INFLUENZA C AND INFLUENZA D VIRUSES

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SUMMARY

Etiology

- Influenza virus C (IVC) and influenza virus D (IVD) are enveloped RNA viruses in the family *Orthomyxoviridae*. They are more closely related to each other than to influenza virus A (IVA) or influenza virus B (IVB), containing seven genomic segments and a hemagglutinin-esterase (*HE*) fusion protein responsible for recognition, binding, and destruction of host receptors.
- IVC appears to be more antigenically stable and slower to evolve compared to other influenza viruses. Re-assortment does not occur between IVC and IVA strains. IVD strains isolated from swine and cattle have been found to frequently reassort with each other.

Cleaning and Disinfection

- Influenza viruses are susceptible to heat, pH extremes, dryness, and are generally unstable in the environment. The ideal replication temperature for IVC is 33˚C, while IVD is capable of growth at 37˚C.
- Influenza viruses are inactivated by sunlight, disinfectants, and detergents, such as sodium deoxycholate and sodium dodecyl sulphate. They can also be inactivated by formaldehyde, glutaraldehyde, beta propio-lactone, and binary ethylenimine in the presence of organic matter. If organic matter has been removed, destruction of the virus can be achieved with phenolics, quaternary ammonium compounds, 5.25% sodium hypochlorite, 2% sodium hydroxide, 4% sodium carbonate dilute acids, and hydroxylamine.

Epidemiology

- IVC is predominantly a human pathogen, causing respiratory disease in infants and children. Infection in pigs, dogs, horses and cattle has also been observed. IVD has been recently discovered and isolated from swine and cattle. Bovines are thought to be the natural reservoir with occasional spillover into swine and humans.
- Transmission of IVC between humans and pigs is strongly suggested but the direction of transmission is unclear. It is not known whether IVD is zoonotic. Experimentally, IVD has been shown to cause clinical disease in ferrets, the preferred animal model for human influenza studies.
- To date, IVC has been isolated only from pigs in China; however, serological evidence of infection has also been found in Japan and Great Britain. IVD has been isolated from pigs in Oklahoma and from cattle in China, France, and the United States, including the states of Minnesota, Kansas, Nebraska, Oklahoma, and Texas.

Transmission

- The primary mode of influenza transmission in swine is direct pig-to-pig contact. Spread of human and swine IVC isolates from experimentally infected swine to naïve swine by direct contact has been reported.
- Influenza can potentially be transmitted by contact with contaminated surfaces, and aerosol transmission has been demonstrated in humans and horses. Close contact during transport and intensive farming practices are thought to facilitate the spread of the virus.

Infection in Swine/Pathogenesis

- Swine experimentally infected with IVC may exhibit normal temperature up to eight days post-exposure and typically fail to exhibit traditional influenza-like symptoms. Several pigs have shown slight dyspnea and increased nasal secretion after intranasal inoculation, clearing quickly or persisting for up to ten days. No other clinical signs of disease have been reported.
- Viruses in the newly proposed *Influenzavirus D* genus may be more pathogenic in swine than IVC. Influenza-like illness was first observed in 15-week-old pigs naturally infected with IVD in April 2011. A retrospective serological analysis of samples from United States swine farms (2010–2012) identified four additional positives via reverse transcriptase polymerase chain reaction (RT-PCR). However, experimental infection in 10-week-old pigs resulted in no clinical signs.

Diagnosis

- Diagnosis of influenza infection is achieved by virus isolation or serological tests, but low viral loads in some samples may necessitate more sensitive methods. Many commonly used cultured cell lines are not capable of propagating IVC.
- Nasal, tonsil, oropharyngeal, pharyngolaryngeal, and tracheal swabs; bronchoalveolar or nasal lavage; and nasopharyngeal aspirates have all been described specifically for IVC isolation. IVD can be readily isolated and cultured in swine testicle (ST) cells.
- Hemagglutination inhibition (HI) assays are often performed with chicken or turkey erythrocytes following virus isolation to identify unknown influenza viruses by antigenic cross-reactivity. Antigenic variation among IVC isolates can be demonstrated with anti-*HE* monoclonal antibodies, and strains from a single lineage are thought to be antigenically indistinguishable. The use of polyclonal serum demonstrates cross-reactivity among all examined human IVC isolates, although the same level of cross-reactivity is not observed between isolates from pigs and humans or between IVC and IVD.
- Conventional and real-time RT-PCR assays are useful for detection of low viral loads in infected tissue or supernatant. Real-time RT-PCR has been used in the detection of all genera of influenza, and several multiplex tests are used in humans for detection of IVC in combination with other respiratory pathogens.
- Serological assays can detect antibodies to specific virion proteins, and are used in the typing of newly identified influenza viruses. Enzyme-linked immunosorbent assays (ELISA), HI, and serum neutralization (SN) have all been described.

Immunity

- IVC may cause persistent infection in pigs, and they can remain infectious for up to 25 days following inoculation with pig or human IVC isolates.
- As IVC predominantly infects humans, no swine vaccines are currently available. Because of its lack of involvement in influenza pandemics, IVC is also not included in seasonal influenza vaccines for humans.

- Pigs infected with IVC experimentally or by contact with infected pigs develop a detectable antibody response within two weeks. However, in one study of pigs infected with a human IVC isolate, a detectable antibody response was found in only two out of six individuals.

Prevention and Control

- Vaccination is the primary method for controlling pathogenic influenza viruses in swine, although this is not currently applicable to IVC or IVD.
- Neuraminidase inhibitors, used in humans to treat IVA and IVB, are ineffective in treatment of IVC infection.
- To prevent and limit influenza infections in swine, common industry biosecurity practices should be in place.

Gaps in Preparedness

- Understanding of IVC continues to lag far behind other influenza viruses, perhaps due to the relatively mild nature of the illness it causes and the lack of rapid IVC-specific diagnostic testing. More information is needed on the interplay between human, swine, canine, equine, and bovine IVC infection.
- Information is also lacking on the recently discovered IVD in swine and cattle. In particular, our current understanding is limited by the paucity of similar isolates available for study. Analyses of the evolutionary rate will be critical in understanding the evolution and host range of this novel virus, and many questions remain regarding pathogenicity, temporal distribution, and zoonotic potential.

OVERVIEW

Influenza viruses, in the family *Orthomyxoviridae*, are diverse, rapidly changing, enveloped RNA viruses with a widespread global presence. The influenza genome is encoded on distinct, independently evolving RNA segments within a matrix protein shell. These segments facilitate the exchange of genetic material between strains in hosts that are simultaneously infected with two or more strains, giving the virus an enhanced ability to produce antigenic variants capable of continually evading host immune systems. Four proposed genera exist: influenza virus A (IVA), influenza virus B (IVB), influenza virus C (IVC), and influenza virus D (IVD). Relatively little is known about IVC and IVD. First identified in humans in 1947, IVC is responsible for mild upper respiratory illness, most commonly in infants and young children. Over 200 isolates of IVC have been identified, and anti-IVC antibodies have been reported in humans, swine, dogs, horses, and cattle. IVD has only been isolated from swine and cattle since its discovery in 2011. Reassortant viruses between different genera are not known to produce viable progeny. Phylogenetic studies suggest that IVC diverged from IVA and IVB prior to their separation, while the newly discovered IVD is most closely related to IVC.

Influenza viruses tend to follow a cyclical pattern with a greater prevalence in winter months. They are sensitive to sunlight, dryness, temperature, and pH changes, and are fairly unstable in the environment. IVC is thought to exhibit slightly less seasonal variation than IVA and IVB, and summer IVC infections in humans do occur. Further, anti-IVC antibodies have been detected in swine in China during all months of the year. IVC can circulate simultaneously with IVA and IVB, and peaks in the rate of infection for each virus do not necessarily overlap. IVC co-infection with other influenza viruses and respiratory pathogens has been observed in swine and humans. Influenza has been identified in every country in the world, and human IVC infection has been reported in many. Seroepidemiological studies indicate that large numbers of humans have been exposed to the virus, and it's estimated that as many as 80% will acquire anti-IVC antibodies during their lifetime. The potential for recurrent IVC infection also indicates that immunity may fail to adequately protect against future exposure. Much less is known about the incidence of the disease in swine, and infection can be subclinical.

Influenza is transmitted by nasopharyngeal exposure during direct contact between animals or humans, contact with contaminated surfaces, and by aerosolized virus. Intensive farming practices and transport likely facilitate the spread of disease among animals, and influenza can spread throughout a herd within days. IVC can spread from person-to-person, pig-to-pig, and between humans and pigs, though directionality of transmission in nature between humans and pigs has not been elucidated. Pigs can potentially infect other pigs for up to 25 days post-exposure. Several organic solvents and detergents effectively inactivate the virus, and a number of chemical disinfectants are capable of destroying the virus in the presence or absence of organic matter.

Diagnosis of influenza infection has been achieved by virus isolation or serological tests, but low viral loads in some samples may necessitate more sensitive methods. Many commonly used cultured cell lines are not capable of propagating IVC, and the virus has traditionally been isolated by inoculation of embryonated chicken eggs. Madin-Darby canine kidney (MDCK) I, human hepatocarcinoma (HuH7), and human malignant melanoma (HMV-II) cells have also been used successfully, despite their limitations. Influenza can be isolated from nasal mucosa, ethmoid, trachea, lungs, tonsils, and regional lymph nodes. Nasal, tonsil, oropharyngeal, pharyngolaryngeal, and tracheal swabs; bronchoalveolar or nasal lavage; and nasopharyngeal aspirates have all been described specifically for IVC isolation. IVD can be readily isolated and cultured in swine testicle (ST) cells.

Hemagglutination inhibition (HI) assays are often performed with chicken or turkey erythrocytes following virus isolation to identify unknown influenza viruses by antigenic cross-reactivity. Antigenic variation among IVC isolates can be demonstrated with anti-HE monoclonal antibodies, and strains from

a single lineage are thought to be antigenically indistinguishable. The use of polyclonal serum demonstrates cross-reactivity among all examined human IVC isolates, although the same level of cross-reactivity is not observed between isolates from pigs and humans or between IVC and IVD.

Conventional and real-time reverse transcription polymerase chain reaction (RT-PCR) assays are more sensitive for detection of low viral loads in infected tissue or supernatant. Real-time RT-PCR has been used in the detection of all genera of influenza, and several multiplex tests are used in humans for detection of IVC in combination with other respiratory pathogens. Serological assays can detect antibodies to specific virion proteins, and are used in the typing of newly identified influenza viruses. Enzyme linked immunosorbent assays (ELISA), HI, and serum neutralization (SN) have all been described.

Illness caused by IVC is generally mild, thus no vaccines are currently available for humans or swine. While the virus is believed to be widespread in humans, little is known about geographic distribution and prevalence in other species. Current trends in IVC research include creation of recombinant viruses with specific protein deletions to further understand viral function at a molecular level. Additionally, the development of more rapid and effective diagnostic tests to facilitate increased surveillance of the virus in humans and livestock will be critical in strengthening our understanding of the virulence and epidemiology of IVC.

LITERATURE REVIEW

1. Etiology

1.1 Key Characteristics

Influenza virus C (IVC) and influenza virus D (IVD) are enveloped viruses in the family *Orthomyxoviridae*. IVC has been classified in its own genus *Influenzavirus C* since 1996.[1] Recent isolation of an influenza virus from a pig in Oklahoma in 2011 has led to discussion and proposal of an additional genus, *Influenzavirus D*.[2]A major distinguishing feature of both IVC and IVD is the hemagglutinin-esterase (*HE*) fusion protein, which is responsible for receptor recognition, viral fusion, and destruction of the host receptor. In contrast, surface spike glycoproteins of both influenza virus A (IVA) and influenza virus B (IVB) consist of hemagglutinin (H) and neuraminidase (N).[3]

Influenza viruses are notorious for the frequent formation of novel antigenic variants that evade host immune systems. This occurs not only through mutation (antigenic drift), but by reassortment and recombination of viral genetic material among strains within a genus (antigenic shift). Division of the influenza genome into segments facilitates this reassortment between strains when a cell is concurrently infected with more than one virus. IVC and IVD contain seven genomic segments, while IVA and IVB each contain eight.[4,5] Structure and composition of IVA, IVB, and IVC are otherwise very similar, though filamentous forms of the virus are more common in IVC.[6]

1.2 Strain Variability

All seven segments of the IVC genome evolved independently of each other,[5] and six different co-circulating lineages of IVC are currently classified based on the *HE* gene[7] found on the fourth segment. This is the most variable among the segments, although immune selection does not appear to play a major role in evolution of the *HE* gene within the individual lines.[5] Exchange of genetic information during mixed infection and emergence of reassortant viruses occurs readily between IVC lineages in vitro[8] and in nature,[9-13] and these events may facilitate a greater spread among human populations.[10-12,14] A 1990 to 1999 surveillance of IVC in humans in Japan showed that 44 out of 45 isolated viruses were reassortants that could be distinguished from reference strains of original lineages.[11] Currently, over 200 isolates of IVC are known.[15]

Among influenza viruses, IVC is comparably more antigenically stable and slower to evolve.[4,10,16] Though antigenic variation has been observed, IVC can survive for long periods of time (≥ 9 years) without changing its antigenicity and genome composition.[17] IVB and IVC have both diverged considerably from IVA, and IVC as a genus is thought to have evolved before the split between IVA and IVB. Modern IVB and IVC reassortants with IVA are unable to produce viable progeny. This was likely facilitated by a period of isolation leading to host-specific adaptive evolution, until the genomic RNA sequence variation was sufficient to prevent viable reassortment among the genera.[18] The nucleoprotein (NP) and matrix (M1) proteins share only a 20–30% homology among the different genera of influenza and are the antigens used to distinguish them.[16]

The novel IVD shares a 50% amino acid homology with human IVC, similar to the level of divergence between IVA and IVB.[16] Analysis of polymerase protein PB1, the most conserved influenza viral protein, indicates that this new virus is more closely related to IVC than to IVA or IVB. The segments of IVC and IVD that contain PB1 share a 72% identity, while the segment containing *HE* has diverged further to 53%.[19] Despite the similarities, this new virus is more distant from other known strains of human IVC. Phylogenetic analysis suggests that this strain diverged from human IVC following the divergence of IVC from IVA and IVB. *In vitro* reassortment studies and serological typing indicate that the virus is also antigenically distinct from human IVC and incapable of producing viable reassortants.[16] An isolate of IVD from swine and another from bovines were found to frequently reassort with each other.[19]

2. Cleaning and Disinfection

2.1 Survival

Influenza viruses are susceptible to heat, pH extremes, dryness, and are generally unstable in the environment.[20] The newly discovered IVD, previously thought to be a subtype of IVC, has demonstrated permissive growth in cell culture at 37˚C, further differentiating it from human IVC.[2] The ideal replication temperature for IVC is 33˚C,[4,10] and variation in the *HE* protein responsible for virus-cell fusion is thought to play a role in the observed differences in replication at various temperatures.[4]

2.2 Disinfection

Influenza viruses are inactivated by sunlight, disinfectants, and detergents,[6] such as sodium deoxycholate and sodium dodecyl sulphate. They can also be inactivated by formaldehyde, glutaraldehyde, beta propio-lactone, and binary ethylenimine in the presence of organic matter. If organic matter has been removed, destruction of the virus can be achieved with phenolics, quaternary ammonium compounds, 5.25% sodium hypochlorite, 2% sodium hydroxide, 4% sodium carbonate dilute acids, and hydroxylamine.[20]

3. Epidemiology

3.1 Species Affected

Originally thought to be an exclusively human disease, IVC was first isolated from pigs in China in 1981.[21] Anti-IVC antibodies had previously been reported in the late 1970s in Japanese cattle without clinical signs[22] and in horses with acute upper respiratory disease in Toronto in the early 1960s.[23] Aside from these isolated cases, no other serological evidence of IVC infection in horses or cattle has been reported. Since then, IVC has also been reported in dogs.[5] While the virus has only been isolated from pigs and humans,[15] anti-IVC antibodies have been detected in dogs[3] and experimental inoculation produces similar symptoms seen in the human manifestation of the disease. Experimental infection in mice, hamsters, monkeys, and rats leads to seroconversion without clinical signs.[24]

While IVC prefers human hosts, the recently identified influenza virus proposed as IVD appears to have a greater tropism for bovines. The prototype virus, originally coined C/swine/Oklahoma/1334/2011 (C/OK), was discovered to be widespread in United States bovine herds in multiple states.[2] This is believed to be the first isolation of an influenza virus from bovines. Since the discovery in 2013, C/OK-like viruses have also been isolated from cattle in China[25] and France.[26] Serological survey is suggestive of the frequent replication and establishment of infection in bovines, indicated by percent positive and mean titers greater than those in humans or swine. At present, bovines are believed to be the natural reservoirs for C/OK with occasional spillover into swine and humans.[2]

3.2 Zoonotic Potential

Humans who work closely with swine and poultry are at greatest risk of zoonotic infection with an influenza virus, and represent a segment of the population in which reassortment events may be more likely to occur.[6] The first suspicion of a connection between influenza in swine and humans occurred during the 1918 pandemic, when both populations became ill simultaneously.[6] Transmission of an influenza virus from pigs to humans was first documented in 1976, and zoonotic infections have occurred throughout the world.[27]

Serological studies in China and Japan indicate that IVC can cause natural infection in pigs. Genetic comparison of human and porcine isolates strongly suggests the occurrence of interspecies transmission of IVC between pigs and humans, though the direction of transmission is unclear.[28] Human IVC isolates are replication competent in experimentally infected pigs.[5,29] Still, the role of swine as a potential animal reservoir host for human IVC has not been proven.[28] Pigs, as well as humans, may be natural reservoirs of the virus.[29] The observation of descendants of human pandemic influenza viral strains persisting in pigs

does indicate that swine may be a reservoir for some influenza viruses, allowing human immunity to wane before reemerging at a later date.[6] However, the diversity of IVA and its animal reservoirs also appear to make it uniquely capable among influenza viruses of causing global human pandemics.[16]

Little is currently known about the epidemiology of IVD, but it also warrants monitoring as a potential zoonotic pathogen. It has been transmitted by contact and can cause clinical disease in ferrets, the preferred animal model for human influenza studies.[4]

3.3 Geographic Distribution

Influenza is a virus of global consequence, causing human infection in every country in the world.[6] IVC also has a wide distribution, with isolates obtained in China, Japan, England, France, Greece, Brazil, South Africa, the United States,[5] Canada, the Philippines, Cuba, Spain, France, Italy, India, Finland,[30] Nigeria,[31] and possibly others. In humans, IVC circulates with IVA and IVB causing minor local epidemics, but has never been implicated in a global pandemic.[16,32] Instead, genetic and antigenic variants of IVC tend to co-circulate within a limited geographic area, during a given time period.[10]

A seroprevalence of 50% or higher has been observed in surveyed human populations in the United States, France, Scotland, Spain, England, Jamaica, Brazil, Japan, the Philippines, and Reunion Island in the southwest Indian Ocean.[33] Antibodies to the virus have been identified in pigs in China,[21] Japan,[29] and Great Britain,[34] but associated clinical disease caused by natural infection has not been reported in these cases.[21,29,34] To date, the only IVC isolation from pigs has been in China.[35]

IVD, initially thought to be a subtype of IVC, has been isolated from pigs in Oklahoma and from cattle in China, France, and the United States, including the states of Minnesota, Kansas, Nebraska, Oklahoma, and Texas.[35] The relatively recent discovery of this virus limits our understanding of its geographic origin and global prevalence, which can be further explored with retrospective studies of archived samples.[26]

IVC generally circulates in winter along with IVA and IVB, causing similar clinical signs.[5,12,36] Studies have shown that some swine influenza viruses circulate year-round, and seasonality may become less noticeable with the advent of indoor confinement systems in swine production.[27] Antibodies to IVC were detected every month of the year in a seroepidemiological study of pigs in China, suggesting the potential for natural infection in populations throughout the year.[21] Similar results were observed in a later study in Japan.[29] Summer infections with IVC have also been observed in humans, albeit less frequently than winter infections.[7,33] It has been suggested that IVC follows a circulation pattern unlike typical IVA infection, persisting throughout the year with some seasonal variation, rather than by re-introduction of new variants every year.[33] Seasonal peaks in IVC infection do not necessarily overlap with peaks in IVA infection.[37]

3.4 Morbidity and Mortality

Between 5 and 20% of humans in the United States are infected each year with some form of influenza. It is estimated that for every 1,000 people infected about 40 require hospitalization and one dies. Globally, seasonal influenza epidemics in humans are responsible for 250,000 to 500,000 deaths every year and millions during less frequent pandemics.[6] A pattern of small biennial human IVC epidemics has been reported in Japan,[37] including 131 cases in Japan in 2004.[12]

Despite its global prevalence, little is known about IVC epidemiology worldwide. Recurrent IVC infection occurs frequently in children and also adults, and the majority of humans develop antibodies to IVC early in life.[11] A serological study of humans in France indicated that as much as 61–70% of the population had been previously exposed to IVC, with the highest rates of positive samples in the 16–30

year-old range.[36] It has been estimated that as many as 80% of humans worldwide will acquire antibodies to IVC during their lifetime.[14]

Serological surveys of pigs at slaughter in China and Japan have shown rates of IVC seropositivity ranging from 3–19%; another study found that nearly 10% of pigs were seropositive for IVC at slaughter in Great Britain.[34] Seroprevalence of IVD in swine tested in the United States is 9.5%, based on the limited amount of data currently available.[19]

4. Transmission

The primary mode of influenza transmission in swine is thought to be through nasopharyngeal exposure by direct pig-to-pig contact.[27] Spread of human and swine IVC isolates from experimentally infected swine to naïve swine by direct contact has been reported.[21,29] Influenza can potentially be transmitted by contact with contaminated surfaces,[6] and aerosol transmission has been demonstrated in humans and horses.[27] Close contact during transport and intensive farming practices are thought to facilitate the spread of the virus.[6] IVC is also transmitted efficiently from person-to-person, and can be maintained by recurrent infections in human populations.[29] In pigs, influenza replicates only in the upper and lower respiratory tracts, thus transmission is able to occur solely via the respiratory route.[27] The virus can spread throughout an entire herd within days and may be spread to adjacent farms by wild boars or other wild animals.[6] Pigs can remain infectious for 25 days after inoculation with IVC.[21]

There is no evidence of IVC being spread through consumption of meat from infected animals.[6] Studies with the highly pathogenic H1N1 strain of IVA have further confirmed the absence of virus in pork and muscle tissue.[27]

5. Infection in Swine/Pathogenesis

5.1Clinical Signs

Influenza viruses are a major cause of acute respiratory disease outbreaks, but infection can also be subclinical. General influenza symptoms in swine include high fever, anorexia, inactivity, huddling, tachypnea, coughing, dyspnea, and labored abdominal breathing.[27] However, these clinical signs have not been attributed specifically to IVC.

Swine experimentally infected with IVC may exhibit normal temperature up to eight days post-exposure and typically fail to exhibit traditional influenza-like symptoms. Several pigs have shown slight dyspnea and increased nasal secretion after intranasal inoculation, clearing quickly or persisting for up to ten days. No other clinical signs of disease have been reported.[21]

Viruses in the newly proposed *Influenzavirus D* genus may be more pathogenic in swine than IVC. Influenza-like illness was first observed in 15-week-old pigs naturally infected with IVD in April 2011. A retrospective serological analysis of samples from United States swine farms (2010–2012) identified four additional positives via RT-PCR. However, experimental infection in 10 week old pigs resulted in no clinical signs.[16]

5.2 Postmortem Lesions

Postmortem lesions specific to IVC infection in swine have not been described, as clinical disease and fatalities are thought to be rare. Gross lesions caused by more pathogenic influenza viruses are observed in the lungs, airways and regional lymph nodes. Affected lung tissue may be firm and purple in color with a sharp line of demarcation between normal lung tissues. Interlobular edema and blood-tinged fibrinous exudate in the airways may also be present. Potential microscopic lesions include lung epithelial necrosis,

denudation of bronchial epithelium, and inflammatory cells, mainly neutrophils, in the airways. Lesions are not always present and are often complicated by intercurrent bacterial infection.[27]

6. Diagnosis

6.1 Clinical History

The first documented case of an influenza virus in swine occurred in 1930, following the suspicions of simultaneous infection of pigs during the human pandemic of 1918. IVC was first isolated from healthy pigs in China in 1981,[21] and IVD was first isolated from swine displaying influenza-like symptoms in Oklahoma in 2011.[2] Up until 1997, IVA subtype H1N1 was the strain almost exclusively attributed to influenza infection in pigs. New strains later emerged featuring genes that were a product of reassortment of human, swine, and avian influenza viruses. As of 2009, the only known influenza viruses endemic in pigs were IVC and IVA subtypes H1N1, H1N2, H3N1, H3N2, and H2N3.[6] Whether or not IVD is also endemic in swine herds remains to be seen.

6.2 Tests to Detect Nucleic Acids, Virus, or Antigens

Historically, clinical diagnosis of influenza has been achieved by virus isolation or serological tests, though it is thought that potentially low viral loads may necessitate more sensitive methods.[5] IVC is not readily isolated and cultured due to the permissibility limitations of many cell lines.[16] Virus isolation has been achieved by inoculation of 8-10 day old embryonated chicken eggs[8,9,11,24,29,34,36,38] and propagation onto Madin-Darby canine kidney (MDCK) I,[3,17,30,33,36,38,39] human hepatocarcinoma (HuH7),[14] and human malignant melanoma (HMV-II) cells.[38] In addition to African green monkey kidney (Vero), MDCK I,[40] and human embryonic kidney (293T) cells,[3] HMV-II cells have also been used for creating and obtaining *in vitro* reassortant viruses.[8] MDCK II,[3] MDCK I,[40] and monkey kidney (LLC-MK_2) cells have been used in IVC plaque assays.[8]

HMV-II cells are more susceptible to IVC than MDCK and LLC-MK_2 cells,[41] but they tend to be slow growing and lose susceptibility to infection over time.[38] Also, even at optimal temperature, IVC exhibits much slower growth on susceptible cells than IVA or IVB.[3] The supplementation of MDCK cells with high concentrations of trypsin can increase their susceptibility to IVC infection.[38] Coating resistant cell lines with bovine brain gangliosides (BBG) can also help to facilitate infection, and has been used in the development of a plaque assay for IVC on MDCK II cells.[3] In contrast, IVD can be readily isolated and cultured in swine testicle (ST) cells.[16]

Hemagglutination inhibition (HI) assays are performed to identify unknown influenza viruses by their antigenic cross-reactivity[16] and separate isolates into antigenic groups. Antigenic variation among IVC isolates can be demonstrated with anti-*HE* monoclonal antibodies.[11,30,37] IVC is known to cause agglutination in chicken or turkey erythrocytes, but not in guinea pig erythrocytes.[12,21,30,41] A radioimmunoprecipitation test (RIP) has also been described to determine which viral components anti-IVC antibodies in pig sera are directed toward.[29]

Conventional reverse transcription polymerase chain reaction (RT-PCR) can be used for detection of viral RNA in infected supernatants.[3,12,17,30] It is worth noting that retrospective RT-PCR diagnosis has been observed in humans following a negative result of virus isolation,[12] although weak positive samples may contain degraded rather than infectious virus.[27] Real-time RT-PCR has also been used in detection of IVA, IVB,[39,42] and IVC,[33,39,43] as well as the recently proposed IVD.[2,16,19,26] Multiplex PCRs have been described, one for the detection of human IVC, rhinovirus, and coronavirus[14] and another for simultaneous detection of IVA, IVB, IVC, adenovirus, and respiratory syncytial viruses A and B.[44]

Electron microscopy studies of cell cultures can tentatively identify characteristics of orthomyxoviruses, while enzymatic assays can further detect traits of a particular genus of influenza. With these methods, RT-PCR is typically performed in tandem for definitive diagnosis.[16]

6.3 Tests to Detect Antibody

Serological assays are a valuable diagnostic tool for the typing of newly identified influenza viruses.[2] Specific antibodies against IVC virion proteins can be detected with enzyme-linked immunosorbent assays (ELISA),[24,29,33] HI,[24,29,33,34] or serum neutralization (SN) tests.[29,33,34] When performed on the same samples, results of HI and SN have shown complete correlation.[29,34] Sensitivity of ELISA is thought to be similar[33] or slightly higher than HI, and a positive result in both assays is sufficient confirmation of infection. Results can also be confirmed by Western blotting.[24]

6.4 Samples

6.4.1 Preferred Samples

Influenza viruses replicate in the epithelial cells of the upper and lower respiratory tract of pigs, thus infectious virus can be isolated from nasal mucosa, ethmoid, trachea, lungs, tonsils, and regional lymph nodes. Bronchoalveolar lavage has been used, as well as nasal, tonsil, and oropharyngeal swabs.[27] Lung tissue and nasal swabs of experimentally infected pigs have successfully been used for real time RT-PCR in detection of IVD.[16] Additionally, nasopharyngeal aspirates, nasal swabs, and nasal washes have been used in human diagnosis.[44] Serum samples from pigs have been used in HI, SN,[34] ELISA, and RIP tests.[29]

6.4.2 Oral Fluids

Isolation of IVC has been achieved with pooled pharyngolaryngeal and tracheal swabs of pigs.[21] Throat swabs have been used in human diagnosis by virus isolation[41] and RT-PCR, [17,31,44] and sputum samples have been used for real-time RT-PCR assays.[39]

7. Immunity

7.1 Post-exposure

IVC may cause persistent infection in pigs, and they can remain infectious for up to 25 days following inoculation with pig or human IVC isolates.[21] The immune response to influenza in swine includes cell-mediated immunity and the production of antibodies,[27] and the response can vary in pigs inoculated with human or pig strains of the virus.[21] Immunity against the specific strain causing the infection may last several years based on human studies, although the exact duration has not been studied in pigs.[27] The observation of IVC reinfection in humans, especially within short periods of time, suggests that infection does not induce sufficient protective immunity in all individuals.[12]

7.2 Vaccines

A variety of seasonal human vaccines are available in the United States for IVA and IVB. These include inactivated trivalent and quadrivalent vaccines, recombinant trivalent vaccines, and live-attenuated quadrivalent vaccines, with delivery by intramuscular injection, intradermal injection, or nasal spray.[45] Similarly, IVA vaccines are available for swine in the United States. Zoetis currently produces the only commercial vaccine offering protection against the pandemic H1N1 virus.[46] As IVC predominantly infects humans, no swine vaccines are currently available. However, because of its lack of involvement in influenza pandemics, IVC is also not included in seasonal influenza vaccines for humans.[5]

7.3 Cross-protection

IVC is much more antigenically stable than IVA, and a high degree of cross-reactivity is observed among isolates of IVC. Antigenic variation among IVC isolates can be demonstrated with anti-*HE* monoclonal antibodies.[11] Analysis with polyclonal serum has indicated cross-reactivity among all examined isolates,

even from different time periods and countries. Another study has shown that 24 Japanese strains from a single lineage, isolated over the course of a decade, are antigenically indistinguishable.[5]

The same level of cross-reactivity is not observed between isolates from pigs and humans. Pigs infected experimentally or by contact with infected pigs all develop a detectable antibody response within two weeks. However, in pigs infected with IVC isolated from humans, a detectable antibody response only occurred in two out of six individuals.[21]

Antibody cross-reactivity has also not observed between IVC and IVD isolates.[19]

8. Prevention and Control

Farm workers, veterinarians, and meat processing workers with increased exposure to swine and poultry represent a heightened risk for potential zoonotic influenza infection and viral reassortment. Surveillance of these populations is of increasing importance in public health.[6] Vaccination is the primary method for controlling pathogenic influenza viruses in swine,[27] although this is not currently applicable to IVC or IVD. Neuraminidase inhibitor, used to treat IVA and IVB, is ineffective in treatment of IVC infection.[37] Personal protective equipment and avoidance of close contact ("within six feet," according to the U.S. Occupational Safety and Health Administration) with affected individuals can help to reduce the risk of infection in susceptible populations.[6]

9. World Organization for Animal Health (OIE) Terrestrial Animal Health Code

Avian influenza and equine influenza are covered in the 2015 OIE Terrestrial Animal Health Code (http://www.oie.int/international-standard-setting/terrestrial-code/access-online/), but there are no recommendations for importation of swine from countries or zones infected with influenza.

10. Gaps in Preparedness

Our understanding of IVC continues to lag far behind other influenza viruses, perhaps due to the relatively mild nature of the illness it causes and the lack of rapid IVC-specific diagnostic testing. Infection in humans can also be accompanied by other respiratory pathogens, further complicating diagnosis and evaluation of clinical symptoms.[32,39,44] Co-infection with respiratory syncytial virus (RSV), adenovirus, rhinovirus, IVA, and IVB often occurs,[14] including a report of the pandemic 2009 IVA (H1N1) combined with an IVC infection reported in a human patient in Spain.[7] Long term monitoring of the virus also occurs infrequently,[30] and inclusion of IVC in respiratory virus testing panels could help to better understand epidemiological and clinical aspects of the disease.[33] The lack of vaccines and rapid clinical diagnostic tests for IVC also limit our ability to respond appropriately to it. Many methods of virus isolation and serological analysis used in research are not designed for clinical use and probably underestimate the actual prevalence of IVC due to low viral loads in respiratory tissues.[5]

A more complete understanding of the effects of IVC in different species is vital in monitoring the spread of the virus. *In vitro* infection of embryonic chickens has demonstrated persistent infection in the lungs,[36] and the interplay between human, swine, canine, equine, and bovine IVC infection is poorly understood. More research is needed on the recently discovered IVD in swine and cattle. It is known to spread among pigs by direct contact and can also be transmitted to and spread among ferrets, the preferred animal model for human influenza infection studies. If this novel virus is capable of reassortment with human IVC strains, it may allow for divergence and greater pathogenicity in humans.[16] Attempts at *in vitro* reassortment with human IVC were unable to produce viable progeny,[2] and sequence analysis of the seven gene segments of known isolates show no evidence of reassortment between IVC and IVD,[26] though the possibility cannot be definitively ruled out. Current understanding is limited by the paucity of similar isolates available for study. Analyses of the evolutionary rate will be critical in understanding the

evolution and host range of this novel virus,[4] and many questions remain regarding pathogenicity, temporal distribution, and zoonotic potential.[25]

Information on human Japanese IVC isolates has been obtained in the last ten years, but data on many other strains dates back to prior decades. Identification of contemporary strains and mutations will be crucial to developing a better understanding of the evolution, distribution, and virulence of the virus.[5] Reverse genetics, specifically the generation of recombinant viruses from cloned cDNA with specific protein deletions, is one method currently being explored in the analysis of the epidemiology and molecular virology of IVC.[10,40] The introduction of viable mutations into the genome will enable further study of the viral life cycle and functions of specific viral proteins as they relate to pathogenicity.[3]

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