2018

Serological Evidence for the Co-Circulation of Two Lineages of Influenza D Virus in Equine Populations of the Midwest United States

Hunter Theodore Nedland
South Dakota State University

SEROLOGICAL EVIDENCE FOR THE CO-CIRCULATION OF TWO LINEAGES OF INFLUENZA D VIRUS IN EQUINE POPULATIONS OF THE MIDWEST UNITED STATES

BY

HUNTER THEODORE MAY NEDLAND

A thesis submitted in partial fulfillment of the requirements of the

Master of Science

Major in Biological Sciences

Specialization in Microbiology

South Dakota State University

2019

SEROLOGICAL EVIDENCE FOR THE CO-CIRCULATION OF TWO LINEAGES OF INFLUENZA D VIRUS IN EQUINE POPULATIONS OF THE MIDWEST UNITED STATES

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Feng Li, PhD — Date
Thesis Advisor

Volker Brozel, PhD — Date
Head, Biology and Microbiology

Dean, Graduate School — Date

ACKNOWLEDGMENTS

I would like to express my most sincere gratitude to my advisor, Dr. Feng Li, who assisted me throughout my master's program. His guidance throughout my project and his support in preparing me for my classwork and research presentations has really allowed me to be highly successful at SDSU. I would also like to thank my committee members, Dr. Natalie Thiex, Dr. Radhey Kaushik, and Dr. Melissa Hauschild-Mork, for being available and helping me to analyze my classwork, my data, and my overall progress. It is impossible to ignore the enormous positive influence all of the students working in Dr. Feng Li's lab have been. Each and every colleague provided much needed support and guidance as I completed my work. A sincere thanks also goes to the Serology lab in the Veterinary Sciences building for providing the samples and related data for this project. I would also like to take the time to thank Jared Wollman who spent at least three semesters helping me with completing the various experiments and procedures for this project. Throughout my Master's work, I also received significant assistance and support from many faculty members in the Department of Biology and Microbiology as well as the Department of Veterinary Sciences. I am extremely grateful for their expertise, support, and overall positivity towards me during my time at SDSU working on my Master's degree. I couldn't have done it without all of the amazing people around me.

TABLE OF CONTENTS

ABBREVIATIONS

CDC = Center for Disease Control

C/JHB = C/human/Johannesburg/1/1966

D/OK = D/swine/Oklahoma/1334/2011

D/660 = D/bovine/Oklahoma/660/2013

DMEM = Dulbecco's Modified Eagle's Medium

DNA = Deoxyribonucleic Acid

HA = Hemagglutinin

HEF = Hemagglutinin-esterase Fusion Protein

HI = Hemagglutinin Inhibition

IAV = Influence A Virus

IBV = Influenza B Virus

ICV = Influenza C Virus

IDV = Influenza D Virus

MDCK = Madin-Darby Canine Kidney

MN = Microtiter Neutralization

NA = Neuraminidase

NP = Nucleoprotein

PA = Polymerase Acidic Protein

PB1 = Polymerase Basic Protein 1

PB2 = Polymerase Basic Protein 2

RNA = Ribonucleic Acid

SDSU = South Dakota State University

TPCK = Tosylsulfonyl Phenylalayl Chloromethyl Ketone

USA = United States of America

WHO = World Health Organization

LIST OF FIGURES AND TABLES

Page

ABSTRACT

SEROLOGICAL EVIDENCE FOR THE CO-CIRCULATION OF TWO LINEAGES OF INFLUENZA D VIRUS IN EQUINE POPULATIONS OF THE MIDWESTERN UNITED STATES

HUNTER THEODORE MAY NEDLAND

2019

Influenza D virus (IDV) is a newly described lineage of the *Orthomyxoviridae* virus family that was first isolated from diseased swine in 2011 and has subsequently been detected in cattle around the world in 2014. In addition, serological evidence for IDV infection in humans has been recently established. Despite all the progress, the full range of susceptible hosts for this novel virus has yet to be determined, but includes swine, bovine, small ruminants and human. This study was designed to determine if equine is a possible host to this newly emerging influenza virus. 364 equine serum samples were collected in 2015 from 141 farms within the Midwestern United States. Serum samples were examined using hemagglutination inhibition (HI) assay against two established IDV lineages (D/OK and D/660) and one IDV-related human ICV lineage (C/JHB). Results of this study showed 44 (44/364, 12%) samples positive for antibodies against D/OK, 39 (39/364, 11%) samples positive for antibodies against D/660, and 41 (41/364,11%) samples positive for antibodies against C/JHB. A subset of these samples was further confirmed via microtiter neutralization (MN) assay. Our data demonstrated that horses are susceptible to two lineages of IDV, and that these viruses were present in equine populations throughout multiple Midwestern states of the United States. These findings

continue to support the need for further surveillance of IDV viruses in agricultural species in order to work toward a better understanding of the full host range and natural reservoirs of influenza D virus.

Chapter 1: Introduction and Background

1. Influenza Virus

Influenza viruses are a group of pathogens responsible for causing infectious disease on a global scale. They belong to the viral family *Orthomyxoviridae* and make up four of the six genera of that family. The four genera of influenza virus are influenza A (IAV), influenza B (IBV), influenza C (ICV), and influenza D (IDV). These genera are classified by analysis of the differences in their matrix proteins and nucleoproteins [21]. Influenza viruses are negative-sense, segmented RNA viruses that require an RNA-dependent viral RNA polymerase to facilitate replication [5, 11, 42]. These viruses are capable of infecting a wide range of hosts including: humans, numerous avian species, bovine, swine, canines, equine, small ruminant species, camels, ferrets, cats, seals, and whales [23, 57, 58]. Infection with influenza virus causes respiratory or intestinal disease in many of these species [3, 10, 18, 24]. This chapter of the thesis will examine influenza viruses in the context of epidemiology and disease surveillance, and how that approach is applied to influenza C virus and the novel influenza D virus.

Influenza A and Influenza B

Influenza A viruses (IAV) and influenza B viruses (IBV) infect a wide range of hosts, both mammalian and avian [4, 18, 27, 47, 57]. The genomes of these related viruses are made up of eight negative-sense strands of RNA. These genomic segments encode for the following proteins: three RNA-dependent RNA polymerase proteins known as polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase

basic protein 2 (PB2), as well as PB1-F2 (a non-structural protein), hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), M1 matrix protein, M2 ion-channel protein, and two additional non-structural proteins known as NS1 and NS2 [11, 27]

IAV subtypes are determined by the variety of the HA and NA glycoproteins expressed on the surface of the virion [3, 31, 41]. There are sixteen HA subtypes (H1-16) and nine NA (N1-9) varieties that can be expressed in combination [27]. This large diversity of antigenic potential allows IAV to reach pandemic levels in human populations, as humans do not develop immunity through exposure to all subtype combinations throughout their lives [26].

IBV is more restricted in its variety, as it does not re-assort with the highly mutable IAV subtypes and does not have any known animal reservoirs, though infection has been recorded in seals [20]. Only two antigenically and genetically different varieties of IBV have been known to circulate around the world since 1983, known as B/Victoria/2/87 and B/Yamagata/16/88 [20, 27]. Though limited in diversity, these lineages cause significant disease in humans and cause epidemic outbreaks approximately every three years in place of an IAV subtype, and are usually included in the mixture of subtypes immunized against in the yearly influenza vaccine [27, 41].

IAV and IBV are the primary causes of influenza infection in humans, and are responsible for the re-occurring seasonal influenza epidemics as well as the sporadic worldwide pandemics [16, 41, 54]. Infection in humans causes general respiratory disease with fever, soreness, and lethargy, with the potential to be more serious in the very young and very old groups. [6, 30]. Seasonal epidemics and the occasional pandemics attributed to influenza infection have significant economic and health-related costs on society, with

notable increases in mortality and economic loss associated with influenza outbreaks [2, 12, 36, 46].

Influenza C Virus

Influenza C virus (ICV) differs genetically from IAV and IBV in that it only contains seven negative-sense genomic RNA segments, and does not contain segments that code for either the hemagglutinin (HA) or neuraminidase (NA) proteins. Instead, ICV contains an RNA segment that codes for the hemagglutinin-esterase fusion (HEF) glycoprotein. This HEF glycoprotein facilitates the function of both the HA and NA proteins of IAV and IBV [61]. Additionally, ICV produces an alternative matrix protein 2 when compared to IAV or IBV, called CM2 [51]. ICV primarily infects humans and has no animal reservoir, though it has been isolated from infected swine and dogs [34, 49, 60]. It is not a pandemic capable virus, though isolated outbreaks can occur, often among children [35, 49]. Infection in humans usually occurs early in life, causes mild cold-like symptoms, and the host develops broadly neutralizing antibodies that usually prevent future infection of any other ICV variant [29, 34]. When serological surveillance was conducted on human populations, one in 1992 in France and another during 2000 in Brazil, high percentages of samples tested showed antibody titers consistent with past infection with ICV [33, 37]. Multiple lineages of ICV appear to co-circulate at any given time, and do exhibit re-assortment [39, 49].

2. Novel Influenza D Virus

In 2011, a viral sample was isolated from diseased swine in Oklahoma that shared 50% homology with known ICV samples, and was originally designated a

separate strain of ICV. Upon further investigation and more sample isolation, it was determined that the samples isolated from swine and bovine hosts represented a new genus of the *Orthomyxoviridae* virus family due to the lack of *in vitro* re-assortment between human ICV samples and the newly isolated animal viruses [23]. Additionally, the newly isolated viruses expressed a unique splicing strategy for the production of the M1 protein [23]. The new genus was named influenza D (IDV) in 2013 and officially accepted by the scientific community as a novel virus genus [32]. Similar to ICV, it has a seven-stranded, segmented, negative-sense RNA genome. IDV is closely related to ICV and expresses the HEF glycoprotein instead of HA and NA proteins found on IAV and IBV [23]. Since its discovery, serological surveillance of human and animal populations has shown evidence of infection with IDV. Its primary reservoir appears to be cattle herds, and it has been isolated from or detected in bovine populations living in multiple countries around the world [8, 9, 14, 15, 23, 32, 38, 58]. Other animal species have been shown to be vulnerable to infection, including sheep, goats, swine, ferrets, and guinea pigs [50]. Serological evidence of infection has also been detected in humans who work closely with cattle [58].

Influenza D Virion Structure

All influenza viruses exhibit a spherical particle shape composed of a host-cell derived lipid membrane with embedded viral surface protein spikes. The virion structure of IDV is very similar to that of ICV. Like ICV, IDV particles express two surface proteins: hemagglutinin-esterase fusion protein (HEF) and CM2. Beneath the lipid membrane the viral structure is made up of a layer of matrix protein 1 (M1) that connects the outer membrane with the inner components of the particle. Within the matrix layer of

the virion lies the negative-sense RNA genome surrounded by nucleoprotein (NP), and attached to each segment of the RNA genome is an RNA dependent RNA polymerase complex. This polymerase complex is a trimeric complex made up of three proteins: polymerase base 1 (PB1), polymerase base 2 (PB2), and polymerase 3 (P3). Each genomic RNA segment and its attached RNA polymerase complex is surrounded by the NP protein to form a structure called the viral ribonucleoprotein (RNP) complex.

Influenza D Genome Structure

The influenza D genome is composed of seven negative-sense single stranded RNA segments. These seven genomic segments produce the nine proteins that make up the IDV particle structure and life cycle.

The three largest segments code for the three proteins that make up the trimeric RNA polymerase complex: PB1, PB2, and P3. The next largest segments codes for the HEF surface protein. The fifth segment of the genome codes for the NP protein.

The sixth genomic segment produces two proteins, the first being the M1 protein that is produced via mRNA splicing. Influenza D is distinguishable from ICV in its M1 splicing method. ICV splices the mRNA of the sixth segment by introducing a termination codon consisting of nucleotides 752, 753, and 982. In contrast, the IDV modifies this splicing strategy for M1 production by adding an additional 4-amino-acid peptide into the preceding exon [23]. The second is the DM2 protein, which is synthesized by the cleavage of the protein produced from the un-spliced mRNA of the sixth segment.

The seventh genomic segment produces non-structural proteins, called nonstructural protein 1 (NS1) and nonstructural protein 2 (NS2). NS1 is produced directly from the seventh segment, while NS2 is produced via mRNA splicing. IDV is similar to other influenza viruses in its NS2 splicing strategy, but it does produce uniquely spliced sequences [23].

Influenza C and D Life Cycle

Due to the fact that IDV has only recently been characterized and described, little is known about the details of its life cycle. As stated above, IDV is most closely related to ICV, and therefore it can be assumed that IDV's life cycle will most closely resemble that of ICV's. Among other influenza viruses, IDV shares the most structural and genetic traits with ICV, and as more work is done to fully characterize the life cycle of IDV, it is likely that many aspects will resemble those found in ICV. The following section will detail the life cycle of ICV, and what is currently known of the IDV life cycle.

Like all influenza viruses, the life cycle of influenza C begins with attachment of the viral particle to a host cell [42]. The major surface glycoprotein of ICV, hemagglutinin-esterase fusion (HEF) protein, mediates the binding of the virus particle to the host cell by recognizing the sialic acid 9-*O*-acetyl-*N*-acetylneuraminic acid (9-*O*-Ac-Neu5Ac) on the surface of epithelial cells [48]. Cells expressing 9-*O*-Ac-Neu5Ac can be found in the respiratory systems of numerous mammal species, which may explain the broad species tropism seen with ICV [56].

Upon attachment to 9-*O*-Ac-Neu5Ac on the surface of the host cell, ICV must gain entry into the cell to continue the life cycle. The entry of the virus particle into the host cell likely occurs via clatharin-mediated endocytosis as reported for other influenza viruses [42]. Endocytosis and entry into the cytoplasm of the host cell requires a low pH environment in order to trigger the next step, cell membrane fusion. Fusion occurs when the cellular endosomal membrane fuses with the viral membrane, and is caused by a low pH environment within the endosome. The low pH environment is important to the fusion process because this induces a structural change in the HEF protein that is necessary for the continuation of the life cycle [17].

The structurally changed HEF protein is cleaved by a host-cell trypsin-like protease to produce two subunit peptides called HEF1 and HEF2. After this, the amino terminus of HEF2 is exposed, allowing for its interaction with the endosomal membrane while its transmembrane domain is embedded in the viral membrane [48]. This interaction with the endosomal membrane allows for both the viral membrane and the endosomal membrane to fuse. Once the membranes have fused, the viral genome and its associated proteins can be released into the cytoplasm of the host cell.

Releasing the viral contents occurs via a process called uncoating. The uncoating process begins with a viral protein called CM2 acting as an ion channel into the interior of the viral particle. Ion channel CM2 allows for hydrogen ions from the endosomal compartment to flow into the virion, lowering the pH and disrupting interactions between multiple copies of the matrix protein 1 (M1) [51]. M1 serves as the internal structure of the virion and the uncoating process is completed by disrupting its interactions [42].

Once the internal contents of the virion are released, the viral RNA genome is transported to the nucleus of the host cell to begin the process of replication. The RNA particles that are transported to the nucleus are surrounded by a protein called nucleoprotein (NP), and the viral nucleoprotein RNA complex is known as RNP [42]. Each viral RNA genome segment also has a RNA polymerase complex bound to its end. This complex is a trimer and is made up of three proteins known as polymerase basic protein 1 (PB1), polymerase base protein 2 (PB2), and P3. The RNA genome segments with their bound RNA polymerase complexes cannot passively diffuse into the nucleus of the host cell, and therefore require active transport through a nuclear pore. In order to be transported, the RNP/polymerase complex requires a nuclear localization signal that is located on the NP protein [52].

To facilitate active transport into the nucleus of the cell, a cellular cargo protein must be recruited to the nuclear localization signal. An importin-family transport protein recognizes the signal carried on the NP protein and allows for the RNP complex to dock to the nuclear pore and be transported into the nucleus for RNA synthesis [52].

Synthesis takes place via a two-step process involving the trimeric RNA polymerase complex. The first step is the transcription of messenger RNA (mRNA) from the imported viral RNA (vRNA), where each genomic sequence is transcribed via a primer dependent mechanism. After the mRNA is transcribed, a full-length positive-sense copy of each of the vRNA genomic sequences is synthesized. Known as complementary RNA (cRNA), these full-length copies serve as a template for the synthesis of additional vRNA genomic segments. Once new vRNA segments are synthesized they are

surrounded with NP proteins that will assist in the trafficking of the new RNP to the cell membrane [42].

The newly synthesized RNP must be exported from the nucleus, and this process is mediated by interactions between the NS2 viral protein and host nucleoporins. Once the RNP has exited the nucleus, they are trafficked to the site of viral assembly on the plasma membrane. Once the RNP has exited the nucleus, it travels to the site of viral assembly, which is made up of lipid rafts coming together on the host cell membrane [42, 52]. The transportation of the RNP to the plasma membrane is thought to be mediated by interactions between the M1 and other viral proteins [17, 51].

The major surface proteins HEF and CM2 are also required for assembly to complete. These proteins are associated into the lipid raft after they are synthesized. Synthesis of HEF and M2 takes place in the cellular endoplasmic reticulum (ER), where they are properly folded and, in the case of HEF, glycosylated [17, 42]. The HEF proteins are then associated into the trimeric protein spike structure and the CM2 proteins are assembled into tetramers. After folding and glycosylation, the proteins undergo further modification in the trans-Golgi network to prepare them for association into the cellular membrane [17]. The HEF and CM2 proteins are directed toward the site of viral assembly by sorting signals contained in both proteins, in addition to the sorting signals each protein contains a signal that directs its association into the lipid rafts that are part of the host cell membrane [51, 52]. These lipid rafts act as the site of virion assembly and are rich in cholesterol and sphingolipids, components important to the construction of the virus particle. Once the proteins have been sorted, HEF is directly incorporated into the lipid raft while CM2 binds with cholesterol molecules that are part of the lipid raft [17].

The binding of CM2 to the cholesterol is thought to aid in virion construction by linking together multiple lipid raft domains on the cell membrane. The CM2 protein is also important in the incorporation of RNP into the developing virion, and virus lacking in CM2 protein developed particles without genomic RNA segments incorporated [17, 51].

The next steps in viral assembly are currently less understood. The current hypothesis is that M1 protein acts as a recruiting signal for non-structural viral proteins as well as the RNP genetic core [42, 52]. The M1 protein itself lacks a membrane targeting signal, so it is hypothesized that the M1 protein interacts in some way with the HEF protein for correct membrane targeting [42]. The M1 recruiting signaling is thought to bring the additional components to the site of viral assembly to fully complete the recruitment of viral proteins.

Once all of the viral proteins and the RNP are recruited and integrated into the host membrane, the process of budding begins. Budding is the outward curving of the host cell membrane that eventually leads to the new virus particle releasing [51]. The current model of influenza budding describes the process beginning with the incorporation of viral proteins into the host cell membrane lipid rafts. This leads to the clustering of lipid rafts at the site of viral assembly as more viral proteins are integrated, causing the membrane to deform [17, 42]. The deformation of the membrane allows for the cytoplasmic tails of the viral proteins to interact, recruiting the RNP genetic core as well as other non-structural viral proteins to the budding virion. As more proteins are recruited and incorporated, the deformation becomes more pronounced until the new virion is ready to cut away from the host cell in a process called scission [42, 52]. The CM2 protein is thought to play a critical role in this process [51]. Finally, after scission is

achieved, the esterase activity of the HEF surface protein may be required to cleave the 9-*O*-acetyl group from 9-*O*-Ac-Neu5Ac to fully release the new virion from the host cell membrane surface [48].

3. Serological Surveillance

Viruses that pose a risk to human or animal health are often under surveillance in order to track the prevalence, variety, changes, and locations of infections [1, 19, 25, 55]. Influenza is under constant surveillance around the world to track changes in the currently circulating virus varieties, determine where outbreaks will occur, and determine the best possible combination of vaccine candidates for the yearly influenza vaccination [7].

Surveillance can be accomplished via serological methods of detecting antibodies in animal samples, genomic sequencing of viral samples, detection of RNA expression, or tracking reports of infection [1, 35, 40, 53]. The World Health Organization (WHO) provides researchers and health workers with a reference guide for the correct conduction of influenza surveillance, titled “Manual for the laboratory diagnosis and virological surveillance of influenza”, and the American Center for Disease Control (CDC) provides an overview on their website of their current efforts in monitoring influenza activity in the USA and around the globe [1, 7].

The active monitoring and documentation of influenza is an on-going global project, and with the recent discovery of the novel genus influenza D, that work must be expanded to include fully elucidating the host range of the potentially harmful new virus. By broadening our understanding of what species of animal are capable of carrying IDV, we can better assess the risks the disease poses to human health and economy.

Chapter 2: Serological evidence for the co-circulation of two lineages of influenza D virus in equine populations of the Midwest United States

Background

The *Orthomyxoviridae* virus family has three influenza genera, A, B, and C, which are classified according to antigenic differences in their nucleoprotein and matrix proteins [42]. The fourth genus of influenza, named influenza D, has been recently described (https://www.cdc.gov/flu/about/viruses/types.htm). Influenza D (IDV) represents a novel type of virus more closely related to influenza C (ICV) than influenza A (IAV) or influenza B (IBV). IDV uses bovine as its primary reservoir, and has been isolated from cattle herds from multiple countries including: China, France, Italy, and the United States [8, 22, 28]. Susceptibility to infection by this novel virus has also been demonstrated in swine, sheep, goats, guinea pigs, and ferrets [43, 50]. In addition, serological evidence for IDV infection in humans has been recently established [58].

The worldwide prevalence and broad species tropism of this new influenza virus represents a growing potential threat to humans and other agricultural species. Many species are vulnerable to influenza infection, including humans; therefore, it is important to identify other potential hosts of this novel type. The primary goals of this study were to investigate the seroprevalence of IDV in American equine populations by conducting multiple serological surveys, as well as studying the antibody prevalence in this animal host of human ICV, which is closely related to IDV.

Materials and Methods

2.1 Cell culture, reference serum, and virus production

Madin-Darby canine kidney (MDCK) cells (ATCC) were cultured using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (PAA Laboratories Inc., Dartmouth, MA, USA) and 1% streptomycin and penicillin (Life Technologies, Carlsbad, CA, USA). Isolated from bovine and swine presenting respiratory disease symptoms, influenza D/bovine/Oklahoma/660/2013 (D/660) and D/swine/Oklahoma/1334/2011 (D/OK) were grown on MDCK cells at 0.01 MOI and incubated at 37ºC with ~5% CO_2 for at least 5 days**.** D/OK and D/660 are representative strains of two antigenic lineages of IDV, D/OK being of swine origin and D/660 being of cattle origin. These two viruses have been found to be antigenically different lineages of IDV, likely due to their host divergence [23]. Influenza C/human/Johannesburg/1/1966, originally isolated from humans, was produced in the same manner.

To better promote viral growth, DMEM maintenance media was supplemented using 1 μg/mL tosylsulfonyl phenylalayl chloromethyl ketone (TPCK) trypsin (Sigma, St. Louis, MO, USA) and 1% penicillin and streptomycin (Life Technologies, Carlsbad, CA, USA). Virus titer ($TCID_{50}$ per ml) was measured using both the Reed and Muench's method and the hemagglutination assay as described in the W.H.O. standard manual[45](www.who.int/influenza/en/).

Reference serum was generated against the three influenza virus strains via rabbit immunization in lab at SDSU (IACUC # 16-027A) and at the Covance Research Products. Covance produced the D/OK reference serum, the D/660 and C/JHB serum was produced

at SDSU. All groups used a nearly identical method of generation involving hyperimmunization of immune-competent rabbits with UV-inactivated virus of the desired lineage formulated in adjuvant. The rabbits were immunized via both the intramuscular and subcutaneous routes. Serum was purified from blood drawn from the rabbits periodically throughout the generation procedure.

2.2 Serology

Hemagglutination inhibition (HI) and MDCK-based Microneutralization (MN) assays were employed as described in the WHO standard manual and previous literature to detect antibody titers [44](www.who.int/influenza/en/), with minor revisions to the MN assay for use with ICV and IDV. Our revision for the MN assay was that virus is cultured on MDCK cells in 96 wells plates in the presence of serially-diluted serum for 120 hours (5 days) to insure that the slow growing ICV and IDV viruses could reach a detectable titer level if they hadn't been neutralized by the antibodies present in the serum. For the assessment of titer, Turkey red blood cells (Lampire Biological Laboratories, Pipersville, PA, USA) were used in both the HI and MN assays. For both the MN and HI assays, a titer of 40 was used as the threshold to describe a positive sample in accordance to the WHO protocols and an additional confirmation study(www.who.int/influenza/en/)[59]. HI and MN assays were tested in duplicate and HI or MN titers were described as the reciprocal of the final serum dilution that blocks viral ability to agglutinate red blood cells or inhibit viral replication, respectively. All samples were assayed in three separate experiments, and the antibody titers were determined as the mean of these triplicate data.

2.3 Serum sample collection

364 equine samples were collected during the summer of 2015 from farms and ranches in six states; Iowa (IA), Minnesota (MN), North Dakota (ND), Nebraska (NE), South Dakota (SD), and Wyoming (WY). These states occupy a generally north-central location of the continental United States of America and share numerous borders with each other. These samples were collected through the Animal Disease Research and Diagnostic Laboratory at South Dakota State University (SDSU)**.** A further 100 equine samples were collected during the summer of 2016 again through the ADRDL at SDSU. The 100 samples were collected from horses from farms or ranches in Iowa (IA), Minnesota (MN), Nebraska (NE), and South Dakota (SD). Anti-sera against two IDV lineages (D/OK and D/660) and one human ICV (C/JHB) was generated from rabbits via immunization as described above, and collected for use as antibody controls and for the purpose of testing cross-reactivity between ICV and IDV.

Results and Discussion

We first investigated the potential cross-reactivity between IDV and ICV by the HI assay. Rabbit reference antisera to two IDV lineages, Influenza D/swine/OK/1334/2011 (D/OK) and Influenza D/bovine/660/2013 (D/660), and one human ICV, Influenza C/Victoria/2/2012 (C/Vic), as well as negative-control sera, were tested. Two IDV lineage-representative viruses, D/OK and D/660 as above, and one human ICV, C/Vic as above, were used in the HI cross-reactivity assay. As summarized in Table 1, the antiserum for the D/660 strain was equally reactive to the D/660 virus and the D/OK virus with an HI titer of 1280 against both, while having no detectable reactivity with C/Vic. Furthermore, the antiserum generated against the D/OK strain was more specific to the D/OK virus, with a HI titer of 2560, but was also cross reactive with D/660 with a titer of 640. The antiserum generated against C/Vic was specific to the C/Vic virus with a HI titer of 2560 with no detectable activity with either IDV lineage. These results confirmed the specificity of our HI assay in the detection of anti-IDV antibodies, demonstrating that it could be used for screening of equine serum samples for antibodies to IDV and ICV. As described below, the possibility of a similar cross-reactive relationship between D/OK and D/660 within equine populations is observed.

Bovine, swine, and small ruminant species are known to host IDV [43]. To determine if the equine population is susceptible to IDV, we tested 364 horse serum samples gathered in 2015 from six states (IA, MN, ND, NE, SD, WY), as well as 100 more samples gathered in 2016 from four states (IA, MN, NE, SD). All of the above samples were also analyzed for the presence of ICV antibodies. All samples were tested for virus specific antibodies via the standard hemagglutinin inhibition assay (HI) in triplicate. Any

serum sample with an average antibody titer greater than or equal to 40 was considered positive for antibodies against that given virus species.

Table 2 details a subset of the HI data from serum samples collected in 2015. Serum samples were grouped by farm of origin, and any farm that was the origin of more than one serum sample and had at least one serum sample test positive for antibodies against at least one of the viral lineages is included in the table. Of the 141 farms that serum was collected from, 47 of them are included in this table. HI data is provided for all three virus lineages tested, and is detailed by one column containing the number of positive serum samples originating from that farm followed by the total number of samples originating from that farm. A second column details the average titer value of the positive samples originating from that farm. The state of the United States in which the farm resides is also included with the serological data. Of the 230 individual serum samples included in the table, 36 tested positive for antibodies against D/OK, 28 tested positive for antibodies against D/660, and 37 tested positive for antibodies against C/JHB.

Farms only represented by one serum sample are not detailed on this table, but the data is as follows: of the 74 single-sample farms, 16 serum samples were positive for antibodies against at least one virus. Of these, 8 were positive for antibodies against D/OK, 11 were positive for antibodies against D/660, and 4 were positive for antibodies against C/JHB.

It should be noted that of 57 total positive samples to IDVs for 2015 serum samples, 23 (23/364; 6.3%) of them were positive for both IDV lineages (D/OK and D/660), while 21 (21/364; 5.8%) and 13 (13/364; 3.6%) tested positive for only D/OK or D/660 lineage,

respectively. Based on the cross-reactivity data of two IDV lineages generated against reference rabbit antiserum (Table 1), we speculated that the 23 horses with detectable antibodies to both lineages were infected largely by D/660 or D/660-like viruses because D/660 antisera recognized both lineages equally well in the HI assay. Furthermore, our observation that 21 horses possessed antibodies only to D/OK seemed to indicate that these animals were infected by D/OK or D/OK-like viruses because rabbit antiserum specific for D/OK has a 4-fold higher HI antibody titer to the D/OK than D/660 (Table 1). Interestingly, we also found 13 horses only seropositive to the D/660 lineage. We suspect that these animals may have been exposed to a D/660-like virus, which may have evolved some mutations abolishing epitopes common to the two lineages tested (D/660 and D/OK). A recent study in Japan has provided some preliminary evidence supporting for the existence of a potential third lineage of IDV [38]. It is also possible that the samples positive for both lineages of IDV could be exhibiting super-infection or co-infection of both D/660 and D/OK. This is difficult to determine due to both the cross-reactive nature of these viral lineages as well as our lack of temporal data related to the samples collected. These hypotheses will be investigated in future studies. In summary, the results of our serology study demonstrated that multiple lineages of IDV already infected horses in the Midwestern region, and that there is possibly some element of super-infection, co-infection, and/or cross-reactivity between the two IDV lineages studied, or there is the potential for the presence of an unidentified IDV lineage related to both D/OK and D/660 circulating among horses.

In Fig. 1, we graphed a 2-D scatterplot containing all 2015 samples from the HI trials that tested positive for at least one of the two IDVs (D/OK and D/660) and one ICV.

The correlation coefficients show that there is strong positive correlation between the antibody titers of the two IDV lineage viruses (D/660-D/OK), with an R value of .541 and a P of $1.53x10^{-8}$. Interestingly, we also found negative correlations between the antibody titers found for our ICV strain and the two IDV strains, with an R of -.364 and a P of $2.86x10^{-4}$ between C/JHB and D/660, and an R of -.435 and a P of $1.06x10^{-5}$ between C/JHB and D/OK.

In addition to 2015 serum samples, we also collected 100 horse serum samples in 2016 to determine the presence of IDV- or ICV- specific antibodies. Compared with 2015 serum samples deriving from aged horses, 2016 serum samples were largely from young ponies in SD and the region. Our result showed 3 (3/100, 3%) samples positive for antibodies against D/OK, 2 (2/100, 2%) samples positive for antibodies against D/660, and 8 (8/100, 8%) samples positive for antibodies against C/JHB (data not shown). The discrepancies observed in the overall seroprevalence between the samples in 2015 and in 2016 may be attributed to a much smaller samples size consisting of samples taken from a much narrower population of horses. The 2016 samples represent both fewer states and a much smaller total number of farms.

To further verify the results observed in the HI assays, the MN assay was used for a select subset of samples. Samples were chosen such that we could confirm both positive and negative results: MN sample groups were made of samples positive for multiple viruses, positive for just one virus, and positive for no viruses. As shown in Table 3 (2015 samples) and Table 4 (2016 samples), the MN assay confirms the results found via HI assay. The titer threshold for confirming a positive sample was considered anything greater than or equal to 40 [43, 44]. Based on the MN results, the HI seroprevalence results appear

to be accurate for all three virus lineages, confirming the presence of IDV and ICV within American equine populations. It should be noted that despite the overall agreement between HI and MN assays several outliers were observed. For example, three samples (IDs# 4, 6, 21) in Table 3 and one sample (IDs# 19, 23), tested positive in the HI assay, did not contain measurable neutralizing antibodies in the MN assay. The discordance may be caused by suboptimal quality of these samples after the long-term storage process. We also found that some samples (IDs 4, 5, 17, 18, 21, and 22 in Table 3 and ID# 71 in Table 4) tested negative by HI, turned out to be positive in the MN assay. This inconsistency is likely due to more sensitive nature the MN assay offers in the antibody detection. Another possibility is that the MN assay could detect other functional antibodies within the horse serum, such as those blocking virus-cell fusion, that could not be detected by HI assay.

In summary, we presented serological evidence that equines are susceptible to IDV and ICV infections. A previous study had showed that ICV antibodies were present in horses [13]. Based on these findings, horses should be added into the host range of this novel influenza virus that already includes bovines, swine, sheep, and goats [8, 15, 28, 43]. Future surveillance of IDV in horse populations is needed, as well as more serological investigations into other species potentially susceptible to influenza viruses to determine the total host range of IDV.

Figures and Tables

Table 1. Cross-reactivity of influenza C and D viruses by HI Assay

	D/OK	D/660	C/Vic
D/660 antiserum	1280	1280	<10
D/OK antiserum	2560	640	<10
C/Vic antiserum	<10	<10	2560

Table 2. Equine serological surveillance of Influenza virus types C and D in 2015

Farm ID	Location	D/OK		D/660		C/JHB	
		Positive\ Total	Average Titer	Positive\ Total	Average Titer	Positive\ Total	Average Titer
1	SD	6 \ 42	51.7	3 \ 42	46.7	14 \ 42	60
2	SD	0 \ 13	–	0 \ 13	–	2 \ 13	40
3	SD	1 \ 11	80	1 \ 11	40	0 \ 11	–
4	SD	0 \ 9	–	1 \ 9	60	2 \ 9	60
5	SD	2 \ 9	40	0 \ 9	–	0 \ 9	–
6	SD	1 \ 7	40	0 \ 7	–	0 \ 7	–
7	SD	1 \ 7	45	0 \ 7	–	1 \ 7	40
8	SD	1 \ 6	60	0 \ 6	–	0 \ 6	–
9	SD	0 \ 5	–	0 \ 5	–	1 \ 5	40
10	SD	1 \ 5	40	0 \ 5	–	0 \ 5	–
11	SD	2 \ 5	80	2 \ 5	50	0 \ 5	–
12	SD	1 \ 5	45	0 \ 5	–	0 \ 5	–
13	SD	0 \ 5	–	0 \ 5	–	1 \ 5	40
14	SD	1 \ 5	80	1 \ 5	80	0 \ 5	–
15	MN	2 \ 4	70	2 \ 4	80	0 \ 4	–
16	SD	0 \ 4	–	0 \ 4	–	2 \ 4	–
17	SD	0 \ 4	–	1 \ 4	40	0 \ 4	–
18	NE	0 \ 4	–	0 \ 4	–	1 \ 4	40
19	MN	4 \ 4	70	4 \ 4	60	0 \ 4	–
20	SD	0 \ 4	–	0 \ 4	–	1 \ 4	40
21	NE	1 \ 4	50	0 \ 4	–	0 \ 4	–
22	SD	0 \ 4	–	1 \ 4	45	0 \ 1	–
23	SD	1 \ 4	60	1 \ 4	80	0 \ 4	–
24	SD	1 \ 4	60	1 \ 4	40	0 \ 4	–
25	SD	1 \ 4	40	1 \ 4	45	0 \ 4	–
26	SD	0 \ 4	–	0 \ 4	–	1 \ 4	40
27	NE	0 \ 3	–	0 \ 3	–	0 \ 3	–
28	SD	1 \ 3	40	0 \ 3	–	0 \ 3	–
29	SD	0 \ 3	–	0 \ 3	–	1 \ 3	40
30	SD	1 \ 3	60	2 \ 3	70	0 \ 3	–
31	SD	0 \ 3	–	1 \ 3	80	0 \ 3	–
32	SD	0 \ 3	–	1 \ 3	40	0 \ 3	–
33	SD	1 \ 2	40	0 \ 2	–	0 \ 2	–
34	WY	0 \ 2	–	0 \ 2	–	1 \ 2	40
35	IA	0 \ 2	–	0 \ 2	–	1 \ 2	80
36	SD	1 \ 2	60	0 \ 2	–	1 \ 2	40
37	SD	0 \ 2	–	0 \ 2	–	2 \ 2	60
38	SD	1 \ 2	40	0 \ 2	–	0 \ 2	–
39	SD	0 \ 2	–	0 \ 2	–	1 \ 2	80
40	SD	1 \ 2	40	0 \ 2	–	0 \ 2	–
41	SD	1 \ 2	60	1 \ 2	80	0 \ 2	–
42	SD	1 \ 2	80	1 \ 2	80	1 \ 2	40
43	SD	1 \ 2	60	1 \ 2	40	0 \ 2	–
44	SD	0 \ 2	–	0 \ 2	–	1 \ 2	40
45	SD	0 \ 2	–	0 \ 2	–	1 \ 2	80
46	SD	1 \ 2	80	1 \ 2	80	0 \ 2	–
47	SD	0 \ 2	–	1 \ 2	40	1 \ 2	40
Total		**36 \ 230**		**28 \ 230**		**37 \ 230**	

Note: Only farms with at least one serum sample positive to at least one of the listed 3 viruses were included. This table did not include the farms that only had one sample. Each farm ID is accompanied by its location by state within the USA. For each farm, the number of samples positive to the virus over the number of total samples, and the average HI titer of the positive samples are shown. A "–" indicates the samples were negative to the virus.

Table 3. Microtiter neutralization confirmation of the HI titers observed for serum samples in 2015

Sample ID	D/OK		D/660		C/JHB	
	HI	MN	HI	MN	HI	MN
1	80	80	80	160	–	–
2	–	–	–	–	–	–
3	80	80	80	160	–	–
4	40	–	40	40	–	80
5	–	–	–	40	–	–
6	40	–	–	–	–	–
7	–	–	–	–	–	–
8	80	160	80	80	–	–
9	80	40	–	–	–	–
10	–	–	–	–	–	–
11	–	–	–	–	–	–
12	–	–	–	–	–	–
13	–	–	–	–	–	–
14	–	–	–	–	–	–
15	–	–	–	–	–	–
16	–	–	–	–	–	–
17	–	–	–	40	–	–
18	–	–	–	40	–	–
19	–	–	–	–	–	–
20	60	40	80	40	–	–
21	40	–	–	40	80	–
22	–	80	60	40	–	–

A "– " indicates the samples were negative to the virus.

Table 4. Microtiter neutralization confirmation of the HI titers observed for serum samples in 2016

	D/OK		D/660		C/JHB	
Sample ID	**HI**	**MN**	**HI**	**MN**	**HI**	**MN**
6	60	160	80	160	–	–
11	–	–	–	–	–	–
12	–	–	–	–	–	–
13	50	80	40	80	–	–
19	–	–	–	–	40	–
21	–	–	–	–	40	80
23	–	–	–	–	40	–
25	–	–	–	–	60	40
30	–	–	–	–	40	160
32	–	–	–	–	60	80
71	–	80	40	40	–	–
91	–	–	–	–	40	40
92	–	–	–	–	–	–
93	–	–	–	–	–	–

A "– " indicates the samples were negative to the virus.

Figure 1. A 2-D Scatter plot of all 2015 samples positive for at least one virus

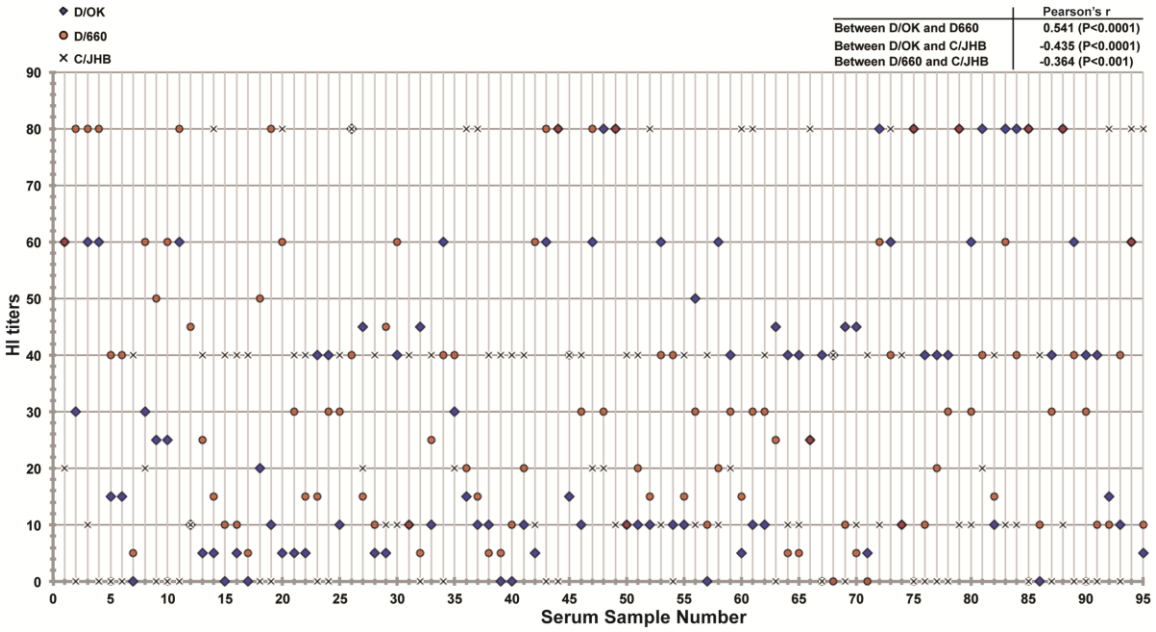

Figure 1. This figure includes all samples from the 2015 sample set that tested positive to at least one of the three viruses tested (D/OK, D/660, C/JHB). These are graphed along the x-axis. Each sample includes three data points, one to represent its tested average titer to each of the three viruses, with titer values graphed on the y-axis. The figure also includes the correlation measurements of the titer values for each virus taken against the others. The correlations show a strong positive relationship between the two IDV lineages (D/OK and D/660) and negative correlations between the ICV lineage and both IDV lineages.

Chapter 3: Summary and Conclusions

Evidence has been presented that two lineages of influenza D virus (IDV) and one lineage of influenza C virus (ICV) are present in equine populations of the Midwestern United States. It has been shown that the antibody titer values measured in this serological study are not due to cross reactivity between ICV and IDV. Both D/bovine/Oklahoma/660/2013 (D/660) and D/swine/Oklahoma/1334/2011 (D/OK) had measurable antibody titers among some of the samples tested, with some samples testing positive for only one of the IDV lineage viruses while others showed antibody titers for both. A significant number of samples tested positive for antibodies against C/human/Johannesburg/1/1966 (C/JHB), a virus not previously described as being capable of infecting horses. The nature of ICV and IDV infection in horses needs to be further explored, especially in relation to close human contact. It needs to be determined if horses could potentially pose a health hazard to humans in relations to infection with these pathogens. The possibility of cross-species infection exists with other influenza families, and IDV broad species tropism could indicate its potential to jump from livestock to humans.

The presence of samples positive for multiple viruses raises interesting questions about the exact nature of IDV infection in horses. Is there another strain of IDV circulating among the equine population that could produce antibodies that are cross-reactive to both D/OK and D/660? Are horses experiencing super-infection or co-infection of these two lineages of IDV? Further development of a lineage-specific diagnosis plus regular monitoring the equine population for IDV infection will provide more clear information on what exactly the relationship between strains of

IDV is, and how that relationship effects the threat the disease poses to livestock and humans. The titer values observed also suggest that horses are vulnerable to active infection with IDV, if this is the case are there clinical symptoms associated with IDV infection in horses? Future work will need to determine if horses are suffering significant morbidity or mortality because of IDV infection. If it is discovered that animals are becoming ill from these infections, treatment and prevention options will need to be assessed and developed to prevent further economic and health effects caused by IDV to equine industry. All of these issues will need to be addressed in future studies to further our understanding of this novel pathogen in equine populations.

Further monitoring of IDV in horses can also help elucidate what evolutionary changes occurred to cause IDV to separate from its close relative ICV. The equine host population of this virus could be an important factor in determining how this strain of influenza developed into a unique family of viruses. Ongoing surveillance of IDV in horse can also be used to track future evolutionary changes, as it may be possible for IDV to be more prone to genetic changes than the relatively stable ICV family.

Further work will also need to include the exploration of additional species as potential hosts for IDV. This project provided more data to expand our understanding of what animals are vulnerable to this infection, but there still exists the potential for more unexamined species to harbor IDV.

In conclusion, evidence has been shown that this newly discovered species of influenza virus is present in equine populations. The exact nature of this virus-host

interaction is not fully understood, but future work can fully clarify what has been discovered through the work done on this thesis.

BIBLIOGRAPHY

1. (2002) WHO manual on animal influenza diagnosis and surveillance
2. Akazawa M, Sindelar JL, Paltiel AD (2003) Economic Costs of Influenza-Related Work Absenteeism. Value in Health 6:107-115
3. Alexander DJ (2000) A review of avian influenza in different bird species. Veterinary Microbiology 74:3-13
4. Boianelli A, Nguyen V, Ebensen T, Schulze K, Wilk E, Sharma N, Stegemann-Koniszewski S, Bruder D, Toapanta F, Guzmán C, Meyer-Hermann M, Hernandez-Vargas E (2015) Modeling Influenza Virus Infection: A Roadmap for Influenza Research. Viruses 7:2875
5. Bouvier NM, Palese P (2008) The biology of influenza viruses. Vaccine 26:D49-D53
6. Canini L, Carrat F (2011) Population Modeling of Influenza A/H1N1 Virus Kinetics and Symptom Dynamics. Journal of Virology 85:2764-2770
7. CDC (2016) Overview of Influenza Surveillance in the United States.
8. Chiapponi C, Faccini S, De Mattia A, Baioni L, Barbieri I, Rosignoli C, Nigrelli A, Foni E (2016) Detection of Influenza D Virus among Swine and Cattle, Italy. Emerg Infect Dis 22:352-354
9. Collin EA, Sheng Z, Lang Y, Ma W, Hause BM, Li F (2015) Cocirculation of Two Distinct Genetic and Antigenic Lineages of Proposed Influenza D Virus in Cattle. Journal of Virology 89:1036-1042
10. Daesub S, Chulseung L, Bokyu K, Kwonil J, Taehoon O, Hyekwon K, Bongkyun P, Jinsik O (2009) Experimental Infection of Dogs with Avian-Origin Canine Influenza A Virus (H3N2). Emerging Infectious Diseases 15:56-58
11. Das K, Aramini JM, Li-Chung M, Krug RM, Arnold E (2010) Structures of influenza A proteins and insights into antiviral drug targets. Nature Structural & Molecular Biology 17:530-538
12. Dawood FS, Iuliano AD, Reed C, Meltzer MI, Shay DK, Cheng P-Y, Bandaranayake D, Breiman RF, Brooks WA, Buchy P, Feikin DR, Fowler KB, Gordon A, Hien NT, Horby P, Huang QS, Katz MA, Krishnan A, Lal R, Montgomery JM, Mølbak K, Pebody R, Presanis AM, Razuri H, Steens A, Tinoco

YO, Wallinga J, Yu H, Vong S, Bresee J, Widdowson M-A (2012) Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. The Lancet Infectious Diseases 12:687-695

13. Ditchfield J, Macpherson LW (1965) Zbitnew A: Upper Respiratory Disease in Thouroughbred Horses: Studies of Its Viral Etiology in the Toronto Area, 1960 to 1963. Canadian journal of comparative medicine and veterinary science 29:18-22
14. Ducatez MF, Pelletier C, Meyer G (2015) Influenza D virus in cattle, France, 2011-2014. Emerging Infectious Diseases 21:368-371
15. Ferguson L, Olivier AK, Genova S, Epperson WB, Smith DR, Schneider L, Barton K, McCuan K, Webby RJ, Wan XF (2016) Pathogenesis of Influenza D Virus in Cattle. Journal of virology 90:5636-5642
16. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, Griffin J, Baggaley RF, Jenkins HE, Lyons EJ, Jombart T, Hinsley WR, Grassly NC, Balloux F, Ghani AC, Ferguson NM, Rambaut A, Pybus OG, Lopez-Gatell H, Alpuche-Aranda CM, Chapela IB, Zavala EP, Guevara DME, Checchi F, Garcia E, Hugonnet S, Roth C (2009) Pandemic Potential of a Strain of Influenza A (H1N1): Early Findings. Science 324:1557
17. Furukawa T, Muraki Y, Noda T, Takashita E, Sho R, Sugawara K, Matsuzaki Y, Shimotai Y, Hongo S (2011) Role of the CM2 Protein in the Influenza C Virus Replication Cycle. Journal of Virology 85:1322-1329
18. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RAM, Pappas C, Alpuche-Aranda CM, xf, pez-Gatell H, Olivera H, xf, pez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD, Boxrud D, Sambol AR, Abid SH, St. George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI, Cox NJ

(2009) Antigenic and Genetic Characteristics of Swine-Origin 2009 A(H1N1) Influenza Viruses Circulating in Humans. Science 325:197-201

19. Gire SK, Goba A, Andersen KG, Sealfon RSG, Park DJ, Kanneh L, Jalloh S, Momoh M, Fullah M, Dudas G, Wohl S, Moses LM, Yozwiak NL, Winnicki S, Matranga CB, Malboeuf CM, Qu J, Gladden AD, Schaffner SF, Yang X, Jiang P-P, Nekoui M, Colubri A, Coomber MR, Fonnie M, Moigboi A, Gbakie M, Kamara FK, Tucker V, Konuwa E, Saffa S, Sellu J, Jalloh AA, Kovoma A, Koninga J, Mustapha I, Kargbo K, Foday M, Yillah M, Kanneh F, Robert W, Massally JLB, Chapman SB, Bochicchio J, Murphy C, Nusbaum C, Young S, Birren BW, Grant DS, Scheiffelin JS, Lander ES, Happi C, Gevao SM, Gnirke A, Rambaut A, Garry RF, Khan SH, Sabeti PC (2014) Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. Science 345:1369
20. Glezen WP, Schmier JK, Kuehn CM, Ryan KJ, Oxford J (2013) The Burden of Influenza B: A Structured Literature Review. American Journal of Public Health 103:e43-51
21. Gorman OT, Bean WJ, Kawaoka Y, Webster RG (1990) Evolution of the nucleoprotein gene of influenza A virus. Journal of Virology 64:1487-1497
22. Hause BM, Ducatez M, Collin EA, Ran Z, Liu R, Sheng Z, Armien A, Kaplan B, Chakravarty S, Hoppe AD, Webby RJ, Simonson RR, Li F (2013) Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. PLoS pathogens 9:e1003176
23. Hause BM, Collin EA, Liu R, Huang B, Sheng Z, Lu W, Wang D, Nelson EA, Li F (2014) Characterization of a Novel Influenza Virus in Cattle and Swine: Proposal for a New Genus in the Orthomyxoviridae Family. mBio 5
24. Herfst S, Schrauwen EJA, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus ADME, Fouchier RAM (2012) Airborne Transmission of Influenza A/H5N1 Virus Between Ferrets. Science 336:1534
25. Heukelbach J, Werneck GL (2016) Surveillance of Zika virus infection and microcephaly in Brazil. The Lancet 388:846-847

26. Igarashi M (2015) Antiviral Drugs Targeting Influenza Virus Surface Proteins: A Computational Structural Biology Approach. Yakugaku Zasshi-Journal of the Pharmaceutical Society of Japan 135:1015-1021
27. Jackson D, Elderfield RA, Barclay WS (2011) Molecular studies of influenza B virus in the reverse genetics era. Journal of General Virology 92:1-17
28. Jiang WM, Wang SC, Peng C, Yu JM, Zhuang QY, Hou GY, Liu S, Li JP, Chen JM (2014) Identification of a potential novel type of influenza virus in Bovine in China. Virus genes 49:493-496
29. Joosting ACC, Head B, Bynoe ML, Tyrrell DAJ (1968) Production of Common Colds in Human Volunteers by Influenza C Virus. British Medical Journal 4:153-154
30. Kaiser L, Fritz RS, Straus SE, Gubareva L, Hayden FG (2001) Symptom pathogenesis during acute influenza: Interleukin-6 and Other cytokine responses. Journal of Medical Virology 64:262-268
31. Kendall PM, Olsen CW, Gray GC (2007) Cases of Swine Influenza in Humans: A Review of the Literature. Clinical Infectious Diseases 44:1084-1088
32. Luo J, Ferguson L, Smith DR, Woolums AR, Epperson WB, Wan X-F (2017) Serological evidence for high prevalence of Influenza D Viruses in Cattle, Nebraska, United States, 2003–2004. Virology 501:88-91
33. Manuguerra J-C, Hannoun C, Aymard M (1992) Influenza C virus infection in France. Journal of Infection 24:91-99
34. Matsuzaki Y, Mizuta K, Sugawara K, Tsuchiya E, Muraki Y, Hongo S, Suzuki H, Nishimura H (2003) Frequent Reassortment among Influenza C Viruses. Journal of Virology 77:871-881
35. Matsuzaki Y, Abiko C, Mizuta K, Sugawara K, Takashita E, Muraki Y, Suzuki H, Mikawa M, Shimada S, Sato K, Kuzuya M, Takao S, Wakatsuki K, Itagaki T, Hongo S, Nishimura H (2007) A Nationwide Epidemic of Influenza C Virus Infection in Japan in 2004. Journal of Clinical Microbiology 45:783-788
36. Molinari N-AM, Ortega-Sanchez IR, Messonnier ML, Thompson WW, Wortley PM, Weintraub E, Bridges CB (2007) The annual impact of seasonal influenza in the US: Measuring disease burden and costs. Vaccine 25:5086-5096

37. Motta FC, Luiz MO, Couceiro JN (2000) Serological analysis reveals circulation of influenza C viruses, Brazil. Revista de Saúde Pública 34:204-205
38. Murakami S, Endoh M, Kobayashi T, Takenaka-Uema A, Chambers JK, Uchida K, Nishihara M, Hause B, Horimoto T (2016) Influenza D Virus Infection in Herd of Cattle, Japan. Emerg Infect Dis 22:1517-1519
39. Odagiri T, Matsuzaki Y, Okamoto M, Suzuki A, Saito M, Tamaki R, Lupisan SP, Sombrero LT, Hongo S, Oshitani H (2015) Isolation and Characterization of Influenza C Viruses in the Philippines and Japan. Journal of Clinical Microbiology 53:847-858
40. Olson DR, Heffernan RT, Paladini M, Konty K, Weiss D, Mostashari F (2007) Monitoring the Impact of Influenza by Age: Emergency Department Fever and Respiratory Complaint Surveillance in New York City. PLOS Medicine 4:e247
41. Osterholm MT, Kelley NS, Sommer A, Belongia EA (2012) Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. The Lancet Infectious Diseases 12:36-44
42. Palese P, Shaw M (2007) Orthomyxoviridae: The viruses and their replication. In: Fields BN, Knipe DM, Howley PM (eds) Field's Virology. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, pp 1647-1689)
43. Quast M, Sreenivasan C, Sexton G, Nedland H, Singrey A, Fawcett L, Miller G, Lauer D, Voss S, Pollock S, Cunha CW, Christopher-Hennings J, Nelson E, Li F (2015) Serological evidence for the presence of influenza D virus in small ruminants. Veterinary microbiology 180:281-285
44. Ran Z, Shen H, Lang Y, Kolb EA, Turan N, Zhu L, Ma J, Bawa B, Liu Q, Liu H, Quast M, Sexton G, Krammer F, Hause BM, Christopher-Hennings J, Nelson EA, Richt J, Li F, Ma W (2015) Domestic pigs are susceptible to infection with influenza B viruses. Journal of virology 89:4818-4826
45. REED LJ, MUENCH H (1938) A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS. American Journal of Epidemiology 27:493-497
46. Reichert TA, Simonsen L, Sharma A, Pardo SA, Fedson DS, Miller MA (2004) Influenza and the Winter Increase in Mortality in the United States, 1959–1999. American Journal of Epidemiology 160:492-502

47. Smith GJD, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Siu Kit M, Chung Lam C, Raghwani J, Bhatt S, Peiris JSM, Yi G, Rambaut A (2009) Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122-1125
48. Song H, Qi J, Khedri Z, Diaz S, Yu H, Chen X, Varki A, Shi Y, Gao GF (2016) An Open Receptor-Binding Cavity of Hemagglutinin-Esterase-Fusion Glycoprotein from Newly-Identified Influenza D Virus: Basis for Its Broad Cell Tropism. PLoS Pathogens 12:e1005411
49. Speranskaya AS, Melnikova NV, Belenikin MS, Dmitriev AA, Oparina NY, Kudryavtseva AV (2012) Genetic diversity and evolution of the influenza C virus. Russian Journal of Genetics 48:671-678
50. Sreenivasan C, Thomas M, Sheng Z, Hause BM, Collin EA, Knudsen DE, Pillatzki A, Nelson E, Wang D, Kaushik RS, Li F (2015) Replication and Transmission of the Novel Bovine Influenza D Virus in a Guinea Pig Model. Journal of virology 89:11990-12001
51. Stewart SM, Pekosz A (2012) The Influenza C Virus CM2 Protein Can Alter Intracellular pH, and Its Transmembrane Domain Can Substitute for That of the Influenza A Virus M2 Protein and Support Infectious Virus Production. Journal of Virology 86:1277-1281
52. Strauss JH, Strauss EG (2008) CHAPTER 4 - Minus-Strand RNA Viruses. In: Strauss JH, Strauss EG (eds) Viruses and Human Disease (Second Edition). Academic Press, London, pp 137-191
53. Trifonov V, Khiabanian H, Rabadan R (2009) Geographic Dependence, Surveillance, and Origins of the 2009 Influenza A (H1N1) Virus. New England Journal of Medicine 361:115-119
54. Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solórzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, García-Sastre A (2005) Characterization of the Reconstructed 1918 Spanish Influenza Pandemic Virus. Science 310:77
55. Wang Q, Ju L, Liu P, Zhou J, Lv X, Li L, Shen H, Su H, Jiang L, Jiang Q (2015) Serological and Virological Surveillance of Avian Influenza A Virus H9N2

Subtype in Humans and Poultry in Shanghai, China, Between 2008 and 2010. Zoonoses and Public Health 62:131-140

56. Wasik BR, Barnard KN, Ossiboff RJ, Khedri Z, Feng KH, Yu H, Chen X, Perez DR, Varki A, Parrish CR (2017) Distribution of O-Acetylated Sialic Acids among Target Host Tissues for Influenza Virus. mSphere 2:e00379-00316
57. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiological Reviews 56:152-179
58. White SK, Ma W, McDaniel CJ, Gray GC, Lednicky JA (2016) Serologic evidence of exposure to influenza D virus among persons with occupational contact with cattle. Journal of Clinical Virology 81:31-33
59. Wood JM, Major D, Heath A, Newman RW, Höschler K, Stephenson I, Clark T, Katz JM, Zambon MC (2012) Reproducibility of serology assays for pandemic influenza H1N1: Collaborative study to evaluate a candidate WHO International Standard. Vaccine 30:210-217
60. Yuanji G, Fengen J, Ping W, Min W, Jiming Z (1983) Isolation of Influenza C Virus from Pigs and Experimental Infection of Pigs with Influenza C Virus. Journal of General Virology 64:177-182
61. Zhang X, Rosenthal PB, Formanowski F, Fitz W, Wong C-H, Meier-Ewert H, Skehel JJ, Wiley DC (1999) X-ray crystallographic determination of the structure of the influenza C virus haemagglutinin-esterase-fusion glycoprotein. Acta Crystallographica Section D 55:945-961