA Kit (Invitrogen), following the manufacturer's instructions. Prior to quantification, 10µL of infected and mock supernatants were 10-fold diluted in PBS supplemented with 0.22µm-filtered bovine serum albumin and a final volume of 100 µL of diluted samples were used for the test.

Nitric oxide quantification by Griess assay

To confirm the RT-qPCR results, the production of nitric oxide was measured in mock and infected supernatants at different time points using the kit Griess Reagent System (Promega) and following the manufacturer's protocol.

Mycoplasma bovis strain expressing the red fluorescent protein mCherry

The *M. bovis* strain RM16 was transformed with plasmid pOGch expressing the red fluorescent marker mCherry. In pOGch, the mCherry coding sequence was cloned downstream of the gentamicin resistance gene *aacA-aphD* to produce a fusion protein (Gch). The mCherry coding sequence was obtained from Torres-Puig *et al*. (73). The primers used for mCherry amplification from the plasmid pCatcherry and the gentamicin marker was amplified with its promoter region from the plasmid pMT85 are available in Table S2 (74). These two regions were assembled by overlap extention PCR using GmF_EcoRI and CherryR_BglII primers. PCRs were performed using the New England Biolabs high-fidelity DNA polymerase. The Gch PCR product was cloned into pGEM-T Easy (Promega) before subcloning at the *Not*I site of p20-1miniO/T plasmid (75) from which the *tet* gene has been extracted by *Pst*I restriction, to generate pOGch. Plasmid constructions were verified by DNA sequencing. Transformation of pOGch in *M. bovis* RM16 strain was done as previously described (76). Gentamicin selected transformants were stored at −80 °C. *M. bovis* mCherry stock cultures were produced in SP4 medium supplemented with gentamicin (50 µg.mL^{-1}) and bacterial titers were determined by counting colony-forming units, as the wild-type strain.

Immunofluorescence and confocal microscopy

For immunofluorescence studies, mock and infected PCLS were fixed with a 4% paraformaldehyde solution (PFA) for 30 minutes at room temperature. The PFA was then replaced with a PBS solution and stored at -20°C until the experiments were carried out. PCLS were permeabilized with 0.5% Triton X-100 in PBS at room temperature (RT) under slow agitation for two hours, followed by one hour of incubation at RT with a blocking buffer (PBS supplemented with 10% horse serum and 0.1% Triton X-100). Primary antibodies were diluted in PBS with 0.1% Triton X-100 and 2% horse serum and incubated overnight at +4°C under slow agitation, followed by five washing steps. The secondary antibodies were diluted in the same buffer used for primary antibodies and were let incubate for two

hours at RT under slow agitation protected from direct light. The nuclei were stained with 1 µg/mL of DAPI stain (Sigma-Aldrich) for ten minutes at RT. Propidium Iodide (PI) (Biorad) staining was done by using PI at a final concentration of 1µg/mL. The PCLS were mounted on microscopy slides using the ProLong Glass Hard Set Antifade mounting medium (Thermo Fischer) and images were captured with a microscope confocal SP8- STED 3X (Leica). Images were analysed with Leica Application Suite X v3.7.2 (Leica) software and Fiji ImageJ (77). The list of primary and secondary antibodies and their dilutions used is available in Table S3.

Electron microscopy

Non-infected and infected PCLS (infected 10^6 TCID50 IDV and/or 10^6 CFU *M. bovis*) were fixed at different time points with 2% glutaraldehyde (EMS) (CliniSciences) in 0.1 M sodium cacodylate (pH 7.4) buffer (CliniSciences) at room temperature. After washing three times in 0.2 M sodium cacodylate buffer, PCLS were post-fixed with 1% aqueous osmium tetroxide (Electron Microscopy Sciences) for 1h at room temperature, dehydrated in a graded series of ethanol at room temperature and embedded in Epon (Sigma-Aldrich). After polymerization, ultrathin sections (100 nm) were cut on a UC7 (Leica) ultramicrotome and collected on 200 mesh grids. Sections were stained with uranyl acetate and lead citrate before observations on a Jeol 1400JEM (Tokyo, Japan) transmission electron microscope equipped with an Orius 1000 camera and Digital Micrograph.

Statistical Analysis

All the experiments were performed with three biological replicates for each condition. Two-way ANOVA with Tukey's multiple comparisons method were performed on GraphPad Prism v9.3.1 (GraphPad Software, San Diego, California USA, http://www.graphpad.com). The represented p-

values in figures are *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. Data are expressed as arithmetic mean values ± standard errors of the means (SEM).

Appendixes

Figure S1. LDH activity measured in infected PCLS

Figure S2. Confocal microscopy studies of IDV and *M. bovis* distribution on PCLS.

Table S1. Primers used for RT-qPCR experiments

Table S2. Primers used for the construction of *M. bovis* fluorescent strain

Table S3. Antibodies and stainings used for immunofluorescence

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Figure captions

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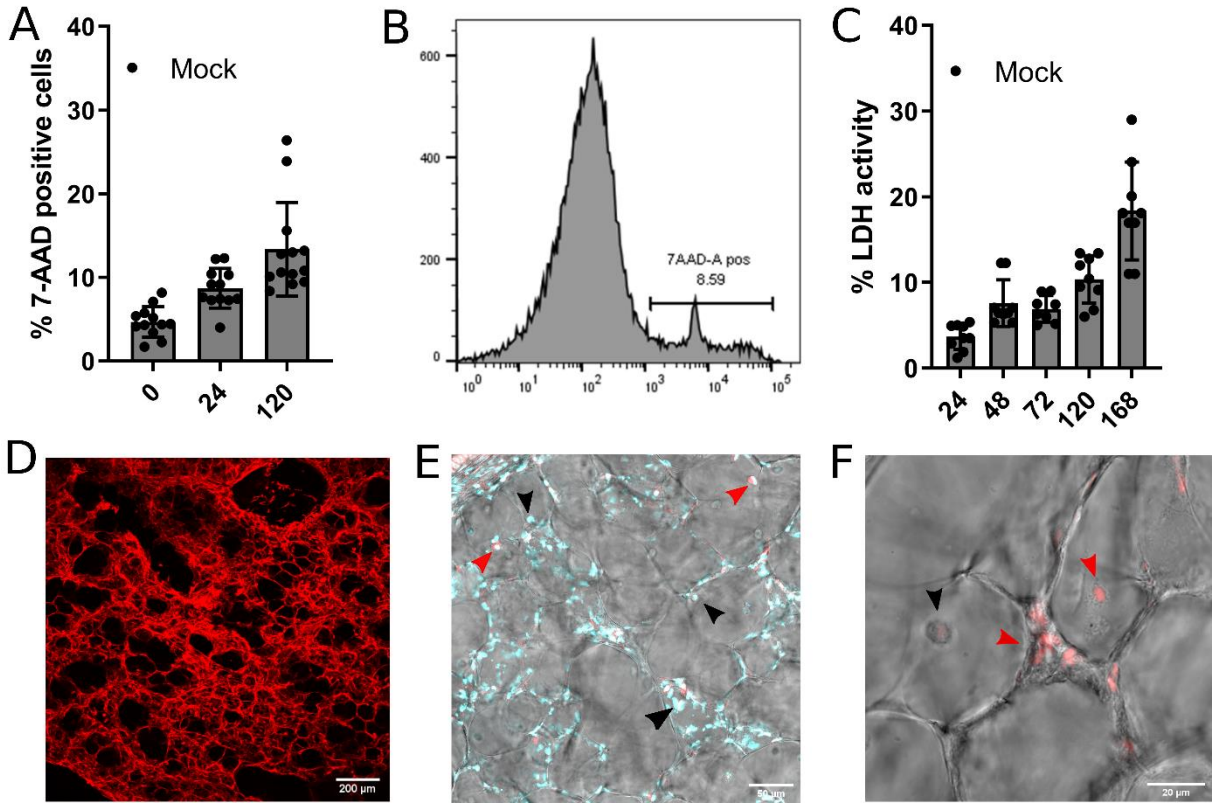

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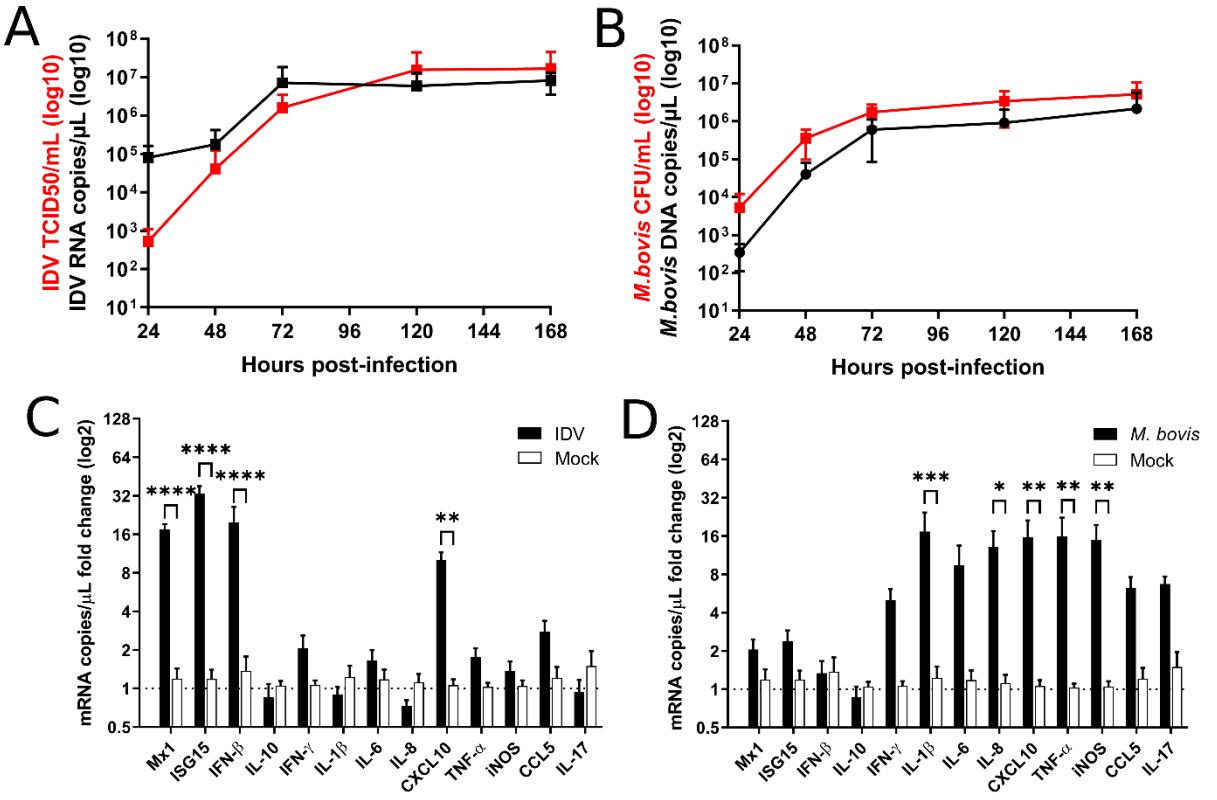

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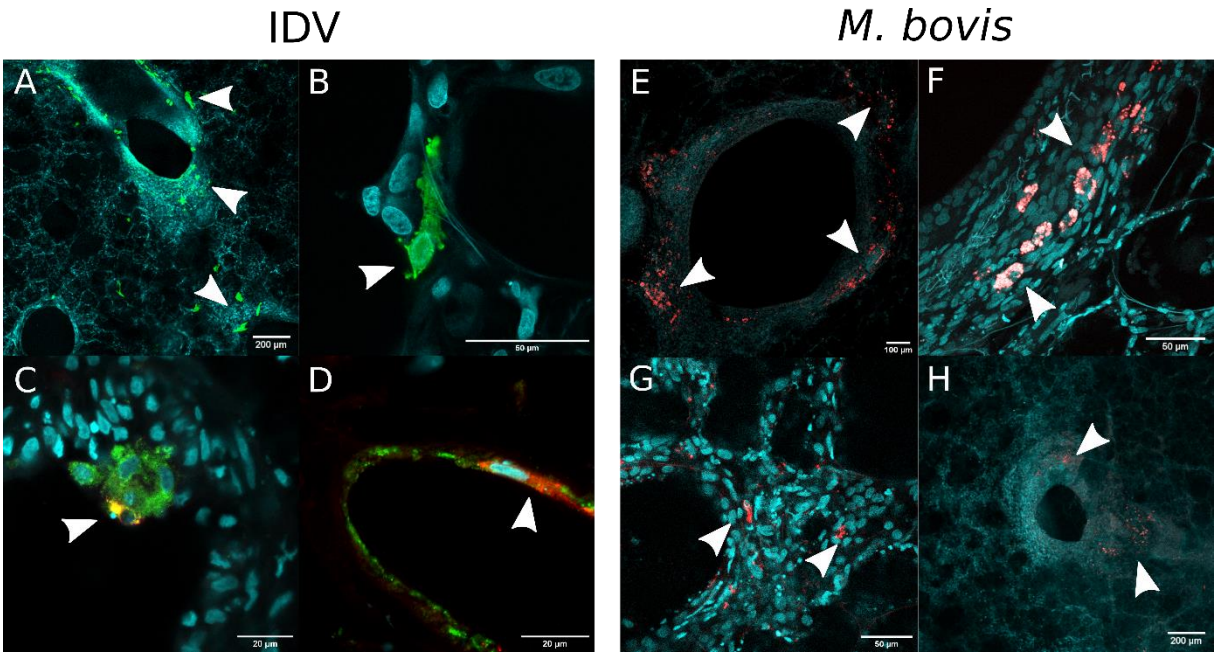

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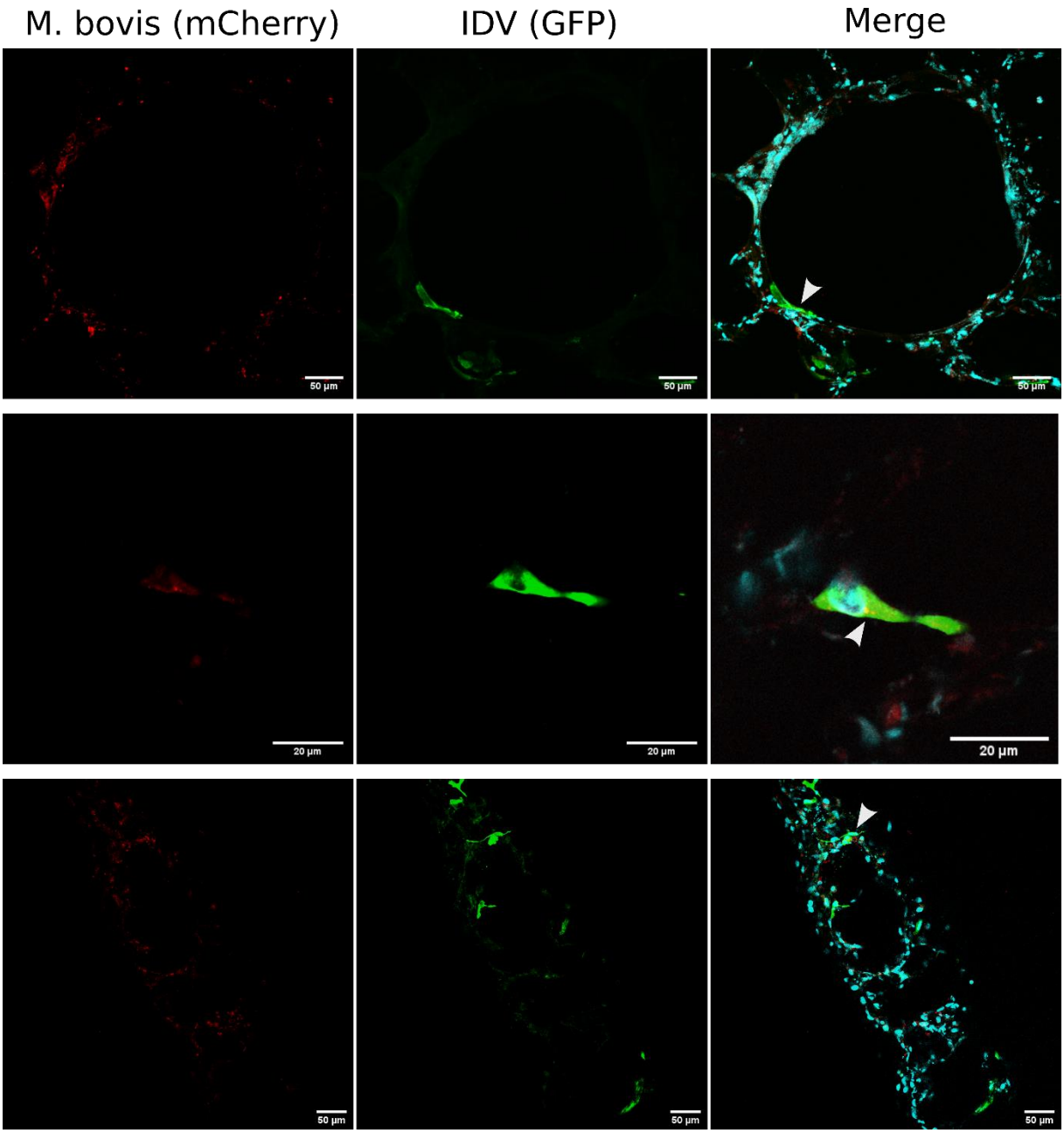

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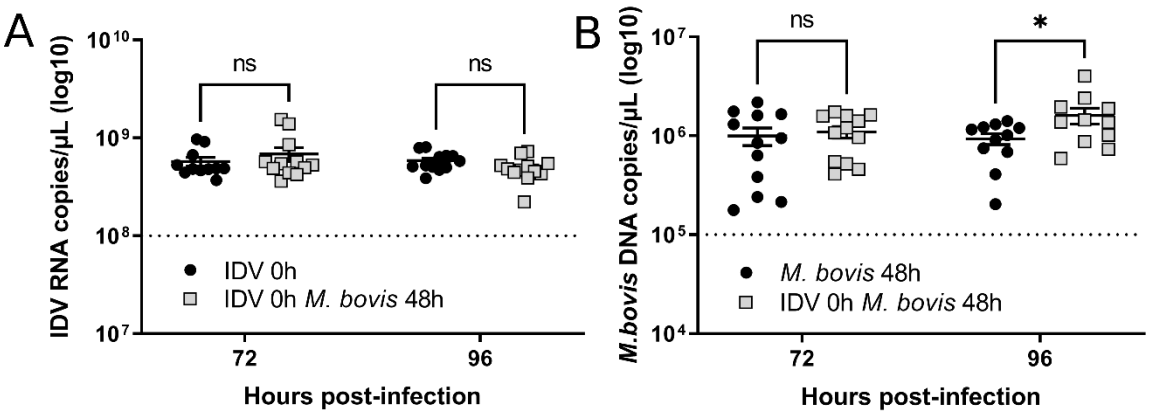

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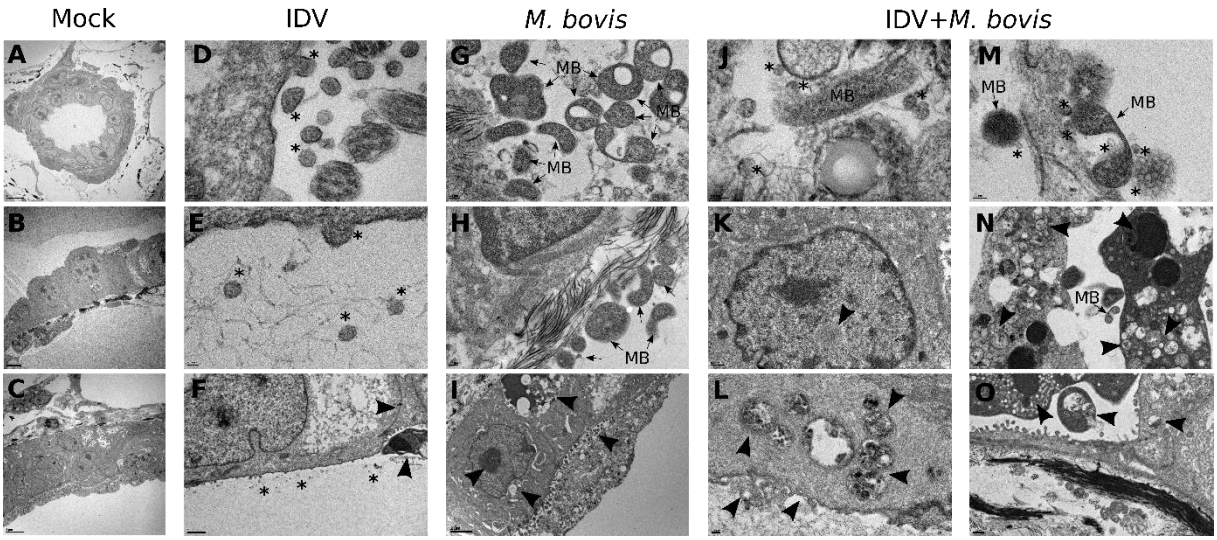

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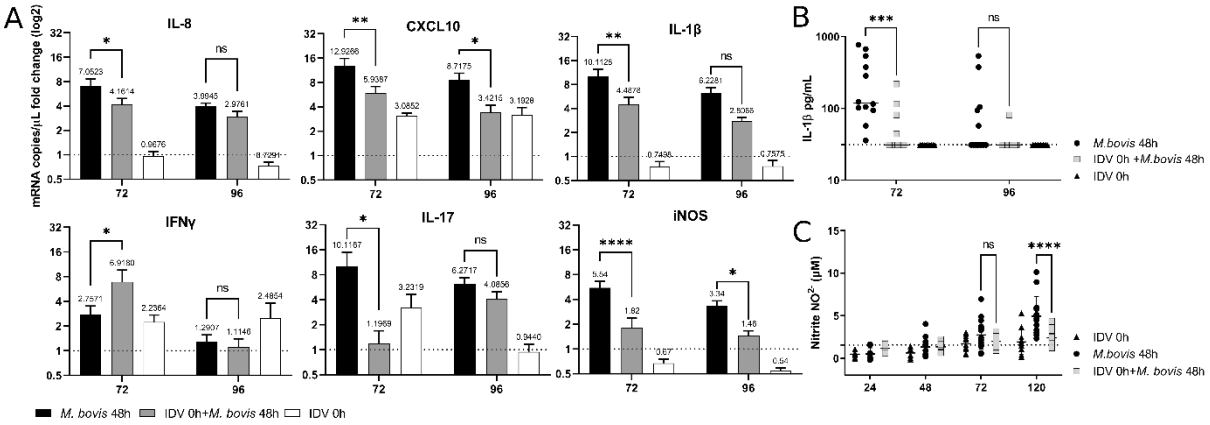

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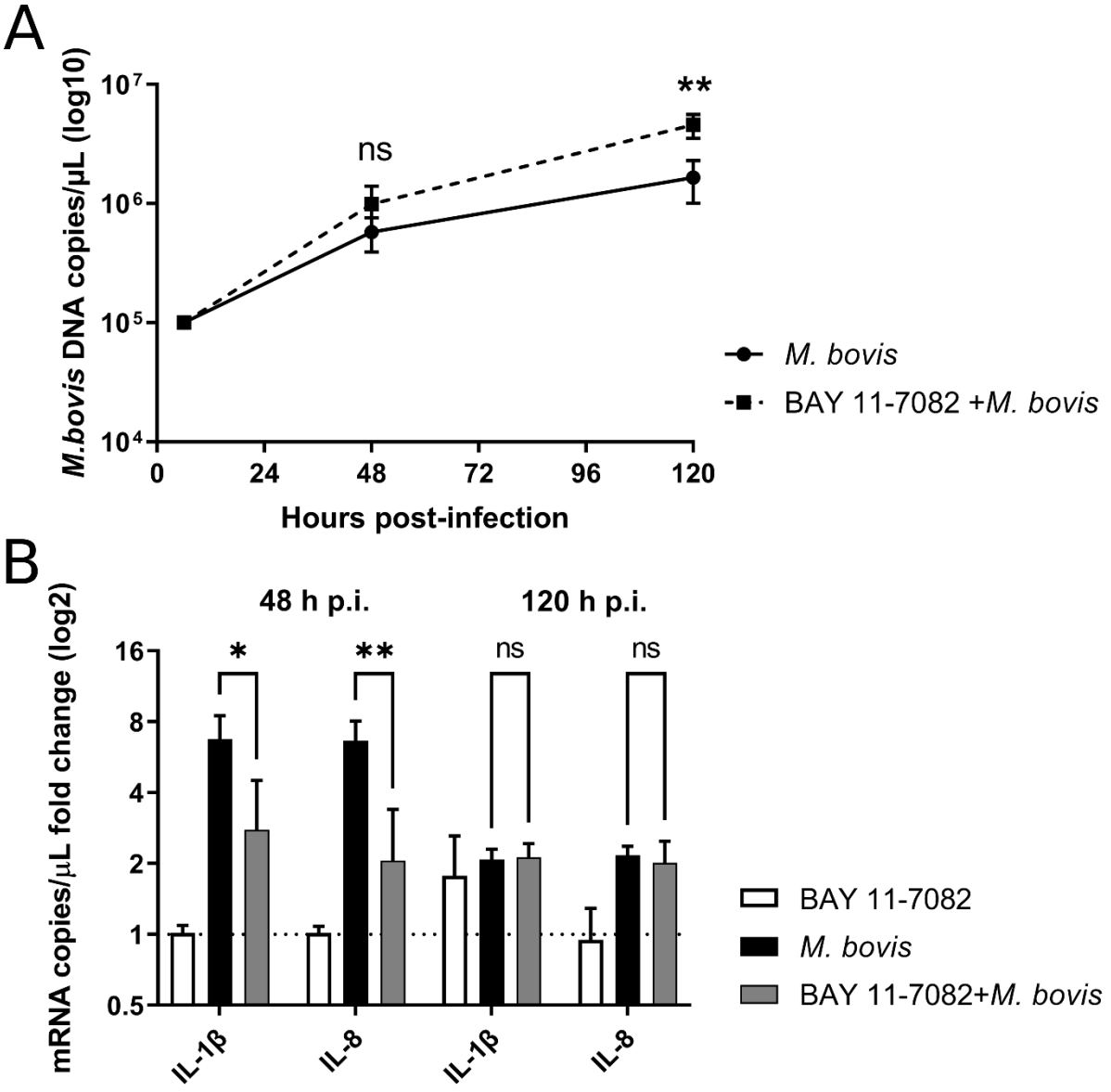

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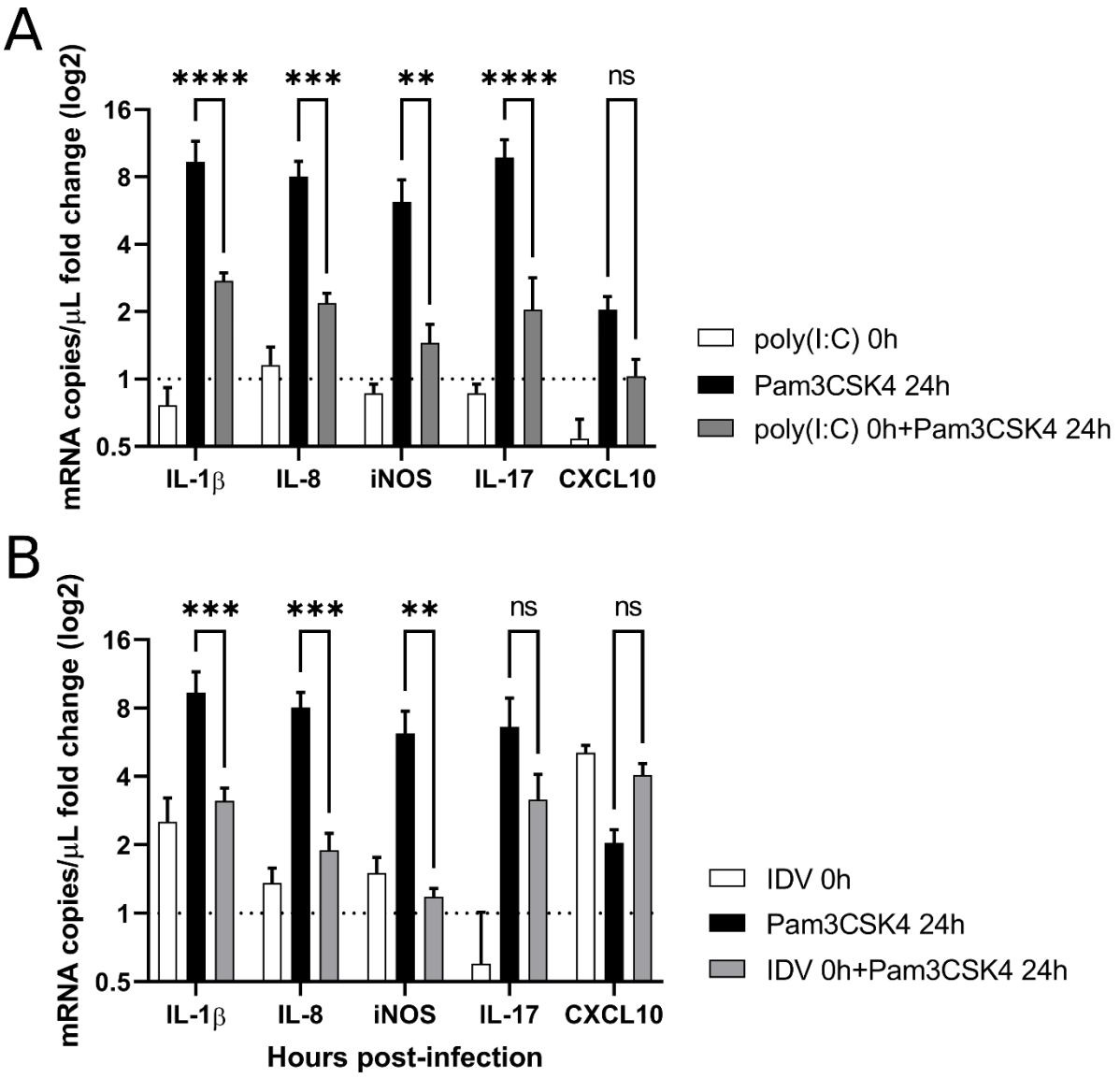

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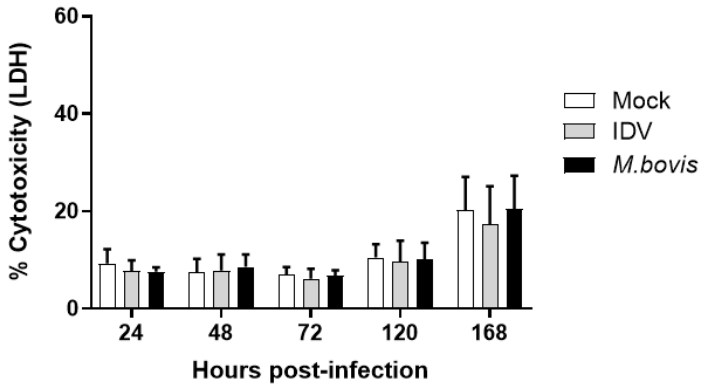
Supplementary material

Figure S1. LDH activity measure in infected PCLS



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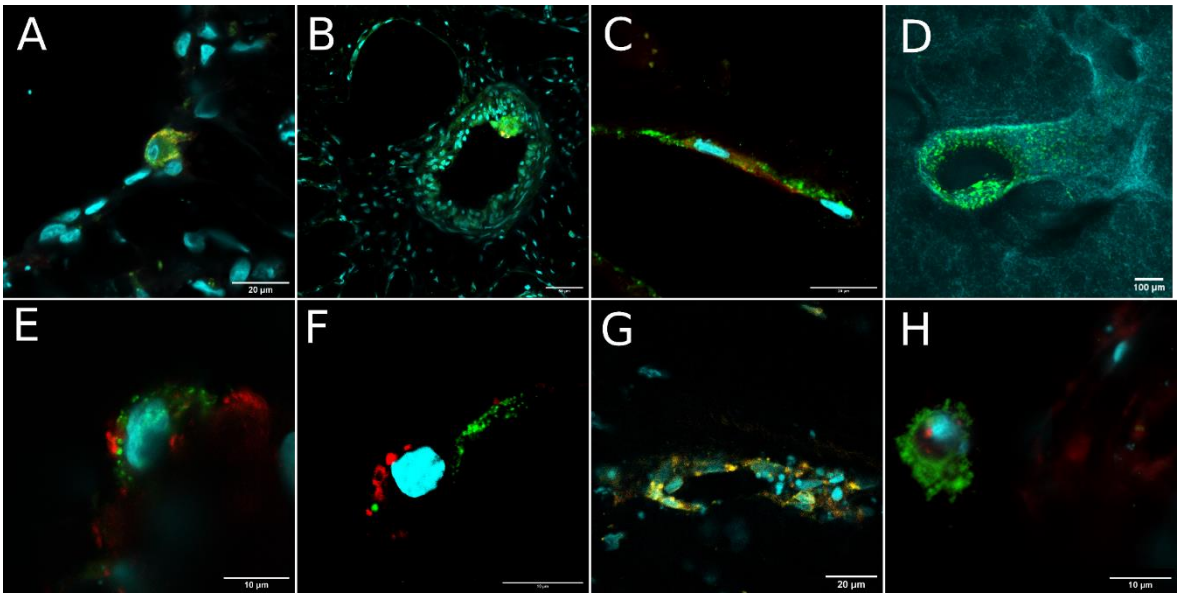


Figure S3. Confocal images of mock PCLS at 48 hours p.i.

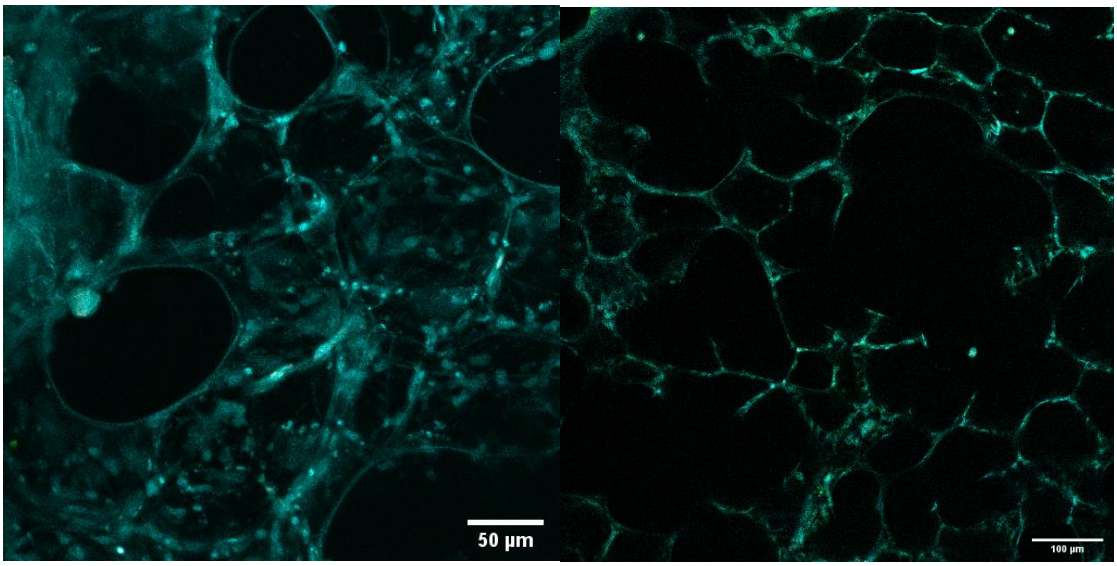
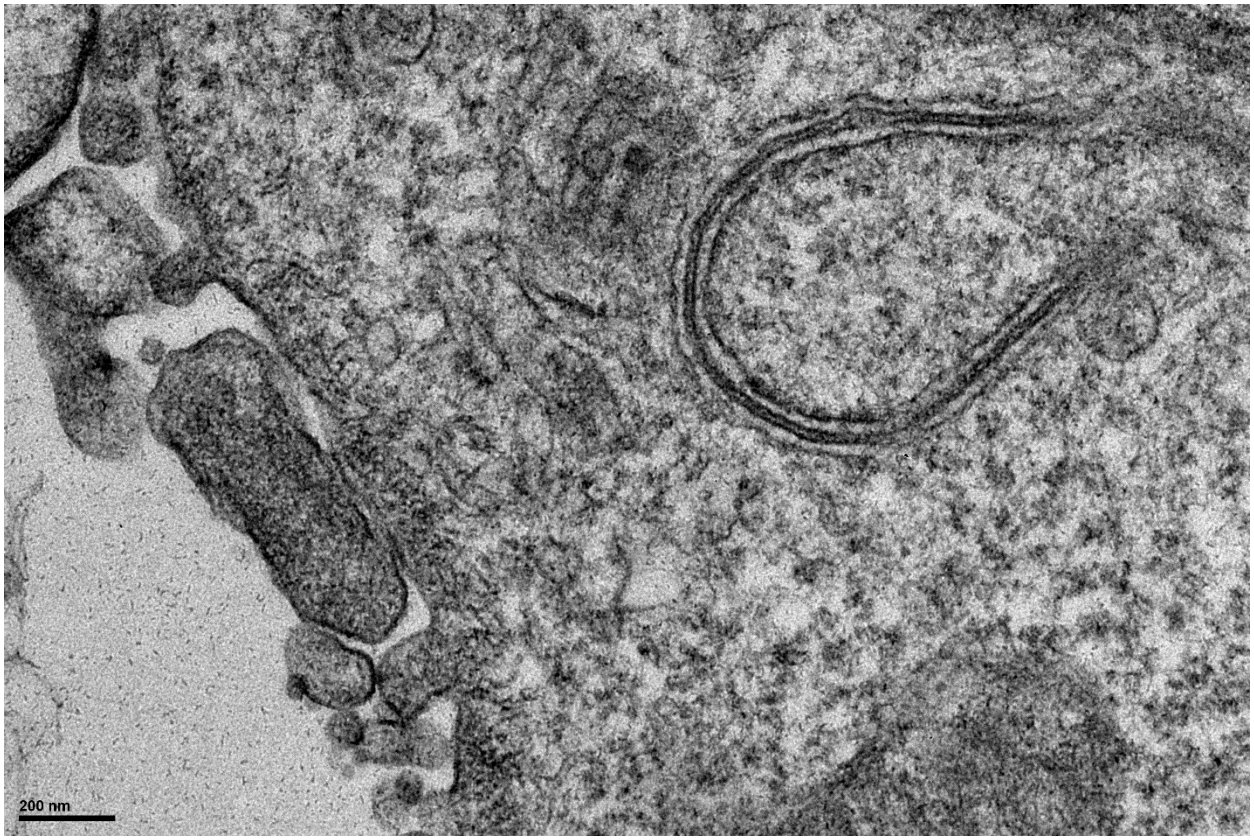


Figure S4. Transmission electronic microscopy image on co-infected PCLS showing a phagophore structure (double-membrane vesicle) at 24 hours p.i. The PCLS were infected with an MOI of 1 (10^6 TCID50/PCLS for IDV, 10^6 CFU/PCLS for *M. bovis*).



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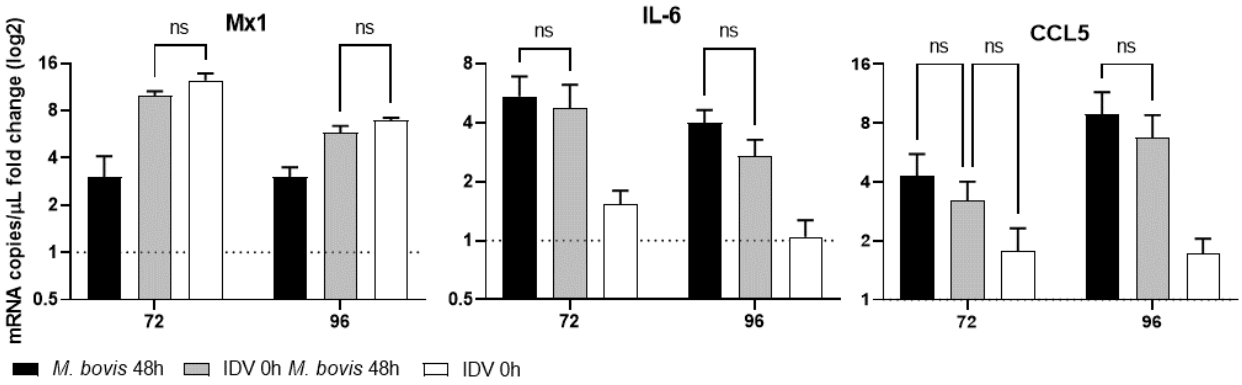

Table S1. Primers used for RT-qPCR experiments

Target	Reference
YWHA7	[1]
GAPDH	[2]
HPRT	[1]
IL-8 (CXCL8)	[1]
Mx1	[1]
ISG15	[1]
IL-10	[1]
IFN-γ	[1]
IFN-β	[1]
IL-6	[1]
CCL5	[1]
TNF-α	[1]
IL-1β	[1]
IP-10 (CXCL10)	[3]
iNOS (NOS2)	[4]
IL-17A	[5]

Table S2. Primers used for the construction of *M. bovis* fluorescent strain

mCherry amplification from plasmid pCatcherry	GmvsCh_F	5'-ATTTATAAAAGGACTTATAAAGATagcaagggcgaggaggataacat-3'
	CherryR_BglII	5'-TTCAGATCTGGATCCTTACTTGTACAGGTCGTC-3'
Gentamicin marker amplification	GmF EcoRI	5'-GATCTGAATTCGCATTTTACACAGGAGTCTGGA-3'

from plasmid pMT85		
	GmvsCh R	5'-ATGTTATCCTCCTCGCCCTTGCTATCTTTATAAGTCCTTTTATAAAT-3'

Table S3. Antibodies and stainings used for immunofluorescence

Target cells	Primary antibody (or staining)	Dilution/ concentration	Secondary antibody	Dilution
IDV	Polyclonal serum anti-IDV nucleoprotein (Rabbit) [6]	1:200	Donkey anti-rabbit 488 (711-546-152, Jacskon Research)	1:2000
IDV	Monoclonal 3G3 [Mouse hybridoma against influenza D virus HE] [7]	1:200	Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 (A-11032 Invitrogen)	1:1000
Club cells	Anti-Clara Cell Secretory Protein Antibody (Rabbit) (Sigma-Aldrich)	1:200	Donkey α rabbit 488 (711-546-152, Jacskon Research)	1:1000
Alveolar macrophages, T cells	MHC Class II DQ antibody | CC158 (Biorad)	1:500	Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 (A-11032 Invitrogen), Goat anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (A-21131 Invitrogen)	1:1000
Endothelial cells	Anti-Von Willebrand Factor antibody (Rabbit) (ab6994, abcam)	1:200	Goat anti-Rabbit IgG (H+L) Secondary Antibody, FITC (65-6111 Invitrogen)	1:1000
Necrotizing cells	Propidium Iodide (Biorad)	1µg/mL		
Nuclei	DAPI (Sigma-Aldrich)	0,5 µg/mL		
β-tubulin	Monoclonal Anti-β-Tubulin−Cy3 antibody produced in mouse (C4585-.2ML) (Sigma-Aldrich)	1:500		

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3.4 Discussion

In this chapter, we presented a study where we used the organotypic PCLS model in order to better characterize IDV and *M. bovis* infections in lung, but also to decipher the molecular mechanisms explaining the impact of a primary IDV infection on *M. bovis* superinfection. PCLS represent a versatile tool that has the huge benefit of meeting the 3Rs principle to reduce the number of animals in *in vivo* experiments. It has been extensively used for lung disease modelling (191–193), including in studies with bovine pathogens (194–196). Additional advantages of this model include the preservation of the 3-dimentional structure and physiological properties of the lung tissue, the preservation of the resident cells that were present in the collected organs, as well as the possibility of doing a large number of biological replicates for different experimental conditions (197). In our model, we also observed by confocal microscopy, the presence of resident immune cells, such as alveolar macrophages and cells expressing MHC-II. In addition, we observed the presence of different cell types, notably epithelial but also endothelial cells. The high complexity of this model is advantageous to mimic the *in vivo* conditions in lung, especially for innate immune studies. However, one of the main inconvenient, unlike immortalized cell lines, is the limited culture time. In our study, the PCLS model showed a good cell viability up to five days, but an increase of cell mortality at day seven. In addition, unlike in *in vivo* models, the adaptive response cannot be studied *ex vivo*.

Before carrying out the co-infections study, the suitability of the model for IDV and *M. bovis* was first assessed for each pathogen individually. Both displayed a good replication profile on lung tissue and they triggered a similar immune response to what observed *in vivo* (23,24). We therefore further investigated the interactions between these two respiratory pathogens. As discussed in the first chapter, primary viral infections can often be predisposing to secondary bacterial superinfections in calves. Therefore, unlike in our previous *in vivo* experimental co-infection where both pathogens were inoculated simultaneously (24), we decided to better characterize the predisposing role of IDV to secondary bacterial superinfections by inoculating first IDV and *M. bovis* 48 hours later on PCLS. We observed by confocal and electron microscopy that both pathogens have a similar tropism and can be also co-localized in close proximity. Therefore, the transmission of both pathogens simultaneously from one co-infected animal to another could be hypothesized. In addition, our transcriptomic

results suggest a viral hijacking of the NF-kB pathway which facilitates a bacterial superinfection, but also a cross-talk between RLRs and TLRs as an additional mechanism. Our results indicate a potential mechanism for the enhanced pathology observed during our previous *in vivo* experimental co-infection with the same pathogens, where animals co-infected with IDV and *M. bovis* had an increase in bacterial colonization of the lower respiratory tract, which was linked to an increase of the clinical scores and gross lung lesions (24). However, in this study animals were inoculated with both pathogens simultaneously.

Taken together, our *in vivo* and *ex vivo* results suggest that IDV could have an impact on BRD in the field, especially when associated with other pathogens. Though, another experimental study showed that calves were first challenged with IDV and subsequently with *M. haemolytica* did not display an increase in clinical signs compare to mono-infections (38). Therefore, the mechanisms of pathogenesis of IDV in co-infection with *M. haemolytica* and other respiratory bacteria remain unsolved and further studies would be useful to gain additional insight into BRD pathogenesis.

Final discussion and perspectives

BRD, IDV and co-infections: what are the future challenges?

In this work, we first reviewed the experimental evidence about the impact of co-infections on BRD by retrieving from scientific literature *in vivo* studies that described the role of primary viral infections on promoting secondary bacterial superinfection in calves. In the second part, we assessed the circulation of the recently discovered IDV and its evolution in cattle herds in Europe from its first detection until present. Finally, in order to clarify IDV role in BRD onset we carried out experimental co-infections in *ex vivo* PCLS model with IDV and *M. bovis*. We will now discuss the findings and perspective based on the initial questions in the introduction chapter.

i) What is the experimental evidence about the impact of co-infections on BRD?

In this work, we retrieved from scientific literature several studies that validated the role of primary viral infections on promoting secondary bacterial superinfection in cattle and we consolidated experimental evidence describing co-infection mechanisms potentiating pneumonia aetiology in cattle. Very few studies described the impact of viral superinfections on BRD, but also mixed bacterial infections, preventing a deep understanding of BRD aetiology. During surveillance, longitudinal studies could also be conducted to observe the dynamics of respiratory outbreaks caused by mixed infections, providing insight about the timing of pathogen introduction during BRD development.

Future challenges for BRD should include an improvement of current viral vaccines, which could limit respiratory outbreak onset in winter seasons. During BRD outbreak, the first choice of treatments remain antibiotics (198–200). Despite a decrease in antibiotic treatments, an increase in antimicrobial resistance has already been highlighted for *P. multocida* and *M. haemolytica* in European countries (201–203) but also in North America (128,204). In addition, in the United States, metaphylaxis has been used as preventive measure to increase animal welfare (205), and it has clearly been associated to an increase in antibiotic resistance for *M. haemolytica* (206,207). Efforts to better understand BRD onset and the role of the respiratory

microbiome in its pathogenesis still have to be made. In addition, to date only limited data exist on viral/viral and bacterial/bacterial co-infections, limiting thus our full comprehension on the pathogenesis mechanisms of BRD. Additional strategies, other than antibiotics, should be investigated in order to reduce the phenomenon of antibiotic resistance. Probiotic administration of *Lactobacillus* strains was evaluated *in vivo* as effective to prevent *M. haemolytica* colonization of the upper respiratory tract to prevent, representing thus a possible alternative in the future as prophylaxis against cattle pneumonia (117). Thus, further studies should assess if future interventions for better BRD management should target the respiratory microbiota, as well as to better understand its role in BRD onset.

In order to better assess BRD treatment, the interactions between BRD pathogens should be studied more in depth; in particular, additional studies about the associations and the exclusions between viruses and bacteria in different farming systems could be useful to better target future interventions against BRD. In addition, a better understanding of the cellular mechanisms and metabolic pathways of the interaction among pathogens could be also useful to identify new possible therapeutic strategies.

Current prophylactic measures in cattle farms include the vaccination mainly against BRSV, BPIV-3 and *M. haemolytica*, however new vaccines against widely diffused pathogens such as *M. bovis* and BCoV should be implemented in order to provide protection against the respiratory disease caused by these pathogens. Prophylactic strategies against newly discovered viruses (i.e. IDV), should also be considered in a near future, however a better risk assessment of the target population would be needed first.

ii) What is IDV prevalence in cattle herds?

Our data show that IDV circulates with high prevalence in cattle population, unlike other domestic species (swine, small ruminants) or wild fauna. We investigated IDV circulation in a few European countries, however its actual prevalence remains unknown in the majority of geographic areas. Due to its active circulation in cattle herds, IDV inclusion in the routine testing for respiratory viruses is therefore recommendable.

Different European projects are currently ongoing to better assess IDV circulation and impact in cattle farms in this geographic area, which are financed by the European Food Safety

Authority (EFSA): GP/EFSA/AFSCO/2017/01 GA04 “Risk assessment for Influenza D in Europe” and GP/EFSA/ENCO/2020/03 “Developing an integrated approach to assess the emergence threat associated with influenza D viruses’ circulating in Europe”, as well as the International Coordination of Research on Infectious Animal Diseases (ICRAD)-ERA NET co-fund ANR-21-ICRD-0007 ‘Deciphering the role of influenza D virus in bovine and human respiratory diseases in Europe’ to study IDV role in respiratory co-infections, but also to assess IDV zoonotic potential. Within these projects, diagnostic tools for IDV detection are being implemented, both for serology and molecular biology techniques (208). In addition, surveillance studies to better understand IDV evolution and the emergence of new clades are also ongoing.

If the upcoming studies will also confirm a high prevalence in bovine population, the question of the future control of the infection should be addressed. So far, IDV alone has not been associated with high pathogenicity (22,23). However, other viruses with relatively low/or not fully understood virulence, like BPIV-3, are inserted in common vaccination programs since the last century due to the experimental evidence of its effects in promoting bovine “Pasteurellosis” (209). Therefore, the possibility of vaccinating cattle against IDV should be discussed in the future. A vaccine was already developed in a research study but it only conferred partial protection in cattle and it has not been commercialized (210).

Before the proposal of any future interventions, a risk assessment for different bovine populations based on age and production system has to be performed in order to correctly address the target of eventual IDV prophylaxis. Some studies suggest that BRD cases occur more frequently in older calves, which could be explained by ongoing exposure to pathogens and waning of maternal immunity, as well as other stress factors with the advancement in the age of the calf (211). However, other studies described a higher risk in pre-weaned calves, when their immune system is still not completely functional and unprimed (4). To date, IDV was detected both in calves, young cattle and cattle of more than one year of age, and both in dairy and beef production systems (155). However, it is still not clear whether any differences of IDV pathogenesis exist based on the age of animals as all the experimental infections were done on young calves so far (22,23).

In literature, in two studies the dynamics of anti-IDV antibodies in calves was monitored. In the first one, the prevalence of maternal antibodies in neonatal calves was measured 24 to 36 hours after birth (176). 94% of 448 sera tested positive for IDV, with a mean titer of 1:410,

showing that maternally-transmitted IDV antibodies are highly prevalent at birth. In addition, cohorts of sera from Mississippi cattle of different age were tested, which included young cattle of 6 month (n=52), 7 month (n=244), 8 month (n=188), heifers of 1 to 3 years (n=64), old cows of 3 to 9 years (n=33) and 9 to 14 year old cows (n=24). The seroprevalence in these groups was 11.5%, 3.7%, 6.9%, 54.7%, 60.6%, 54.2%, respectively. This suggests that IDV maternal antibodies tend to decrease with age in 6 to 8 month old calves but they then increase starting at one year of age, when animals are exposed to the pathogen. In a second study, calves were tested at one week of age and they were then retested 3 months later. While 98% of sera from 1 week old calves tested positive, the prevalence in the same animals decreased to 76% of the same calves 3 months later, confirming also a decrease of maternal antibodies with age (150). Young cattle could therefore be considered a highly susceptible population for IDV. However, upon experimental reinfection with IDV, existing antibodies could not protect cattle from reshedding the virus after a second challenge 22 days later (212). Therefore, adult seropositive cattle could also be potentially reinfected with IDV. Overall, these data do not allow to draw solid conclusions about a potential cattle group at higher risk for IDV infection. Epidemiological studies done on cattle of different age could help to answer to this question. Longitudinal studies to assess the length of the antibody-mediated protection could be useful to better understand the dynamics of IDV infection and reinfection in cattle. In addition, further studies are needed to fully understand if IDV immune response is only antibody-mediated or not.

iii) How did IDV evolve since its discovery?

IDV displayed relatively rapid evolution in the last decade after its discovery. IDV genetic drift, the introduction of new clades on the continent and multiple reassortment patterns shape the increasing viral diversity observed in the last years. The consequences of reassortment on viral fitness remain unknown for IDV. Interestingly, the evolutionary rate of IDV HEF was higher than its human counterpart ICV, but lower than IAV and IBV HA, which represent a known public health threat. This could indicate that the novel IDV is still not fully adapted to cattle population, from which the majority of available sequences are derived. Sequences from animal species other than swine are currently missing and could provide additional insight into IDV evolution in other hosts. Molecular screening of IDV in other species than

cattle could help could help gain insight into the emergence of new clades, but also into eventual spread to new hosts. In particular, molecular surveillance in species for which only serology results are available could be useful in order to validate serology data, such as for humans and camelids, but also wild fauna. This could also help understanding IDV intercontinental spread and origins, which remains an important unanswered question. The first description of IDV in cattle population was assessed in 2003 in the United States by a serological study conducted on a cohort of sera collected from 1977 to 2010 (149), and starting from 2005, an increase in IDV human seropositivity was highlighted (82), it is likely that humans could have been exposed as accidental hosts following IDV circulation at high prevalence in cattle (zoonotic transmission), rather than a conversely species jump in the opposite direction (anthroponotic transmission) with a consequent adaptation in cattle. However, it is also possible that IDV circulation in humans started in other geographical areas before cattle and remained undetected for several years. IDV circulation in cattle before 2003 in other geographic areas is currently unknown, and serology studies using cohorts of human and cattle sera collected from the same timeframe of the putative IDV spread, as well as serology studies on other animal species that were not considered so far, could help to provide a better understanding of the role of humans in the virus origin and transmission.

iv) What is the impact of the novel respiratory IDV on BRD in cattle?

We experimentally studied IDV co-infection with *M. bovis* in organotypic PCLS model in order to better understand IDV impact on BRD aetiology. In our study we characterized IDV tropism in lung, which was only partially known so far, unveiling an important replication in bronchioles, which could be responsible for the observed clinical signs *in vivo* (22,23). In addition, we could co-localize IDV and *M. bovis* in the same cell types in lung tissue, but also in close contact one with another. The aggregation of the viral particles and bacteria could potentially facilitate the transmission of both pathogens from a co-infected animal to another, but the biological signification of these particular structures remains to be further investigated. In our study we also better characterized IDV immune response in lung tissue. We observed that IDV interferes with the NF-kB pathway, as shown by our transcriptomic

results, which is a mechanism that is commonly exploited by several viruses to evade the immune response (213). IDV matrix protein was shown to degrade TRAF6 in human HEK-293T cells, which is known to play a pivotal role in the NF-kB activation (214–216) but also in the IFN-I pathway (217). The degradation of TRAF6 upon primary IDV infection could be a mechanism that explains the down-regulation of the TRL2-induced cytokines via the TRAF6/NF-kB pathway, however further studies are granted to better understand the exact molecular mechanisms of IDV antagonism of this specific immune pathway.

Importantly, we found that the down-regulation of NF-kB pathway has an effect on promoting *M. bovis* replication. As a matter of fact, despite pro-inflammatory cytokines are responsible for tissue damage and disease, they also play an important role in counteracting the replication of pathogens. We could demonstrate this for *M. bovis* by decreasing the levels of pro-inflammatory cytokines by inhibiting the NF-kB pathway with a synthetic agonist. This is in agreement with what was observed for the human pathogen *Mycoplasma pneumoniae* in the mouse model, where mice unable to produce IL-1β had delayed bacterial clearance in the lungs (218). Furthermore, our results suggest that one of the molecular basis for the synergistic effect of IDV and *M. bovis* co-infection could be due to a cross-talk between the activated PRRs. Indeed, we found that a primary activation of RIG-I/MDA5 pathway inhibits the TLR2 activation cascade, therefore blocking the cytokine cascade that is required to clear the bacterial infection, which is also in agreement with results already observed in bone marrow-derived macrophages in mice (219). Similarly, MyD88 was observed to be a negative regulator for the TLR3/TRIF pathway in mice corneal epithelium (220) but it was also shown to inhibit TRIF-mediated IFN-β and RANTES by suppressing IKKε-dependent IRF3 phosphorylation in macrophages (221).

The role of human influenza virus in predisposing and aggravating secondary bacterial infections were extensively investigated, especially in mice models. Previously described pathogenic mechanisms of human IAV in mice model include neutrophil impairement in lung, associated with increased susceptibility to *Streptococcus pneumoniae* (222) and neutrophil chemoattractants deficience that resulted in the inability to resolve *S. pneumoniae* superinfection (223). In other studies, experimental pneumococcal superinfections aggravation were associated to IFN-I presence, as shown by the increased survival of Ifnar-/- mice compared to wild-type mice (224,225) and to decreased Th17 response (226), as shown

by the impaired *S. aureus* clearance in IL-17R(-/-) mice compared to wild-type. Similarly, in our study we observed that IDV infection decreased IL-17 mRNA of 10-fold in superinfection, however the immune cell recruitment in lung, which plays a very important role in the outcome of the coinfection, cannot be studied in our model and it represents one of its main limits.

In future studies, the role of IFN-I presence on bacterial superinfections should be studied within the BRD complex. Several studies have already documented in mice models that the production of IFN-I was detrimental during bacterial infection due to the suppression of IL-17, which was the case with *Listeria monocytogenes* (227,228), *S. aureus* (224,229) but also *Mycobacterium tuberculosis* (230,231). However, in other cases the presence of IFN-I was protective against secondary bacterial superinfections, such as with *S. pneumoniae* (232–234), due to the promotion of tissue integrity.

We only described a non-additive response for the RIG-I/MDA5 pathway, however TLR/RLR interference could also occur for other receptors, which could be interesting to study for other pathogens that activate different immune pathways (i.e. bacteria expressing LPS, which are recognized by TLR4). In addition to RLRs and TLRs antagonism, some still unidentified immune pathways could be responsible for the observed synergism of other pro-inflammatory cytokines, such as IFNγ, which was shown to be upregulated during IDV and *M. bovis* coinfection both in the PCLS model but also *in vivo* (24). The simultaneous induction of the RIG-I/MDA5 and TLR2 pathways could therefore lead to exacerbated IFNγ, which could be considered a potential marker of coinfection with this couple of pathogens.

Taken together, these results suggest that IDV likely plays a role in initiating BRD in cattle, representing another possible threat to cattle health. However, the mechanisms of pathogenesis of IDV in co-infection may be specific to the bacteria, as suggested by the absence of enhanced disease when calves were co-infected with IDV and *M. haemolytica* (38). The synergy between IDV and *M. bovis* was observed during *in vivo* co-infection, where animals co-infected with IDV and *M. bovis* had an increase in bacterial colonization of the lower respiratory tract, which was linked to an increase in the clinical scores and gross lung lesions compared to the group challenged with *M. bovis* alone (24). In this study, both pathogens were inoculated simultaneously and the innate immune response was made on bronchoalveolar lavages, explaining therefore the possible differences in transcriptomic

results. However, in both studies an up-regulation of IFNγ was observed, underlining that PCLS model is suitable for co-infection studies in cattle. Indeed, PCLS represents a versatile tool that has the huge benefit of meeting the 3Rs principle to reduce the number of animals in in vivo experiments. It has been extensively used for lung disease modelling (191–193), including studies with bovine pathogens (194–196). Additional advantages of this model include the preservation of the 3-dimentional structure and physiological properties of the lung tissue, the preservation of the resident cells that were present in the collected organs, as well as the possibility of doing a large number of biological replicates for different experimental conditions (197). However, the major inconvenient is represented by the limited time of culture, as shown by our viability studies. In addition, it is also impossible to study the adaptive immune response which have very important roles in the outcome of viral and bacterial infections and are critical to fully understanding viral and bacterial co-infection pathogenesis.

Our experimental study shows that IDV promotes *M. bovis* superinfections. An interesting perspective could be to study also IDV predisposing role to secondary viral infections in cattle. As IDV was shown to counteract the immune response to aggravate bacterial superinfections, it would be important to understand if IDV promotes bacterial superinfections also in other hosts that are suspected to be susceptible to IDV, like swine and humans. A study carried out in mice investigated the role of IDV in predisposing secondary *Staphylococcus aureus* superinfections. Mice were first inoculated with IDV and seven days later they were challenged with *S. aureus*, however IDV seemed to have a protective effect on co-infected animals by increasing the survival rate and recovery compared to the *S. aureus* group alone (235). The authors proposed that the antiviral response (notably the IFN-I activation) was responsible for protecting the host against these potentially deadly outcomes. Despite this, the only mice models that were developed for IDV lack of clinical signs, making them inadequate models to investigate BRD mechanisms (185,235). On the other hand, these models could be useful to study IDV impact on human respiratory bacterial superinfections. However, IDV pathogenesis in humans remains still unknown to date.

It would be interesting to repeat the experiment with other bacterial pathogens or by decreasing the delay of the superinfection. This is in contrast to what reported for IAV primary infection and *S. aureus* superinfection in the same model, where IAV is known to prime and predispose mice to secondary pneumonia (236,237). In this case IAV solicited host immune

factors were similar to IDV, triggering the induction of ISGs and antiviral proteins; however, concurrent with their antiviral effect, however, IFN-I production can decrease important antibacterial immune responses and neutrophil-recruiting chemokines (237), similarly to what observed with IDV and *M. bovis* co-infection in bovine PCLS. Therefore, the outcome of co-infections seems to be host- and pathogen-dependent, therefore future studies should focus on the interspecies variability to IDV immune response in order to better establish if IDV primary infections can predispose bacterial superinfections in other hosts and can be therefore be considered a threat also in other species than cattle. Future studies should also consider to test other bacterial pathogens that are known to become opportunistic and cause pneumonia after a primary viral infection.

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