Novel Influenza D virus: Epidemiology, pathology, evolution and biological characteristics

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Introduction

Influenza A virus (IAV) represents a major public health concern, both for its pandemic potential and for the impact of seasonal influenza. The recurrence of IAV infections is due to antigenic drift of its major glycoprotein hemagglutinin (HA), i.e., amino acid exchanges that weaken binding of antibodies generated during a previous IAV infection. The main animal reservoir of IAV is waterfowl, which harbor viruses having all possible combinations of 16 HA and 9 neuraminidase (NA) subtypes. Only 3 HA (H1, H2, and H3) and 2 NA (N1 and N2) subtypes have caused human epidemics, defined as sustained, widespread, person-to-person transmission. Influenza epidemics usually occur if an IAV from animals enters the immunologically naive human population. This is often facilitated by antigenic shift, the reassortment of gene segments between 2 different viruses. This may occur in pigs that can be infected by both avian and human viruses. In addition, avian influenza viruses often spread to poultry, where multiple subtypes of IAVs currently circulate all over the world, including H7N9 and H5N1 that pose the highest threat to public health. Of note, due to the rapidly increasing numbers of infections of humans with the newly emerged highly pathogenic (HP) H7N9 influenza virus, the potential of H7N9 as the causative agent of a new human pandemic is of great concern. Furthermore, there have been many reassortment events introducing various neuraminidase (NA) genes into the genetic backbone of the classical Asian HP H5N1. This led to novel H5Nx variants, such as H5N2, H5N5, H5N6 H5N8, H5N9 (among others) that still pose a potential threat to both poultry industry and public health. Two Influenza A-like viruses were recently also detected in bats, but since their HA and NA lack hemagglutination and neuraminidase activity, the hallmark of IAV, they might probably not easily cross the species barrier.

In contrast, humans are the primary reservoir of Influenza B virus (IBV). The virus infects both the upper and lower respiratory tract and causes symptoms like IAV. However, IAV and IBV have significant differences in evolution and epidemiology; the limited subtype diversity and gene reassortment may be the reason for the narrower host range of IBV. IBV has similar genome structure as IAV, and antigenic drift of HA also occurs, but is more slowly compared with IAV. Influenza C virus (ICV) causes infection of the upper respiratory tract, especially in children, but clinical symptoms are typically mild. Serological studies indicate that ICV has a worldwide distribution and that most humans develop antibodies against the virus early in life. ICV is much more antigenically stable than IAV,
and a high degree of cross-reactivity is observed among isolates of ICV.\textsuperscript{17} The reservoir of influenza C virus are humans, but the virus also infects pigs and some strains could be experimentally transmitted from pig to pig.\textsuperscript{18} The genome of ICV consists of 7 gene segments and encodes only one glycoprotein (HEF), which combines both the function of HA and NA of IAV or IBV.\textsuperscript{19} The existing ICV strains are subclassified into 6 genetic and antigenic lineages, but reassortment between viruses of different lineages occurred frequently, and newly emerged reassortant viruses replaced previously circulating ones and became the epidemic strains.\textsuperscript{20}

A novel Influenza C-like virus, recently isolated from clinically-ill pigs and cattle became the founding member of a new genus of the \textit{Orthomyxoviridae}, Influenzavirus D (IDV, https://talk.ictvonline.org/ictv-reports/ictv_online_report/). Considering the zoonotic potential, the interspecies transmission and the important public health significance of influenza viruses, it is urgently required to understand the characteristic of this novel virus. In this review, we summarize the epidemiology, pathology and molecular biology of IDV and consider its relevance for human and veterinary medicine.

**Epidemiological and pathological characteristics of Influenza D virus**

In 2011, an influenza C-like virus was isolated from clinically-ill pigs with influenza-like symptoms in the Midwest region of the USA.\textsuperscript{21} Subsequently a very similar virus was isolated from other regions of the USA, Mexico, Asia (China, Japan) and Europe (France, Italy) in cattle, a species that was never considered to be susceptible to Influenza virus infection.\textsuperscript{22-27} In addition, antibody for IDV were also detected in small ruminants from USA and Canada.\textsuperscript{28} To date, cattle are thought to be the primary natural reservoir for IDV according to epidemiological, serological and pathological studies.\textsuperscript{24,25,29,30} Phylogenetic analysis showed that these strains have only 50% overall amino acid identity to human influenza C viruses, a divergence like that observed between influenza A and B viruses. Accordingly, no cross reactivity was observed between the new virus strains and human ICV antisera. ICV also exhibits a new mechanism for generating the M1 protein and does not reassort with human influenza C virus and generate viable progeny.\textsuperscript{21,30} Based on these differences to ICV this virus was classified by the International Committee on Taxonomy of Viruses (ICTV) as a new genus of the family \textit{Orthomyxoviridae}, named influenzaivirus D.

Several studies have investigated the epidemiology of IDV in swine, cattle, human and small ruminants (Fig. 1). In pig serum samples the seropositivity rate in hemagglutination inhibition assays is low (9.5%),\textsuperscript{21} indicating that the virus circulates in pigs, but is not widespread. The prevalence of IDV in cattle is higher, especially in calves. Two studies describe that \textasciitilde{}95% of newborn calves had high levels of maternal antibodies against IDV, which subsequently decreased in the next 6 months, causing increased susceptibility to IDV.\textsuperscript{25,31} It thus seems likely that cattle are most sensitive to IDV by 6 months of age. These susceptible populations of young cattle might create a permissive environment for IDV infection that allows active transmission and generates the virus reservoir. Analyses of archived sera suggested that IDV has been circulating in the US cattle population (Nebraska and Mississippi) since at least 2003.\textsuperscript{25,31} A nationwide serological survey in Japan reports an average 30% seropositivity rate in cattle, but the positivity rates varied among regions.\textsuperscript{27,32} Infections of cattle with a very similar virus have been also reported for China and Europe (France and Italy) suggesting that IDV likely circulates widely in the world.\textsuperscript{22,24,26}

The intercontinental transmission and high prevalence of IDV, especially in cattle highlight its potential threat to other agricultural animals and humans. A serological survey was conducted to investigate the seroprevalence of IDV in small ruminants (sheep and goats) and poultry (chicken and turkey). The results show seropositivity rates of 5.2% for sheep and 8.8% for goats.
indicating that both are susceptible to IDV, while all tested poultry serum samples were negative for IDV antibodies.28

Several surveys for infections of humans with IDV have also been conducted. Only ICV, but not IDV was identified by RT-PCR in archived human respiratory samples from Scotland.33 However, serological surveys suggest that IDV might infect humans. Screening of serum samples indicate that 1.3% of the general human population have hemagglutination inhibiting antibodies against IDV.21 Another 2 studies analyzed the seroprevalence against IDV in humans with occupational exposure to cattle. Whereas one study reported only 1% seroprevalence among 741 humans, another study from Florida reported a very high seropositivity rate (>95%) among 35 persons working with cattle.35 In the latter study a rather high seroprevalence (18%) was also determined for non-cattle-exposed individuals. However, it was not excluded that the high seropositivity rate is (at least partly) due to antibodies made during an ICV infection since they cross-react with IDV. Clearly, more studies are required to estimate the percentage of humans infected with IDV. Furthermore, it would be interesting to know whether a high prevalence of IDV in humans correlates with an IDV outbreak in cattle in the same area. A newly developed real-Time RT-PCR assay might be a valuable tool for the detection of IDV strains.36 Furthermore, it is unknown whether IDV causes disease in humans and is transmitted from person to person.

The pathology and mode of transmission of IDV was investigated by experimental infection of cattle and swine, but also of guinea pigs and ferrets. In pigs IDV replicates in nasal turbinates and virus shedding was detectable in nasal swabs. The virus was also transmitted to naive animals by direct contact. However, virus was not detected in trachea and lungs, indicating that virus replication was limited to the upper respiratory tract. No clinical symptoms and lesions typical of influenza were observed.21

After experimental infection with IDV in cattle, virus can be detected both in upper and lower respiratory tract and transmitted to contact animals. Highest viral titers were present in nasal turbinates, decreasing in tissue lower in the respiratory tract. Virus infection causes seroconversion both in infected and contact animals, and also cause neutrophil infiltration in infected animals. However, the clinical signs (rhinitis and tracheitis) were rather mild in IDV-infected cattle, but statistically verified in comparison to uninfected controls.30,37,38 The mild symptoms associated with experimental infection conflict with the observation that IDV is associated with a more pronounced disease in the field. This might be due to differences in infection routes and doses or husbandry conditions. However, it was also suggested that IDV alone is not sufficient to cause disease.23 IDV infections might be part of the bovine respiratory disease (BRD) complex, which requires infection by one (or more) respiratory virus that predisposes the host for secondary bacterial colonization of the respiratory tract. Indeed, a recent metagenomic study showed that IDV, bovine adenovirus 3 and bovine rhinitis A virus were the top 3 viruses associated with BRD in dairy calves.23 Previous studies have reported that IDV detection rates are higher in clinically sick cattle than in apparently healthy cattle, and tended to be significantly associated with (BRD).23,30,38 An inactivated vaccine was recently developed, but it only partially protected cattle in challenge experiments.37

The ferret is the best small mammalian model since it recapitulates the hallmarks of human influenza virus infection, such as clinical symptoms and airborne transmission. After experimental infection with IDV, virus can be detected in nasal turbinates, but not in the lower respiratory tract of the ferret. The virus was transmitted to sentinels by direct contact, but not by aerosol. Similar with swine infection, no clinical symptoms and lesions typical of influenza were observed.21 In another study ferrets were exposed to fomites soaked in nasal discharge from infected calves, but they did not seroconvert and did not shed the virus.38

Experimental infections were also conducted with guinea pigs. In contrast to ferrets, IDV replicates in both the upper and lower respiratory tract, including the lung, but no clinical symptoms were observed. IDV could transmit from infected to sentinel animals by direct contact, but not by aerosol.39

**Genome structure of Influenza D virus and encoded proteins**

IDV has an identical genome structure as ICV, i.e. it consists of 7 gene segments containing negative-sense single-stranded RNA (Fig. 2). This contrasts with the genome of IAV and IBV that consists of 8 gene segments. Each of the 7 segments of ICV contains a non-coding (NC) region both at its 5’ and 3’ end. The first 11 nucleotides (3’-C/UCGUAUUCGUC-5’) at the 3’ end and the last 12 nucleotides (5’-AGCAGUG-CAG-3’) at the 5’ end are identical between the 7 gene segments, except position 1 at the 3’ terminus, which is either cytosine or uridine.21 The 3’ and 5’ sequences of IDV are also identical to those of ICV, except for the nucleotide at position 5 at the 3’end, which is adenine in IDV, but cytosine in ICV. These conserved regions form “panhandle” structures by partial inverted complementarity between the 5’ and
3’ NC regions. This unique structure serves as the promotor for transcription of mRNAs and plays a pivotal role in genome replication and packaging. In addition, a uridine-rich region at the 5' end of each segment in both ICV and IDV serves as a template for the poly A tail present at the 3' end of each mRNA. The 5' and 3' NC regions plus terminal coding sequences of each segment also contain the required information for packaging of viral RNPs into virus particles. The high similarity of the conserved sequences at the ends of the gene segments suggests the potential for viral segment reassortment between ICV and IDV in nature. However, this was not observed under experimental conditions; IDV strains as well as ICV strains can exchange gene segments only with each other. 

Very little experimental work has been performed on the function of the individual proteins of both ICV and IDV, but they have been extensively studied for IAV. The 3 longest segments produce the proteins PB2, PB1, and PA/P3, respectively, which build the heterotrimeric polymerase complex that performs replication and viral mRNA synthesis. PB1 contains the polymerase active site (which is conserved in PB1 of IDV), whereas PB2 and PA/P3 comprise, respectively, cap-binding and endonuclease domains required for initiation of transcription by cap-snatching, a process during which a short nucleotide sequence is cleaved from the 5’ end of cellular mRNAs. The endonuclease active site of PA/P3 is identical between IDV and other influenza viruses. The guanine-binding residues of cap-binding domain are conserved between IDV and IAV, but the phosphate-binding residues are different. The protein encoded by the third segment is named P3 (instead of PA as in the case of IAV and IBV) since P3 of ICV does not exhibit a negative isoelectric point. However, the predicted isoelectric point of P3 of IDV is 6.2, which is between those of the influenza A/B (~7.2) and influenza C virus (~5.2).

The fourth segment encodes the glycoprotein HEF, the sole spike of the viral envelope, which is described in detail below. The fifth segment produces the nucleoprotein NP, which together with the polymerases and the vRNAs constitutes the viral ribonucleoprotein complex (vRNPs). The sixth segment encodes 2 proteins, the matrix protein M1, a peripheral membrane protein that covers the viral membrane on its inside, and CM2, a small transmembrane protein that exhibits proton-channel activity. In contrast to IAV, the larger protein M1 is translated from a spliced RNA, but details of splicing differ between ICV and IDV. The ICV M segment
produces the M1 protein by introducing a termination codon into the mRNA, whereas splicing of the IDV M segment adds an additional 4-amino-acid peptide into the preceding exon. The unspliced mRNA is translated into the precursor protein p42, which contains an internal signal peptide that is subsequently cleaved to generate the integral membrane protein CM2. 30,47 The seventh segment produces the 2 non-structural proteins NS1 and NS2 also by splicing. The unspliced mRNA is translated into the NS1 protein that counteracts the cellular interferon response 48,59 and the spliced mRNA translates into the shorter NS2 protein, which is also designated nuclear export protein (NEP) since it mediates the nuclear export of RNPs. 49

Origin and evolution of Influenza D virus

The amino acid sequence identity between IDV and ICV is 50% for the whole genome and thus much lower in comparison to the 95% identity observed among the 6 lineages of human ICV. PB1, the most conserved viral protein (~70% identity relative to ICV, but only ~40% with IAV and IBV), is frequently used to evaluate the evolutionary relationship among influenza viruses. 21,50 Here we constructed the maximum-likelihood phylogenetic tree based on nucleotide sequences of PB1 that include influenza A, B, C and D viruses to shed light on the evolutionary relationship between them (Fig. 3A). As expected, IDV clusters most closely with ICV suggesting...
that both viruses had a common ancestor. Using the PB2, P3, NP, M and NS genes for sequence comparison also indicates that IDV is derived from human ICV. In addition, Bayesian analysis was conducted to address the origin and evolutionary history of IDV (Fig. 3B). The mean time for the most recent common ancestor (t-MRCA) for ICV (based on the HEF gene sequence) was estimated at 1896.2 of the common era (A.D.), which coincides with a previous study, indicating that our analysis is unbiased. The t-MRCA of the HEF gene of ICV and IDV was estimated at 482 (A.D.) (95% HPD, 758 (B.C.) to 1468.8 (A.D.)), about 1534 y ago, which is a little earlier than previously reported. Based on the comparison of the nucleotide sequence of the HEF gene, 2 distinct co-circulating lineages represented by D/swine/Oklahoma/1334/2011 and D/bovine/Oklahoma/660/2013 have been identified (Fig. 3B), which frequently reassort with each other. They show antigenic cross-reactivity, but it is reduced up to 10-fold between both prototype viruses using heterologous compared with homologous antiserum. We also estimated that the 2 lineages of IDV share a most recent common ancestor (t-MRCA) just about 44.6 y ago, suggesting that these 2 lineages emerged only recently. Whether another new lineage will emerge needs to be monitored. The mean substitution rate for the HEF gene of IDV was calculated by Bayesian analysis to be $1.54 \times 10^{-3}$ (95% HPD, $5.4 \times 10^{-4}$ to $2.7 \times 10^{-3}$) per site and per year. It is thus faster than estimated for HEF of ICV alone analyzed in a previous study, suggesting that the substitution rate for HEF from IDV gene may be faster than HEF from ICV. Thus, continuous monitoring of the evolution of IDV needs to be conducted in the future.

**The HEF glycoproteins of ICV and IDV**

**Comparison of the crystal structures and protein modifications**

The hemagglutinating glycoproteins of influenza viruses are typical type I membrane proteins with a cleavable N-terminal signal peptide, a large ectodomain, one transmembrane region and a short cytoplasmic tail (reviewed in). Crystallography revealed that the overall structures as well as folds of individual segments of HA and HEF are quite similar. They form a mushroom-shaped trimer consisting of a membrane-near stalk (containing the regions involved in membrane fusion, such as heptad repeats and the fusion peptide) and a globular head domain carrying the receptor-binding site. HEF contains an additional bulge located at the lower part of the globular domain that carries the esterase region not present in HA. Another particular feature of influenza C virus particles not observed for influenza A and B virions is the arrangement of spike proteins in a reticular, mainly hexagonal structure. The formation of a regular arrangement of HEF trimers on the plasma membrane might induce membrane curvature required for virus budding. Whether HEF of IDV also forms regular arrays in the viral membrane cannot be deduced from the currently available EM micrographs.

HEF of ICV and IDV have 55% amino acid identity and an almost identical structural fold. Accordingly, the locations of the 6 disulphide bonds that stabilize the globular head of HEF and the one that connects HEF1 and HEF2, are completely conserved. Similar are also the parts of HEF not present in the crystalized ectodomain, such as the length of the transmembrane region (26 amino acids) and the very short, positively charged cytoplasmic tail (RTK in ICV and KK in IDV). Both HEFs contain cysteine residues at the end of the transmembrane region, one in ICV and 2 in IDV. The cysteine of ICV HEF is stoichiometric acylated with stearic acid, a modification that (modestly) affects virus replication and the membrane fusion activity of HEF.

A large part of the surface of HEF is covered by carbohydrates. The location of 5 glycosylation sites is completely conserved between ICV and IDV HEF, 2 sites are shifted by 10 or 12 residues (marked as gray balls in Fig. 4). Interestingly, HEF of ICV contains a glycosylation site near the receptor-binding domain that is not present in IDV HEF (Fig. 4, S176). Instead, IDV HEF has one additional glycosylation site near the esterase domain (Fig. 4, N330) and another one in the shorter $\alpha$-helix of HEF2 (N58). Furthermore, the consensus sequence compiled from the 27 HEF sequences in the database revealed another glycosylation site at N233 which is located in the 230-helix that confines the upper part of the receptor binding site (marked as red ball in Fig. 4, see also below and Fig. 5). This glycosylation site is present in 21 HEF sequences, but not in the prototype strain D/swine/Oklahoma used for crystallisation of HEF. This glycosylation site, if used, might restrict access of HEF to its receptor and by its deletion the virus might acquire a broader cell tropism. Interestingly, experimental adaption of the H5N1 IAV to airborne transmission between ferrets led to loss of a glycosylation site located at a very similar position in HA, i.e. above the receptor binding site. Whether the other carbohydrates affect vital functions of HEF, such as proteolytic processing, accessibility of the receptor binding or esterase domain or might shield antibody epitopes, as demonstrated for HA is also not known.
Comparison of the receptor-binding and -destroying sites

HA of IAV uses terminal N-acetylneuraminic acid (Neu5Ac) as receptor determinant and the glycosidic bond of Neu5Ac influence the host specificity. Avian influenza viruses usually bind to Neu5Ac-α2,3-gal while mammalian influenza viruses attach to Neu5Ac-α2,6-gal. In contrast, HEF of ICV and IDV requires an acetylated derivative of Neu5Ac, namely N-acetyl-9-O-acetylneuraminic acid (9-O-Ac-Neu5Ac), but HEF binds to its receptor regardless of whether 9-O-Ac-Neu5Ac is attached via an α-2,6 or α-2,3 linkage to the following galactosyl-residue. IDV HEF also tolerates small substitutions at the C5 position, such as esterification with glycolic acid instead of acetic acid.

The receptor-binding site of HEF from both ICV and IDV is located near the top of the globular head in a shallow cavity, surrounded by residues from 4 secondary structure elements: the 170-loop, 190-loop, 230-helix and 270-loop (Fig. 5A). The most prominent difference between HEF of ICV and IDV is an open channel in the receptor-binding site of ICV HEF. The negatively-charged residue D269 and the positively-charged residue K235 form a salt bridge, pulling the 270-loop up to attach to the 230-helix. At equivalent positions in IDV HEF the amino acids T239 and A273 are present that cannot form a salt bridge. Thus, helix 230 is not connected with loop 270 and HEF of IVD therefore possesses an open channel (Fig. 5B). It has been suggested that the more open receptor binding cavity of IDV HEF is the basis for the broader cell tropism of IDV but definitive proof of this assumption requires a detailed analysis of the ligand binding characteristics of ICV HEF.

Both ICV and IDV HEF bind to trachea sections from human, swine and bovine origin indicating that the relevant receptor determinant is present in all 3 species. However, binding does not necessarily prove that the cells can be infected. This requires (at least) attachment to a receptor capable to undergo clathrin-mediated endocytosis and exposure of HEF to acidic pH to trigger its membrane fusion activity.

In agreement with its receptor binding specificity, the receptor-destroying activity of HEF is an esterase that cleaves acetyl from the C9 position of terminal 9-O-Ac-Neu5Ac residues. The esterase domain is the structurally most conserved part of HEF; the position of the catalytic triad characteristic for serine hydrolases (serine 57, aspartic acid 356 and histidine 359) is identical in ICV and IDV HEF. Interestingly, the HEF esterase of IDV is very active, even at 4°C. Only if the catalytic triad is inactivated HEF can hemagglutinate erythrocytes and binds to MDCK cells or bovine submaxillary mucins, which are enriched in 9-O-Ac-Neu5Ac.

Comparison of the proteolytic cleavage sites

To catalyze virus entry by membrane fusion HEF must be proteolytically processed into the larger HEF1 and the membrane-bound HEF2 subunits. The molecular features of the cleavage site determine which of the many trypsin-like serine proteases recognize the precursor protein and the expression of this protease determines whether a certain cell type produces infectious virus.
particles. HEF from both ICV and IDV possess a monobasic cleavage site, a single arginine residue at the C-terminus of HEF1. HEF is in this respect similar to HAs from mammalian and low pathogenic avian influenza A viruses. Replication of these viruses is restricted to the site of virus infection, usually the respiratory tract. Spread to other organs or systemic infection, as it happens for highly pathogenic avian influenza virus having a multibasic cleavage site between HA1 and HA2, does not occur.65

Interestingly, the amino acids surrounding the monobasic cleavage site differ between ICV and IDV HEF. HEF from ICV contains 3 closely spaced basic residues at the C-terminus of HEF2 (TVTKPSR), whereas HEF from IDV contains only 2, widely spaced basic residues (RTLTPATR). In contrast, the N-terminal amino acids of the HEF2 subunit are completely conserved between ICV and IDV HEF (IFGIDDLI), but very different from the respective, strictly conserved amino acids of HA2 (GLFGAIAGFIIE). The N-terminal amino acids of HEF2 and HA2 function as the fusion peptide, which is completely buried inside the trimeric structure of HA, but partly surface-exposed in HEF.54,57

The enzymes that activate HEF have not been identified, but in the case of human IAV and IBV, HAs with a monobasic cleavage site are proteolytically activated by members of the TMPRSS (transmembrane protein serine protease) family, mainly by TMPRSS2 and HAT (human airway trypsin-like protease), but a few HA subtypes are cleaved by TMPRSS4 and matriptase. These enzymes are expressed along the exocytic pathway or at the plasma membrane of human bronchial and tracheal epithelial cells. The homologous enzymes in swine have very similar characteristics with respect to cleavage specificity and (sub)cellular expression; information about the respective enzymes of bovine respiratory cells are not available.65

Since the amino acids surrounding the cleavage site are part of the protease recognition motif it will be interesting to identify the enzymes which process HEF of ICV and IDV and analyze their expression in cells of the respiratory epithelium. Since only cells expressing the appropriate protease release infectious virus particles knowledge about the processing enzyme might be instrumental to explain the differences in cell tropism of IAV/IBV and ICV/IDV (upper versus lower respiratory tract) and host range of ICV and IDV (human and swine vs. bovine and swine). However, proteases secreted by certain bacteria might also activate the viral fusion proteins, as has been demonstrated for HA of IAV.66

Temperature sensitivity of HEF

HEF of ICV exhibits intrinsic temperature sensitivity. Folding and trimerization of the molecule, surface expression and membrane fusion is less efficient at 37°C than at 33°C.67 Probably as a consequence, ICV grows to higher titers in cell culture and chicken embryos at 33°C compared with 37°C.68 This temperature sensitivity might be one of the factors that prevent replication of ICV in the lung that has a higher temperature compared with the upper respiratory tract.

Figure 5. Comparison of the receptor-binding sites of ICV and IDV HEF. Surface presentations of the receptor-binding site of HEF from ICV (A) and IDV (B). The parts of the HEF1 molecule involved in receptor-binding (the 170 loop, the 230 helix, the 270 and 290 loops) are colored yellow. The 5 residues F127 (C HEF: Y127), W185 (C HEF: L184), Y231 (C HEF: Y227), F229 (C HEF: F225) and F297 (C HEF: F293) that form the bottom of the cavity are colored red. Note that K235 (blue) and D269 (red) form a salt bridge in ICV HEF that connects helix 230 with loop 270. At equivalent positions of IDV HEF A273 and T239 (colored red) are present that are not able to form a salt bridge. As a result, the receptor cavity of IDV HEF is more open and thus might allow binding of more 9-O-Ac-Neu5Ac derivatives. The figure was created with PyMol from PDB files 5e64 and 1flc and rendering was done with Blender.
However, this temperature sensitivity is not preserved in IDV since the virus replicates efficiently at 37°C.21

In summary, the structures of HEF of ICV and IDV are (almost) identical. Notable exceptions are the more open receptor-binding cavity in ICV HEF, the location of certain glycosylation-binding sites and the amino acids surrounding HA cleavage site. Whether these features affect the host range of ICV and IDV needs to be explored.

**Does Influenza D virus represent a public health threat?**

Using the (very limited) information available on IDV we now assess the public health relevance of this emerging pathogen. The presence of antibodies against IDV in humans, especially with cattle exposure suggests that the virus can infect humans.35 Likewise, ferrets, surrogates for human influenza virus infection, are also susceptible. Is it conceivable that IDV might acquire the ability to also invade the lower respiratory tract possibly creating more severe symptoms? In contrast to ICV, IDV replicates well in cell culture at 37°C,21 the prevailing temperature in the lung. Likewise, the more open receptor binding cavity in HEF of IDV suggests that the virus can infect a wider range of cells compared with ICV.27

Indeed, HEF of IDV binds to human trachea sections and the virus shows a wider tropism in cell culture.27 Thus, there seems to be no inherent blockade that prevents IDV from invading the lower respiratory tract and to do so only a few adaptive genetic changes might be required.

However, very few amino acid exchanges are observed between the proteins of circulating viruses (both in ICV and IDV) suggesting that virus evolution is slow. Furthermore, the only surface glycoprotein HEF is antigenically stable indicating that antibody escape mutants might not occur frequently. Thus, infection with IDV (like infections with ICV) might cause long lasting immunity. The reason for the slow evolutionary change of ICV and IDV proteins is unknown. It might be either a low error rate of the polymerases and/or that viral proteins do not tolerate many amino acid changes without compromising their functionality.

The relevance of IDV for veterinary medicine needs to be further explored, i.e. whether the virus can cause disease on its own or only as part of the bovine respiratory disease (BRD) complex. Since IDV (in contrast to the other Influenza viruses) was isolated only a few years ago one might argue that its pathogenic potential is rather limited. However, one could also imagine that the pathogen may have spread to swine and cattle only recently, possibly from another still unknown natural animal reservoir. In addition, considering the proposed role of swine as the “mixing vessel” for reassortment between avian and human influenza A viruses to generate genetically novel, often pandemic viruses,69 one might envision that pigs play a similar role in IDV ecology. Because swine are also sensitive to both ICV and IDV,18,21 continuous monitoring of pigs for reassortment of IDV is advisable. It is also unknown whether there are more genotypes of influenza C- and influenza D-like viruses circulating in cattle or other hosts. Further epidemiology survey of different animals should be conducted by veterinarians.

In summary, despite its zoonotic potential, the significance of IDV for public health seems to be rather low provided that the pathogenic potential and the antigenic stability of the virus does not change drastically. However, since properties and changes of influenza viruses are hard to predict, closer surveillance of the virus, especially in humans, is needed and efforts should be made to design a prototype vaccine that protects humans in case of an outbreak.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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