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Molecular characterization of the novel respiratory Influenza D virus detected in French calves

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To my family;

To all nice people I've met in Toulouse;

Special thanks to the team "FluD"

that always patiently helped me

in learning new things

and gave a huge contribution to this work.

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List of abbreviations

BCoV: Bovine Coronavirus

BRDC: Bovine Respiratory Complex Disease

BRSV: Bovine Respiratory Syncytial Virus

BVDV: Bovine Viral Diarrhea Virus

Ct: Threshold cycle

HA: Hemagglutinin

HEF: Hemagglutinin esterase fusion

HI assay: Hemagglutination inhibition assay

IAV: Influenza A virus

IBR: Infectious Bovine Rinotracheitis

IBV: Influenza B virus

ICV: Influenza C virus

IDV: Influenza D virus

MN assay: Microneutralization assay

MP: Matrix protein

NA: neuraminidase

NGS: Next Generation Sequencing

NP: Nucleoprotein

NS: Nonstructural protein

ORF: Open Reading Frame

PB1: Polymerase basic protein 1

PB2: Polymerase basic protein 2

P3: Polymerase protein 3

PI3: Parainfluenza-type3

RNP: Ribonucleoprotein

RT-qPCR: Retrotranscription quantitative polymerase chain reaction

t-MRCA: Mean recent common ancestor time

UHA: Hemagglutination units

Abstract

In 2011 in the United States an Influenza C-like virus was isolated from swine samples (Hause et al., 2013). Further studies better characterized this novel virus and discovered that it had only 50% of genetic identity with human infecting Influenza C virus and was not able to reassort not only with ICV but also with the other two genera of the Influenza viruses family, the *Orthomyxoviridae*. 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 in the Influenza virus family. The new genus is currently known as Influenza D virus, extending therefore the *Orthomyxoviridae* from six to seven genera.

So far, IDV has been detected in different animal species and on almost all continents. Some studies describe its molecular detection but many serologic evidences for anti-IDV antibodies presence are also available. At the moment the susceptible species seem to be cattle, swine, small ruminants and it appears that horses could be infected as well. Whether humans are likely to be potential hosts of IDV is still unclear: studies in the ferret model (where IDV replicates efficiently) as well as IDV receptors characterizations suggest that humans may be susceptible, but serious serological evidences are still lacking to evidence the species jump (Hause et al, 2013; Song et al, 2016; Eckart 2016; White et al, 2016).

In France, IDV was identified in 2011-2014 in cattle with respiratory clinical signs in Saone-et-Loire (Ducatez et al, 2015). However, the extent of IDV circulation in French cattle and small ruminants was not known. The aim of this work was therefore to detect and characterize IDV in French calves to assess the virus prevalence and genetic diversity and also evaluate IDV seroprevalence in cattle and small ruminants in 5 French departments (Vendée, Bretagne, Nord, Aveyron and Ariège).

Riassunto (Italian)

Recentemente negli Stati Uniti (2011) è stato isolato un nuovo virus Influenzale da un tampone nasale prelevato da un suino che presentava sintomi respiratori (Hause et al., 2013). Il virus che aveva identità genetica maggiore è risultato essere il virus dell'Influenza C (famiglia degli *Orthomyxoviridae*), motivo per cui venne provvisoriamente chiamato "Influenza C-like". Il virus dell'Influenza C (che solitamente causa infezioni delle vie respiratorie nell'uomo) aveva però in comune solo il 50% del suo genoma con il nuovo virus isolato. La grande distanza genetica e il fatto che questo non cross-reagisse con gli altri 3 generi di virus Influenza hanno permesso quindi di classificarlo come un nuovo genere di questa famiglia, denominandolo così virus dell'Influenza D (IDV, Influenza D virus).

Ulteriori studi fatti in seguito hanno consentito di isolare il virus Influenzale per la prima volta dal bovino e di verificare la sua grande diffusione in questa specie. Questi infatti, fino ad allora, non erano mai stati ritenuti possibili ospiti di questi patogeni respiratori. Al contrario, dai dati epidemiologici oggi disponibili si ritiene molto probabile che sia proprio il bovino l'ospite principale del virus, anche se l'assenza di dati non permette ancora di poterlo definire o meno un reservoir. Il virus dell'Influenza D è stato subito scoperto essere piuttosto diffuso: è stato difatti trovato (identificazione molecolare del virus o degli anticorpi contro il virus) in quasi tutti i continenti e in diverse specie animali (bovino, suino, piccoli ruminanti come capra e pecora, camelidi, equini ecc).

Per il momento il suo potenziale zoonotico è ancora ampiamente in discussione perché manca la prova fondamentale, cioè l'isolamento del virus dall'uomo. Le indagini serologiche per rilevare gli anticorpi anti-IDV nell'uomo hanno dato risultati incostistenti, tuttavia esperimenti di immunoistochimica hanno rivelato che il virus è in grado di legarsi ai recettori cellulari dell'apparato respiratorio in tessuti umani. Il fatto che il suino sia una specie sensibile al virus giustifica lo studio del suo potenziale zoonotico poiché si teme che in seguito a un riassortimento (come accadde per il virus dell'Influenza A H1N1 nel 2009) ci possa essere un adattamento all'uomo. Inoltre, il virus più geneticamente simile (Virus dell'Influenza C) causa patologia nell'uomo. Per

il momento non ci sono ancora prove che il virus possa passare all'uomo ma si tende ad avere una certa attenzione a monitorarne l'evoluzione e il tropismo di specie proprio per questo motivo.

Da infezioni sperimentali e indagini epidemiologiche sembrerebbe che il virus abbia un particolare tropismo per i vitelli, individui giovani (<6 mesi) e ancora non pienamente immunocompetenti che sono spesso soggetti a sindromi respiratorie negli allevamenti soprattutto di natura intensiva (BRDC, Bovine Respiratory Complex Disease). Queste sindromi hanno un'eziologia multifattoriale e non sono quasi mai provocate da un solo agente patogeno ma da co-infezioni tra virus respiratori e tra virus e batteri. Studi di metagenomica hanno cercato di capire se il virus dell'Influenza D fosse coinvolto in qualche modo in questa sindrome respiratoria e i dati suggeriscono un'associazione, poiché è stato rilevato con una certa frequenza in co-infezioni con altri patogeni respiratori. Se il virus contribuisca a peggiorare la sindrome nei bovini o meno, non è ancora certo; le prossime indagini saranno certamente volte a studiare i meccanismi di interazione tra questi virus includendo sicuramente anche il nuovo virus respiratorio isolato recentemente.

Viste le premesse qui sopra elencate, questo lavoro è stato volto a capire se IDV circoli attualmente in Francia, sede del laboratorio dove ho svolto il lavoro per la tesi magistrale. Il virus è stato isolato per la prima volta in Europa proprio in Francia nel 2012 (Ducatez et al., 2015) e si è quindi voluto studiarne, oltre all'eventuale circolazione, anche l'evoluzione, caratterizzandolo molecolarmente.

I. Introduction: Influenza D virus, a novel member of the *Orthomyxoviridae* family

1.1 The discovery of the virus

The Influenza D virus was first isolated in April 2011 from nasal swabs of swine exhibiting influenza-like symptoms[1]. The samples were tested for Influenza A virus but they resulted negative, therefore an isolation on cell culture using the ST swine testicle cell line was performed. At day 3 the isolated virus caused cytopathic effects similar to those caused by Influenza viruses, thus it was observed through the EM (Electron Microscopy) and it displayed morphological characteristics of a virus belonging to the family of the Influenza viruses, the *Orthomyxoviridae*. Enzymatic assays were also performed and they showed a viral weak neuroaminidase activity but a detectable O-acetylesterase activity, suggesting that it could have been a member of the genus Influenza C. However, the RT-qPCR resulted negative also for Influenza B and Influenza C viruses. Afterwards, the viral genome was sequenced by using the Ion Torrent technology and genetic analyses were performed. A first BlastP search of the ORFs identified the human Influenza C virus as the most similar genome and this led to provisionally designe it as C/swine/Oklahoma/1334/2011. Despite this, the identity of PB1 (which is considered as the most conserved protein among Influenza viruses) was only of 69-72% when compared to the PB1 protein of Influenza C virus and of 39-41% with Influenza A and B virus PB1, so this led to think that the isolated virus was likely to belong to a new genus which could be much more similar to Influenza C than to Influenza A or B. In addition, the new virus HE protein shared 53% identity with Influenza C virus HE (Hemagglutinin esterase). The HE protein allows Influenza C virus to bind to the receptor and to destroy it through the acetylesterase activity and perform the fusion with the membrane. On the contrary, this type of activity in Influenza A and B viruses is achieved through two separate proteins which work in cooperation, the NA (neuraminidase) and HA (hemagglutinin).

[1] Hause B.M., Ducatez M., Collin E.A., Ran Z., Liu R., et al. (2013) Isolation of a Novel Swine Influenza Virus from Oklahoma in 2011 Which Is Distantly Related to Human Influenza C Viruses. PLoS Pathogens 9(2): e1003176

Identity analyses were also performed for other proteins such as NP (nucleoprotein) and M (matrix): these two are highly conserved within a same Influenza genus (higher than 85% of genetic identity) but they are less conserved among the different Influenza genera (about 20-30%) and this makes them the ideal markers for a distinction among the three genera. In particular, the authors found that the NP of the isolated virus had an 38-41% homology in amino acid sequence with the protein of Influenza C virus. The non-coding regions of the genomes were analyzed as well, and as they are normally highly conserved it was significant to find a single nucleotide difference at position 5 from the 3' end and another polymorphism at the first position of the 3' end, showing that the isolated virus had biological differences as compared to ICV. To understand if the virus is seroprevalent in humans, an HI assay (Hemagglutination inibition assay) was performed using strains from the three genera of Influenza; only 4 on 316 sera resulted in having detectable HI titers, so the researchers concluded that 1,3% of seroprevalence was an inconclusive result to determine an effective circulation among humans.

A following study[2] then tried to investigate if the virus was present in non-swine species. Since C/OK was demonstrated to be clearly biological distinguishable from human ICV, the authors searched for the virus in 45 tissue samples or swab of cattle presenting respiratory symptoms, finding a 18% of positivity. The viral genome was sequenced using Ion Torrent technology and three genetically different sequences were identified. These were later designated as C/bovine/Oklahoma/660/2013 (C/660), a lineage coming from a bovine in Oklahoma, and the other two coming from Minnesota: C/bovine/Minnesota/628/2013 and C/bovine/Minnesota/729/2013, thus C/628 and C/729. By amplifying the non coding regions at 3' and 5' the authors also discovered that there were different splicing strategies concerning the NS and the M segments by comparing them with the C/Yamagata/1/88, underlining again a genetic difference between C/OK and the existing human lineages of ICV. In the same study the authors also tried to assess if the new isolated viruses were able to reassort with ICV by performing in vitro reassorting experiments. ST cells were used and they

[2] 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.

were co-infected with two representative lineages of human Influenza C virus, C/OK and the lineage C/660 but the experiment showed the inability of both to reassort with the human viruses. In the same study the authors also performed the agar gel immunodiffusion assay (AGID) in order to assess if the new virus reacted with IAV, IBV and ICV antisera but no recognition of the C/OK antigens was detected.

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 in the Influenza virus family. The new genus is currently known as Influenza D virus, extending therefore the *Orthomyxoviridae* from six to seven genera. The proposal was justified by the following reasons, which can resume the assertions listed in the introduction above: the Influenza D virus is distantly related to ICV, but also IAV and IBV, it did not prove reassortment with ICV in in vitro coinfections, 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 proteins. What is more, the virus circulates in cattle, which is rarely infected by IAV, IBV or ICV and above all it is not prevalent in human population. The virus was also found to be prevalent in other small ruminants and it was detected in cattle and swine both in Europe and in Asia, as it will be discussed in the next chapters.

Family: *Orthomyxoviridae*	(7 Genera)
Genus: *Alphainfluenzavirus*	(1 Species)
★ **Species:** *Influenza A virus*	
Genus: *Betainfluenzavirus*	(1 Species)
★ **Species:** *Influenza B virus*	
Genus: *Deltainfluenzavirus*	(1 Species)
★ **Species:** *Influenza D virus*	
Genus: *Gammainfluenzavirus*	(1 Species)
★ **Species:** *Influenza C virus*	
Genus: *Isavirus*	(1 Species)
★ **Species:** *Salmon isavirus*	
Genus: *Quaranjavirus*	(2 Species)
Species: *Johnston Atoll quaranjavirus*	
★ **Species:** *Quaranfil quaranjavirus*	
Genus: *Thogotovirus*	(2 Species)
Species: *Dhori thogotovirus*	
★ **Species:** *Thogoto thogotovirus*	

Figure 1: The *Orthomyxoviridae* family as for the International Committee on Taxonomy of Viruses. Source: https://talk.ictvonline.org/taxonomy/

1.2 Viral structure and genomic organization of Influenza D virus

Similarly to ICV, the IDV genome is composed of 7 segments containing ssRNA in negative sense, unlike IAV and IBV, whose genome is composed of 8 segments. Their size all range from 0,76 to 2,32 kB, whereas the total genome size is about 10 kB. At the beginning and at the end of each segment a non-coding region is present; in particular, there are the same 11 nucleotides for all the seven segments at the 3' except for the first base, which can be either a cytosine or a uracile (3'-C/UCGUAUUCGUC-5'), and of 12 nucleotides at the 5' (5'-AGCAGUAGCAAG-'3). At 5' of each ORF there is a short tail rich of uridines that serves as a template for the polyA activity and information for packing the viral RNPs into viral particles are also present in the terminal non-coding regions. To be infectious, each progeny virion needs to pack all the 7 RNA segments but, as stated in a recent article[3], it seems that despite IDV contains 7 segments it organizes its genome into eight ribonucleoprotein complexes, suggesting therefore that all Influenza viruses prefer to organize their RNA segments into eight RNP indipendently from their genome size.

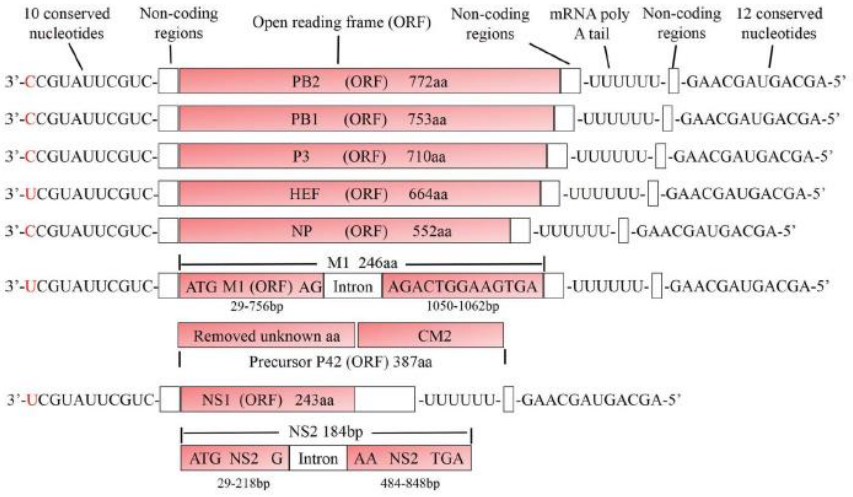


Figure 2: Structure of the 7 Influenza D virus genome segments. Source: Su S., Fu X., Li G., Kerlin F. & Veit M. (2017) Novel Influenza D virus: Epidemiology, pathology, evolution and biological characteristics, Virulence, 8:8, 1580-1591

[3] Nakatsu S., Murakami S., Shindo K., Horimoto T., Sagara H., Noda T., Kawaoka Y. Influenza C and D Viruses Package Eight Organized Ribonucleoprotein Complexes (2018) Journal of Virology. vol. 92 no. 6 e02084-17

For Influenza viruses genera, all the segments that compose the genome encode for proteins which are necessary for the viral replication. To do this, each RNA segment binds to an RNA-dependent RNA polymerase and to several copies of the nucleoprotein NP, thus forming the ribonucleoprotein complex (RNP) that allows the viral transcription and replication. These RNPs are contained into a lipidic envelope that underlays the M1 protein and it has the function to help the maintenance of the virus shape. The glycoprotein HEF is also inserted in this envelope, together with an ion-channel protein CM2. The three longest segments encode for the proteins PB1, PB2 and P3 which form the polymerase complex necessary for the mRNA synthesis and for the viral replication. The active site of the polymerase is PB1 while the other two units have the function of cap-binding (PB2) and the performance of the endonuclease activity (P3). The NS segment encodes for two non-structural proteins, NS1 and NS2: the unspliced RNA is firstly translated into NS1 and then after a process of splicing the shorter protein NS2 is produced.

In Influenza D virus a particular role is played by HEF, the Hemagglutinin Esterase Fusion, the major glycoprotein present on the virus surface. This protein is the direct responsible for the receptor recognition and binding, its destruction and the fusion between the virions and the host cell membranes. What is more, as demonstrated in a study conducted by Yu J et al[4], HEF also seems to be involved in the viral resistance to relatively high temperatures and acid pH. In this experiment, the authors treated an IDV strain for 60 minutes at 53°C and exposed it to a 3,0 pH for 30 minutes and they applied the same procedure to make a comparison with all the other Influenza genera. While IAV, IBV and ICV were inactivated after the treatment, IDV was the only that was able to survive for 120 minutes at 53°C and was not inactivated after the low pH exposure. To demonstrate the role of HEF the authors constructed a chimeric IAV virus inserting the HEF gene: this showed similar characteristics of resistance to IDV, since it could not be inactivated by the temperature and it resulted more stable to the low pH than non-chimeric IAV.

[4] Yu J., Hika B., Liu R., Sheng Z., Hause B.M., Li F., Wang D. (2017). The hemagglutinin esterase fusion glycoprotein is a primary determinant of the exceptional thermal and acid stability of influenza D virus. mSphere 2: e00254-17.

1.3 Origin, evolution and currently circulating lineages

Considering their similarity as concerns the genetic and morphological structure, some authors agreed in saying that IDV probably originated from ICV (Su S. et al, 2017). Despite the two genomes have only about 50% of similarity, the analyses based only on PB1 revealed a 70% of identity between the two genera. PB1 is the most conserved nucleotide sequence among the Influenza viruses, whereas the most variable region is represented by HEF. For this reason, these two are often used in combination for phylogenetic analyses in order to reveal the genetic differences among Influenza genera. When making a phylogenetic tree analysis, IDV lineages clearly cluster with ICV strains, whereas IBV clusters with IAV, proving that Influenza D virus probably had a common ancestor with Influenza C virus. The authors obtained the same results also by comparing PB2, P3, NP and NS sequences.

As for the current knowledge, there are two genetically distinct lineages circulating worldwide, the first one was first isolated from swine and the second one from cattle: these are represented by D/swine/Oklahoma/1334/2011 (D/OK), the first ever lineage of IDV isolated in 2011, and D/bovine/Oklahoma/660/2013 (D/660). D/OK and D/660 represent therefore two distinct antigenic groups but at the same time they show antigenic cross-reactivity and they are reported to frequently reassort with each other. There is then a third Japanese strain that clusters indipendently from the first two[5] strains isolated in Europe and America and it is represented by two lineages, both isolated from bovine in 2016: D/bovine/Ibaraki/7768/2016 and D/bovine/Miyazaki/B22/2016. These two Japanese strains appear together in the same independent cluster, suggesting that Influenza D virus has evolved in this country in a different way since many years. The phylogenetic tree below (which is based on the HEF analysis) shows the three clusters representing the three current known circulating lineages of Influenza D virus.

[5] Murakami, S., Endoh, M., Kobayashi, T., Takenaka-Uema, A., Chambers, J. K., Uchida, K., Horimoto, T. (2016). Influenza D virus infection in herd of cattle. Japan. Emerging Infectious Diseases, 22(8), 1517–1519.

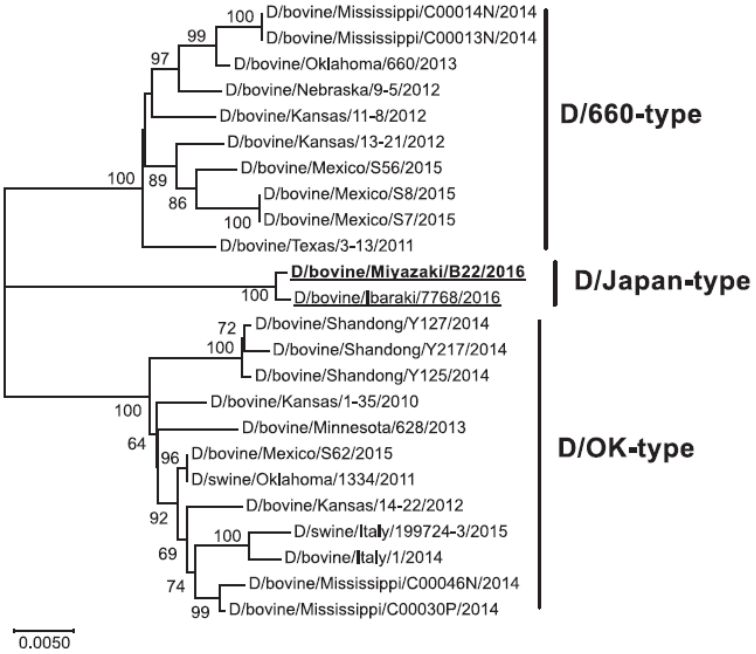


Figure 3: Phylogenetic tree of Influenza D virus. Source: Mekata H., Yamamoto M., Hamabe S., et al. Molecular epidemiological survey and phylogenetic analysis of bovine influenza D virus in Japan (2017). Transboundary Emerging Diseases; 00:1–6

(Su S. et al, 2017) estimated the t-MRCA, the mean time of the most recent common ancestor, both for ICV and IDV but also for the two lineages of IDV, D/OK and D/660. As for their Bayesian analysis, the t-MRCA for ICV and IDV would be at 482 A.D., thus 1534 years ago. At the same time, the t-MRCA of the two lineages of IDV was estimated to be only about 45 years ago, indicating that they emerged only in recent times. Finally, the authors calculated the mean substitution rate for the gene HEF of IDV as well and it resulted to be 1.54×10-3 (95% HPD, 5.4x10-4 to 2.7x10-3). This value seems to be more significant than a previous one which was calculated for ICV[6], which was assessed to be 4.87x10-4 (95% HPD: 4.12x 10-4 to 5.66 x 10-4). These findings could be interpreted as a faster evolution of IDV than ICV, meaning that new lineages are likely to emerge also in short times. A continuous molecular monitoring is thus necessary in order to detect this possibility.

[6] Gatherer D. Tempo and mode in the molecular evolution of influenza C (2010). PLoS Currents; 2:RRN1199. PMID:21127722

1.4 A worldwide distributed virus: viroprevalence, seroprevalence and molecular epidemiology of susceptible species

So far, the Influenza D virus has been detected in different animal species and on almost all continents. Some studies describe its molecular detection but many serologic evidences for anti-IDV antibodies presence are also available. At the moment the susceptible hosts seem to be cattle, swine, small ruminants and it appears that horses could be infected as well. Whether humans are likely to be potential hosts of IDV, this will be extensively discussed in the next chapter. Since the virus was not discovered very long time ago, little is known on its host species tropism, but despite the virus was first isolated from swine, bovines are currently considered to be the natural reservoir of this virus. As a matter of fact, various studies underline a high prevalence of IDV in cattle, whereas historically the bovine has never been found to be a potential host of the Influenza genera. In addition, serologic tests, such as the HI assay, showed that IDV is present in swine but not as widespread as for the bovine (Hause et al, 2013).

1.4.1 Cattle

As regards cattle, there are various studies that prove IDV circulation in North America, Asia, Europe and Africa. In the United States there are serologic evidences for the virus to be circulating from at least 2003 and 2004 in Nebraska[7] and in Mississipi State[8], but also in Oklahoma (Hause et al, 2013). Some findings suggest that IDV is present in other States as well (Hause et al, 2014). Besides the antibody detection, the virus was also found by qPCR in some nasal swabs with a positivity of 4,8% on a total of 208 samples[9].

[7] Luo J., Ferguson L., Smith D.R., Woolums A.R., Epperson W.B., Wan X.F. Serological evidence for high prevalence of Influenza D viruses in cattle, Nebraska, United States, 2003–2004 (2017). Virology; 501:88-91. PMID:27888742

[8] Ferguson L., Eckard L., Epperson W.B., Long L.P., Smith D., Huston C., Genova S., Webby R., Wan X.F. Influenza D virus infection in Mississippi beef cattle (2015). Virology; 486:28-34. doi:10.1016/ j.virol.2015.08.030. PMID:26386554

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

In Asia, IDV was first detected in China by RT-PCR from nasal swabs of apparently healthy cattle but it was found in low prevalence (0,66%). The isolated viruses were then designated as Bovine/Shandong/Y127/2014, Bovine/Shandong/Y125/2014 and Bovine/Shandong/Y127/2014[10]. Three years later another study showed a higher prevalence (12,8%) of IDV detection, still by RT-PCR from nasal swabs of asymptomatic animals. The authors also found the virus in serum samples of the same animals with a prevalence of 7,8%[11]. In Japan, a first study identified anti-IDV antibodies by HI assay with a positivity of 30,5%, using the antigens of the two lineages, D/OK and D/660[12]. A following study took into consideration nasal swabs from both asymptomatic (n=205) and symptomatic herd cattle (n=172) and the authors detected IDV by RT-PCR in 2,1% of asymptomatic animals and in 1,7% of animals with clinical signs[13].

As for Europe, the virus has been currently detected in Italy, Ireland, France and Luxembourg. In a study conducted on Irish cattle the virus was found with a higher prevalence: 320 nasal swabs from animals with clinical symptoms were analyzed by RT-PCR and the positive samples for IDV resulted to be 18[14] (5.6%). In a recent study[15] IDV presence was investigated in cattle farms in Luxembourg: in 2016, 450 serum samples were collected from asymptomatic animals and they were screened by HI assay. A seroprevalence of 80,1% was found with HI titers ranging from 20 to 1280. The authors reported that bovine individuals were older than 6 months old, therefore it is unlikely that the detected antibodies were maternally derived. The authors reported also that the medium age of seropositive cattle was (60 months) was significantly higher than the medium age of seronegative animals (which was 41

[10] Jiang, W. et al. Identification of a potential novel type of influenza virus in Bovine in China (2014). Virus Genes, 49(3), pp.493-496.

[11] Zhai S., Zhang H., Chen S., Zhou X., Lin T., Liu R., et al. Influenza D Virus in Animal Species in Guangdong Province, Southern China (2017). Emerging Infectious Diseases;23(8):1392-1396.

[12] Horimoto T., Hiono T., Mekata H., Odagiri T., Lei Z., Kobayashi T., et al. Nationwide Distribution of Bovine Influenza D Virus Infection in Japan (2016). PLoS ONE 11(9): e0163828.

[13] Mekata H., Yamamoto M., Hamabe S., et al. Molecular epidemiological survey and phylogenetic analysis of bovine influenza D virus in Japan (2017). Transboundary Emerging Diseases; 00:1–6.

[14] Flynn O., Gallagher C., Mooney J., Irvine C., Ducatez M., Hause B., McGrath G., Ryan E. Influenza D Virus in Cattle, Ireland (2018). Emerging Infectious Diseases Vol. 24, No. 2

[15] Snoeck J.C., Oliva J., Pauly M., Losch S., Wildschutz F., Muller C.P., Hübschen J.M., Ducatez M. Influenza D Virus Circulation in Cattle and Swine, Luxembourg, 2012–2016 (2018). Emerging Infectious Diseases, Vol. 24, No. 7

months old, p<0,001). In addition, most of the samples originated from cattle born in Luxembourg (90%) but anti-IDV antibodies were found also in other individuals who were born in other countries such as France, Belgium and Germany.

As concerns France, IDV was detected in various bovine samples of 2011 to 2014, including nasal swabs, lung fragments and trans-tracheal aspiration liquids. A positivity of 4,5% was detected and the cycle threshold values ranged from 15 to 35, with a positive sample of the year 2011, one of 2012 and four of 2014[16].

In Italy, IDV was first detected by RT-PCR in 2 nasal swabs (out of 150) from cattle in Northern Italy in the Po Valley[17]. Further studies[18] allowed to better understand the dynamic of IDV circulation by applying molecular and serological assays. Between 2014 and 2016, 895 samples (which included 744 nasal swabs and 151 lung tissues) were collected from 574 different farms in Northern Italy. 58 samples (6,5%) were IDV positive by Real-Time RT-PCR and in 20 of the 32 identified outbreaks animals were co-infected with other viral respiratory pathogens that can be normally found in a context of BRDC. The virus was isolated from different samples on cell culture and the viral genomes were sequenced by NGS technology. According to the phylogenetic analyses, all the isolated viruses clustered with the first detected virus D/swine/Oklahoma/1334/2011. IDV antibodies to IDV were also searched by performing HI assay in 420 serum samples from 42 dairy farms in the province of Mantova (Northern Italy). Positive samples were found in all the units and the global seroprevalence resulted 92,4%.

A study of 2017[19] took into consideration African sera collected from 1991 to 2015 coming from Morocco and West Africa. The authors performed HI assay to detect

[16] Ducatez M., Pelletier C., Meyer G. Influenza D Virus in Cattle, France, 2011–2014 (2015), Emerging Infectious Diseases www.cdc.gov/eid Vol. 21, No. 2

[17] Chiapponi C., Faccini S., De Mattia A., Baioni L., Barbieri I., Rosignoli et al. Detection of Influenza D Virus among Swine and Cattle, Italy (2016). Emerging Infectious Diseases, 22(2), 352-354.

[18] Rosignoli C, Faccini S, Merenda M, Chiapponi C, De Mattia A, Bufalo G, et al. Influenza D virus infection in cattle in Italy [in Italian] (2017). Large Animal Review;23:123–8.

[19] Salem E., Cook E., Lbacha H., Oliva J., Awoume F., Aplogan G.L. et al. Serologic Evidence for Influenza C and D Virus among Ruminants and Camelids, Africa, 1991–2015 (2017). Emerging Infectious Diseases;23(9):1556-1559.

anti-IDV antibodies by using the antigen D/bovine/France/5920/2014. The samples coming from Morocco (n=200), Benin (n=207) and Togo (n=201) were found to be positive with a seroprevalence, of 35%, 1,9% and 10,4% respectively, demonstrating the Influenza D virus circulation in these countries from at least 2012. The seroprevalence in cattle sera from Morocco was then also confirmed by performing a microneutralization assay. No IDV positivity was found in samples coming from Côte d'Ivoire. In the same study also sera coming from Kenya were screened by HI assay, in particular 938 cattle sera and 293 camel sera. All the bovine sera resulted negative.

1.4.2 Camelids

As concerns the camelid samples of the study discussed right above (Salem et al. 2017), these showed a 99% seroprevalence by using the two antigens D/bovine/Nebraska/9-5/2012 and D/bovine/France/5920/2014. They were therefore also tested for Influenza C virus by using the lineage C/Victoria/1/11 and in this case the positive proportion was 94%, suggesting a cross-reactivity between these two viruses. The results remained uncertain also after absorption with one virus before re-performing the test with the other because most antibodies were lost in both cases. Therefore, camels could be a new discovered host for the Influenza C virus but also a possible one for the new emerging IDV.

1.4.3 Swine and feral swine

Even if the novel Influenza D virus was first isolated from swine samples, it has never been found in high prevalences in this species as in bovines. Up to now the few evidences for its circulation in swine livestock come from Italy, Luxembourg, China and the United States, where a 2,8% of seroprevalence was found on 220 sera in 2011 when IDV was discovered (Hause et al., 2013). The virus was molecularly detected in high prevalence from some nasal swabs in China, with a positivity of 36,8%, with 7 positive samples by RT-PCR out of the 19 analyzed (Zhai et al., 2017). Anti-IDV antibodies were also searched in swine sera samples of Côte d'Ivoire but these all resulted negative (Salem et al., 2017).

IDV was detected in Italy in 2015 from a nasal swab of a swine presenting Influenza symptoms (Chiapponi et al., 2016) and in a following study[20] its circulation in Northern Italy was confirmed by analyzing 845 samples of symptomatic animals coming from 448 herds in the Po Valley, one of the most important regions in Europe for swine production. A total of 21 samples were found positive (2,3%) by RT-PCR, which included 14 nasal swabs, 3 lung samples and 4 oral fluids. The isolated viruses were named D/swine/Italy/199724-3/2015, D/swine/Italy/354017/2015 and D/swine/Italy/173287/2016. The auhors also searched for anti-IDV antibodies by HI assay in 3106 sera collected at the slaughterhouse, finding a positivity of 364 (11,7%) samples. The sera with the lowest HI titers (20) were then confirmed by microneutralization assay.

To verify IDV circulation in Luxembourg, serum samples from pigs were collected at 2 slaughterhouses. In particular, the authors of the study[21] screened by HI assay 258 samples collected in 2012 and 287 samples collected in 2014 and 2015. In addition, they also analyzed by PCR 232 nasal swabs collected in 2009 and 427 collected in 2014 and 2015. The seroprevalence resulted 0% in the samples of 2012, whereas it increased at 5,6% in samples collected in the following years. The viral presence in nasal swabs was very low in quantity and also in prevalence, resulting 0% in samples from 2009 and 0,7% in samples from 2014 and 2015.

In a recent study[22] IDV was also found in wild feral swine populations in the United States. 256 sera were collected during 2012 and 2013 from Hawaii (n = 73 samples), North Carolina (n = 64), Oklahoma (n = 49), and Texas (n = 70) and were analyzed by HI assay. Two different antigens were tested: D/bovine/C00046N/Mississipi/2014 (D/OK) and D/bovine/C00013N/Mississipi/2014 (D/660). The authors found a 19,1% seroprevalence to both the antigens, in particular 39 samples were positive for D/13N

[20] Foni E., Chiapponi C., Baioni L., Zanni I., Merenda M., Rosignoli C., Kyriakis C.S., Luini M.V., Mandola M.L., Bolzoni L., Nigrelli A., Faccini S., Influenza D in Italy: towards a better understanding of an emerging viral infection in swine (2017). Scientific Reports, 7: 11660

[21] Snoeck J.C., Oliva J., Pauly M., Losch S., Wildschutz F., Muller C.P., Hübschen J.M., Ducatez M. Influenza D Virus Circulation in Cattle and Swine, Luxembourg, 2012–2016 (2018). Emerging Infectious Diseases, Vol. 24, No. 7

[22] 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., Influenza D Virus Infection in Feral Swine Populations, United States (2018). Emerging Infectious Diseases Vol. 24, No. 6.

(15%) and 18 were positive for D/46N (7%). The authors also wanted to evaluate if the sampled feral swines were also positive for IAV, since both swine and feral swine are susceptible for this virus as already reported in literature[23]. Of the 256 samples 5,1% (n=13) were positive for IAV and of these 13, 5% were also IDV positive. To better understand this possible coinfection, the authors examined the 13 IAV-positive samples with 83 additional serum samples of 2012-2013 (which were already known to be IAV-positive) and tested them for both the IDV lineages. Taken together, of these 96 tested sera 41 were positive for D/13N (42,7%), 9 were positive for D/46N and 5 samples were positive for both lineages.

1.4.4 Small ruminants

Since Influenza D virus has been discovered to be widespread in cattle, the hypothesis that small ruminants could be potential hosts has then emerged. To confirm this, some authors considered the research of anti-IDV antibodies also in goat and sheep sera in order to determine whether these species could be infected as well. (Quast et al., 2015) in their study[24] analyzed by HI assay 472 sera of sheep coming from 111 farms located in the Midwest States in the USA and the results showed 29 positive samples (6,1%), 12 of which had a HI titer higher than 80. Goat samples were also tested (27 coming from 5 farms in South Dakota and Montana) and 7 of them were seropositive (25,9%).

In China (Zhai et al., 2017) the virus was found in native hybrid white goat serum samples in high prevalence (33,8%, 27 positive samples out of 80). The authors indicated that 20% of the individuals were severely diseased, suggesting that IDV in its most acute phase can become viremic; what is more, in the same study a rectal swab from a hybrid black goat goat was found positive.

[23] Martin B.E., Sun H., Carrel M., Cunningham F.L., Baroch J.A., Hanson-Dorr K.C., et al. Feral swine in the United States have been exposed to both avian and swine influenza A viruses (2017). Applied Environmental Microbiology; 83:e01346–17.

[24] Quast M. et al. Serological evidence for the presence of influenza D virus in small ruminants (2015). Veterinary Microbiology 180 (2015) 281–285

1.4.5 Equines

So far, only one study about IDV in equine populations[25] has been published. Nedland et al. investigated by HI assay 364 horses serum samples collected in 2015 and 100 of 2016, gathered from six different States (USA). They tested the sera in triplicate and considered the treshold of 40 to define positive samples. Taken together, they found 57 positivities (6,3%) for both the lineages D/OK and D/660; the human Influenza C virus C/Victoria/2/2012 was tested as well and it was found a 8% seropositivity, showing the absence of cross-reactivity between the two viruses. The results here found by HI assay were then verified and confirmed by MN (microneutralization) assay. The authors of the study conclude therefore that both ICV and the novel Influenza D virus circulate in the equine population of USA, including also this animal species in the list of the IDV susceptible ones.

[25] Nedland H., Wollman J., Sreenivasan C., et al. Serological evidence for the co-circulation of two lineages of influenza D viruses in equine populations of the Midwest United States (2018). Zoonoses Public Health; 65: e148–e154.

II. Pathobiology of Influenza D virus in bovine species

2.1 Biochemistry of the HEF ectodomain and receptor binding mechanism

Influenza D virus, similarly to Influenza C virus, owns only one major surface glycoprotein: HEF, the hemagglutinin-esterase-fusion. The other two members of the *Orthomyxoviridae*, on the contrary, arrange two surface glycoproteins: HA (hemagglutinin), which binds to the host cell receptors and mediates the membrane fusion, and NA (neuraminidase), which has the function of destroying the receptor and releases the new virion particles. Thus, in ICV and IDV these activities are accomplished by the only protein available on the surface. The considered part of HEF is the ectodomain, this means that the domain that extends itself outside of the cell, in the extracellular space. The majority of its external surface is covered with carbohydrates and the protein continues then with a transmembrane region and a short tail located in the cytoplasm. By studying the molecular structure of IDV and comparing it to HEF of ICV some authors noticed (Su S. et al., 2017) that ICV contains one more glycosilation site near the binding domain of the receptor than IDV, which could normally restrict the viral access to the receptor. Thus, by eliminating this glycosylation site the novel virus could have acquired a broader cell tropism. We currently know the specific receptor of IDV thanks to a study[26] which investigated the receptor-binding characteristics of HEF using glycan microarrays. The authors tested 610 different glycans and verified to which of these the viral fusion protein annealed best. It resulted that HEF only binds strongly to 9-O-Ac-Sia glycan derivatives. Furthermore, it resulted that the fusion protein of IDV binds much more robustly to this receptor than ICV does. In the same study also host tissue tropism was investigated: different trachea tissue sections were stained with both ICV and IDV HEF protein and the experiment was performed on human, swine and bovine tissues. Surprisingly, all three exhibited positive staining for the receptor 9-O-Ac-Sia, but the signal was more evident for the swine and bovine and in these two species the signal

[26] Song H., Qi J., Khedri Z., Diaz S., Yu H., Chen X., et al. An Open Receptor-Binding Cavity of Hemagglutinin-Esterase-Fusion Glycoprotein from Newly-Identified Influenza D Virus: Basis for Its Broad Cell Tropism (2016). PLoS Pathog 12(1): e1005411.

was not limited to the epithelial surface but it was also detectable more in depth in the tracheal mucosa.

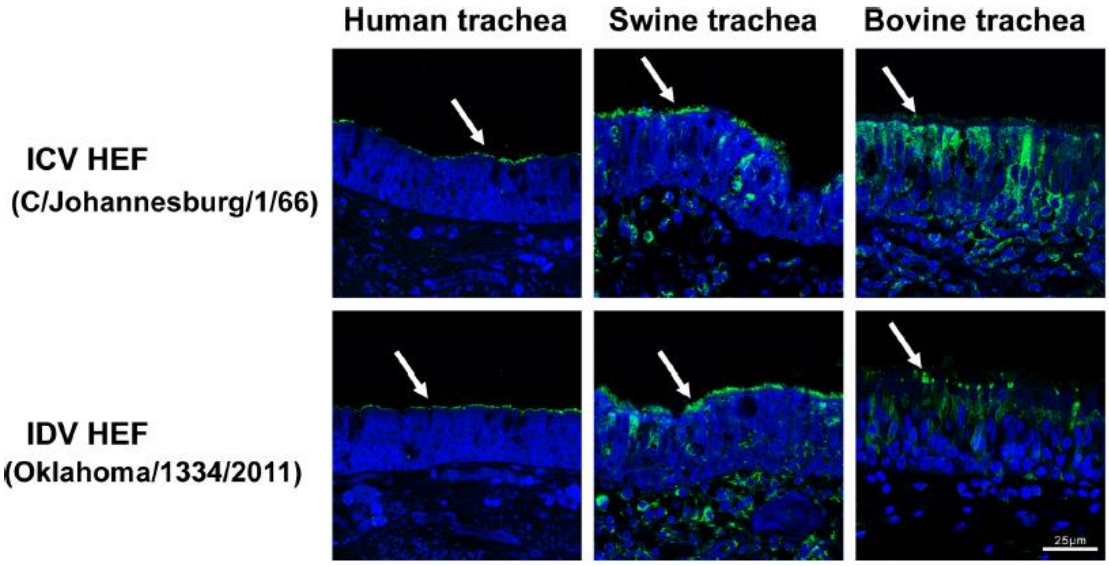


Figure 4: Staining of human, swine and bovine trachea with either ICV or IDV HEF protein. Paraffinized tissue sections were stained with recombinant HEF-mut protein derived from the baculovirus expression system. Specific staining by recombinant protein (green) is indicated by white arrows. Nuclei were counterstained with DAPI (blue). Source: Song H., Qi J., Khedri Z., Diaz S., Yu H., Chen X., et al. An Open Receptor-Binding Cavity of Hemagglutinin-Esterase-Fusion Glycoprotein from Newly-Identified Influenza D Virus: Basis for Its Broad Cell Tropism (2016). PLoS Pathogens 12(1): e1005411.

This finding is important because it shows the molecular basis for the potential human infection with Influenza D virus. Not only humans possess the specific receptor but it has also been demonstrated that IDV is effectively able to bind to the epithelial trachea, one of the target organ for the Influenza viruses. Despite this, it is always important to remember that even if the virus is able to bind to the receptor it does not mean that the cell can be necessarly infected.

To attach to the receptor, ICV and IDV require an acetylated derivate named N-acetyl-9-O-acetylneuraminic acid (9-O-Ac-Neu5Ac) which can bind to both α-2,6 or α-2,3

linkages to a galactosyl residue. On the contrary, avian IAV usually bind to Neu5Ac-α2,3-gal and mammalian IAV to α2,6-gal. Some structural studies were performed and it was shown that the receptor binding site of IDV HEF is more open than ICV HEF and this extra space could allow for different glycan linkages which suggest binding to receptors of different hosts. In addition, (Song H. et al., 2016) suggested this open cavity to be a possible cause of the broad cell tropism of IDV.

Once HEF is attached to the receptor, HEF must be splitted into two units by a tripsin-like serin protease to enter the cell by membrane fusion, but these are still not clearly identified neither in bovine nor in humans. For HA of IAV and IBV, these enzymes are expressed on the membranes of human bronchial and tracheal epithelial cells and they are very similar to those present in swine. The proteases recognize specific amino acids around the cleavage site and consequently HEF is processed into HEF1, a larger unit which has a single arginine residue at the C-terminus, and HEF2, a smaller subunit. Only the cells that express these specific proteases can allow the viral particles to enter and therefore to replicate, making the infection process start. (Su S. et al., 2017) therefore suggest that different processing enzymes could be another reason to explain the different cell tropism among the four Influenza genera, but also within different tissues of a same species (i.e. upper and lower respiratory tracts).

2.2 Cattle as the primary host: IDV transmission and clinical signs

So far, epidemiological studies suggest that even if the virus was first isolated from pigs, these are not suspected to be the primary host of the novel Influenza D virus. A few pieces of evidences rather lead to considering cattle as the real responsible for maintaining the virus in the environment, such as the results of the viral molecular detection and the antibody research. However, it is still not clear wheter cattle can be considered a primary host or a natural reservoir, since IDV ecology and its mainteinance in the enviroment is still not fully understood. As a matter of fact, effective circulation of the virus in both swine and bovine species has been shown but with different prevalences. To resume what was discussed in the introductive

chapter, even if IDV circulates in swine it is not as widespread as in cattle: the current data for swine show a seroprevalence of 9,5% (Hause et al., 2013) and 11,7% (Foni at al., 2017), whereas for the bovine in some cases it was shown to reach the 30,5% (Horimoto at al., 2016) and 35% (Salem et al., 2017). Also, in neonatal calves in the USA it reached the 95-98% of seroprevalence. What makes the virus circulate widespreadly is still unknown but it could be due to poor biosecurity and to intensive farming practices.

To understand the dynamics of the viral transmission, experimental infections were performed. Since IDV was first isolated from pigs, the first study was planned in order to understand the mode of transmission among these animals.

In a first study (Hause et al., 2013) IDV was inoculated intranasally and it was detectable from 3 to 10 days post-infection by PCR in nasal swabs. The infected pigs did not show any clinical signs and the hystopathological analysis on lungs did not reveal typical Influenza lesions. On day 14 all the infected pigs had anti-IDV antibodies and a half of pigs exposed by direct contact seroconverted. By this study it emerged that the virus can be transmitted to seronegative pigs by direct contact and that the kinetics of the virus replication is slower for Influenza D virus than for IAV.

A few years later (Ferguson et al., 2018) IDV transmission was then evaluated among feral swine, which often share the same pathogens. The authors inoculated intranasally the strain D/46N to 12 trapped feral swine and 8 were used as contact animals, exposed to the infected ones. None of the infected feral swine showed any clinical sign and the virus was detectable in nasal swabs from day 3 to 7 days post-infections. 63% of the animals seroconverted at day 5, whereas the remaining seroconverted at day 7. Half of the contact animals were infected by donor animals and, at day 3, 4 infected animals showed viremia; one contact animal showed viremia at day 7. Even if after a week the virus was no more detectable in nasal swabs, it still was present with a high titer in the soft palate and in the low trachea. Taken together the study showed that IDV can be transmitted to feral swine by direct contact similarly to the domestic swine and with limited clinical signs. At the epidemiological level, a higher seroprevalence was observed in wild feral swine compared to domestic swine, which could be due to increased IDV transmission by direct contact, as they

are mobile and have the possibility to come in contact with a large range of other feral swine but also of various animals (including bovine, the natural reservoir of IDV), both wild and domesticated.

As IDV concerns bovine species, two studies were performed in order to understand the dynamics of viral transmission in cattle and they both used calves (6 months old individuals or less) as a model. In the first study[27] nine seronegative calves were included and the authors infected intranasally two of them. A control group inoculated with PBS was included as well and all the animals were monitored for 21 days (at days 1, 2, 4, 6, 9 and 21 nasal swabs, rectal swabs and blood were collected; temperature, heart rate and lung ausculation were taken twice a day). The day after the inoculation the two infected calves were paired to 2 non-inoculated seronegative calves each to see if IDV could be transmitted by direct contact. The results of the study showed that the infected animals already produced anti-IDV antibodies 6 days after the inoculation with titers of 1:20 and 1:80; at day 9 the titer was 1:320 and at day 21 1:640. All nasal swabs collected from day 1 to 6 were positive to Influenza D virus. All the three contact animals seroconverted after 9 days with HI titers of 1:10 and 1:20; the third one seroconverted at day 13 with a HI titer of 160. IDV was also detected in nasal swabs of contact animals at day 6, showing that IDV does not replicate efficiently in calves but it also efficiently transmits to direct contact animals.

The second part of the experiment aimed at observing the clinical signs and the pathological signs that would appear after infection. Of the infected calves, one was noticed to have dry cough at day 2 and nasal discharge at day 5; the second one had lung ausculation score 1 at day 2 and the third calf did not show any symptoms. One of the calves belonging to the contact group had detectable lung ausculation score 1 at day 4, even before that the virus was detectable in nasal swabs; a second calf had nasal discharge at day 9 and serous ocular discharge at day 13 and the third one had only nasal discharge at day 9. An immunoistochemical assay was also included in the study and it revealed a significant tracheal inflammation in the infected calves,

[27] Ferguson L., Olivier A.K., Genova S., Epperson W.B., Smith D.R., Schneider L., Barton K., McCuan K., Webby R.J., Wan X-F. Pathogenesis of influenza D virus in cattle (2016). Journal of Virology 90:5636 – 5642.

characterized by a significant neutrophilic infiltration in the tracheal epithelium. In the lung, areas containing degenerated neutrophils were observed, together with mucus infiltration. Immunostaining was used to detect the antigen, which resulted to be present in nasal turbinates, trachea and bronchus but was not detectable in the lungs. To summarize, this study shows that Influenza D virus can be transmitted among calves by direct contact and it causes mild respiratory disease in the infected animals, associated with inflammation signs in the respiratory tract. The higher viral titers were detected in nasal turbinates and they constantly decreased in the lower respiratory tract. According to the authors, an important role could be played by the route of transmission because contact animals had more marked clinical signs than the inoculated animals, even if the neutrophilic infiltration was much more evident in the inoculated calves. In this case, the contact animals were probably infected through fomites or contaminated aerosol droplets.

In another experimental study[28] (originally designed to test a vaccine) it was observed that the viral replication is limited to the upper respiratory tract and the infected calves developped rinithis and tracheitis. These signs observed in the 2 experimental infections described above are less marked than those observed in the field, in calf-rearings. According to some authors (Su S. et al., 2017) this could be due to the differences in husbandry conditions, to the infection routes and above all to the doses of exposure. Furthermore, it seems that IDV alone is probably not sufficient to cause disease but it could be part of the BRDC (Bovine Respiratory Disease Complex) complex, as it will be discussed in the next paragraph.

To make a comparison of Influenza D virus transmission among different species, it seems that this virus can be transmitted by direct contact among swine and in a very similar way among feral swine and in calves. Looking at viral titers and the duration of shedding it seems that IDV is less efficiently transmitted among swine than calves.

[28] Hause B., Huntimerc L., Falkenbergc S., Henningsona J., Lechtenbergd K., Halburc T. An inactivated influenza D virus vaccine partially protects cattle from respiratory disease caused by homologous challenge (2017), Veterinary Microbiology 199, 47–53.

2.3 Is IDV alone sufficient to cause clinical disease?

As for the studies published so far, it seems that Influenza D virus in bovine species is likely to have an association with the BRDC (Bovine Respiratory Disease Complex). The BRDC is one of the most important diseases of economic importance of cattle, causing high mortality both in dairy and in beef cattle. In fattening units, where meat is produced, BRDC is mostly seen at the beginning of the productive cycle and it is considered as the main cause of morbility and mortality. In dairy farms, on the contrary, respiratory infections mostly occur in enzootic forms in calves on their development stage, above all in pre- and post-weaning periods. In these cases, the mortality rate is not elevated but this pathology frequently evolves in a chronic respiratory disease that causes lesions which remain persistent at the parenchymal lung level. In addition, BRD can cause a significant delay of the growth and a subsequent delay of the reproductive phase. In addition, it seems that such lesions in the respiratory tract are likely linked to a significant reduction of the production potential in adult phase[29]. At first, the pathology is triggered by a less efficient immune response which is caused by enviromental and managerial risk factors. Among these, the most frequently are recognized are transport stress, overcrowding, mixing of individuals of different age or origin, poor ventilation and elevated humidity. To these predisposing situations viral infections of the upper respiratory tract follow. These infections promotes then the proliferation and the following colonization of the lower respiratory tract by bacteria which are normally present in nose and pharynx. The first signs manifested by infected individuals are general signs such as fever, depression, lack of appetite until more specific respiratoy symptoms like rapid breathing, coughing, and serous discharge (or blood discharge in the most acute cases).

The BRDC is defined as a “Complex disease” because it usually involves a combination of viral and bacterial agents, although also parasitic and fungal agents can be present as well. Some of the viral pathogens associated with this disease seem to be the Bovine Respiratory Syncytial Virus (BRSV), some viruses belonging to Adenovirus

[29] Rosignoli C, Faccini S, Merenda M, Chiapponi C, De Mattia A, Bufalo G, et al. Influenza D virus infection in cattle in Italy [in Italian] (2017). Large Animal Review; 23:123–8.

family, ParaInfluenza virus 3 (PI3), the Bovine Viral Diarrhea Virus (BVDV), Bovine Coronavirus (BCoV) and Infectious Bovine Rhinotracheitis virus (IBR). The most common bacterial agents that play an important role in this syndrome are *Pasteurella multocida*, *Mannhemia haemolytica*, *Histophilus somni* and *Mycoplasma bovis*.

So far, it is not simple to define a clear etiological role for Influenza D virus because it was detected in animals presenting different pathological conditions. For instance, various studies report IDV detection in bovine presenting respiratory symptoms; however, the virus has been found in healthy animals as well (even if with lower prevalences and higher Ct in the qPCR analysis). In a study (Ferguson et al., 2015), IDV was found in 16 of 55 sick calves (29,1%) and in 2 out of 82 healthy calves (2,4%). In another study (Ducatez et al., 2015) IDV was detected in 6 samples, 4 of those were coinfected with *Mannheimia haemolytica*, *Pasteurella multocida, Histophilus somni*, Bovine respiratory syncytial virus, and/or Bovine Herpesvirus 1. Despite this, the authors also detected IDV in two samples that were collected from animals presenting clinical signs, but for which no coinfections were detected. Also, in the experimental infections discussed above it was shown that calves inoculated only with IDV show some degree of pathology.

In a study conducted in Italy, with the purpose of IDV surveillance in bovine populations (Rosignoli et al., 2017), the virus was found both in cattle affected by BRDC and in healthy animals; with a significantly higher proportion in BRDC-affected than in healthy cattle ($p<0,05$). In fact, a viroprevalence of 8% was found in diseased individuals (48 positive samples out of 603) whereas 3,4% (10/292) of animals without respiratory symptoms were positive. According to the authors, these data tend to support the hypothesis of a potential association between BRDC and IDV. In the same study, IDV was identified in 32 outbreaks of respiratory disease, 5 in beef fattening units and 27 in dairy herds. In 20 outbreaks (62.5%) IDV was the only respiratory virus detected, while in 12 outbreaks (37.5%) the infection was mixed with other major viral respiratory pathogens normally involved in BRDC (BVDV, BRSV, BoHV-1, PI3V, BCoV).

A study[30] tried to identify the most common viral agents associated with the BRD including the Influenza D virus for the first time. By using NGS technology (Illumina HiSeq), which enables to sequence the genome of all viruses included in the sample, the authors made a metagenomic analysis to identify all the viruses in nasopharyngeal swabs involved in 50 calves with BRD and in 50 healthy individuals used as controls. The advantage of using this kind of technology includes the possibility of analyzing a huge amount of sequences and to eventually identify new viruses belonging to the virome. Also, it allows to estimate the viruses genetic material present in higher "quantity" and the viruses that are a minor part of the virome. In the study, IDV sequences D/OK-type were identified together with sequences from 9 other respiratory viruses. The pool of samples that contained IDV was used to amplify the virus sequences by PCR and then a further deep sequencing was performed to characterize its full genome. The complete genome characterization generated 304 reads which were sufficient to cover 72% of the genome. On the whole, of the 50 diseased calves 7 individuals were IDV positive, which was represented by the 14% of the total RNA used for the analysis, showing that the virus may be involved in the BRD complex. Surprisingly, even if this novel virus was detected in these samples, the viruses typically associated with BRD were not found in a statistically significant proportion and they were limited to Bovine adenovirus 3 (48%) and Bovine rhinitis A virus (30%).

In another study[31], using a similar metagenomic approach, the authors analyzed the virome of nasal swabs collected from both symptomatic and healthy cattle. On the whole the authors detected 21 viruses but only 3 resulted to be strongly associated with BRD. The most commonly identified were the Bovine rhinitis A virus (52,7%), Bovine rhinitis B virus (23,7%), Bovine Coronavirus (24,7%) and the authors found the novel Influenza D virus to be strongly associated as well. Other BRD-commonly associated viruses were also detected but less frequently (Bovine Viral Diarrhea virus,

[30] Ng T.F.F., Kondov N.O., Deng X., Van Eenennaam A., Neibergs H.L., Delwart E. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. (2015). Journal of Virology 89:5340 –5349.

[31] Mitra N., Cernicchiaro N., Torres S., Li F., Hause B. M. Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus (2016). Journal of General Virology, 97, 1771–1784.

Bovine Herpesvirus 1, Bovine Respiratory Syncytial virus and Bovine Parainfluenza virus 3). In this case IDV was detected in 8 nasal swabs of symptomatic animals but also in 3 nasal swabs of cattle showing no apparent respiratory disease.

Influenza D virus has been demonstrated to cause clinical disease alone in experimental infections; for instance, in their study Ferguson et al. (2016) noticed that the contact animals manifested clinical symptoms, even more marked than animals which were inoculated with the virus. In addition, molecular findings showed that some IDV positive samples coming from animals showing respiratory diseases were not coinfected with other pathogens usually present in the BRD complex (Mitra et al., 2016, Ducatez et al., 2015). At the same time, Ferguson et al., 2016 propose that the virus alone is not sufficient to cause disease, but it would cause inflammation that could facilitate the coinfection by other pathogens. Thus, in their opinion, IDV would not be able to cause clinical disease in cattle in the field without concurrent infections. This opinion is supported by the two metagenomic studies discussed above, which tend to correlate Influenza D virus in the BRD complex and even suggest an etiological role of the virus in the disease. To summarize, IDV is able to cause clinical disease alone as demonstrated by experimental infections and has been detected in asymptomatic animals in the field: its implication in the BRD has still to be fully demonstrated. More epidemiological and metagenomic studies are surely needed to figure this relationship out.

2.4 Calves seem to be more susceptible to IDV infection than adults

According to some authors, IDV prevalence in cattle is higher in young bovine individuals than in adults; this would be due to the high levels of maternal anti-IDV antibodies acquired at birth that decrease throughout the months, causing an augmented susceptibility and therefore creating a permissive enviroment that allows the virus to replicate and to be actively transmitted. Thus, calves in particular could be considered the natural reservoir more than cattle in general.

To confirm this hypothesis, two studies monitored the evolution and the dynamic of antibodies in calves. A first study (Ferguson et al., 2015) verified the prevalence of maternal antibodies in neonatal calves 24-36 hours after birth. In the experiment, 448 serum samples were tested using the antigen D/bovine/Mississipi/C00046N/2014 and 94% of the samples were seropositive against IDV, with a mean titer of 1:410. The authors also tried to verify if the infection occurred before animals arrived in calf-facilities or once they were already in. To do this, a paired sampling was performed in two lots of calves: at the arrival, by HI assay, 21% of the individuals belonging to the first lot (n=19) were seropositive with a mean anti-IDV titer of 1:80, whereas 22,2% of the second one (n=27) were seropositive with a mean titer of 1:127. A week later, the sampling was repeated and 56% and 33% of respectively the first and second lots were positive with a mean titer of 1:113. Of these two lots, 7 nasal swabs were also collected for IDV molecular detection. If none of these were positive at arrival, 2 were positive a week later, suggesting that the infection occurs after calves arrived in the facility.

At birth, calves absorbe passively maternal antibodies through the colostrum, therefore the immunoglobulins (in particular IgGs) have to pass the intestinal epithelium but this process lasts only the first 24-36 hours. The half-life of these immunoglobulins is normally 21 to 35 days, thus conferring a passive immunity that lasts 3 to 4 months. After this period, calves tend to lose this protection: as a matter of fact, if at birth 94% of the calves of this study were seropositive only 3,7% to 11% of the individuals aged 6 to 8 months were seropositive. Cattle of 1 year of age reacquires protection because there is an increase in anti-IDV antibodies: 66.7% of the 1 years old calves (n=42) were seropositive.

The authors of this study also tried to better understand IDV seroprevalence in individuals of different ages, since the dynamics of passively acquired antibodies is pathogen dependent. 605 bovine sera (from 2004 to 2006) were categorized in 6 groups according to the age: 6 months (n=52), 7 months (n=244), 8 months (n=188), 1-3 years old (n=64), 3-9 years old (n=33) and 9-14 years old (n=24). The seroprevalence of these groups was found to be respectively 11.5%, 3.7%, 6.9%, 54.7%, 60.6% and 54.2%. This seems to suggest that after 6 months of age calves tend to lose anti-IDV antibodies, which are then reacquired when the individuals grow and encounter an IDV infection. The authors propose also an another concurrent factor: the stressful transportation in high number of calves to the facilities, which would contribute to lower their immunological defenses against the infection.

The high level of maternal anti-IDV antibodies was then confirmed in another study (Luo J. et al., 2017), which analyzed sera of 2003 and 2004 from various farms located in Nebraska. The tested animals were one week old and were retested when they were 3 months old. HI assay was performed to detect the antibody levels. In this case the authors used the antigen D/bovine/Mississippi/C00046N/2014 (D/46N) and found that 98% of the sera from 1 week old individuals were positive against D/46N (mean titer 1:648). 76% of the same calves 3 months later remained seropositive, with titers ranging from 1:10 to 1:640. Neonatal calves have thus high levels of maternal antibodies which decrease throughout the months, but in this case no serological analyses of calves aged more than 6 and 8 months were performed.

III. Zoonotic potential of the novel emerging Influenza D virus

3.1 Ecology of Influenza viruses and their zoonotic potential: the key role of birds and swine

Since this novel virus demonstrated to have similar features to Influenza C virus, a particular attention has been given to its characterization. ICV is thought to have its natural reservoir in humans and it usually causes infection of the upper respiratory tract in children, causing clinical symptoms that can vary from mild, such as cough and fever, to more severe. Influenza B virus normally circulates among humans as a seasonal pathogen, but neither IBV nor ICV have a pandemic potential compared to that of Influenza A virus. This last one recognizes wild acquatic birds as a natural reservoir, even if specific strains have mammals as host (i.e. horses and dogs)[32]. If wild birds (such as ducks, geese, swans, waders and gulls) come in contact with domestic poultry, both directly or indirectly through faeces, these can spread virus and cause low or highly pathogenic disease. In this last case, it can cause up to 90% of mortality[33]. Normally these avian Influenza viruses infect only birds but occasionally they can also infect humans that are in close contact with these infected birds. For example, in some Asian countries, the IAV subtypes H5N1 and H7N9 are associated with human infection in the context of live poultry markets.

In addition to this, and most importantly, swine IAV can more easily transmit to humans because they have similar receptors. Since swine carry both avian and human-like receptors, in this species reassortments can occur and this can cause epidemics, or at worst, also pandemics. A concrete example comes from the pandemic in 2009 by H1N1, for which there were strong evidences of its emergence in humans after its transfer from swine.

[32] Parrish C.R., Murcia P.R., Holmes E.C. Influenza virus reservoirs and intermediate hosts: dogs, horses, and new possibilities for influenza virus exposure of humans (2015). Journal of Virology 89:2990–2994.
[33] https://www.vetinst.no/en/surveillance-programmes/avian-influenza-in-wild-birds

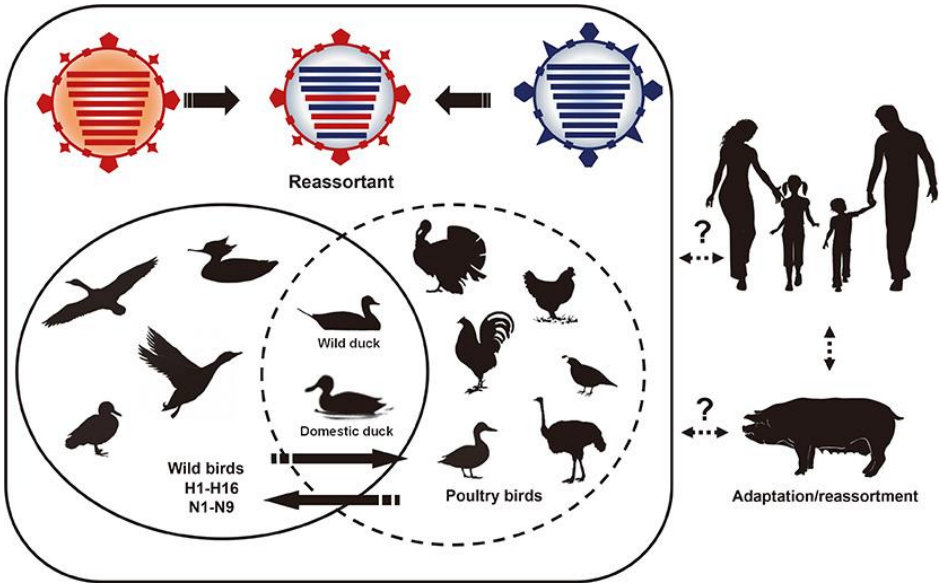


Figure 5: Transmission and spread of avian influenza viruses from wild to domestic birds and potential spill to humans. Source: Pascua P.N., Choi Y.K. Zoonotic infections with avian influenza A viruses and vaccine preparedness: a game of "mix and match" (2014). Clinical and Experimental Vaccine Research. Jul;3(2):140-148

In reality, pigs are also susceptible to Influenza B[34] and C[35] viruses, even if they are not thought to be their natural reservoirs. When pigs were discovered to be a host of the new Influenza D virus, which is distantly related to human ICV, concerns about its zoonotic potential arose in this case as well. Even if ICV is predominantly a human pathogen it has also been isolated from this animal species and its transmission between humans and swine has been suggested but the direction of transmission is still unclear, thus unresolving if it can be considered zoonotic or anthroponotic[36]. Some authors (Su S. et al., 2017) suggest to continue monitoring a possible future

[34] Zhiguang R. et al., Domestic pigs are susceptible to infection with influenza B viruses (2015) Journal of Virology. JVI.00059-15.
[35] Lambert T., Killoran K., Leedom Larson K.R. Influenza C and influenza D viruses (2016). Swine Health Information Center and Center for Food Security and Public Health. http://www.cfsph.iastate.edu/pdf/shic-factsheet-influenza-cd.
[36] Lambert T., Killoran K., Leedom Larson K.R. Influenza C and influenza D viruses (2016). Swine Health Information Center and Center for Food Security and Public Health. http://www.cfsph.iastate.edu/pdf/shic-factsheet-influenza-cd.

reassortment of ICV and of IDV, since swine is susceptible to these two Influenza genera. In fact, since IDV can infect pigs, it is necessary to continuosly monitor its evolution in this species in order to verify if it can play a role of "mixing vessel" between swine and humans, as for Influenza A virus.

3.2 Serologic studies conducted on human serum samples

Soon after the virus isolation from pigs, researchers tried to rapidly understand if the virus was also circulating among humans. As discussed in the introductive chapter, it was soon clear that IDV had very similar characteristics to Influenza C virus, a pathogen that has humans as its natural reservoir. Thus, in order to understand this, serologic assays for the research of anti-IDV antibodies were conducted on human serum samples. A first study (Hause et al., 2013) analyzed 316 sera from Canadian people collected during 2007-2008 and 2008-2009 Influenza seasons. Sera were analyzed by HI assay by using a C/OK-type strain. Of these samples, 4 were positive for anti-IDV antibodies, 3 of them with a titer of 20 and one with a titer of 40 but the first 3 also had high titers (160, 320, 1280) for Influenza C virus. The strain C/Yamagata/10/1981 was used to verify if the positive samples were actually false positives due to a cross-reaction between ICV and IDV. The last sample with a titer of 40 did not show positivity with the ICV strain. Since the total seropositivity was only 1,3%, the authors concluded that the virus was unlikely to circulate among humans. In her PhD thesis[37], L. E. Eckard analyzed by HI assay 741 samples from adults aged more than 50 years. All the collected sera originated from two different seasons (2008-2009 and 2012-2013) and they belonged to people who lived in a community that had a high probability of exposure to cattle. Of all the samples, 8 were weakly positive (thus 1%) and to see if this was due to a cross-reaction with ICV all the samples were preabsorbed with C/Victoria strain before being retested. The preabsorption with ICV managed to remove all IDV positive antibodies except for one

[37] Eckard Laura Evelyn, "Assessment of the Zoonotic Potential of a Novel Bovine Influenza Virus" (2016). Theses and Dissertations (ETD). Paper 388. http://dx.doi.org/10.21007/etd.cghs.2016.0405.

sample, confirming the cross-reactivity between the two viruses. Another study[38] assessed the seroprevalence in a human cohort. The study was performed in Florida (United States) and the authors collected 35 cattle-exposed and 11 non-cattle-exposed adult serum samples and tested them by HI assay and MN assay using the antigen D/Bovine/Kansas/1-35/2010. The seroprevalence was much higher than that reported in the previous two studies, with a positivity of 91% by HI assay among cattle exposed individuals and 97% by MN assay. The reported seroprevalence among non-exposed individuals was 75% by HI assay and only 18% by MN assay. Overall, the global reported seroprevalence among humans was 94%. In order to properly evaluate the results obtained in this study, it has to be taken into consideration that the authors did not preabsorbe sera with Influenza C virus, which is already known and demonstrated to cross-react with IDV in human species. This feature (together with the limited number of analyzed samples) can explain such a high seroprevalence detected among human individuals exposed to cattle in Florida, which is in strong contrast to the results obtained in the two previous studies. (Eckard L., 2016) took into consideration a bigger sample size from the same period of the study conducted in Florida and also in this case the sera came from people who were likely exposed to cattle. Her results were inconclusive to assess the circulation of IDV among human population, at least in the United States, in agreement with the previous study (Hause et al., 2013). However, this does necessarily imply that if the serum samples of the study of White et al. were preabsorbed with ICV they would have shown such a very low seroprevalences as in the other two cases.

To date, no studies were published about IDV seroprevalence in humans outside of the United States. In Europe, only one study[39] about IDV in humans was carried out but in this case it dealt with its molecular detection in respiratory samples. In 2016 Smith et al. indeed analyzed 3300 respiratory samples of Scottish patients collected between 2006 and 2008. The authors were unable to detect Influenza D virus, whereas ICV was detected with a low frequency (0,2%). The authors reported that

[38] White S., Mac W., McDaniel C. J., Grayd G. C., Lednickya J.A., Serologic evidence of exposure to influenza D virus among persons with occupational contact with cattle (2016), Journal of Clinical Virology 81, 31–33.

[39] Smith D.B., Gaunt E.R., Digard P., Templeton K., Simmonds P., Detection of influenza C virus but not influenza D virus in Scottish respiratory samples (2016). Journal of Clinical Virology 74, 50–53.

the real frequencies were probably higher but they failed in detecting it because of a probable RNA degradation of samples due to the prolonged storage.

3.3 Animal models to assess IDV zoonotic transmission

Even though there are different animal models to study Influenza infection, the animal of choice for all the Influenza genera remains the ferret. Its susceptibility for these viruses was discovered in the 1930's[40] and it owns lots of advantages if compared to mice, which are normally the first choice in different fields of clinical research due to their low cost and easy handling. For Influenza viruses, ferrets remain the best choice because they manifest almost all clinical symptoms that affect also humans following the infection. For instance, after experimental infections some of the observed clinical signs are sneezing, nasal discharge, lethargy, fever, weight loss. Virus shedding is also detected. This can be explained (at least in part) because they own the cellular receptors for Influenza viruses in the respiratory tract, thus allowing viral binding. Apart from clinical signs, other advantages of this animal model are their ability to transmit the virus and, above all, their susceptibility to human Influenza viruses without a prior adaptation. However, their use in research is not free from different technical and scientific bias; for example, their genetic heterogeneity and their availability are some limiting factors, together with the requirement of a complex husbandry facility and caging system.

Since their discovery as models, ferrets have been studied for Influenza A and Influenza B virus infections. As far as Influenza C virus is concerned, even if it was discovered in 1947[41] there is still no animal model developped in order to study the infection in humans (Eckard, 2016). When IDV was first isolated, the ferret was used as a model in order to assess if the virus was able to infect and to replicate in this species (Hause et al., 2013), thus suggesting a possible zoonotic transmission. In this study, ferrets were inoculated intranasally with IDV and the virus was detectable

[40] Oh D.Y., Hurt A.C. Using the Ferret as an Animal Model for Investigating Influenza Antiviral Effectiveness (2016). Frontiers in Microbiology.7:80. 0

[41] Crescenzo-Chaigne C., van der Werf S. Rescue of Influenza C Virus from Recombinant DNA (2007). Journal of virology, p. 11282–11289.

after 3 days in nasal washes and after 7 days in ferrets that were exposed to the inoculated animals. In this case ferrets did not show any respiratory signs and the histopathological analysis of lung tissues did not reveal the typical Influenza lesions. (Eckard L., 2016) performed an experimental infection with three ferrets using IDV; since no animal models are available for human ICV study, infections with this virus were performed as well. The experiment was carried out using the lineages D/OK for IDV and C/Victoria and C/Kowloon for ICV. After the infection, viral replication, transmission and seroconversion were evaluated in ferrets. The study demonstrated that IDV is able to replicate in this animal model and its maximum shedding was achieved at day 4 post-infection in two ferrets infected with D/OK and one ferret infected with C/Kowloon, whereas the infection was cleared by day 6. The virus was not detected in ferrets infected with C/Victoria lineage, neither in directly infected animals nor in the contact group. As for D/OK, all three ferrets of the contact group shed an avarage of 10^2.5 TCID50/mL virus titer. Only one contact ferret exposed to C/Kowloon had detectable virus. No clinical signs were reported neither for ICV infections nor IDV infection, results which are in agreement with the previous study (Hause et al., 2013). HI assays were performed 14 days post infection to assess if the infected ferrets seroconverted: for D/OK no anti-IDV antibodies were detected neither in infected nor in the contact group animals, even if virus had been detected. On the contrary, seroconversion was detected in ferrets infected by C/Kowloon and also in the contact group individuals. Even if viral shedding of C/Victoria was not observed in infected ferrets, anti-ICV antibodies were detected in these same animals, suggesting that they could be susceptible to the virus despite the absence of viral shedding. Even if ferrets are a great model for Influenza A virus, the results of this study show a certain incongruence between virus replication and seroconversion which, as the author stated, were confirmed in a second repeated experiment. What emerges from this study is that ferrets are not the ideal model for Influenza C virus infections and maybe not even the ideal one for the novel Influenza D virus. Better results could be perhaps obtained after a virus adaptation in this animal species, following multiple viral replication cycles.

Together with ferrets, another animal model to study Influenza D virus infection has been recently developed. In a study, IDV replication and transmission was investigated in a guinea pig model[42]. In a 3-week experiment, the animals were divided into 3 experimental groups: one was used to study the growth kinetics of the virus, which consisted of 10 directly inoculated animals and 3 controls. The guinea pigs were inoculated with the bovine IDV lineage D/bovine/Oklahoma/660/2013. A second group was used to study the direct contact transmission and the last one to investigate the aerosol transmission. For these two last experimental groups, 6 guinea pigs were inoculated intranasally with the same virus, 6 remained uninfected; to study the aerosol transmission, 3 inoculated animals were put into a cage that separated them from 3 other non-inoculated animals. The cage permitted the aerosol droplets to pass but did not allow direct contact between guinea pigs.

The inoculated animals of the first kinetics study group did not show any clinical sign but the virus was detectable in nasal washes for the first 7 days post infection, showing that IDV is able to replicate in the upper respiratory tract. In addition, the virus was also found in lung tissues, indicating that in this animal IDV is able to replicate in all the respiratory tract; on the contrary, in ferrets the virus was only detected in the upper respiratory tract and not in lungs. HI assays were performed in order to evaluate the seroconversion in the inoculated animals: after 7 and 9 days post infection specific anti-IDV antibodies were detected in sera of the directly infected animals, showing that guinea pigs are a potential good model to study Influenza D infection. As regards the direct contact transmission, two of the three sentinels acquired the infection from the directly infected guinea pigs and the viral presence was verified in nasal washes; the same three animals seroconverted 14 days after exposure to infected animals. This result thus shows that IDV can be transmitted between guinea pigs by direct contact. None of the three aerosol-exposed animals had detectable virus in nasal washes but they all seroconverted at day 14 post exposure.

[42] Sreenivasan C., Thomas M., Sheng Z., Hause B.M., Collin E.A., Knudsen D.E.B., Pillatzki A., Nelson E., Wang D., Kaushik R.S., Li F. Replication and transmission of the novel bovine influenza D virus in a guinea pig model (2015). Journal of Virology 89:11990 –12001.

Overall, guinea pigs seem to be a good animal model to study Influenza D virus infection since it is able to replicate not only in the upper respiratory tract but also in the lower one. Also, the virus was transmitted among animals by direct contact and both the infected and contact animals seroconverted. However, pathogenicity features normally caused by Influenza viruses infection (such as fever and weight loss) are missing in this model, showing that either humans are potentially missing any clinical sign as well or, on the contrary, guinea pigs do not reflect the clinical symptoms that could affect humans if infected with IDV. Data about eventual pathology and symptoms in humans after Influenza D virus infection are completely missing until now.

3.4 IDV: a possible health risk for humans?

Taking everything into consideration, up to now there is little evidence that proves that humans are likely to be infected with Influenza D virus. Anti-IDV antibodies have been found in human sera after cattle exposure with very low seroprevalences and the chosen animal model to study the mechanisms of Influenza virus infection in humans, the ferret, is susceptible to the infection but does not show any clinical sign. Despite this, as reported in the second chapter, IDV is able to bind to the 9-O-Ac-Sia receptor in human trachea, as shown by immunochemical staining experiments. However, once the HEF-domain is bound to the receptor it is not clear if humans own the tripsin-like serin proteases that cut the HEF protein into two subunits and allow the virus entrance in the cell. These proteases have not still been characterized neither for humans nor for bovines.

As demonstrated by the molecular study that calculated the t-MRCA (ref. Second chapter), this virus probably originated from the human infecting Influenza C virus about 1500 years ago, showing that it can still be considered as a “young virus” and maybe by continuing its evolution it could rapidly adapt to humans.

If the virus spread was obstacled in the primary host, cattle, the potential to extend its infection to other species, and eventually also to humans, would be reduced. There are already some studies that consider the development of a vaccine for cattle[43][44] and other studies about the identification of new antiviral drug targets (i.e. M2 ion channel blocker) are still ongoing[45].

Most importantly, there are various concerns about the fact that IDV is able to infect swine, since it was already demonstrated for Influenza A virus that this animal has a role of "mixing vessel" that allows to create reassortants able to infect humans too. Up to now, seroprevalence in human serum samples has only been searched in the United States in two studies and not in Europe, Africa or Asia; therefore, seroprevalence in humans could be underestimated. The absence of an effective seroprevalence in humans and the fact that the virus has not been detected in human samples do not allow to consider Influenza D virus a zoonosis to date. More studies to investigate its zoonotic potential are currently taking place.

[43] Hause B., Huntimerc L., Falkenbergc S., Henningsona J., Lechtenbergd K., Halburc T. An inactivated influenza D virus vaccine partially protects cattle from respiratory disease caused by homologous challenge (2017), Veterinary Microbiology 199, 47–53

[44] Wan Y., Kang G., Sreenivasan C., Daharsh L., Zhang J., Fan W., Wang D., Moriyama H., Li F., Li.Q. A DNA vaccine expressing consensus hemagglutinin-esterase fusion protein protected guinea pigs from infection by two lineages of influenza D virus (2018) Journal of Virology. JVI.00110-18.

[45] Kesinger E., Liu J., Jensen A., Chia C.P., Demers A., Moriyama H. Influenza D virus M2 protein exhibits ion channel activity in Xenopus laevis oocytes (2018). PLoS ONE 13(6): e0199227.

IV. State of the art and aim of the work

IDV was first isolated in April 2011 from nasal swabs of swine exhibiting influenza-like symptoms in the USA (Hause et al, 2013). Next generation sequencing allowed for a full genome characterization: IDV harbors a segmented single stranded negative sense RNA genome (7 segments) sharing 50% identity with influenza C virus (ICV) genome. Antigenically, influenza A, B, C, and D viruses/antisera were shown not to cross-react (Hause et al, 2013). 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 in the Influenza virus family. The new genus is currently known as Influenza D virus, extending therefore the *Orthomyxoviridae* from six to seven genera.

So far, IDV has been detected in different animal species and on almost all continents. Some studies describe its molecular detection but many serologic evidences for anti-IDV antibodies presence are also available. At the moment the susceptible species seem to be the bovine, the swine, the small ruminants and it appears that horses could be infected as well. Whether humans are likely to be potential hosts of IDV is still unclear: studies in the ferret model (where IDV replicates efficiently) as well as IDV receptors characterizations suggest that humans may be susceptible, but serious serological or virological evidences are still lacking to evidence the species jump (Hause et al, 2013; Song et al, 2016; Eckart 2016; White et al, 2016).

In France, IDV was identified in 2011-2014 in cattle with respiratory clinical signs (Ducatez et al, 2015). However, the extent of IDV circulation in French cattle and small ruminants was not known. The aim of the master's project is therefore to (i) detect and characterize IDV in French calves to assess the virus prevalence and genetic diversity and (ii) evaluate IDV seroprevalence in cattle and small ruminants in 5 French regions (Vendée, Bretagne, Nord, Aveyron and Ariège).

V. Materials and methods

5.1 Sample collection in French calf farms

In February 2018, 3 veal calves' farms were visited and nasal swabs were collected for IDV screening. The farms were located in the Occitanie region in France (southwest of France), nearby the city of Toulouse. Calves were between 2 and 4 weeks old and they had limited respiratory signs in all the three farms that were visited. In total, 145 nasal swabs were collected: 50 in the first farm (Farm A), 45 in the second one (Farm B) and 50 in the last one (Farm C). Swabs were inserted deep in the calf nostril and rotated for 30 seconds on the nasal mucosae. Then, they were placed in PBS and kept at 4°C from the farm to the laboratory (a few hours maximum) before they were stored at -80°C before further testing.

5.2 IDV detection through RT-qPCR

Viral RNA was extracted using the Qiagen Viral RNA minikit (Qiagen) following the manufacturer instructions and Real-time RT-PCR was carried out to detect Influenza D virus. The Real-Time PCR was performed by using the QuantiNova Probe RT-PCR Kit (Qiagen) with a Taqman protocol assay, as already described in a previous study (Ducatez et al., 2015). The primers used in the assay are designed to amplify a small portion of the polymerase gene PB1 (second segment of IDV genome), which is one of the most conserved genes among Influenza viruses. Samples with a Ct (threshold cycle) lower than 40 were considered positive.

D/OK Forw	5'-GCT GTT TGC AAG TTG ATG GG-3'
D/OK Rev	5'-TGA AAG CAG GTA ACT CCA AGG-3'
D/OK Probe	5'-TTC AGG CAA GCA CCC GTA GGA TT-3'

Table 1: Primer and probe sequences used for the Real-Time RT-PCR screening

5.3 Virus isolation on cell cultures

Virus isolation on cell cultures was carried out for some of the positive swabs with the lowest Ct value (A13, B19, B20). IDV genomes of remaining samples were directly sequenced from extracted viral RNA. Both cell lines were tested for *Mycoplasma spp*. before use. Uninfected cells were harvested using Gibco™ DMEM high glucose medium implemented with Penicillin-Streptomycin and 10% of fetal calf serum. Infected samples were filtered with a 0,22µm filter and they were first inoculated on 80% confluent hRT-18g cell line (Human colorectal adenocarcinoma), whereas the second passage was then performed on ST cells (swine testis). All samples were tested in duplicate and with three different dilutions (1:2, 1:10, 1:50). Cells were infected using the medium Gibco™ Opti-MEM™ Reduced Serum Medium. The medium was implemented with TPCK trypsin (1µg/ml, Thermo Fisher Scientific, USA), Amphotericin B (2.5 µg/ml, Sigma-Aldrich, USA), BM-cyclin (15 µg/ml, Sigma-Aldrich, USA) and Cyprofloxacin (10 µg/ml, Sigma-Aldrich, USA). After the infection, cells were incubated 4 days for each passage at 37°C and then supernatant was collected and used to amplify the seven gene segments of the virus.

5.4 Sequencing and phylogenetic analyses

The whole genome of the isolated virus was amplified by RT-PCR and then molecularly characterized by using Sanger sequencing technology. The RT-PCR was performed using the QIAGEN OneStep RT-PCR Kit (Qiagen) using the primers reported in Table 2. Samples were sequenced on a 3130XL Applied Biosystems capillary sequencer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were manually checked with BioEdit v7.1 and compared with nucleotide sequence data available on GenBank. Multiple sequence alignments were generated using ClustalW (Thompson, 1994). Maximum Likelihood trees were created using MEGA v7, testing each time for each gene the best DNA model before performing the phylogenetic analysis. To construct the phylogenetic trees the Bootstrap statistical analysis was included and 1000 replicates were used.

Hemagglutininesterase (HEF)	1F 5'-AGCATAAGCAGGAGATTTTCAAAG-3' 745R 5'-GCACTACATGCTTGTTGC-3' 667F 5'-GTTTGTGGGACTGAGCAATC-3' 1350R 5'- CCCTGCTTGCGGTATTATC-3' 1267F 5-'CCCAAGTATGGCAGATG-3' 2042R 5'- GCAAGGAGATTTTTTCTAAGATT-3'
Matrix (MP)	8F 5'-GCAGAGGATATTTTTGACGC-3' 670R 5'-CCCATATGCTATTCTTGCCAG-3' 602F 5'-AAAAAAGAGGCCCAGGCAC-3' 1212R 5'-GCAAGAGGATTTTTTCGCG-3'
Nucleoprotein (NP)	1F 5'-GGCATAAGCAGGAGATTATTAAGC-3' 949R 5'-TAAAGGCTCTTACTCCAGAATA-3' 849F 5'-GCCTTGGTCAATGTGGCTG-3' 1717R 5'-GGGGACTGCAACAGAACCA-3'
Nonstructural protein (NS)	8F 5'-GCAGGGGTGTACAATTTCAAT-3' 804R 5'-TCGAAACTGACTTGATTTCATCC-3'
Polymerase basic 1 (PB1)	1F 5'-GGCATAAGCAGAGGATTTTAT-3' 736R 5'-TTTTCCTCTTTCTCCGTC-3' 631F 5'-AAAAATGAAGTCTCCAACATTG-3' C/OK-Rev 5'-TGAAAGCAGGTAACTCCAAGG-3' C/OK-Fwd 5'-GCTGTTTGCAAGTTGATGGG-3' 2317R 5'-GATTTTTCTGTTATTAAACAACGC-3'
Polymerase basic 2 (PB2)	1F 5'-GGCATAAGCAGAGGATGTC-3' 882R 5'-CCCTTATCTTCTCTGCTGG-3' 796F 5'-AAAAGAAGAGAGATGTTAGAGC-3' 1776R 5'-TTTTACCCATTATCAAAGCAGG-3' 1724F 5'-GAAAGAATAAACACTGATGATG-3' 2353R 5'-GAGGATTTTTTCAATGTGCTTC-3'
Polymerase 3 (PB3)	1F 5'-GGCATAAGCAGGAGATTTA-3' 759R 5'-TTTTCTTCTAGATGTTCCAGTTTGA-3' 677F 5'-AAAAGAAATCAGGCTGAATGC-3' 1467R 5'-CCAAACAAACAGTCAGTTGA-3' 1394F 5'-CCCGGAAAGGTCAAGATAG-3' 2184R 5'-GGAGATTTTTAACATTACAAGGCC-3'

Table 2: Primers used to amplify and sequence the whole genome of Influenza D virus

5.5 HI assay (Inhibition of Hemagglutination assay)

The HI assay is a serologic test that can be performed using viruses that own envelope proteins called hemagglutinins (HA). These proteins bind to the sialic acid receptors on the cells and are able to bind to erythrocytes and as a result a lattice is formed and it can be viewed as a diffused red solution. These characteristics allow to determine the presence of Influenza viruses in a sample, since the formation of the lattice depends on the concentrations of the virus. Multiple diluitions are included in the assay in order to determine the viral titer.

Veterinary diagnostic laboratories from 5 French departments (Bretagne, Nord, Vendée, Ariège and Aveyron) provided us with cohorts of about 500 sera per department (in total n=2337) from cattle collected in 2013-2015. About 20 samples per farm were collected, for a total of 100 farms. Apart from cattle, sera from small ruminants (goats and sheep) were also analyzed in order to assess if IDV circulates in France among these species (in total n=1814), as already described in the United States (Quast et al., 2015). These sera were originally collected for the surveillance of IBR (Infectious Bovine Rinotracheitis) and, in order to avoid interferences with maternal antibodies, only animals aged more than 1 year were tested.

In this work HI assay (Inhibition of Hemagglutination) was performed using the antigen D/bovine/France/5920/2014 and 1% horse RBC (Red Blood Cells) solution, following the OIE protocol on HI test for swine influenza virus antibodies detection. The RBC solution was prepared with fresh blood right before starting the test. To determine the viral titer before starting the HI assay, an HA assay (Hemagglutination assay) was performed in order to prepare a standardized solution of virus. In this assay, a solution of 8 UHA/50µl of IDV was prepared and it was used to determine the presence of anti-IDV antibodies in the analyzed sera. Before testing sera with HI assay, pre-treatments on these were carried out in order to eliminate the possibility of having non-specific inhibitors, thus improving the sensitivity of the assay. Briefly, sera were treated with RDE (Receptor Destroying Enzyme) and incubated overnight at 37°C. The following day they were inactivated at 56°C for 30 minutes.

Sera were then hemabsorbed adding packed horse red blood cells and incubated 1 hour at 4°C with slow shaking before they were centrifuged 10 minutes at 1500 rpm. To do the HI assay, the treated sera were 2-fold diluted with PBS (Phosphate-buffered saline) and they were inoculated with the prepared solution of virus with a titer of 8 UHA/50µl. Sera with the viral solution were incubated 30 minutes at room temperature and then the 1% solution of horse RBC was added. An incubation at 4°C for 45 minutes followed and then the results were read and interpreted. A serum sample was determined as seropositive when the HI titer was greater than or equal to 1:20.

VI. Results: IDV circulation in French ruminants

6.1 Molecular screening of nasal swabs

By molecular screening with Real-Time RT-PCR, nasal swabs collected from three different calves farms were tested for the presence of IDV. As result, this virus was detected in all the three farms that were visited but with a very different prevalence. In the first farm (Farm A), less than half of the sampled calves were IDV positive (40% of nasal swabs). The Ct (threshold cycles) of the analyzed samples ranged from 23 to 37,8. Calves belonging to the second farm (Farm B) had a very high viroprevalence: almost all sampled individuals were IDV positive with a total viroprevalence of 89%. In addition, the Ct were lower than in the first farm, ranging from 19,46 to 31,08. It can be therefore assumed that the infection was widespread in the whole farm and calves were probably sampled in correspondence of the maximum viral shedding. In the third farm (Farm C) the situation was different and only 16% of nasal swabs were positive. The Ct were higher than the other two farms, ranging from 33,74 to 36,57. Prevalences and Ct ranges are summarized in Table 3 below.

	Farm A	**Farm B**	**Farm C**
Nr. of collected nasal swabs	Nr=50	Nr= 45	Nr= 50
% of positive swabs	**40%**	**89%**	**16%**
Ct values range by Real-Time RT-PCR	23 to 37,8	19,46 to 31,08	33,74 to 36,57

Table 3: Viroprevalences and Ct values of the positive swabs for Influenza D virus

6.2 HI assay results: IDV seroprevalence in French cattle

Together with molecular detection and characterization of the isolated IDV strains, seroprevalence of IDV in French cattle was determined as well. 2337 sera collected in 2013-2015 coming from 5 different French departments were analyzed by HI assay in order to detect anti-IDV antibodies. In all the departments positive samples were found, both from cattle and from small ruminants. Seroprevalences in cattle ranged from 38% to 70%, whereas it was much lower in small ruminants, ranging from 1% to 10%. The p-value was calculated in order to confirm that seroprevalences were significantly higher in bovine species ($p<0,05$).

In Nord department, 38% of seroprevalence was found in cattle, 10% in sheep and 6% in goats. In Ille-et-Vilaine, 48% of cattle was seroprevalent, 3% of sheep and 9% of goats. In department of Vendée only bovine serum samples were available and no small ruminants sera were therefore analyzed. In this department it was found a high IDV seroprevalence, i.e. 70%. In Aveyron department 68% of cattle was seropositive, 1% of sheep and 5% of goats. Finally, in Ariège department 53% of cattle was seropositive but no goat serum samples were analyzed; only sheep sera were available and a seroprevalence of 8% was found.

These departments are well distributed along the whole French Country: the Northern department borders Belgium, whereas one of the two departments located in the South borders with Spain. This suggests that IDV circulates in the whole French Country and is not limited to a particular area. In Ille-et-Vilaine and Vendée departments very high seroprevalences were found; this could be explained because of the several livestock exchanges between these two areas. All the results are graphically simplified in the picture in the following page.

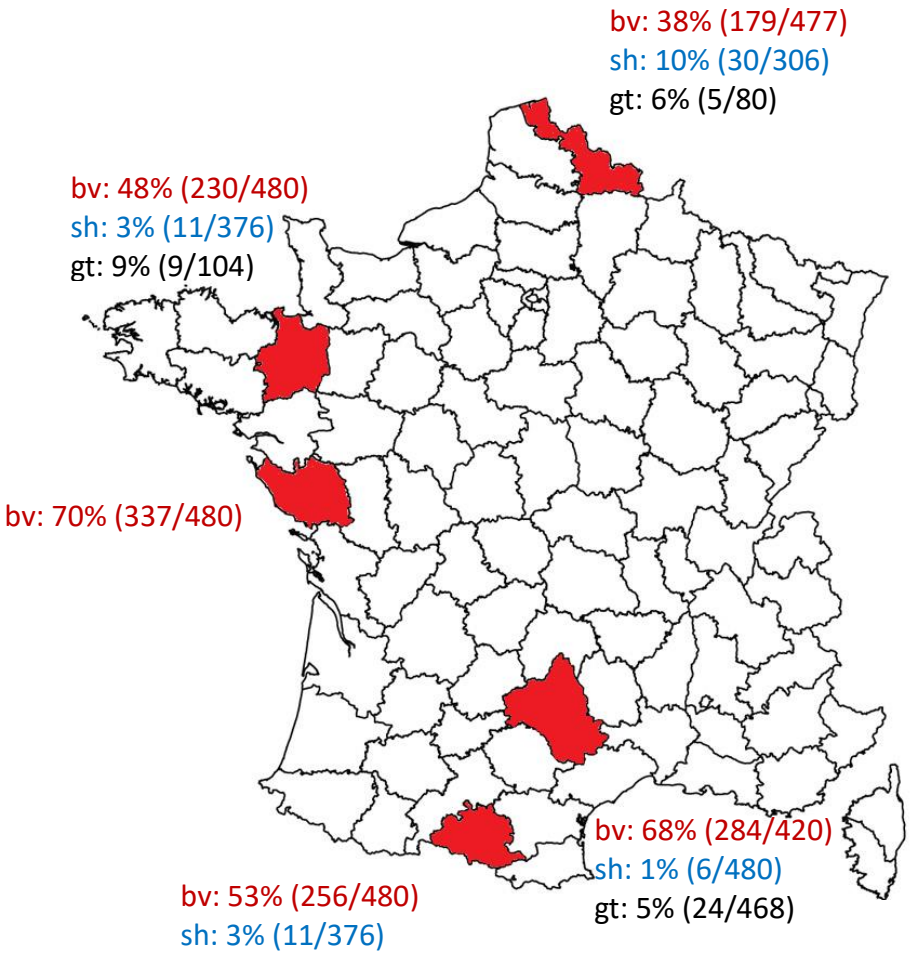
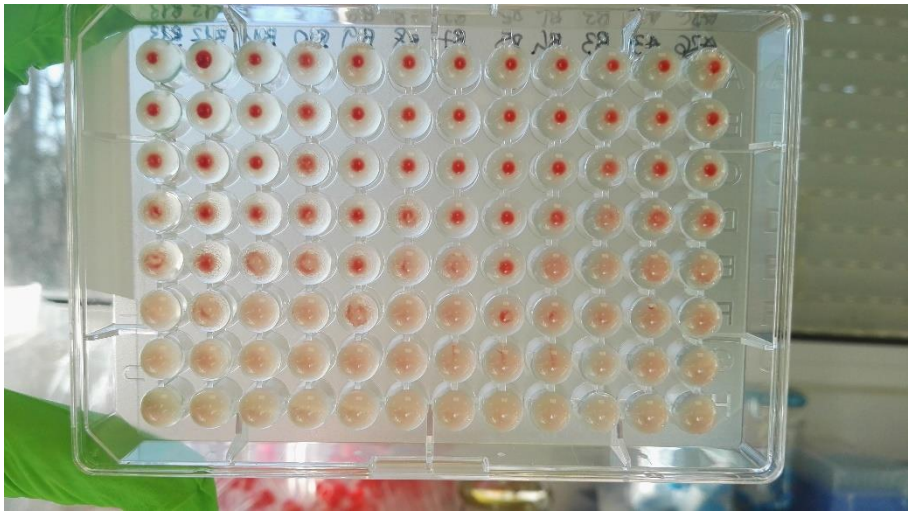


Figure 6: Graphical representation of different IDV seroprevalences in the 5 French departments that were tested. Department's names are from top to bottom: Nord, Ille-et-Vilaine, Vendée, Aveyron, Ariège.



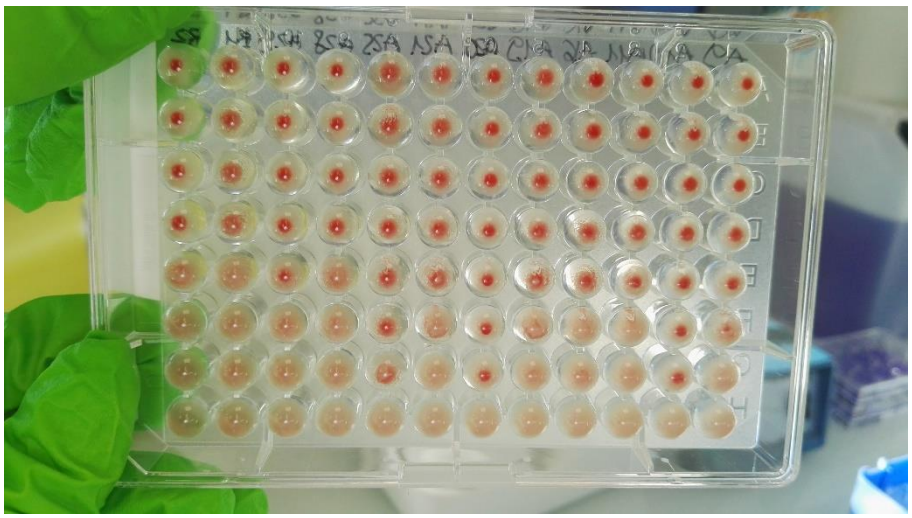


Figure 7: Results of some of the HI assays that were performed on French cattle sera

VII. Results: Full genome molecular characterization of the isolated viruses

7.1 Phylogenetic analyses of Influenza D viruses

In this part of the work, different Influenza D viruses were isolated from positive samples collected in the three farms that were visited. For each virus, the whole genome was sequenced using Sanger sequencing technology. IDV genome, as already described in the introductive chapter, is composed of 7 gene segments. The first three segments encode for the polymerase complex (PB1, PB2 and P3), NS encodes for the non-structural protein, NP encodes for the nucleoprotein, MP encodes for the proteins forming the matrix and, most importantly, HEF encodes for the Hemagglutinin esterase fusion, responsible for the viral binding to receptors and therefore playing a key role in infecting hosts.

In the first farm, only one IDV complete genome (which was named A13) was amplified and succesfully sequenced, since only one sample had a low Ct value that permitted further analyses. Another sample (A31) had a high Ct (=36,92) and only two segments could be sequenced, NS and HEF. In the third farm Ct values of nasal swabs were even higher and it was not possible to obtain a complete genome with Sanger sequencing technology; though, for one sample (C3) three segments were succesfully sequenced (NS, MP and HEF). For samples collected from the second farm the situation was different because the low Ct values and the high number of positive samples allowed to isolate and sequence 4 different complete IDV genomes (B19, B20, B31, B32).

Figure 8: Results of a gel electrophoresis showing the full genome amplification of Influenza D virus isolated from individual B31.

From left to right: 100 bp ladder, HEF gene (3 amplicons), MP gene (2 amplicons), NP gene (2 amplicons), PB1 gene (3 amplicons), PB2 gene (3 amplicons), P3 gene (3 amplicons), 100 bp ladder.

For each of the seven IDV segments phylogenetic analyses were performed. Sequences obtained in this part of the work were aligned with all the sequences available on GenBank for each gene. The aim of the phylogenetic analyses was to confirm if these isolated viruses belonged to D/OK-type, which is the only one that is currently known to circulate in Europe. However, in 2012 in France a lineage which differs from the others isolated in Italy, France and Ireland was identified; this same lineage clusters with another one that was detected in Ireland in 2014, still from bovine species, thus suggesting that in Europe there could be two circulating lineages: a major circulating lineage (D/OK-type) and this genetically distinguishable lineage (also different from the D/660 and Japan lineages) with only two sequences available at the moment: D/bovine/France/2986/2012 and D/bovine/Ireland/007780/2014. On the contrary, in the United States both D/OK-type and D/660-type seem to be present; all IDV isolated in Japan seem to belong to another lineage which is genetically different from the other two (Japan-type).

As for the analyses carried out in this study, for most of the segments also the Chinese IDV strains seemed to genetically differ from D/OK-type and D/660-type and also from the Japanese ones, forming a different cluster. This was however not observed for instance for the HEF gene: IDV lineages (detected in the Guangdong and Shandong provinces) cluster with D/OK-type and are not too genetically distant from D/swine/Oklahoma/1334/2011. This last lineage is important because it contains the first IDV isolate and it represents the D/OK-type. On the contrary, the genome of reference for the D/660-type is D/bovine/Oklahoma/660/2013.

All segments of all Influenza D viruses characterized here seem to belong to D/OK-type; no segments belonging to the D/660-type were found. A part from this work, no reassortments were found neither in the previous study in France (Ducatez et al., 2015), nor in Italy and Ireland. This means that, up to now, no reassortments among IDV lineages have been detected in Europe. This is in strong contrast with the results obtained in the United States, where an important number of reassortments among IDV lineages has been found even in a limited cohort of positive samples (Collins et al., 2015).

The remaining phylogenetic trees of all other segments are available in supplemental material section.

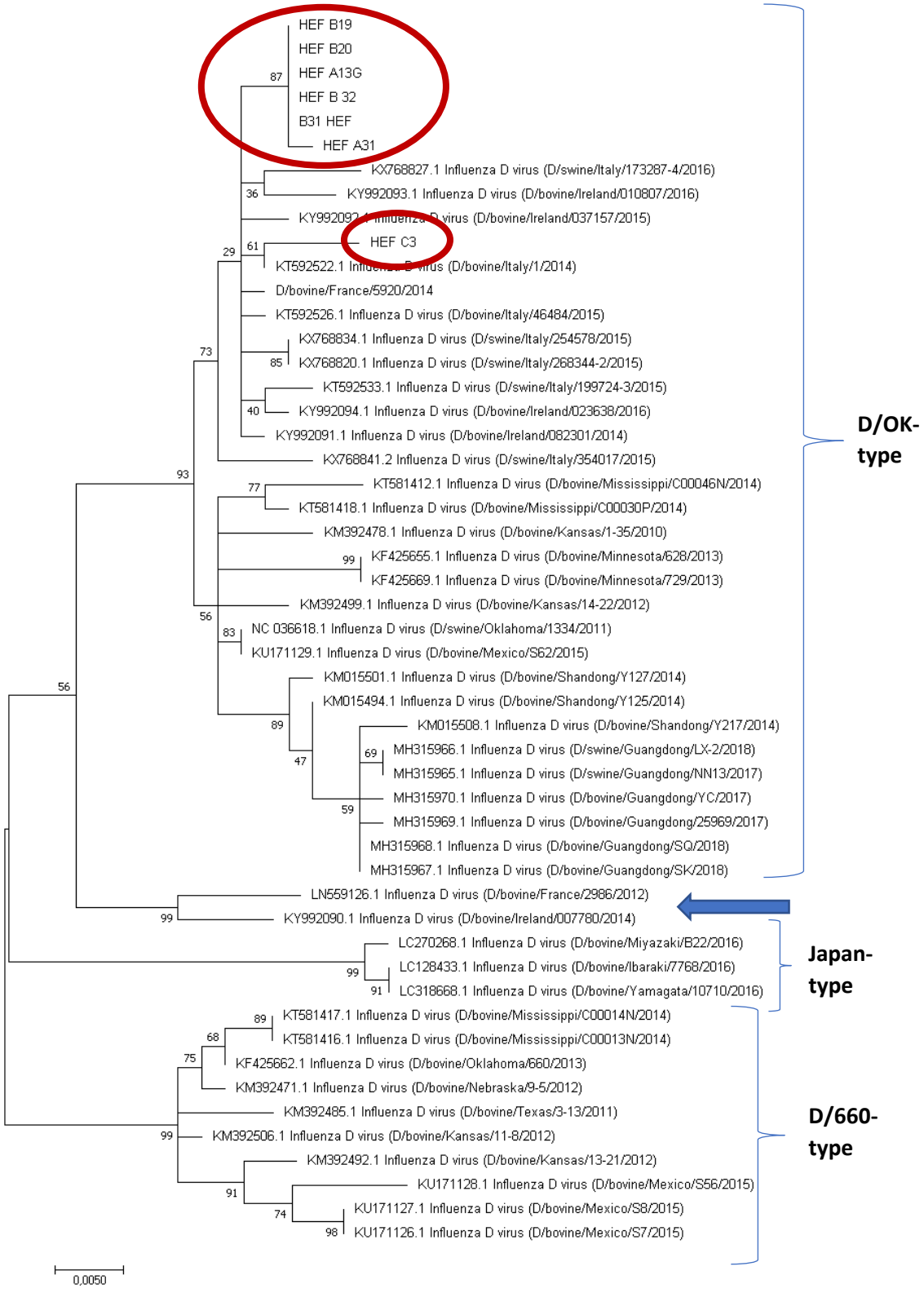


Figure 9: Phylogenetic tree of HEF segment; Influenza D viruses characterized in this piece of work were highlighted in the red circle. The arrow indicates the two viruses from France and Ireland that are genetically different from the D/OK-type.

Changes at amino acids level were also taken into consideration. Nucleic sequences of the alignment used to construct HEF phylogenetic tree were translated into amino acids; the aim of this analysis was to see if there were any changes in IDV sequences detected in the three calves farms compared to IDV detected in the rest of Europe and other continents. In addition, HEF mutations at amino acid level that could modify its structure and therefore eventually its virulence were also searched.

In two positions, an amino acid mutation was found and it was referable only to the 7 HEF segments sequenced in this work. Thus, they could be considered as a molecular marker of IDV strains detected in French calves in 2018. No differences were found among IDV sequenced from extracted viral RNA and IDV sequenced after being isolated on cell culture; this shows that, as for this segment, no changes occurred at amino acid level after cell lines infection. On the contrary, in four other positions an amino acid mutation was common to all European IDV, ie viruses detected in Ireland, Italy and France. The only exceptions were the two genetically different strains D/bovine/France/2986/2012 and D/bovine/Ireland/007780/2014, thus showing that in this case this difference with the other European isolates occurs also at the amino acid level.

Aa Position in HEF	Type of mutation	Specificity of the mutation
Pos 56	T → I	French calves 2018
Pos 400	Y → H	Most French calves 2018
Pos 289	A → V	Most European IDV
Pos 409	K → R	All European IDV
Pos 563	I → L	All European IDV
Pos 652	A → V	All European IDV

Table 4: Amino acidic mutations in isolated IDV genomes

For positions 289 and 400 the mutation did not occur for HEF sequenced from farm C, which from phylogenetic analyses resulted to be genetically distinguishable from IDV detected in farm A and B.

The lack of data did not allow to predict how these mutations could affect HEF in comparison with other IDV lineages of previous works. By consulting the article by Song et al. (2016), which contains relevant information about HEF structure and amino acid sequence, it seems that these mutations are not implied in particular changes of HEF function and its effective structure. In this publication D/swine/Oklahoma/1334/2011 was used, therefore changes in amino acid sequence that affected HEF structure could actually have occurred in these newly isolated IDV from French calves.

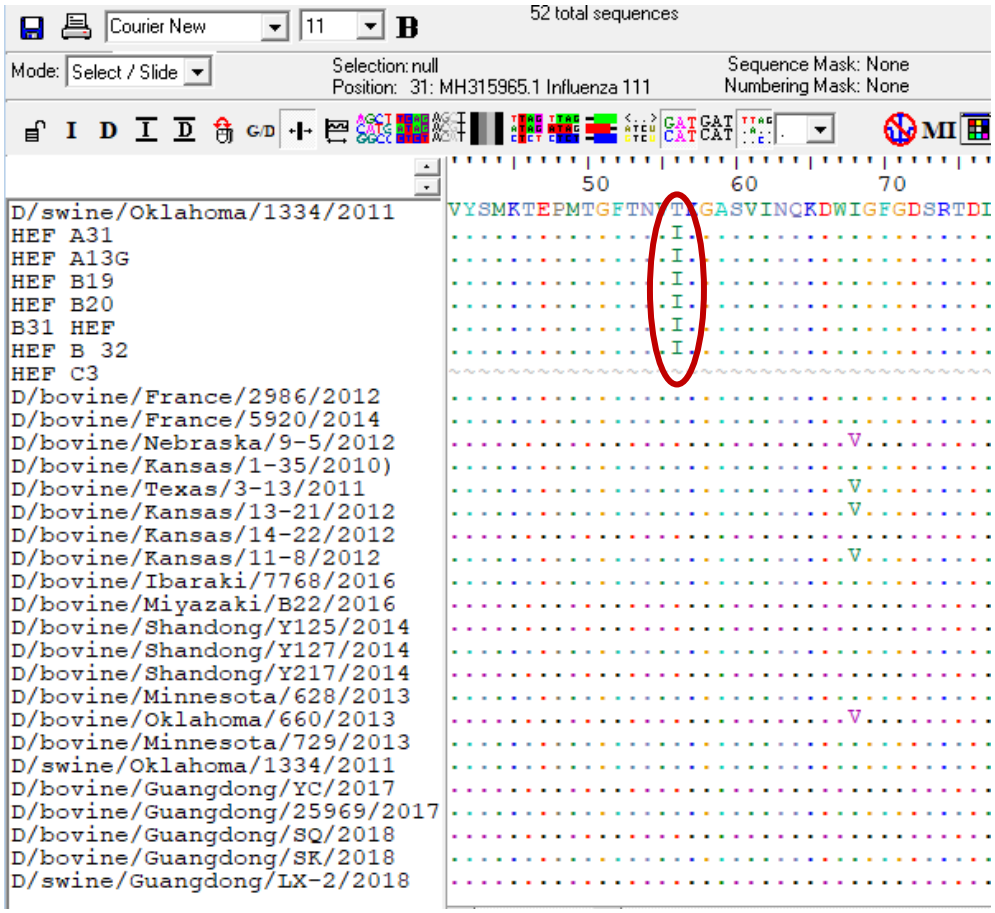


Figure 10: alignment of HEF sequences translated into amino acids by using BioEdit v7.1; in the red circle the mutation pos56 T→I specific of IDV isolated from French calves farms in 2018.

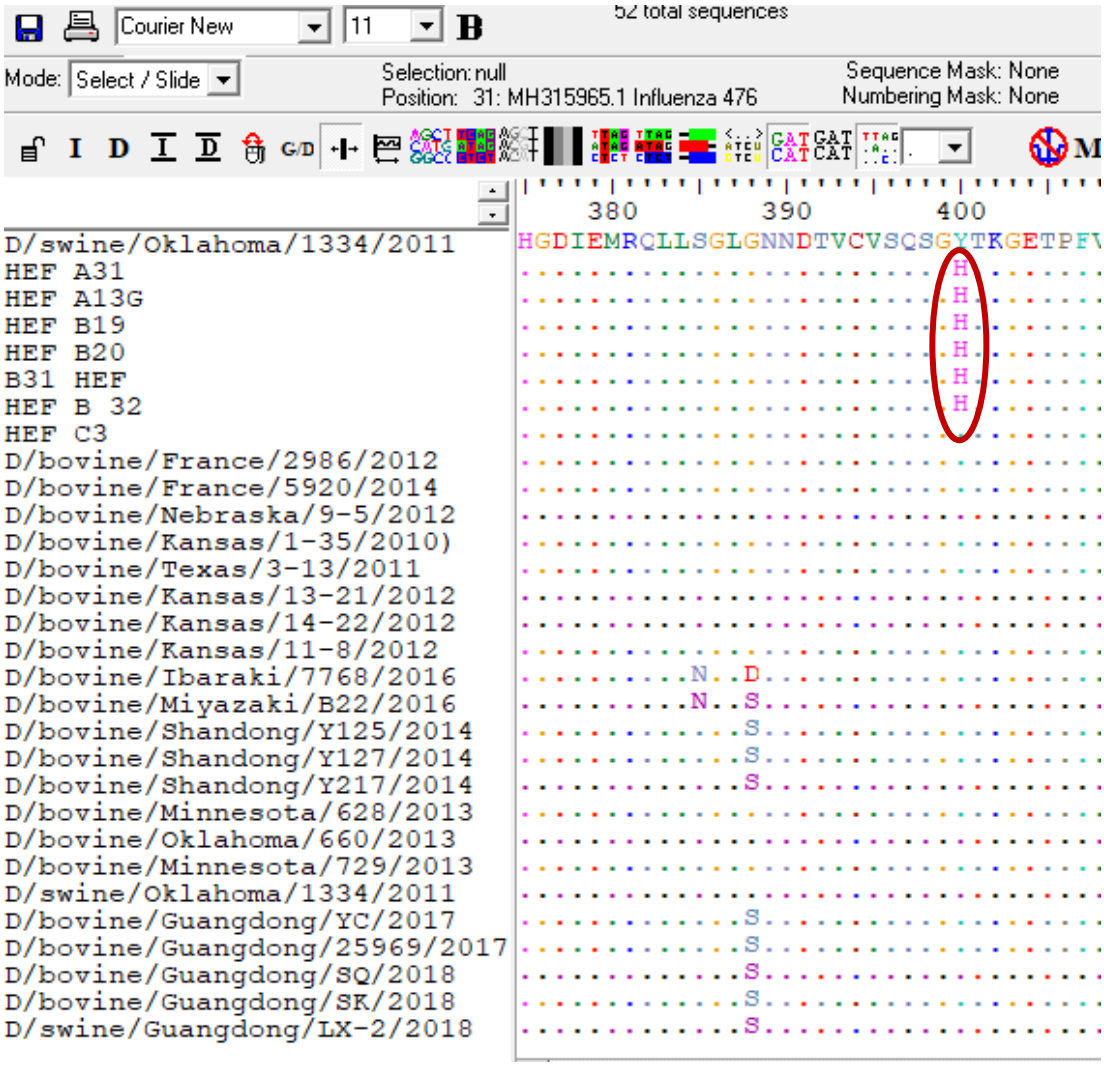


Figure 11: alignment of HEF sequences translated into amino acids by using BioEdit v7.1; in the red circle the mutation pos400 Y→H specific of IDV isolated from French calves farms in 2018.

Pictures of remaining European strain-specific mutations are available in supplemental material section.

7.2 Characterization of the isolated lineages: genetic differences intra- and inter farms

Distance matrices were built in order to assess genetic distance among the sequenced viruses. In particular, the aim of these analyses was to determine the genetic differences among viruses that were isolated in the same farm but also to compare viruses isolated from different farms. The limit of this investigation is the low number of sequenced genomes, which were, as described in the previous chapter, seven (two of which were only partial).

Segment	Viruses from farm B	Genetic distance	Genetic identity	Viruses from farm A	Genetic distance	Genetic identity
MP	B19-B20-B31-B32	0,0%	100%			
NS	B19-B20-B31-B32	0,0%	100%	A13-A31	0,0%	100%
HEF	B19-B20-B31-B32	0,0%	100%	A13-A31	0,2%	99,8%
P3	B19-B20-B31-B32	0,2%	99,8%			
NP	B19-B20	0,3%	99,7%			
PB1	B19-B20	0,4%	99,6%			
PB2	B19-B20	0,8%	99,2%			

Table 5: Intrafarm genetic distances between IDV isolates

The table above shows the results of the genetic distance analysis based on the distance matrix. Comparisons were only possible for farm B and for farm A but in this last case only for two genes, since only NS and HEF were sequenced for A31 specimen. Intrafarm genetic distance was not calculated for farm C since only one genome was sequenced. The results show a very low genetic variability among viruses isolated in the same farm from different calves, showing that probably there was the exact same virus circulating and infecting calves in the same farm and not two lineages at the same time. In farm B, the most variable segments resulted to be genes codifying for the polymerase complex, whereas HEF, NS and MP were the most conserved ones. Also in farm A, HEF and NS showed a very high conservation and low genetic distance.

Segment	Inter-farms comparisons	Genetic distances	Genetic identities
HEF	A-B	0,2%	99,8%
	B-C	1,2%	98,8%
	A-C	1,4%	98,6%
NP	A-B	0,4%	99,6%
P3	A-B	0,4%	99,6%
PB1	A-B	0,4%	99,6%
PB2	A-B	0,6%	99,4%
MP	A-B	0,8%	99,2%
	B-C	0,8%	99,2%
	A-C	0,8%	99,2%
NS	A-B	0,8%	99,2%
	B-C	1,3%	98,7%
	A-C	1,3%	98,7%

Table 6: interfarm genetic distances between IDV isolates

The table above shows different results when comparing genomes of viruses isolated in different farms. From the analysis it emerges that IDV isolated in farm A and in farm B do not show a high variability among each other: the most different genes (MP and NS) have a genetic distance of 0,8%, whereas HEF for istance is almost identical in viruses from the two farms.

On the contrary, when taking into consideration IDV detected in farm C this seems to be slightly different from IDV from farm A and farm B. This difference can be observed in the underlined values in the table above and the same result also emerged from the phylogenetic analysis that was performed. For farm C only one partial genome was sequenced, i.e. segments MP, HEF and NS. MP shows the same variability among the three farms but HEF and NS of farm C are clearly genetically distinguishable from the same segments in the other two farms.

This means that, as for these partial results, IDV viruses from farms A and B are very similar and in the phylogenetic tree it can be observed that they belong to the same cluster. However, IDV isolated from farm C seems to be genetically different, even if it still belongs to D/OK-type lineage. This difference can be graphically seen in the phylogenetic tree of HEF already shown in paragraph 7.1 (page 64) and also in the tree representing the phylogenetic analysis of the NS fragment below:

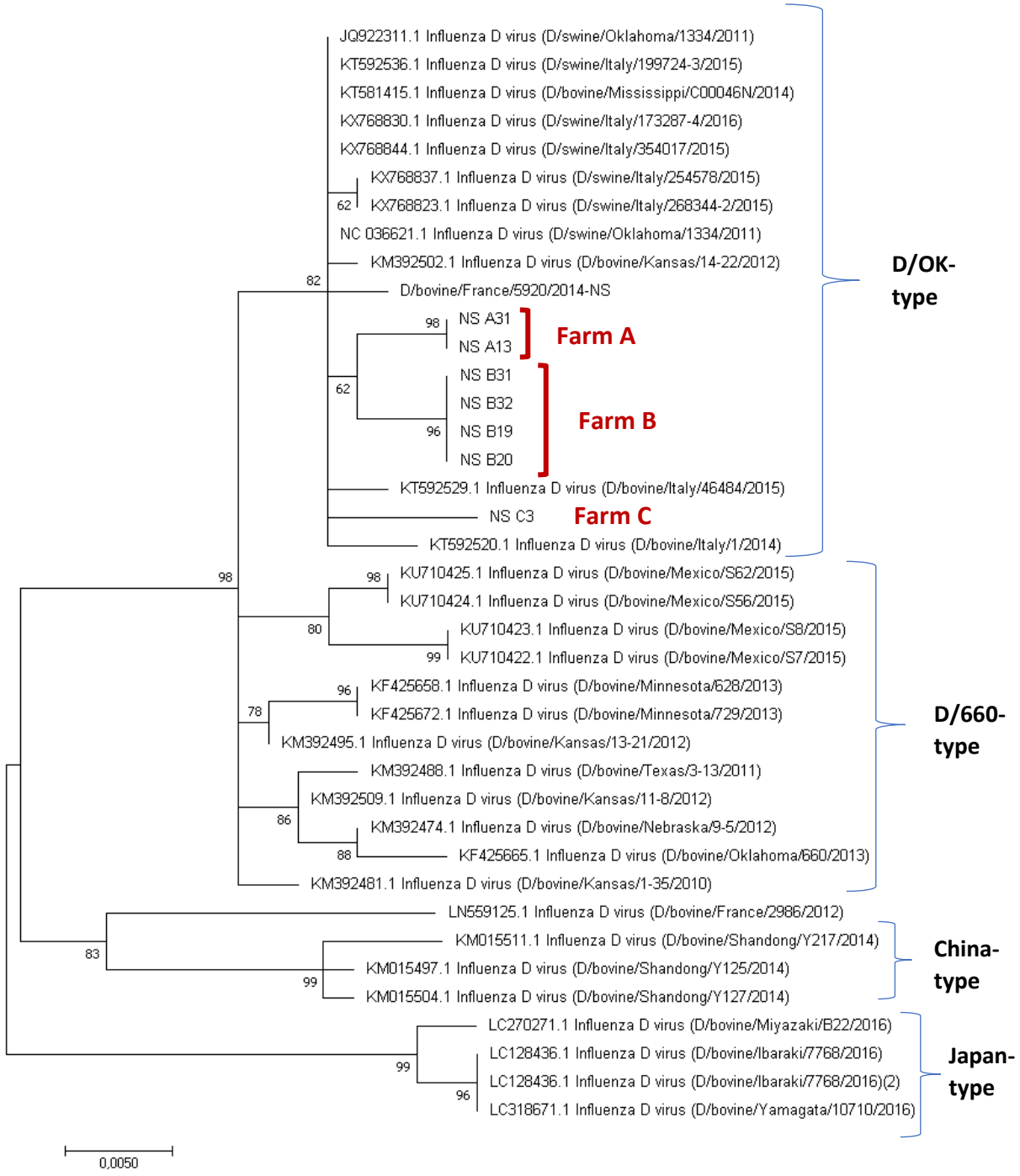


Figure 12: Phylogenetic tree of the NS segment (Nonstructural protein); in the figure all IDV sequences from the three different farms are highlighted

VIII. Discussion

The aim of the work was to determine if the novel respiratory Influenza D virus, detected in Europe in 2012 for the first time, was currently circulating in French cattle. To achieve this, two different approaches were used: anti-IDV antibodies detection, thus serology, and virus detection from nasal swabs. The first approach implied a high number of samples coming from the whole Country, whereas for the second one only 150 samples were collected.

The results of serologic analyses indicate that IDV is highly seroprevalent in French cattle and in all departments that were tested; values are similar to those of other publications, for instance to the United States, where an avarage of 45% IDV seroprevalence was found in cattle (Hause et al., 2015). It can be observed that even if the virus is so widespread it was so far not detected; different hypotheses can be made to explain this. For example, the virus had maybe not been detected earlier because it does not cause severe respiratory signs and it could have been confused with other several respiratory pathogens that normally affect cattle farms. Experimental monoinfections with IDV on bovine species confirmed that the virus alone does not cause particularly severe clinical signs (Ferguson et al., 2016). Or simplier, the virus may not have been detected earlier because its existence was unknown and therefore cattle had never been tested for its presence.

Still considering pathological features of IDV infection, the results obtained in this work are in contrast with a previous study (Ferguson et al., 2016) in which authors reported that calves were protected from IDV infection by maternal anti-IDV antibodies at birth (in their study they found a 94% IDV seroprevalence in neonatal calves) and these levels tend to decrease after 3-4 months, thus conferring a more susceptibility to the viral infection. On the contrary, in this work it was shown that IDV can efficiently infect also very young calves: in fact, calves that were sampled in the three farms were only 2 to 4 weeks old. Farmers reported that all of them had taken colostrum, therefore they were likely immunologically protected. Thus, it remains unclear why these young calves showed respiratory signs, even if limited, and high viral shedding even if they had maternal antibodies protection. Concurring

factors can be the different IDV lineage or, more probably, stressful conditions of animals that had just arrived in facilities that could have temporarly caused an immune depression.

On the other side, direct viral isolation from nasal swabs collected in calves farms allowed a full molecular characterization, giving important information about IDV genetics and evolution. Phylogenetic analyses showed that all viruses isolated from the three farms belong to D/OK-type lineage. On the basis of these results there is evidence that IDV is currently circulating among French calves. Despite this, it is important to remember that samples came from a limited geographic area and may not therefore represent the real circulation of the virus in French cattle but only a "portrait" of the situation that was found in farms when nasal swabs were collected. In addition, samples were collected in a restricted period of time: the first two farms were visited the same day at the end of February 2018, whereas samples of the third farm were collected a week after in March 2018. The second farm was visited when the virus probably had its maximum shedding among calves, whereas for instance in the third farm samples were collected when the infection was almost over. The first farm had an avarage situation as compared to the other two. This can explain the different results that were found among farms located in such a restricted geographical area. A correlation among seroprevalence (thus the research of anti-IDV antibodies) and virus detection was not feasible because of the young age of animals, which would have shown false positive results due to maternal antibodies interference.

In this work, no reassortments between different IDV lineages were found; this is in contrast with findings published in the United States (Collins et al., 2015), where authors reported several reassortments in IDV positive samples collected from cattle. These results tend to suggest that in Europe there is only one major lineage circulating (D/OK-type), in contrast to United States where both lineages are present (D/OK- and D/660-type). As a matter of fact, all IDV isolated in Ireland, Italy and France seem to be highly genetically similar and researchers never found reassortments in any of the viruses detected in these countries.

Despite this, there are two viruses that differ from all IDV detected in Europe until now, i.e. D/bovine/France/2986/2012 and D/bovine/Ireland/007780/2014. These two do not fully cluster with D/OK-type viruses but they belong to the same phylogenetic group, despite the relatively high genetic distance. Missing data do not allow to conclude that they effectively represent a second different lineage that might be circulating in Europe and is still not present in the United States.

The higher diversity of IDV lineages in the United States could be due to the different farming system, which consists of very big independant units of livestock, which are commonly called feedlots, where thousands of animals are gathered together and this might increase chances for viruses to reassort. At the same time, it could be hypothesized that in Europe the only major lineage circulating could be due to livestock exchanges among different countries, thus transporting the same virus in a different place. For instance, in Italy beef calves are normally imported from France where they are born (some of the most imported breeds are French meat breeds such as Charolaise, Limousine or Blonde d'Aquitaine). After they are weaned they are then exported to Italy where they are fattened. This could explain, for example, why Italian and French IDV show such a high genetic identity; this finding emerged also from analyses made in this work, where all sequenced IDV segments majorly clustered with IDV detected in Italy.

As for other pathogens, another unknown aspect is why in two different continents (America and Europe) it was detected almost the exactly same virus, which for certain segments share 0% of genetic distance. These two geographic areas are physically separated and no livestock exchanges occur. In this case it could be speculated that a third host could have actively transported the virus and it could be questioned if it could have been humans. This high similarity among Influenza D viruses is still not detected on Asian countries: in Japan it seems to be present a genetically different type which forms a third IDV lineage, which in this work was named Japan-type on phylogenetic analyses. For some segments, IDV isolated in China seem to belong to D/OK-type, but in the majority of cases they are genetically distant and are grouped in a different cluster.

More IDV isolates need to be sequenced in order to clarify this aspect and to confirm if there is effectively a highly omogeneity of IDV circulating in Europe. Another aspect that has still to be clarified is if French swine and cattle share the exact same lineage, which is the case in Italy (Rosignoli et al., 2017).

IX. Conclusions

The results obtained in this work demonstrate the current circulation of the novel emerging respiratory Influenza D virus in French ruminants. Serologic results show that this virus is spread in almost the whole French country with high seroprevalences in bovine species and low seroprevalences in small ruminants. The virus was detected in February 2018 in the Occitanie region in the South-west of France with high viroprevalence in three different calves farms. High viral shedding detected from collected nasal swabs suggest that this virus could have a particular tropism for young calves. Its isolation and molecular characterization allowed to discover that the virus belongs to the European lineage D/OK-type and it shows a high genetic identity with IDV detected in Northern Italy. No-reassortants with other IDV lineages were found. More studies should be performed to isolate and characterize more IDV lineages to verify this effective genetic uniformity of IDV circulating in European cattle.

X. Supplemental material

Phylogenetic trees of IDV segments:

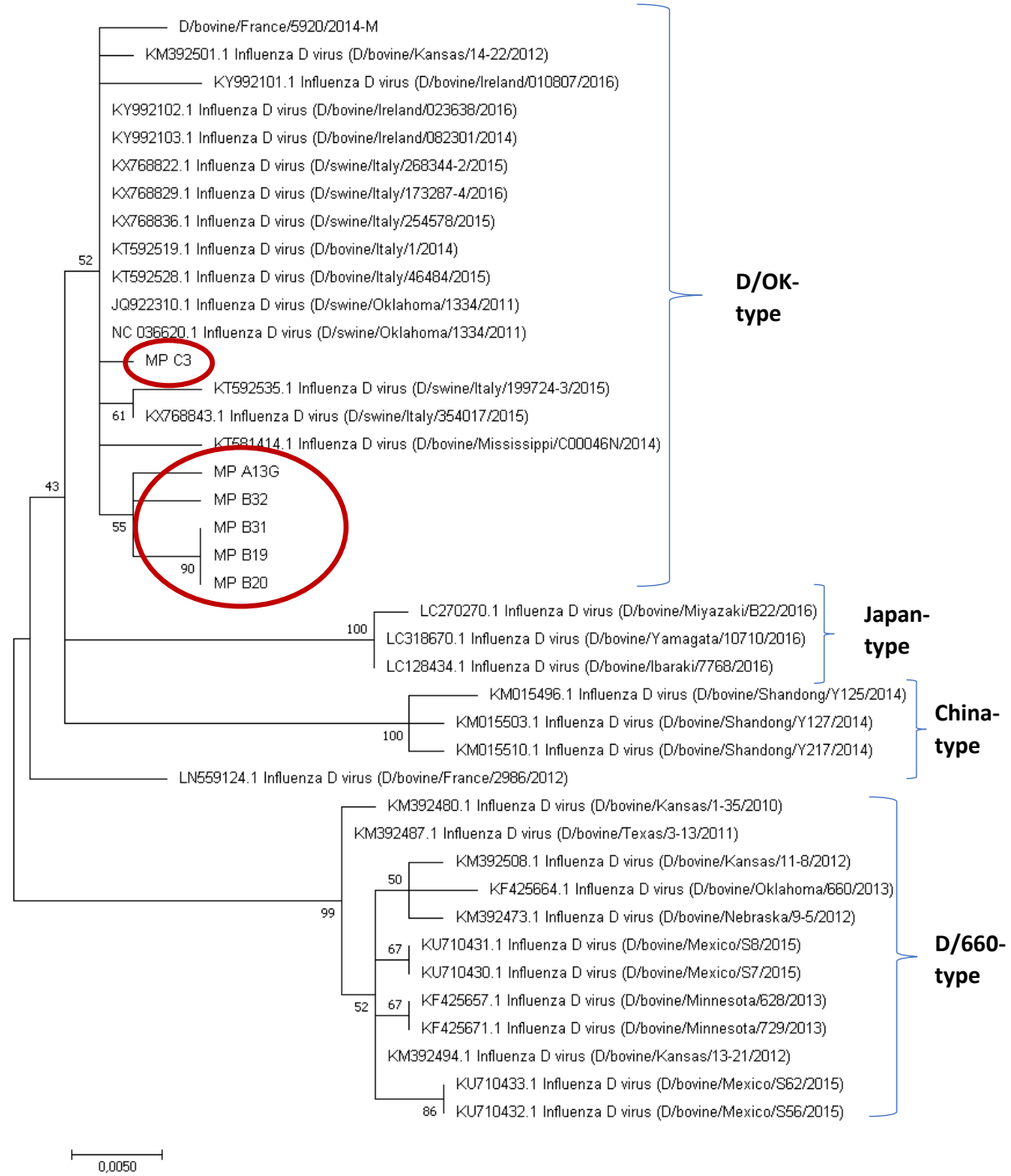


Figure 13: Phyogenetic tree of IDV MP segment

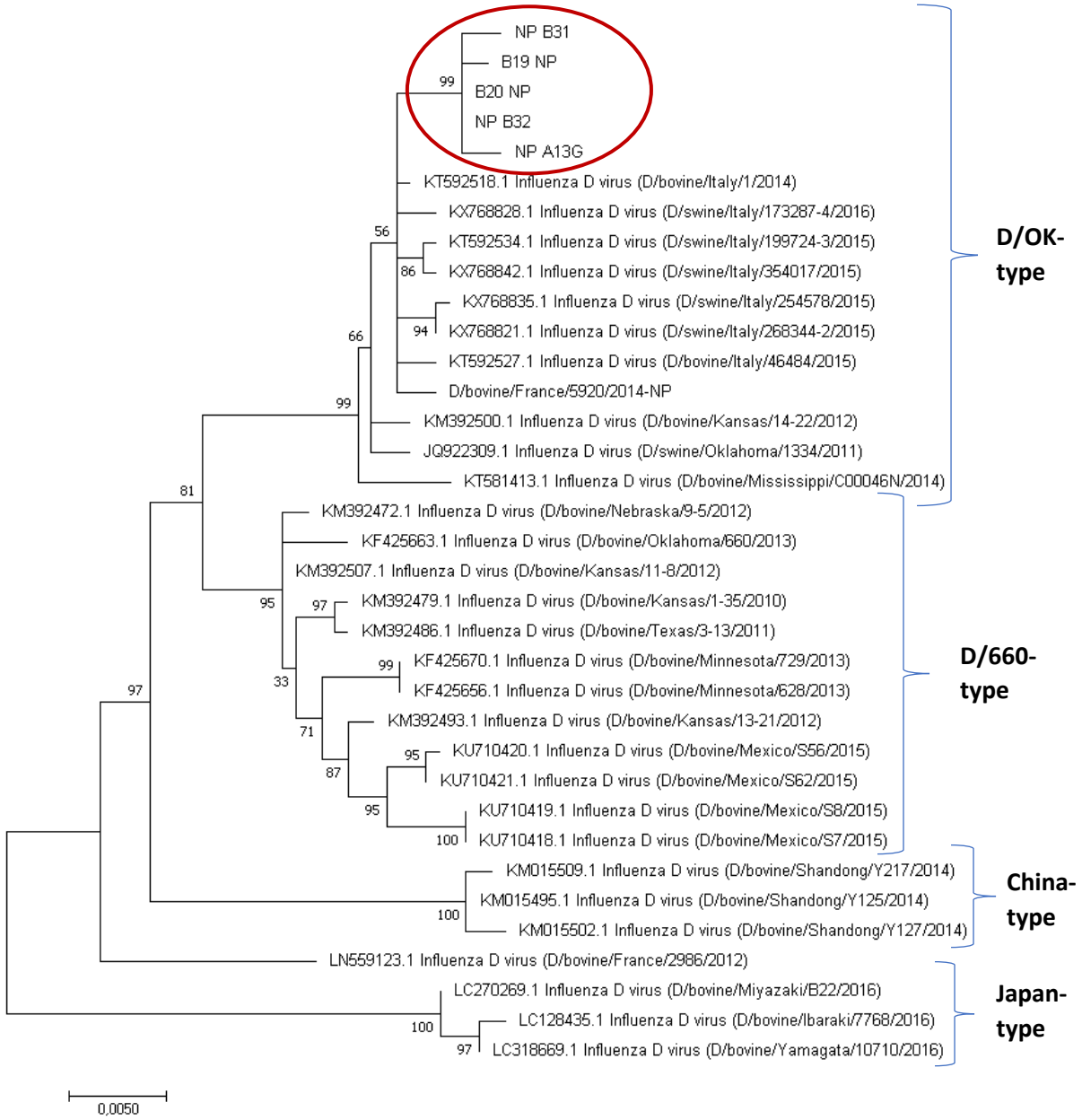


Figure 14: Phyogenetic tree of IDV NP segment

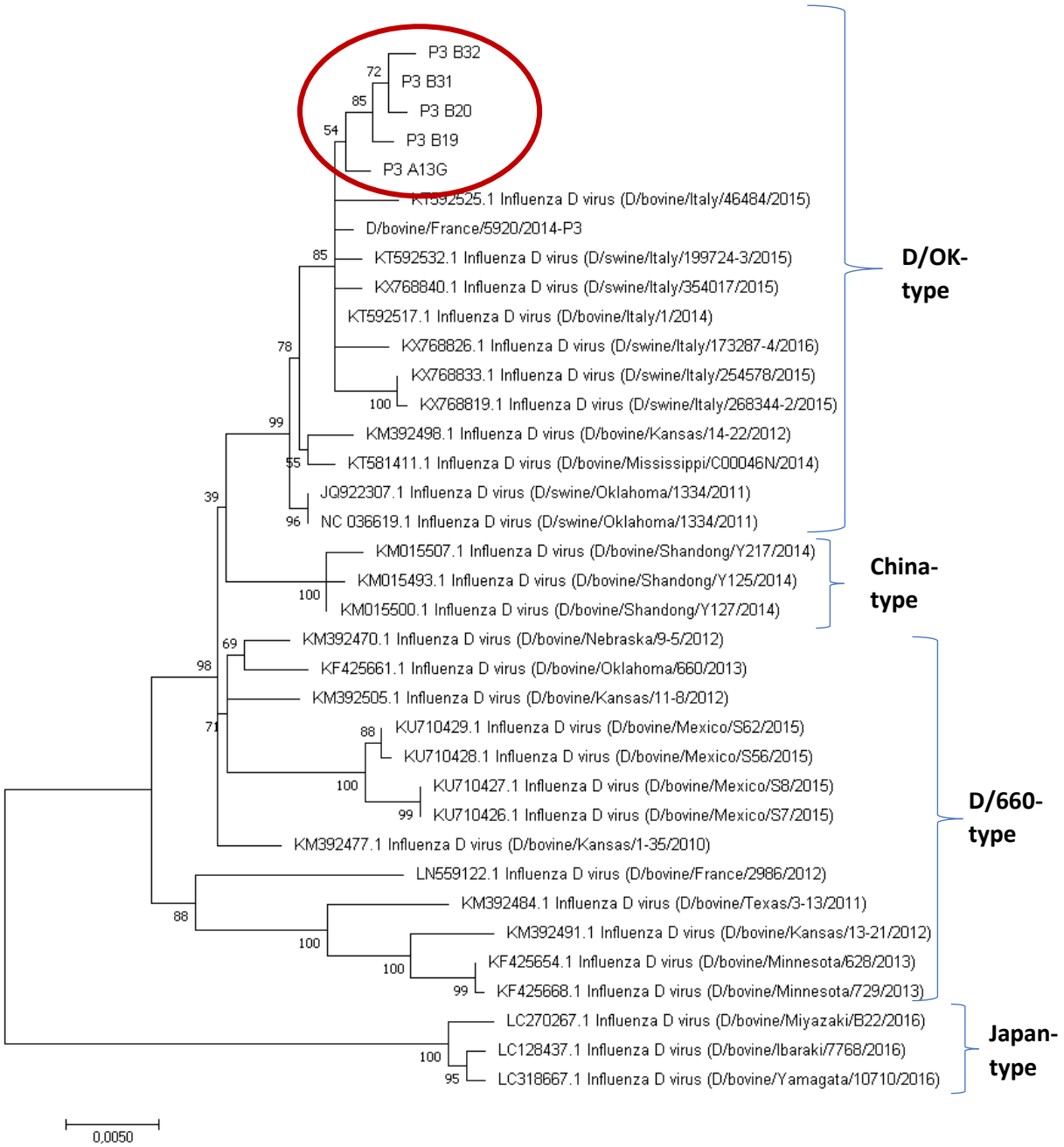


Figure 15: Phyogenetic tree of IDV P3 segment

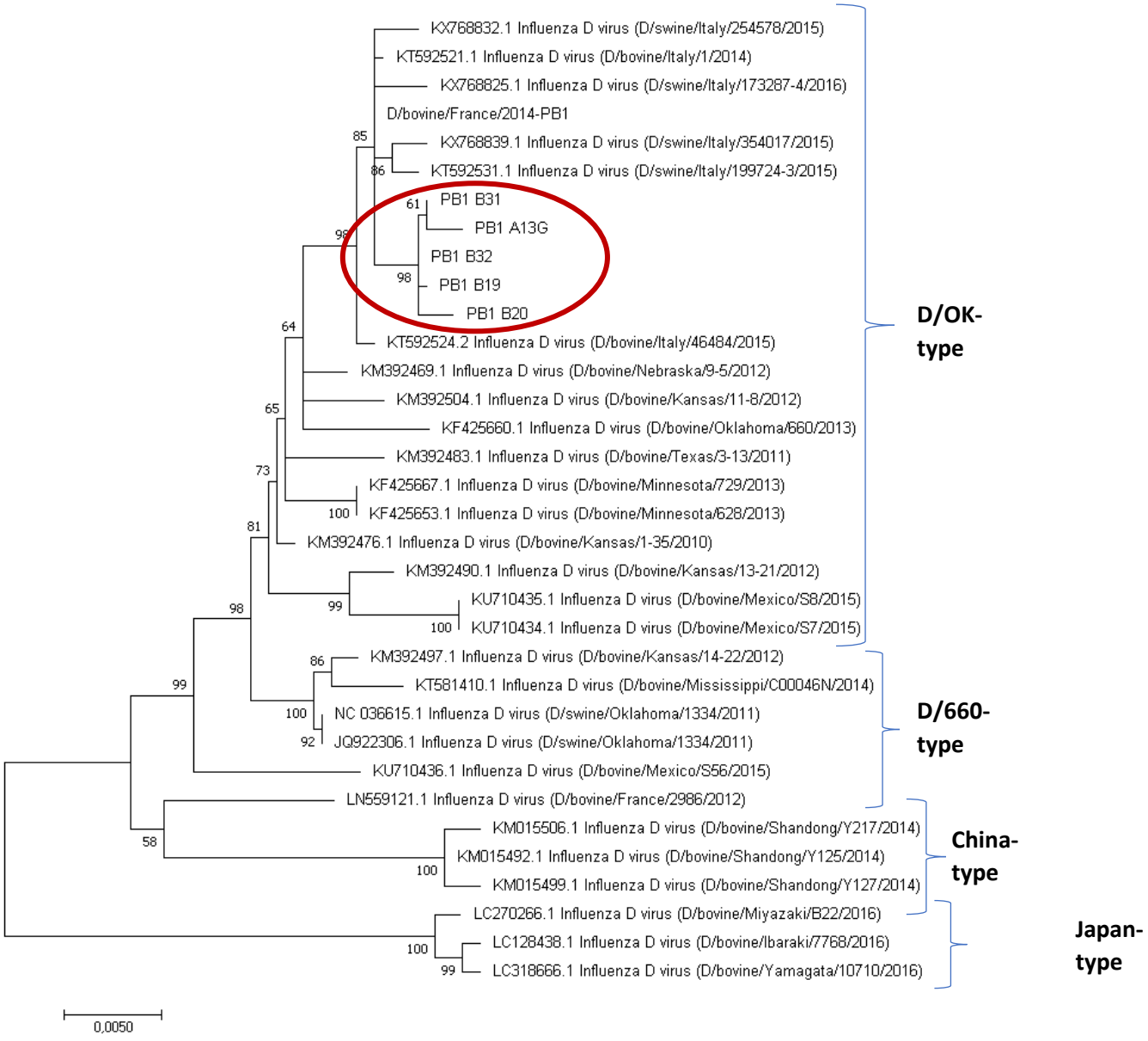


Figure 16: Phyogenetic tree of IDV PB1 segment

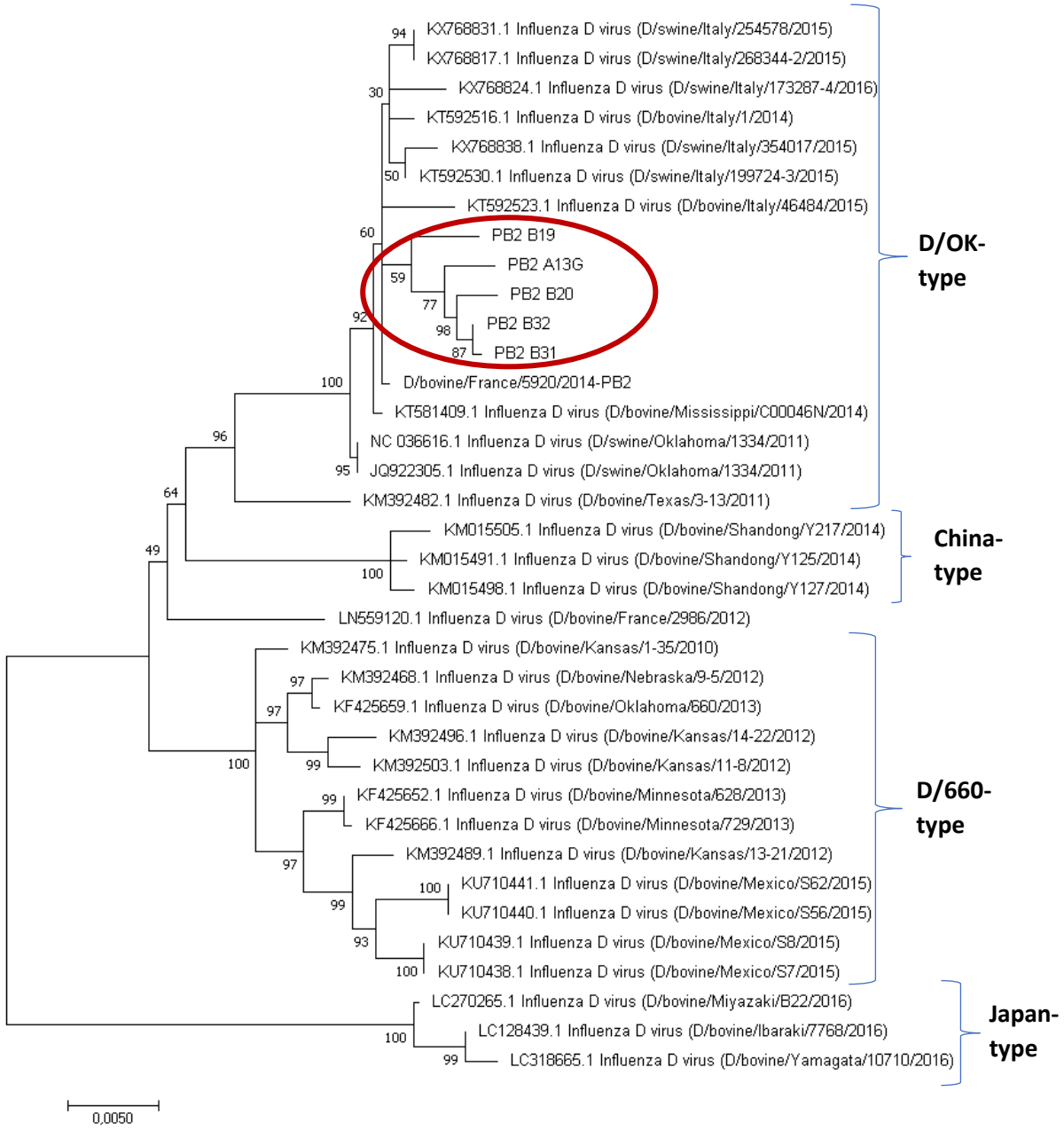


Figure 17: Phyogenetic tree of IDV PB2 segment

Amino acid alignments showing European lineage-specific mutations (ref. Pag. 67):

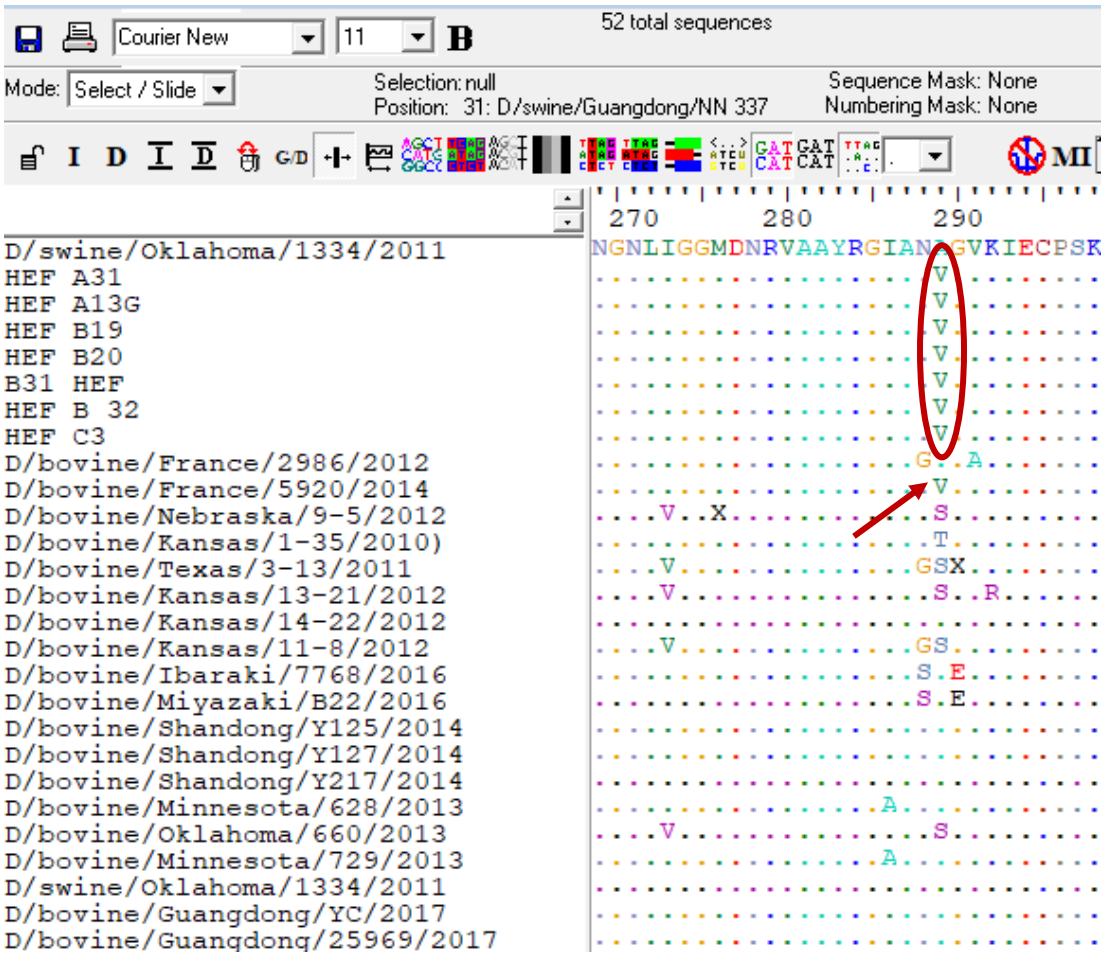


Figure 18: In the picture above: alignment of HEF sequences translated into amino acids by using BioEdit v7.1; in the red circle the mutation pos289 A→V specific of all IDV detected in Europe with the exception of the strain D/bovine/France/2986/2012 which is genetically distant to D/OK-type

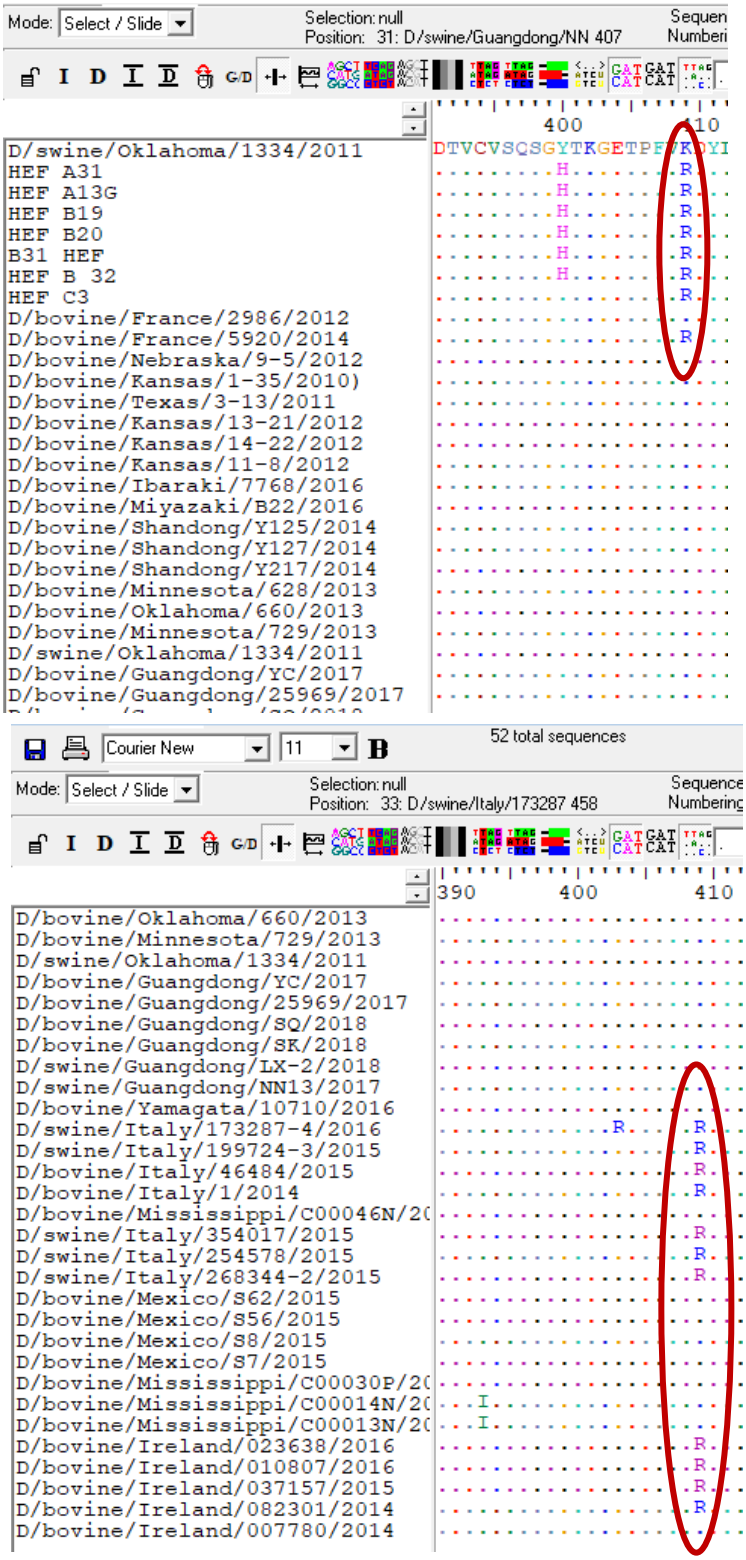


Figure 19: In the picture above: alignment of HEF sequences translated into amino acids by using BioEdit v7.1; in the red circle the mutation pos409 K→R specific of all IDV detected in Europe with the exception of the strain D/bovine/France/2986/2012 and D/bovine/Ireland/007780/2014, which are genetically distant to D/OK-type

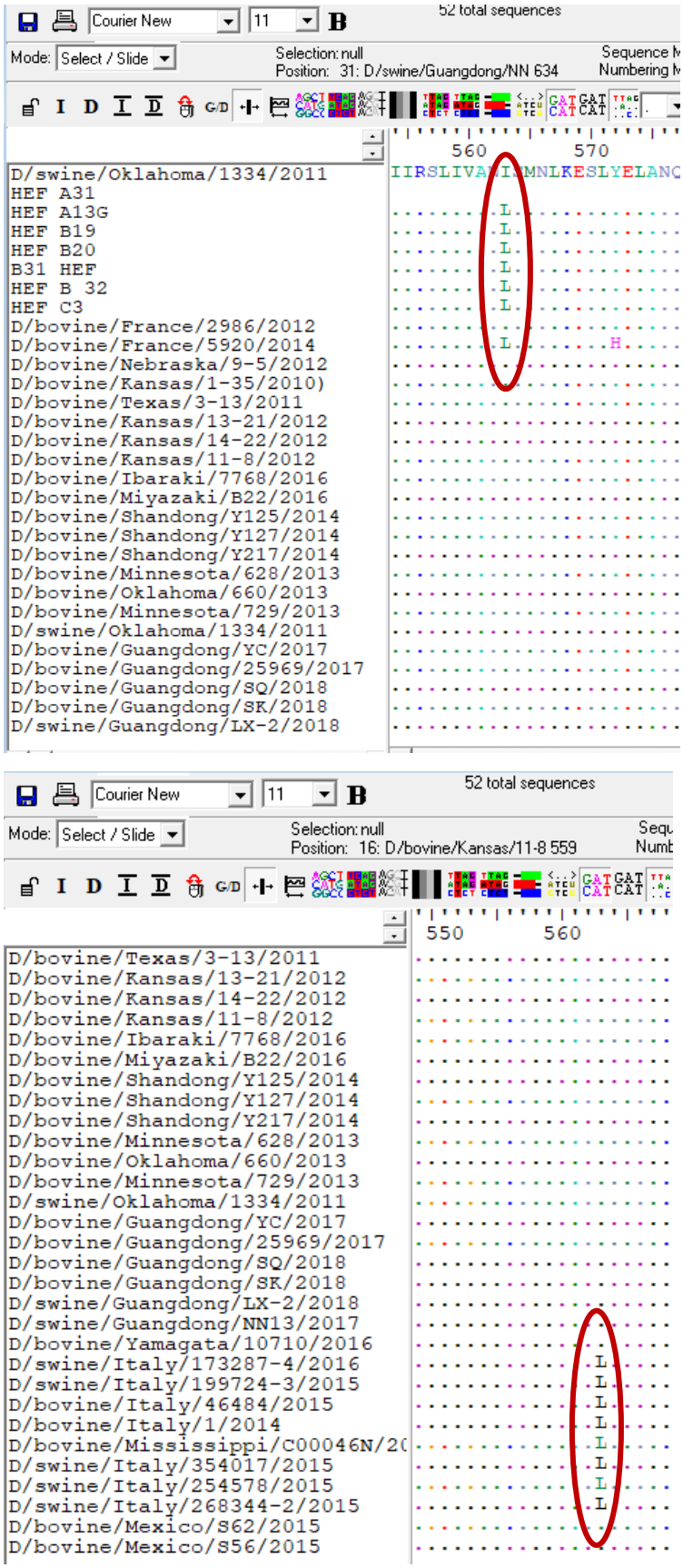
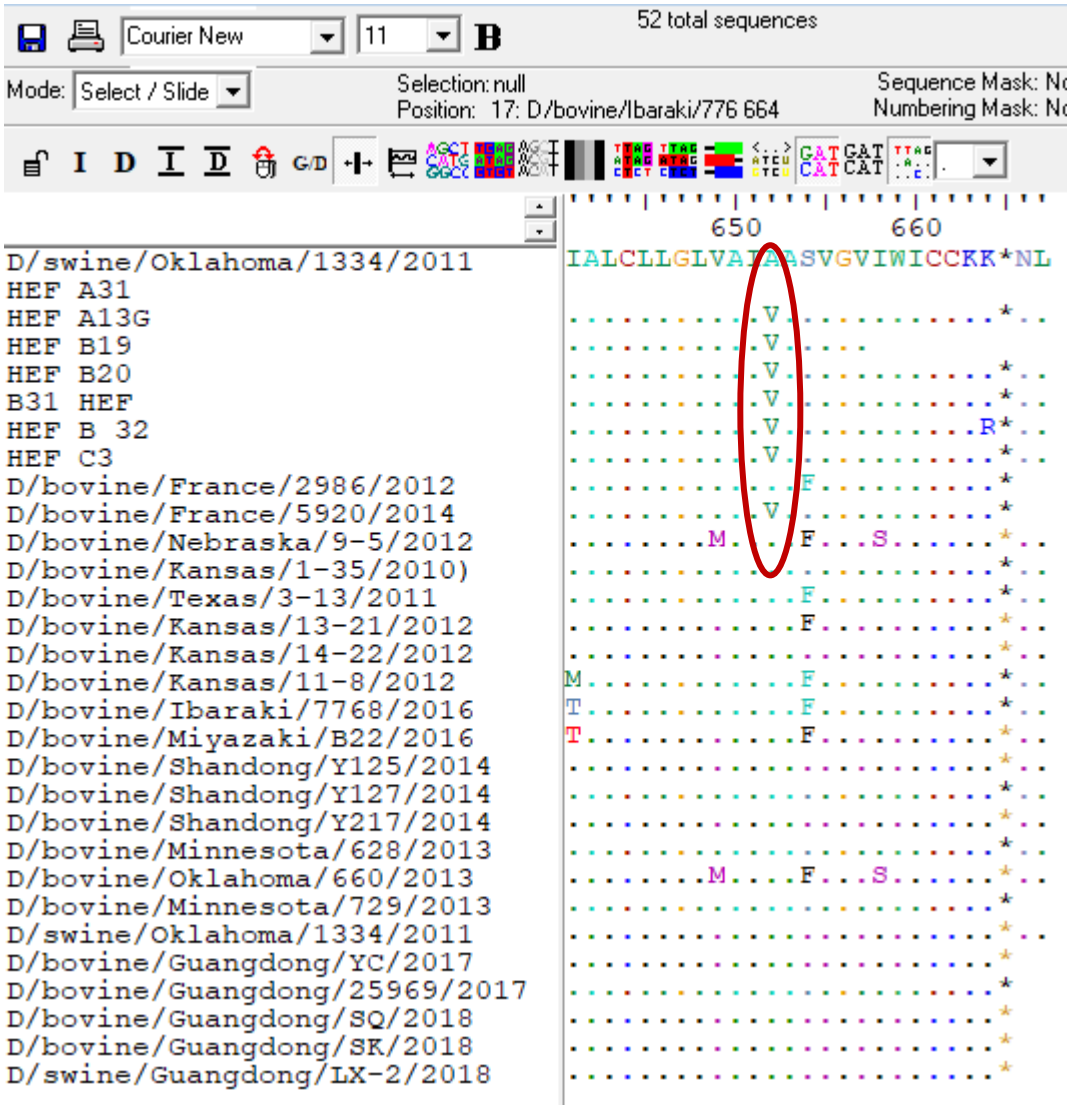


Figure 20: In the pictures at side: alignment of HEF sequences translated into amino acids by using BioEdit v7.1; in the red circle the mutation pos563 I→L specific of all IDV detected in Europe


Courier New
11
52 total sequences
Mode: Select / Slide
Selection: null
Position: 17: D/bovine/Ibaraki/776 664
Sequence Mask: No
Numbering Mask: No
650
660
IALCLLGLVAIAASVGVIWICCKK*NL
D/swine/Oklahoma/1334/2011
HEF A31
HEF A13G
HEF B19
HEF B20
B31 HEF
HEF B 32
HEF C3
D/bovine/France/2986/2012
D/bovine/France/5920/2014
D/bovine/Nebraska/9-5/2012
D/bovine/Kansas/1-35/2010)
D/bovine/Texas/3-13/2011
D/bovine/Kansas/13-21/2012
D/bovine/Kansas/14-22/2012
D/bovine/Kansas/11-8/2012
D/bovine/Ibaraki/7768/2016
D/bovine/Miyazaki/B22/2016
D/bovine/Shandong/Y125/2014
D/bovine/Shandong/Y127/2014
D/bovine/Shandong/Y217/2014
D/bovine/Minnesota/628/2013
D/bovine/Oklahoma/660/2013
D/bovine/Minnesota/729/2013
D/swine/Oklahoma/1334/2011
D/bovine/Guangdong/YC/2017
D/bovine/Guangdong/25969/2017
D/bovine/Guangdong/SQ/2018
D/bovine/Guangdong/SK/2018
D/swine/Guangdong/LX-2/2018

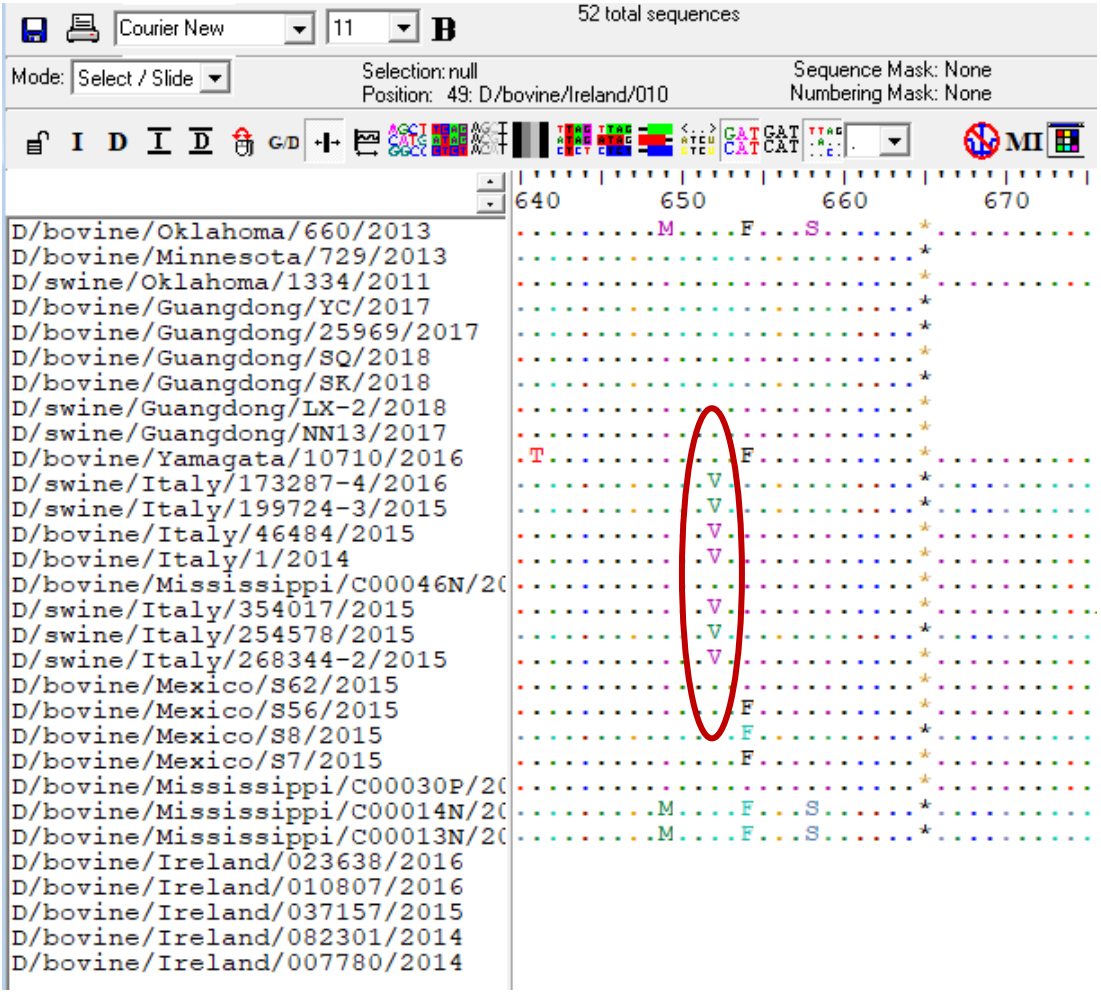


Figure 21: In the pictures above: alignment of HEF sequences translated into amino acids by using BioEdit v7.1; in the red circle the mutation pos563 I→L specific of all IDV detected in Europe with the exception of the strain D/bovine/France/2986/2012 which is genetically distant to D/OK-type

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XII. Webography

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