PROF. CLAUDE SAEGERMAN (Orcid ID : 0000-0001-9087-7436)

Article type : Original Article

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

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

[1] Fundamental and Applied Research for Animal and Health (FARAH) Center, University of Liège, B-4000 Liège, claude.saegerman@uliege.be

[2] INRAE UMR 1225 IHAP-ENVT, 31076 Toulouse, France, maria.gaudino@envt.fr; gilles.meyer@envt.fr; mariette.ducatez@envt.fr

[3] Biovet inc. Saint-Hyacinthe, Québec, Canada, Christian.Savard@biovet-inc.com; andre.broes@biovet-inc.com; Olivier.Ariel@biovet-inc.com

[#]The two first authors contributed equally to the work.

*Corresponding author: claude.saegerman@uliege.be; mariette.ducatez@envt.fr

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/TBED.14085

Short running title: IDV in respiratory disease of dairy cattle

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 was 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.

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 United States of America (Hause et al., 2013) and further, in cattle and pigs from Europe and Asia (e.g. Dane et al., 2019; Snoeck et al., 2018; Zhai et al., 2017; Chiapponi et al., 2016; Murakami et al., 2016; Ducatez et al., 2015). 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. Gaudino et al., 2020; O'Donovan et al., 2019; Oliva et al., 2019; Silveira et al., 2019; Dane et al., 2019; Murakami et al., 2019; Gorin et al., 2019; Flynn et al., 2018; Snoeck et al., 2018; Ferguson et al., 2018; Nedland et al., 2018; Salem et al., 2017; Ferguson et al., 2015). 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. Salem et al., 2019; Ferguson et al, 2016), but the virus also likely plays a role in the bovine respiratory disease complex, so-called BRDC (e.g. Zhang et al., 2020; Zhang et al., 2019; Mitra et al., 2016; Ng et al., 2015). 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 a., 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 para-influenza 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 is 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 et al. 2019) supporting this initial hypothesis. Despite this, no experimental data is 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 have 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 and Lowen 2014). However reassortment events between influenza genera cannot occur (Hause et al., 2014; Gao et al., 2019).

To date, IDV circulation has only been described in Western Canada and data about its presence in the eastern part of the country is 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.

MATERIALS AND METHODS

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 5000 dairy cattle farms are registered (on average, each farm contains 70 dairy cows, which produce 600,000 liters of milk by year). Animals stay on farm during the year. Samples consisted in nasal or naso-pharyngeal 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.

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/).

Samples preparation

First 500 µL of PBS are added to the swabs, which are then vortexed vigorously. Nucleic acids were extracted from 200µl 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.

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.

Whole genome sequencing

Whole genome of five IDV positive samples was amplified by RT-PCR with QIAGEN OneStep RT-PCR Kit (Qiagen) by using primers described in (Ducatez et al., 2015). PCR products were sequenced with Sanger technology (Eurofins, GATC, Germany) 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.

Statistical analysis

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 (Petry & 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 (Petry and Watson, 2016). 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, College Station, Texas, USA). The limit of significance was 0.05.

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 ≤ 0.2 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 > 0.05) 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 < 0.05). Goodness of fit was assessed using the Hosmer–Lemeshow goodness-of-fit test (Petrie and Watson, 2013). All analyses were performed using Stata SE 14.2 (StataCorp, College Station, Texas, USA). The limit of significance was 0.05.

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).

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, the Youden's index "J" is frequently used in conjunction with the ROC curve analysis to estimate the best cut-off (Petrie & Watson, 2013), with:

Youden's index = sensitivity + specificity − 1 **[Equation 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, i.e. that the test is perfect.

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., 1983; Saegerman et al., 2004; Speybroeck et al., 2004; Saegerman et al., 2011). Using Salford Predictive Modeler (SPM) 8.3.2. (Minitab LLC, Stade College, PA, USA) (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., 1983). 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.

RESULTS

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.

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

Legend: Np, Number of positive animals; CI, confidence interval; BCoV, bovine coronavirus; BRSV, bovine respiratory syncitial 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.

The frequencies between the identification of pathogens alone or in association with other selected pathogen(s) are highly correlated (Pearson correlation coefficient = 0.92; p-value = 0.0002). 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 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	-

Legend: 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.

Correlation between selected pathogens in respiratory disease

In order to visualize the possible correlation between selected pathogens associated to BRDC of Canadian dairy cattle, a matrix of binary Jaccard similarity coefficients was calculated (**Figure 2**). The six most important correlation (> 0.2) 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*.

IDV is more associated to 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 0.2 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 = 0.03), *M. bovis* with an odd ratio of 2.63 (95% CI: 1.42-4.88; p-value = 0.002) 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 = 0.80).

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.

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:

OPS = [(Presence of BRSV=1)*(OR_{BRSV})] + [(Presence of *Mycoplasma bovis*=1)*($OR_{Mycoplasma\ bovis}$)]

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.

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**.

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/1M-H/2019	April 2019	Mauricie	MT246273-79
D/bovine/ Québec/3M-B/2020	January 2020	Québec	MT246286-92
D/bovine/ Québec/4Q-J20/2020	January 2020	Montérégie	MT246293-99

* indicates that partial genome was obtained.

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/1M-H/2019 (**Figure 5**). The NP gene segment of these 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.

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 (EFSA, 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 and colleagues (2020) 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 of recent field study evidenced that IDV viral 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 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 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 the presence of any specific individual virus or combination of viruses (Zhang 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 program, 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 intra-nasally 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 (Schultz-Cherry, 2015; Nickbakhsh et al., 2019). 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 coinfection/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 Netea et al., 2016; Kumar et al. 2018). Indeed, a look at potential epigenetic changes and cellular metabolism modifications would be of high interest in further respiratory coinfections (Kumar 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 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).

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

We would like to thank all farmers that participated to the study. This work was partly funded by the French National Agency for Research, project ANR-15-CE35-0005 "FLUD". M. Gaudino is supported by a PhD scholarship of the Animal Health Department of INRAe and of the Région Occitanie.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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.

ORCID

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

FIGURES CAPTION

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

Figure 2. Matrix of binary Jaccard similarity coefficients between influenza D virus and selected pathogens in respiratory disease of Canadian dairy cattle (N = 883)

Legend: Colour code for the coefficients in function of the increasing importance of the binary similarity: green to red. 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.

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 syncitial virus; BVDV, bovine viral diarrhoea virus; BoHV-1, bovine herpesvirus 1.

Figure 4. Receiver operating characteristic curve of the overall pondered score of IDV

Legend: 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.

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.

REFERENCES

Antonis, A.F.G., de Jong, M.C., van der Poel, W.H.M., van der Most, R.G., Stockhofe-Zurwieden, N., Kimman, T., & Schrijver, R.S. (2010). Age-dependent differences in the pathogenesis of bovine respiratory syncytial virus infections related to the development of natural immunocompetence. J Gen Virol., 91(10), 2497-506. doi: 10.1099/vir.0.020842-0.

Bailey, E.S., Fieldhouse, J.K., Alarja, N.A., Chen, D.D., Kovalik, M.E., Zemke, J.N., Choi, J.Y., Borkenhagen, L.K., Toh, T.H., Lee, J.S.Y., Chong, K.S., & Gray, G.C. (2020). First sequence of influenza D virus identified in poultry farm bioaerosols in Sarawak, Malaysia. Trop Dis Travel Med Vaccines, 6, 5. doi: 10.1186/s40794-020-0105-9.

Borkenhagen, L.K., Mallinson, K.A., Tsao, R.W., Ha, S.J., Lim, W.H., Toh, T.H., Anderson, B.D., Fieldhouse, J.K., Philo, S.E., Chong, K.S., Lindsley, W.G., Ramirez, A., Lowe, J.F., Coleman, K.K., & Gray, G.C. (2018). Surveillance for respiratory and diarrhoeal pathogens at the human-pig interface in Sarawak, Malaysia. PLoS One, 13(7), e0201295. doi: 10.1371/journal.pone.0201295.

Breiman, I., Friedman, J.H., Olsen, R.A., & Stone, C.J. (1984). Classification and Regression Trees. Wadsworth International Group Belmont, Monterey, CA, USA.

Caswell, J.L., & Archambault, M. (2007). *Mycoplasma bovis* pneumonia in cattle. Anim Health Res Rev., 8(2), 161-186. doi: 10.1017/S1466252307001351.

Chamorro, M.F., & Palomares. R.A. (2020). Bovine Respiratory Disease Vaccination Against Viral Pathogens: Modified-Live Versus Inactivated Antigen Vaccines, Intranasal Versus Parenteral, What Is the Evidence? Vet Clin North Am Food Anim Pract., 36(2), 461-472. doi: 10.1016/j.cvfa.2020.03.006.

Chiapponi, C., Ducatez, M,, Faccini, S., Foni, E., Gaudino, M., Hägglund, S., Luppi, A., Meyer, G., Moreno, A., Näslund, K., Nemanichvili, N., Oliva, J., Prosperi, A., Rosignoli, C., Renault V., Saegerman, C., Sausy, A., Snoeck, C., Valarcher, J.F., Verheije, H., & Zohari S. (2020). Risk assessment for influenza D in Europe. EFSA supporting publication 2020:EN-1853.17pp. doi:10.2903/sp.efsa.2020.EN-1853.

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

Chiapponi, C., Faccini, S., Fusaro, A., Moreno, A., Prosperi, A., Merenda, M., Baioni, L., Gabbi, V., Rosignoli, C., Alborali, G.L., Cavicchio, L., Monne, I., Torreggiani, C., Luppi, A., & Foni, E. (2019). Detection of a New Genetic Cluster of Influenza D Virus in Italian Cattle. Viruses, 11(12), 1110. doi: 10.3390/v11121110.

Chung, N.C., Miasojedow, B., Startek, M., & Gambin, A. (2019). Jaccard/Tanimoto similarity test and estimation methods for biological presence-absence data. BMC Bioinformatics, 20(15), 644. doi: 10.1186/s12859-019-3118-5.

Collin, E.A., Sheng, Z., Lang, Y., Ma, W., Hause, B.M., & Li, F. (2015). Cocirculation of two distinct genetic and antigenic lineages of proposed influenza D virus in cattle. J Virol., 89(2), 1036-1042. doi: 10.1128/JVI.02718-14.

Dane, H., Duffy, C., Guelbenzu, M., Hause, B., Fee, S., Forster, F., McMenamy, M.J., & Lemon, K. (2019). Detection of influenza D virus in bovine respiratory disease samples, UK. Transbound Emerg Dis., 66(5), 2184-2187. doi: 10.1111/tbed.13273.

Ducatez, M.F., Pelletier, C., & Meyer, G. (2015). Influenza D virus in cattle, France, 2011-2014. Emerg Infect Dis., 21(2), 368-371. doi: 10.3201/eid2102.141449.

Ellis, J.A. (2010). Bovine Parainfluenza-3 Virus. Vet Clin North Am Food Anim Pract., 26(3), 575-93. doi: 10.1016/j.cvfa.2010.08.002.

European Food Safety Authority. (2016). Annual report of the Emerging Risks Exchange Network 2015. EFSA supporting publication 2016:EN-1067. 36 pp. https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/sp.efsa.2016.EN-1067.

Ferguson, L., Eckard, L., Epperson, W.B., Long, L.P., Smith, D., Huston, C., Genova, S., Webby, R., & Wan, X.F. (2015). Influenza D virus infection in Mississippi beef cattle. Virology, 486, 28-34. doi: 10.1016/j.virol.2015.08.030.

Ferguson, L., Olivier, A.K., Genova, S., Epperson, W.B., Smith, D.R., Schneider, L., Barton, K., McCuan, K., Webby, R.J., & Wan, Y.-F. (2016). Pathogenesis of Influenza D Virus in Cattle. J Virol., 90(12), 5636-5642. doi: 10.1128/JVI.03122-15.

Ferguson, L., Luo, K., Olivier, A.K., Cunningham, F.L., Blackmon, S., Hanson-Dorr, K., Sun, H., Baroch, J., Lutman, M.W., Quade, B., Epperson, W., Webby, R., DeLiberto, T.J., & Wan X-F. (2018). Influenza D Virus Infection in Feral Swine Populations, United States. Emerg Infect Dis., 24(6), 1020-1028. doi: 10.3201/eid2406.172102.

Flynn O., Gallagher C., Mooney J., Irvine C., Ducatez M.F., Hause B., McGrath G., & Ryan E. (2018). Influenza D Virus in Cattle, Ireland. Emerg Infect Dis., 24(2), 389-391. doi: 10.3201/eid2402.170759.

Gao, S., Zhang, W., Lu, C., Cao, M., Cen, S., Peng, Y., & Deng, T. (2019). Identification of a Type-Specific Promoter Element That Differentiates between Influenza A and B Viruses. J Virol., 93(23), e01164-19. doi: 10.1128/JVI.01164-19.

Gaudino, M., Moreno, A., Snoeck, C.J., Zohari, S., Saegerman, C., O'Donovan, T., Ryan, E., Zanni, I., Foni, E., Sausy, A., Hübschen, J., Meyer, G., Chiapponi, C., & Ducatez, M.F. (2020). Emerging influenza D virus infection in European livestock as determined in serology studies: are we underestimating its spread over the continent? Transbound Emerg Dis. In press. doi: 10.1111/tbed.13805.

Gorin, S., Fablet, C., Quéguiner, S., Barbier, N., Paboeuf, F., Hervé, S., Rose, N., & Simon, G. (2019). Assessment of Influenza D Virus in Domestic Pigs and Wild Boars in France: Apparent Limited Spread within Swine Populations despite Serological Evidence of Breeding Sow Exposure. Viruses, 12(1), 25. doi: 10.3390/v12010025.

Goris, K., Uhlenbruck, S., Schwegmann-Wessels, C., Köhl, W., Niedorf, F., Stern, M., Hewicker-Trautwein, M., Bals, R., Taylor, G., Braun, A., Bicker, G., Kietzmann, M., Herrler, G. (2009). Differential sensitivity of differentiated epithelial cells to respiratory viruses reveals different viral strategies of host infection. J Virol., 83(4), 1962-1968. doi: 10.1128/JVI.01271-08.

Grissett, G. P., B. J. White, & R. L. Larson. (2015). Structured Literature Review of Responses of Cattle to Viral and Bacterial Pathogens Causing Bovine Respiratory Disease Complex. J. Vet. Intern Med., 29(3), 770-780. doi: 10.1111/jvim.12597.

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

Hause, B.M., Collin, E.A., Liu, R., Huang, B., Sheng, Z., Lu, W., Wang, D., Nelson, E.A., & Li., F. (2014). Characterization of a novel influenza virus in cattle and Swine: proposal for a new genus in the Orthomyxoviridae family. mBio, 5(2), e00031-14. doi: 10.1128/mBio.00031-14.

Hause, B.M., Huntimer, L., Falkenberg, S., Henningson, J., Lechtenberg, K., & Halbur, T. (2017). An inactivated influenza D virus vaccine partially protects cattle from respiratory disease caused by homologous challenge. Vet Microbiol., 199, 47-53. doi: 10.1016/j.vetmic.2016.12.024.

Hayakawa, J., Masuko, T., Takehana, T., & Suzuki, T. (2020). Genetic and antigenic characterization and retrospective surveillance of bovine influenza D viruses identified in Hokkaido, Japan from 2018 to 2020. Viruses, 12(8), 877. doi: 10.3390/v12080877.

Hodgins, D.C., Conlon, J.A., & Shewen. P.E. (2002). Chapter 12. Respiratory Viruses and Bacteria in Cattle. *In*: Polymicrobial Diseases. Brogden, K.A., Guthmiller, J.M., editors. Washington (DC), ASM Press.

Kumar, N., Sharma, S., Barua, S., Tripathi, B.N., & Rouse, B.T. (2018). Virological and Immunological Outcomes of Coinfections. Clin Microbiol Rev., 31(4), e00111-17. doi: 10.1128/CMR.00111-17.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol., 35(6), 1547-1549. doi: 10.1093/molbev/msy096.

Kumari, A., & Kumar, B. (2019). Emerging Influenza D Virus Threat: What We Know so Far! J Clin Med., 8(2), 192. doi: 10.3390/jcm8020192.

Lekeux, P. (1995). Bovine respiratory disease complex: an European perspective. The Bovine Practitionner, 29, 71-75. https://orbi.uliege.be/handle/2268/6486.

Liu, R., Sheng, Z., Huang, C., Wang, D., & Li, F. (2020). Influenza D virus. Curr Opin Virol., 44, 154-161. doi: 10.1016/j.coviro.2020.08.004.

McGuirk, S.M. (2008). Disease management of dairy calves and heifers. Vet Clin North Am Food Anim Pract., 24(1), 139-153. doi: 10.1016/j.cvfa.2007.10.003.

Mitra, N., Cernicchiaro, N., Torres, S., Li, F., & Hause, B.M. (2016). Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus. J Gen Virol., 97 (8), 1771-1784. doi:10.1099/jgv.0.000492.

Murakami, S., Endoh, M., Kobayashi, T., Takenaka-Uema, A., Chambers, J.K., Uchida, K., Nishihara, M., Hause, B., Horimoto, T. (2016). Influenza D Virus Infection in Herd of Cattle, Japan. Emerg Infect Dis., 22(8), 1517-1519. doi: 10.3201/eid2208.160362.

Murakami, S., Odagiri, T., Keskes Melaku, S., Bazartseren, B., Ishida, H., Takenaka-Uema, A., Muraki, Y., Sentsui, H., & Horimoto, T. (2019). Influenza D Virus Infection in Dromedary Camels, Ethiopia. Emerg Infect Dis., 25(6), 1224-1226. doi: 10.3201/eid2506.181158.

Murakami, S., Sato, R., Ishida, H., Katayama, M., Takenaka-Uema, A., & Horimoto, T. (2020). Influenza D virus of new phylogenetic lineage, Japan. Emerg Infect Dis., 26, 168-171. doi: 10.3201/eid2601.191092.

Nedland, H., Wollman, J., Sreenivasan, C., Quast, M., Singrey, A., Fawcett, L., Christopher-Hennings, J., Nelson, E., Kaushik, R.S., Wang, D., & Li, F. (2018). Serological Evidence for the Co-Circulation of Two Lineages of Influenza D Viruses in Equine Populations of the Midwest United States. Zoonoses Public Health, 65(1), e148-e154. doi: 10.1111/zph.12423.

Netea, M.G., Joosten, L.A.B., Latz, E., Mills, K.H.G., Natoli, G., Stunnenberg, H.G., O'Neill, L.A.J., Xavier, R.J. (2016). Trained immunity: a program of innate immune memory in health and disease. Science, 352(6284), aaf1098. doi: 10.1126/science.aaf1098.

Ng, T.F.F., Kondov, N.O., Deng, X., Van Eenennaam, A., Neibergs, H.L., & Delwart, E. (2015). A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. J Virol., 89(10), 5340-5349. doi: 10.1128/JVI.00064-15.

Nickbakhsh, S., Mair, C., Matthews, L., Reeve, E., Johnson, P.C.D., Thorburn, F ., von Wissmann, B., Reynolds, A., McMenamin, J., Gunson, R.N., Murcia, P.R. (2019). Virus-virus

interactions impact the population dynamics of influenza and the common cold. Proc Natl Acad Sci U S A., 116(52), 27142-27150. doi: 10.1073/pnas.1911083116.

Nissly, R.H., Zaman, N., Ibrahim, P.A.S., McDaniel, K., Lim, L., Kiser, J.N., Bird, I., Chothe, S.K., Bhushan, G.L., Vandegrift, K., Neibergs, H.L., & Kuchipudia S.V. (2020). Influenza C and D viral load in cattle correlates with bovine respiratory disease (BRD): Emerging role of orthomyxoviruses in the pathogenesis of BRD. Virology, 551, 10-15. doi: 10.1016/j.virol.2020.08.014.

Odeón, A.C., Kelling, C.L., Marshall, D.H., Estela, E.S., Dubovi, E.J., & Donis, R.O. (1999). Experimental infection of calves with bovine viral diarrhoea virus genotype II (NY-93). J Vet Diagn Invest., 11(3), 221-228. doi: 10.1177/104063879901100303.

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

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

Petrie A, Watson P. (2013). Statistics for veterinary and animal science. This edition, John Wiley & Sons, Ltd, West Sussex, UK, 391 pages.

Preux, P.M., Odermatt, P., Perna, A., Marin, B., & Vergnenégre, A. (2005). Qu'est-ce qu'une régression logistique ? Rev. Mal. Respir., 22(1), 159-162. doi: RMR-02-2005-22-1-0761-8425-101019-200512206.

Quast, M., Sreenivasan, C., Sexton, G., Nedland, H., Singrey, A., Fawcett, L., Miller, G., Lauer, D., Voss, S., Pollock, S., Cunha, C.W., Christopher-Hennings, J., Nelson, E., & Li, F. (2015). Serological evidence for the presence of influenza D virus in small ruminants. Vet Microbiol., 180(3-4), 281-285. doi: 10.1016/j.vetmic.2015.09.005.

Saegerman, C., Porter, S.R., & Humblet, M.F. (2011). The use of modelling to evaluate and adapt strategies for animal disease control. Rev Sci Tech. 2011, 30(2), 555-569. doi: 10.20506/rst.30.2.2048.

Saegerman, C., Speybroeck, N., Roels, S., Vanopdenbosch, E., Thiry, E., & Berkvens, D. (2004). Decision support tools for clinical diagnosis of disease in cows with suspected bovine spongiform encephalopathy. J Clin Microbiol., 42(1), 172-178. doi: 10.1128/jcm.42.1.172-178.2004.

Saade, G., Deblanc, C., Bougon, J., Marois-Créhan, C., Fablet, C., Auray, G., Belloc, C., Leblanc-Maridor, M., Gagnon, C.A., Zhu, J., Gottschalk, M., Summerfield, A., Simon, G., Bertho, N., & Meurens, F. (2020). Coinfections and their molecular consequences in the porcine respiratory tract. Vet Res., 51(1), 80. doi: 10.1186/s13567-020-00807-8.

Salem, E, Hägglund, S, Cassard, H, Corre, T, Näslund, K, Foret, C, Gauthier, D, Pinard, A, Delverdier, M, Zohari, S, Valarcher, JF, Ducatez, M, & Meyer, G. (2019). Pathogenesis, Host Innate Immune Response, and Aerosol Transmission of Influenza D Virus in Cattle. J Virol., 93(7), pii, e01853-18. doi: 10.1128/JVI.01853-18.

Salem, E., Cook, E.A.J., Ait Lbacha, H., Oliva, J., Awoume, F., Aplogan, G.L., Hymann, E.C., Muloi, D., Deem, S.L., Alali, S., Zouagui, Z., Fèvre, E.M., Meyer, G., & Ducatez, M.F. (2017). Serologic Evidence for Influenza C and D Virus among Ruminants and Camelids, Africa, 1991-2015. Emerg Infect Dis., 23(9), 1556-1559. doi: 10.3201/eid2309.170342.

Schultz-Cherry, S. (2015). Viral interference: the case of influenza viruses. J Infect Dis., 212(11), 1690-1691. doi: 10.1093/infdis/jiv261.

Silveira, S., Falkenberg, S.M., Kaplan, B.S., Crossley, B., Ridpath, J.F., Bauermann, F.B., Fossler, C.P., Dargatz, D.A., Dassanayake, R.P., Vincent, A.L., Canal, C.W., & Neill, J.D. (2019). Serosurvey for Influenza D Virus Exposure in Cattle, United States, 2014-2015. Emerg Infect Dis., 25(11), 2074-2080. doi: 10.3201/eid2511.190253.

Snoeck, C.J., Oliva, J., Pauly, M., Losch, S., Wildschutz, F., Muller, C.P., Hubschen, J.M., & Ducatez, M.F. (2018). Influenza D virus circulation in cattle and swine, Luxembourg, 2012-2016. Emerg Infect Dis., 24, 1388-1389. doi: 10.3201/eid2407.171937.

Speybroeck N, Berkvens D, Mfoukou-Ntsakala A, Aerts M, Hens N, Van Huylenbroeck G, & Thys T. (2004). Classification trees versus multinomial models in the analysis of urban farming systems in Central Africa. Agric. Syst., 80, 133-149. doi: 10.1016/j.agsy.2003.06.006.

Steel, J., & Lowen, A.C. (2014). Influenza A virus reassortment. Curr Top Microbiol Immunol., 385, 377-401. doi: 10.1007/82_2014_395.

Steinberg D, & Colla P. (1997). CART-Classification and Regression Trees. Instruction manual, Salford Systems, San Diego, CA.

Taylor, J.D., Fulton, R.W., Lehenbauer, T.W., Step, D.L., & Confer, A.W. (2010). The epidemiology of bovine respiratory disease: What is the evidence for predisposing factors? Can Vet J., 51(10), 1095-1102. PMC2942046.

Trombetta, C.M., Marchi, S., Manini, I., Kistner, O., Li, F., Piu, P., Manenti, A., Biuso, F., Sreenivasan, C., Druce, J., & Montomoli, E. (2019). Influenza D Virus: Serological Evidence in the Italian Population from 2005 to 2017. Viruses, 12(1), pii: E30. doi: 10.3390/v12010030.

White, S.K., Ma, W., McDaniel, C.J., Gray, G.C., & Lednicky, J.A. (2016). Serologic evidence of exposure to influenza D virus among persons with occupational contact with cattle. J Clin Virol., 81, 31-33. doi: 10.1016/j.jcv.2016.05.017.

Zhai, SL, Zhang, H, Chen, SN, Zhou, X, Lin, T, Liu, R, Lv, DH, Wen, XH, Wei, WK, Wang, D, & Feng, Li. (2017). Influenza D virus in animal species in Guangdong Province, Southern China. Emerg Infect Dis., 23,1392-1396. doi: 10.3201/eid2308.170059.

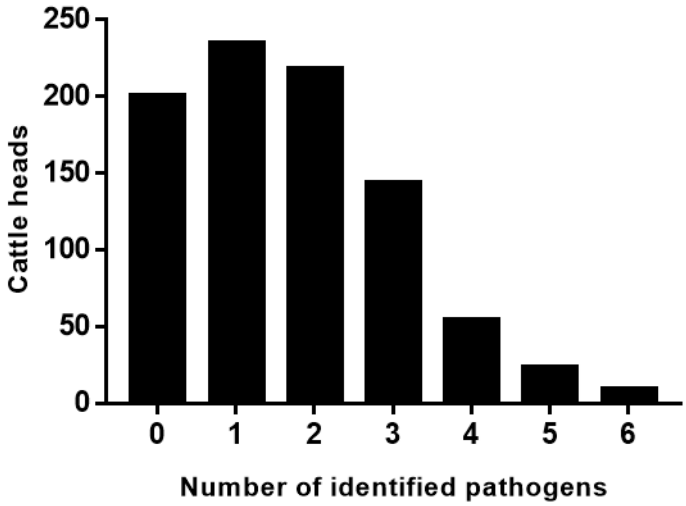
Zhang, M., Hill, J.A., Fernando, C., Alexander, T.W., Timsit, E., van der Meer, F., & Huang, Y. (2019). Respiratory viruses identified in western Canadian beef cattle by metagenomic sequencing and their association with bovine respiratory disease. Transbound Emerg Dis., 66(3), 1379-1386. doi: 10.1111/tbed.13172.

Zhang, M., Hill, J.E., Alexander, T.W., & Huang, Y. (2020). The nasal viromes of cattle on arrival at western Canadian feedlots and their relationship to development of bovine respiratory disease. Transbound Emerg Dis. In press. doi: 10.1111/tbed.13873.

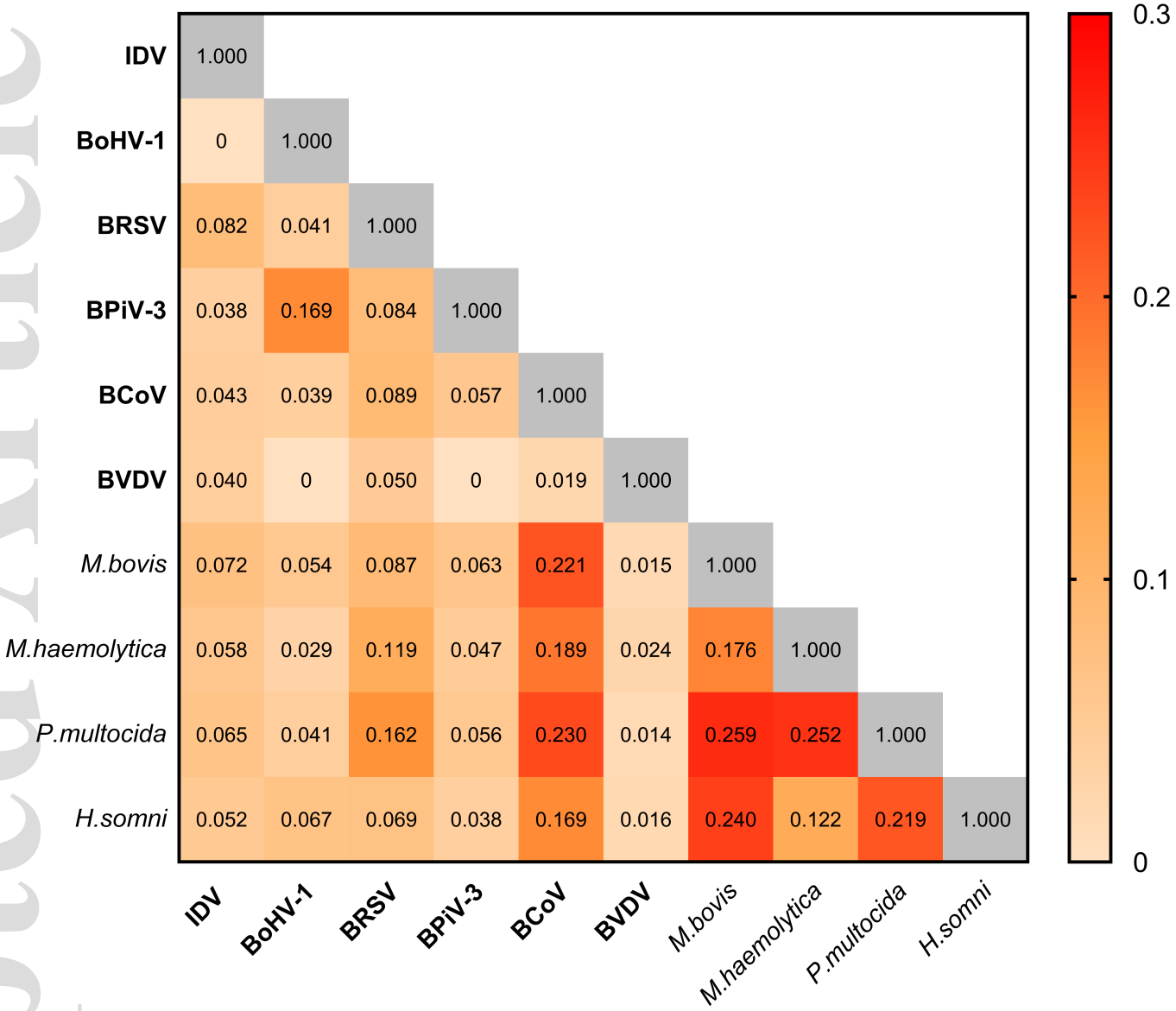
Zhang M., Huang Y., Godson DL., Fernando C., Alexander TW., & Hill JE. (2020). Assessment of Metagenomic Sequencing and qPCR for Detection of Influenza D Virus in Bovine Respiratory Tract Samples. Viruses, 12(8), 814. doi: 10.3390/v12080814.

Zhang, M., Hill, J.E., Godson, D.L., Ngeleka, M., Fernando, C., & Huang, Y. (2020). The pulmonary virome, bacteriological and histopathological findings in bovine respiratory disease from western Canada. Transbound Emerg Dis., 67(2), 924-934. doi: 10.1111/tbed.13419.

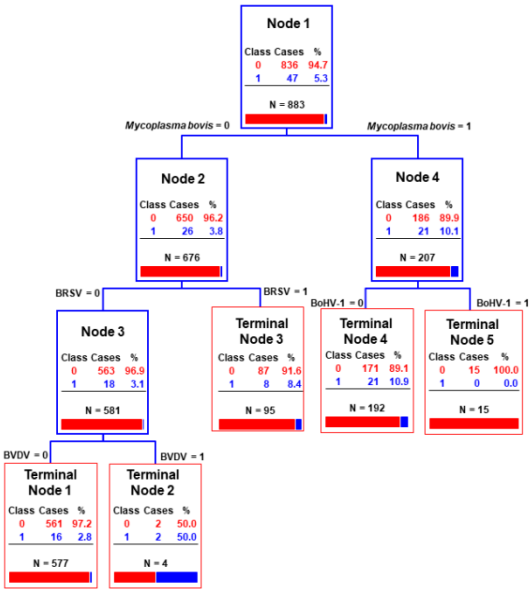
Zhang, X., Outlaw, C., Olivier, A.K., Woolums, A., Epperson, W., & Wan X.F. (2019). Pathogenesis of co-infections of influenza D virus and *Mannheimia haemolytica* in cattle. Vet Microbiol., 231, 246-253. doi: 10.1016/j.vetmic.2019.03.027.



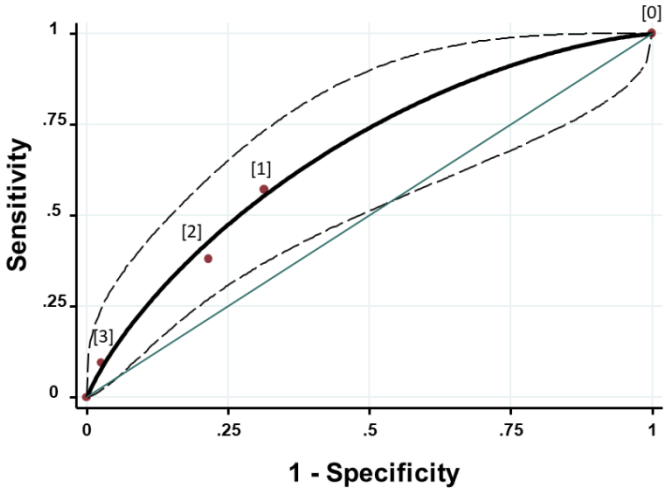
tbed_14085_f1.tif



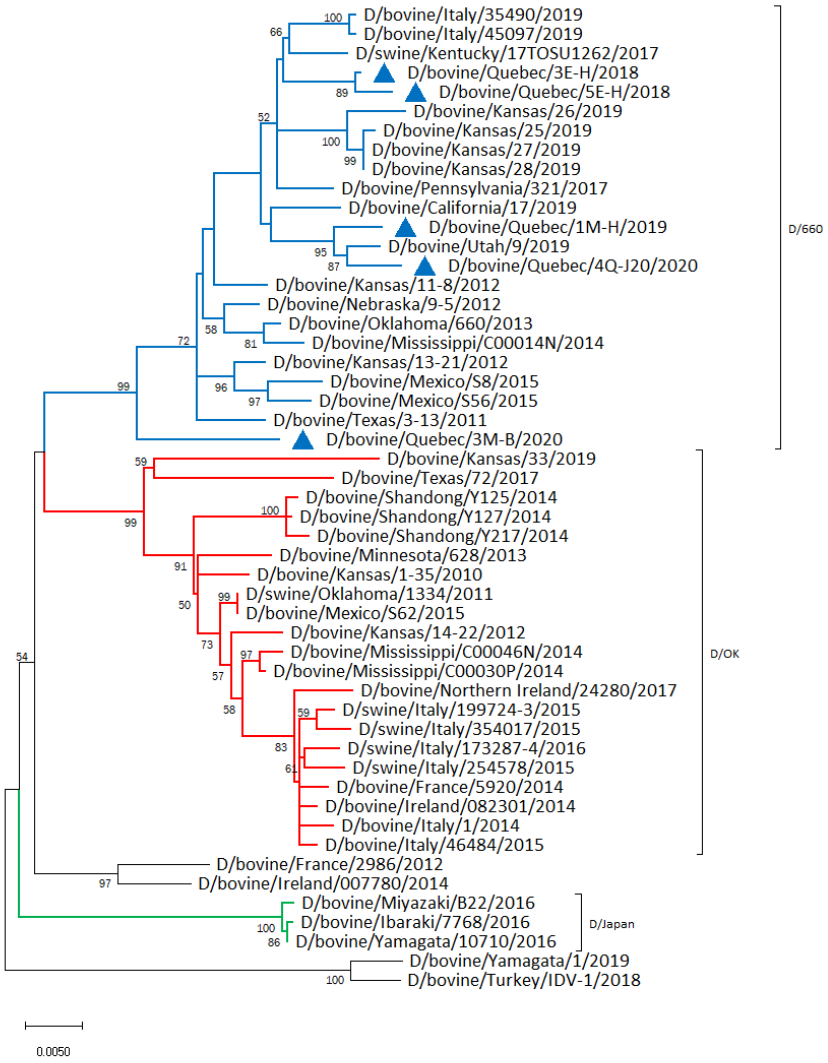
tbed_14085_f2.tif



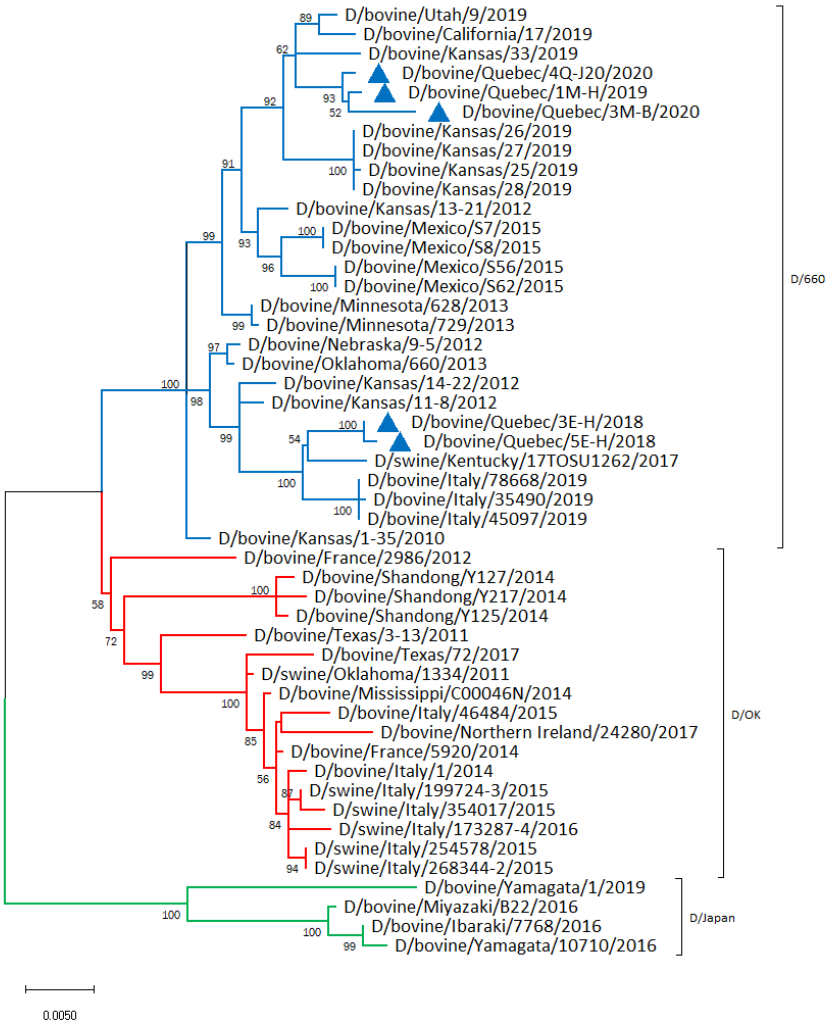
tbed_14085_f3.tif



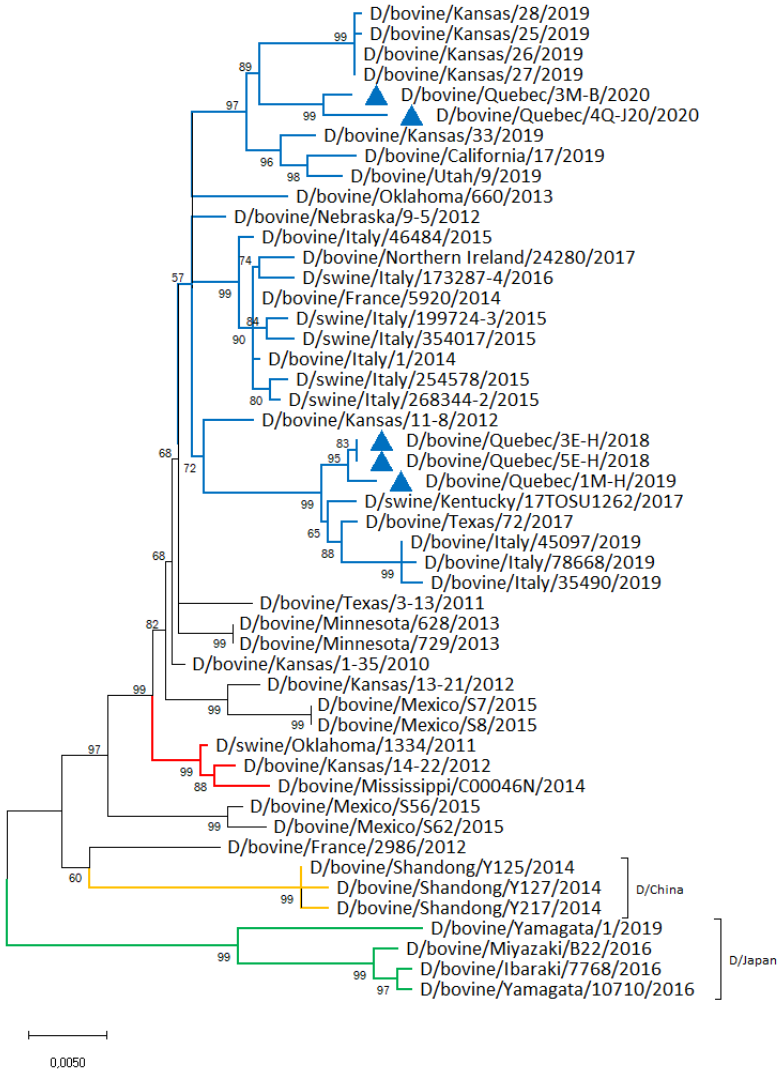
tbed_14085_f4.tif



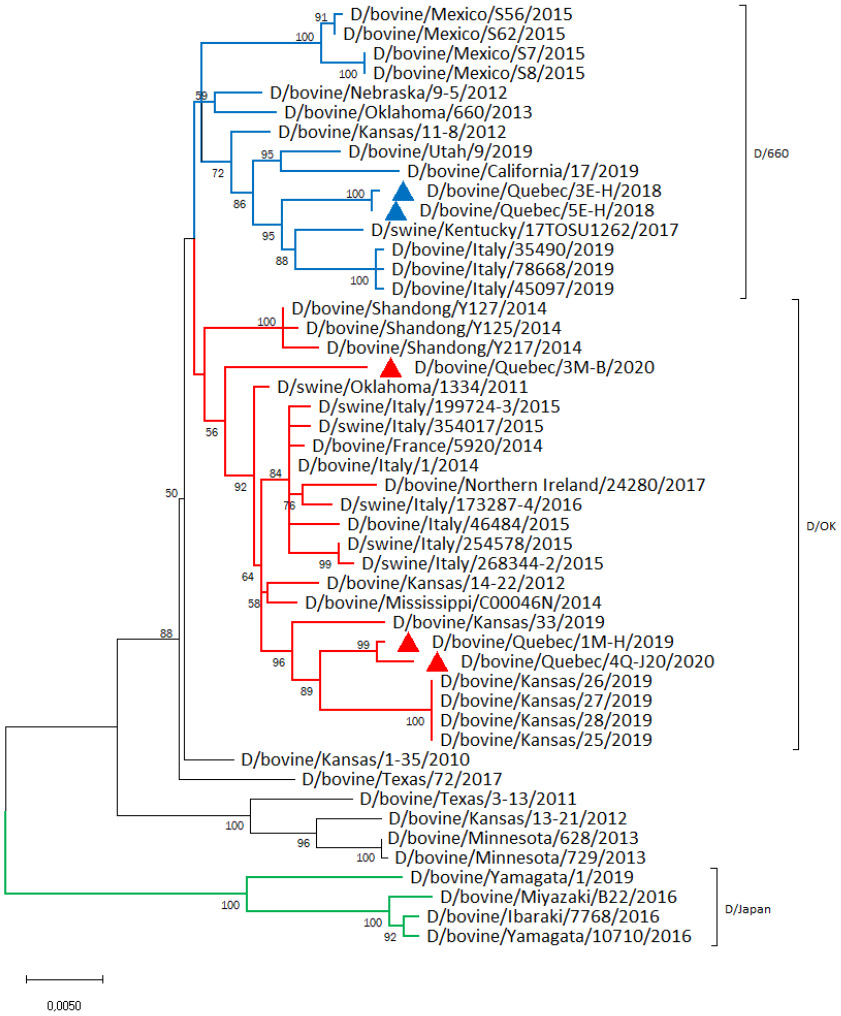
tbed_14085_f5a.tif



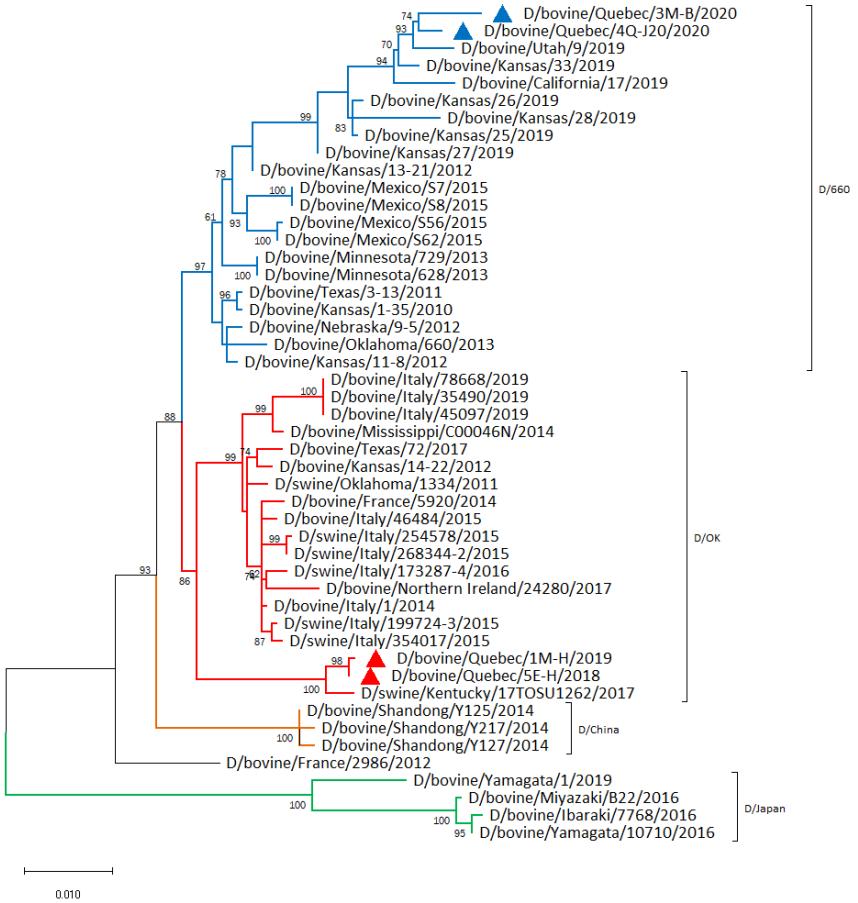
tbed_14085_f5b.tif



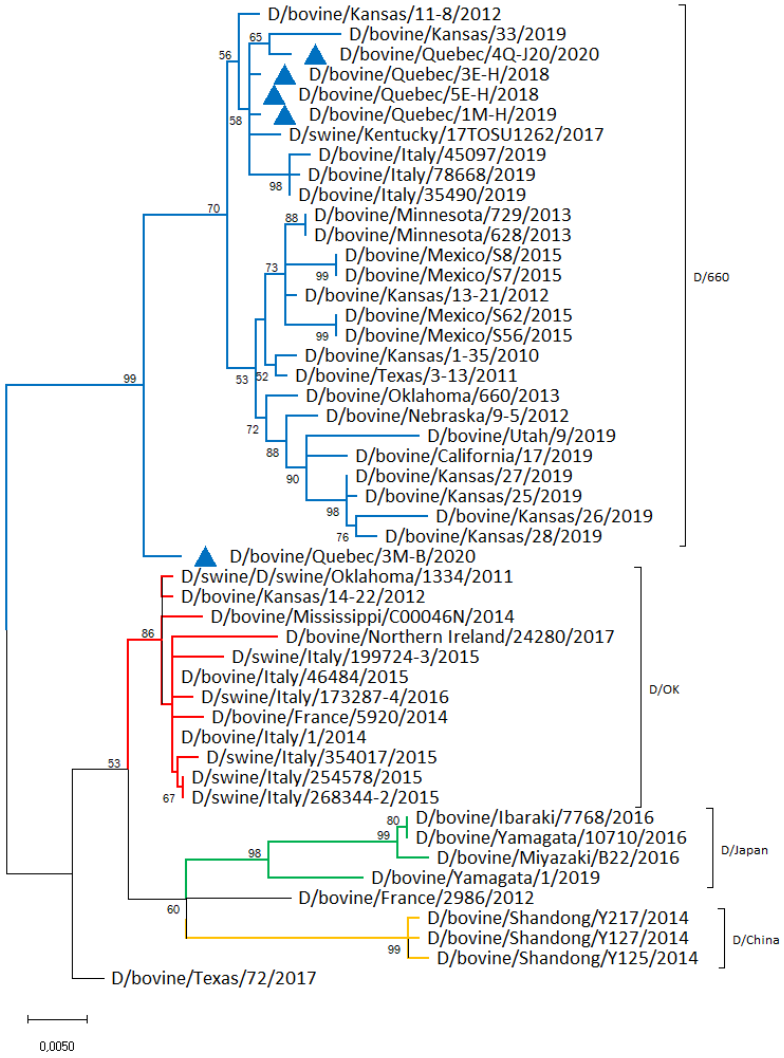
tbed_14085_f5c.tif



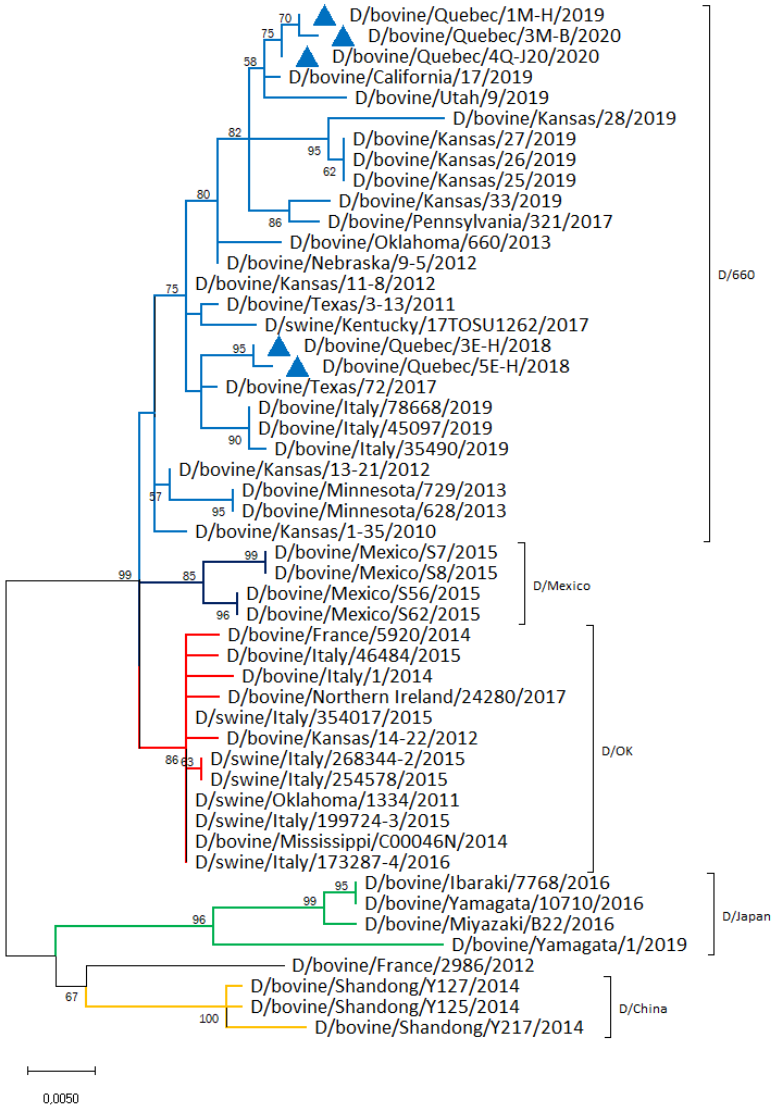
tbed_14085_f5d.tif



tbed_14085_f5e.tif



tbed_14085_f5f.tif



tbed_14085_f5g.tif