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Replication, Virulence, and Pathogenesis of Influenza Viruses

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REPLICATION, VIRULENCE, AND PATHOGENESIS OF INFLUENZA VIRUSES

BY

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VIRUSES

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Biological Sciences, specialization in Microbiology and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidates are necessarily the conclusions of the major department.

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Dedicated to

My Beloved parents, Sreenivasan Chembil Krishnan & Sarojini Seethedath Mani

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ABBREVIATIONS

%: Percentage

°C: Degree centigrade

α-SMA: alpha-smooth muscle actin

BHK: Baby hamster kidney cells

bLf: bovine lactoferrin

BVD: bovine viral diarrhea

BRSV: Bovine respiratory syncytial virus

CL-43: collectin-43

CL-46: collectin-46

DNA: Deoxyribonucleic acid

dsRNA: Double-stranded RNA

ELISA: Enzyme-linked Immunosorbent Assay

FACS: Fluorescence-activated cell sorting

FBS: Fetal Bovine Serum

FISH: Fluorescent in-situ hybridization

HA: Hemagglutinin

HEF: Hemagglutinin esterase fusion

HPAIV: Highly pathogenic avian influenza virus

IAV: Influenza A virus

IBV: Influenza B virus

ICV: Influenza C virus

IDV: Influenza D virus

IBR: Infectious bovine rhinotracheitis

IFA: Indirect Immunoflourescence Assay

IHC: Immunohistochemistry

ITS: Insulin Transferrin Selenium

MAL-II: *Maackia amurensis* lectin-II

MDBK: Madin-Darby bovine kidney cells

MDCK: Madin-Darby Canine Kidney Epithelial cells

mRNA: Messenger ribonucleic acid

M: Matrix protein

NP: Nucleoprotein

NA: Neuraminidase

NANA: N-acetyl neuraminic acid

NS1: Non-structural protein 1

NS2: Non-structural protein 2

PA/P3: Polymerase acidic protein

PB1: Polymerase basic protein 1

PB2: Polymerase basic protein 2

PI3: parainfluenza-3

PRRSV: Porcine reproductive and respiratory syndrome virus

qRT-PCR: Quantitative Reverse Transcriptase- Polymerase Chain Reaction

SIV: Swine influenza virus

ssRNA: Single-stranded RNA

TEER: Transepithelial electric resistance

WHO: World Health Organization

ZO-1: Zona occludens-1

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ABSTRACT

REPLICATION, VIRULENCE, AND PATHOGENESIS OF INFLUENZA VIRUSES

CHITHRA C. SREENIVASAN

2018

Influenza D virus (IDV) is a novel influenza virus that infects cattle and swine, with cattle as its primary host species. The goal of our first study was to investigate the replication and transmission of bovine IDV in guinea pigs. Following direct intranasal inoculation of animals, the virus was detected in nasal washes of infected animals during the first 7 days post-infection. High viral titers were obtained from nasal turbinates and lung tissues of directly inoculated animals. Further, bovine IDV was able to transmit from the infected guinea pigs to sentinel animals by means of contact and not by aerosol dissemination under the experimental conditions tested in this study. Despite exhibiting no clinical signs, infected guinea pigs developed seroconversion and the viral antigen was detected in lungs of animals by immunohistochemistry. The observation that bovine IDV replicated in the respiratory tract of guinea pigs was similar to observations described previously in studies of gnotobiotic calves and pigs experimentally infected with bovine IDV but different from those described previously in experimental infections in ferrets and swine with a swine IDV, which supported virus replication only in the upper respiratory tract and not in the lower respiratory tract, including lung. Our study established that guinea pigs could be used as an animal model for studying this newly emerging influenza virus. Influenza D virus isolated from the cattle and swine populations from North America and Eurasia shares 50% homology to the human influenza C virus. The goal of our second study was to investigate the replication kinetics and virulence of bovine and swine

influenza D isolates (96-98% homology), in comparison to human influenza C in guinea pigs. Despite the similarity, both bovine and swine IDVs differ antigenically and genetically and belong to two different lineages. Guinea pigs upon intranasal inoculation of D/bovine/660/Oklahoma/2013 (bovine IDV), D/swine/1334/Oklahoma/2011 (swine IDV) and C/ Victoria/2012 (human ICV) did not exhibit any clinical signs. However, all the infected animals seroconverted at 7 days post-infection (dpi). Guinea pigs infected with ICV did not shed the virus in nasal washes at 1 dpi and only 2/8 shed virus at 3 dpi. In contrast, in bovine IDV infected group, 9/10 animals shed the virus in nasal washes at 1 dpi, while the swine IDV group (8/8) began to shed the virus only at 3 dpi. Hence, the disparity in the virus-shedding pattern of swine IDV could be an adaptation lag due to the subtle difference in receptor binding specificity and virus tropism. Deep RNA sequencing of viral genomes in the nasal washes, receptor binding preference, and structural modeling of receptor binding domain of hemagglutinin-esterase fusion protein are currently underway to identify the key factors and mechanisms involved in the differential replication kinetics, viral tropism, pathogenesis of the bovine and swine influenza D viruses. Further, our third project was aimed at developing a good primary culture system from swine for studying the virulence and pathogenesis. Influenza viruses are a group of respiratory pathogens that have evolved into four different types: A, B, C, and D. One common feature is that all four types are capable of replication and transmission among pigs. Human respiratory primary epithelial cell culture has been recently utilized to examine the replication and pathogenesis of influenza A viruses. However, little has been made in the development of the autologous cell culture system from swine to study influenza viruses. Here we describe the development of primary epithelial cells from swine

nasal turbinates, trachea and lungs and determine their utility in the replication of four types of influenza viruses. Phenotypic characterization using immunocytochemistry coupled with flow cytometry analysis showed that cytokeratin was expressed at high levels in swine nasal turbinates, trachea, and lung cells, while the relatively low abundance of other epithelial cell markers (desmin, α-SMA, and vimentin) was detected. In addition, all three swine cells were found able to undergo the polarization as measured by trans-epithelial electrical resistance (TEER) and expression of tight junction proteins including claudin-1, -3, Zona occludens protein -1 (ZO-1) and occludin-1. These results strongly suggest that the developed swine primary cells possess common characteristics of epithelial cells. Furthermore, sialic acid receptor profile analysis through lectin binding assay with Sambucus Nigra Lectin (SNA) and Maackia Amurensis Lectin II (MAL-II) demonstrated that three swine primary epithelial cells expressed higher levels of alpha 2,6 linkage sialic acid (SNA) than alpha 2,3 linkage sialic acid receptors (MAL-II). Finally, all three primary cells supported the replication of Influenza A, B, C and D viruses to an appreciable level, but virus type-dependent replication kinetics were observed. Overall, these swine respiratory primary cells showed epithelial phenotype and are suitable for studying the comparative biology and pathobiology of four types of influenza viruses.

Chapter 1. Introduction and objectives

Influenza viruses are negative-sense, single-stranded RNA viruses classified in the Orthomyxoviridae family. There are four recognized genera of influenza viruses, designated influenza A (IAV), influenza B (IBV), influenza C (ICV) and influenza D (IDV) (https://www.cdc.gov/flu/about/viruses/types.htm, Accessed April 24, 2018). IAV and IBV have 8 negative-sense, single-stranded RNA segments, whereas ICV and IDV have only 7 segments. Influenza D virus (IDV) originally isolated from an ailing swine from Oklahoma showing respiratory illness has been provisionally described as influenza C like virus, as the virus was more homologous to influenza C virus than influenza A and B viruses. This newly emerged virus has been officially approved by the international committee of Taxonomy of Viruses (ICTV) and categorized as the fourth genus in the Orthomyxoviridae family in 2016 [2]. After IDV was isolated from swine, serological screening in pigs showed 9.5 % seroprevalence in the mid-west swine populations [2]. Further, serological screening in bovines showed a much higher prevalence in bovine herds and reverse transcription RT-PCR on bovine respiratory disease submission samples, led to the isolation of several similar strains of IDV [2]. Genetic and antigenic analyses demonstrated that the IDVs cannot undergo reassortment with ICV and have peculiar splicing pattern of NS and M protein [2]. More detailed studies have revealed that there are two lineages of IDV, represented by D/OK (D/swine/Oklahoma/1334/2011) and D/660 (D/bovine/Oklahoma/660/2013), of which D/660 is predominant lineage in the USA. A third lineage D/bovine/Ibaraki/7768/2016 was discovered later, in Japan [3].

A little is known about the virulence, pathogenicity, and transmission of IDV. Previous research demonstrated that swine IDV was found to infect ferrets and pigs and

can also be transmitted to naïve animals by contact. Swine IDV was able to replicate in nasal turbinates and shed detectable levels of virus in nasal washes. However, swine IDV was not detected in trachea and lungs in either ferrets or pigs indicating its inability to replicate in the lower respiratory tract. The animals seroconverted but neither ferrets nor pigs developed clinical symptoms and lesions typical of Influenza [4]. Ferrets have been used as surrogates for human influenza infection and pathogenesis. The ability of swine IDV to replicate in the upper respiratory tract of ferrets may indicate the zoonotic potential of these viruses.

The objective of our first study is to examine viral replication, transmission, and virulence properties of the novel bovine IDV in guinea pigs. Our study is based on the following considerations. First, guinea pig has been used extensively as an alternate mammalian animal model for studying the pathogenesis of many influenza viruses including IBV [5-7]. Second, this animal model was susceptible to infections by Influenza A virus subtypes and demonstrated virus replication in lungs. Third, guinea pigs were able to transmit human influenza viruses from one animal to another [7]. Fourth, they are easier to handle and house, and less expensive compared to ferrets. Importantly, guinea pigs share similar airway hyperresponsiveness and have bronchus-associated lymphoid tissue as humans [8, 9]. Finally, despite being highly similar to a swine IDV strain used previously for ferret and pig studies, the bovine IDV selected for this study is a representative strain of another antigenic lineage different from swine IDV, possessing some distinct variations in the HEF protein, a major mediator of host range and viral tropism [10]. Further, the bovine lineage has been found as the predominant lineage in US herds. In this study, guinea

pigs will be intranasally inoculated with bovine IDV to study the virulence and also the transmissibility of the virus by contact and aerosol.

The virus ecology of influenza D has been expanding since it was discovered in 2011-2013 and several strains of IDV have been isolated from different parts of the North America, and Eurasia. Serological evidence of IDV was found in small ruminants (goats and sheep), buffaloes, equines, and camelids [11-13]. Influenza D antibodies have also been detected from occupational workers which implicates the public health importance of this newly emerged virus [14]. Even though the majority of IDVs isolated from North America belong to the D660 lineage, it is interesting to note that recent isolates of IDVs of Eurasian origin were mostly of D/OK lineage [3, 13, 15, 16]. As stated earlier, humans are the primary host for ICV, but ICV has also been isolated from pigs and the directionality of transmission still needs to be addressed. Like ICV, IDV also uses the HEF protein, for the virus entry and exit, and these proteins share a conserved enzymatic site and divergent receptor binding sites. IDV exhibits broad host tropism, and IDV HEF has exceptional acid and thermal stability compared to ICV [17]. Based on these phenotypic characteristics, we wanted to identify the molecular factors/ determinants responsible for the broad host tropism of IDV compared to ICV. The second thing is, HEF of the IDVs belonging to two lineages may have genetic and antigenic differences. So the goal of our second study is to investigate the differences in the virulence, and pathogenicity of these two lineages in the guinea pig model.

Apart from the in-vivo studies to characterize the IDV and ICV, we also wanted to develop a good in-vitro primary cell culture system to study the influenza viruses as most of the current research and commercial vaccine production depends on continuous cell

culture. Several cell lines such as Madin Darby canine kidney cells (MDCK), Vero, MRC-5 and baby hamster kidney (BHK) cells, have been used extensively for the influenza virus growth [18-20]. Primary cells mimic the physiological properties in-vivo and hence is the best in-vitro model to study the mechanistic details of the normal or diseased conditions of the body. Primary cell cultures had been an excellent in-vitro system to study the virulence and pathogenetic characteristics of the influenza viruses. Influenza studies using primary cell culture from humans and swine has been utilized for studying the virulence, and receptor binding specificities of the viruses from different host origin [21-25]. Several studies have been conducted on swine tracheal/bronchial/lung epithelial cells to study the pathogenesis and anti-viral responses at the transcriptional and translational level as the swine species share the most anatomical and physiological characteristics to humans [26-28] Pigs are the mixing vessels of influenza viruses and harbor receptors for both the avian and human influenza viruses. Recently, it was found that domestic pigs are susceptible to influenza B and C viruses [29-31]. Further influenza D has been initially isolated from swine [4]. Taken together, pigs can be infected with all four types of influenza viruses. The goal of our third study is to develop, characterize and study the utility of the primary swine respiratory epithelial cells derived from the from upper and lower respiratory tract (nasal turbinates, trachea and lungs) of a gnotobiotic piglet on the differential replication of four types of the influenza compared to MDCK cells at two different temperatures, 33°C and 37°C.

Objectives:

I. To evaluate guinea pigs as model mammalian host for the novel bovine IDV

    a. Checking the viral growth kinetics

b. Direct contact transmission of IDV

c. Aerosol/droplet transmission of IDV

II. To compare the virulence and pathogenesis of bovine and swine IDVs to human ICV in guinea pig model

a. Swine and bovine HEF belong to two lineages based on their genetic and antigenic differences: Whether they have any difference in pathogenesis and tissue tropism?

b. To determine the sialic acid population and distribution in guinea pigs

c. To identify the molecular determinants/factors responsible for the differential replication and tissue tropism of IDV compared to ICV

III. Development and characterization of porcine primary airway epithelial cells: differential replication of four influenza virus types.

Chapter 2. Influenza epidemiology, clinical features, and host spectrum

2.1 Epidemiology and clinical features of influenza

Influenza disease recognized as early as the 16th century causes two forms of infections globally 1) epidemic (seasonal or interpandemic) caused by the influenza A and B type viruses and sporadic pandemics caused by the influenza A viruses. Influenza viruses evolved to form four different types and several subtypes, infecting multiple mammalian species worldwide, including humans. Over time, we have seen some new mammalian hosts such as bats, seals, and whales added to the host spectra of influenza [32-36].

Influenza is an acute respiratory infection affecting humans of all age groups, birds and animal populations all over the world. Each year, influenza causes a high rate of hospitalization and death in humans, particularly among high-risk people such as elderly patients, pregnant women, immune-compromised individuals, and young children. According to WHO factual sheet, 3 to 5 million cases of seasonal epidemics occur worldwide and 290,000 to 650,000 deaths are attributed to influenza infection annually (http://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal) accessed April 29, 2018). Apart from the seasonal epidemics, several pandemics emerged during the last century. The first pandemic 'Spanish flu' occurred in 1918, caused by Influenza A subtype H1N1, followed by 'Asian flu' in 1957-58 and 'Hong Kong flu' in 1968-69. More recently, a pandemic caused by the swine-origin H1N1 virus was reported in 2009 [37]. Individuals with pre-existing conditions such as diabetes, heart diseases and chronic pulmonary diseases and physiological conditions such as pregnancy are prone to influenza-related complications. Most common clinical symptoms include sore-throat, high temperature, cough, and body pain. Influenza complications include pneumonia, myocarditis,

pericarditis, and death. In the 2009 pandemic, gastrointestinal signs such as abdominal pain, and dysentery were also present along with respiratory symptoms [38].

Influenza A infections were reported in humans, pigs and birds globally, caused by different subtypes. In humans, influenza B causes seasonal epidemics. Since Pdm2009, few other strains emerged that can cause infections in humans. These include a new variant of H3N2 (H3N2v), which derived its matrix gene segment from H1N1pdm2009 virus causing infections in both children and adults during 2011-13 [39]. More recently, the avian H7N9 strain has emerged as a major threat in China [40]. Surveillance reports from 2017-18 revealed that influenza A H3N2 predominated among infections, along with influenza B viruses since last March 2018 (https://www.cdc.gov/flu/weekly/index.htm).

Influenza viruses belong to Orthomyxoviridae family and are negative sense single-stranded viruses affecting several different types of hosts. So far there are four different types of influenza: A, B, C, and D (https://www.cdc.gov/flu/about/viruses/types.htm; http://www.who.int/en/news-room/fact-sheets/detail/influenza-(seasonal) accessed, April 29,2018). Influenza A is the most common subtype affecting the diverse population of animals and poultry such as waterfowls, domesticated birds, swine, seals, human, equines, canines. The factors that govern the virulence, pathogenicity and interspecies transmission of influenza viruses could be multifactorial, which includes viral as well as host factors. Among the viral factors, HA glycoprotein is the most important one determining the host range and interspecies transmission. Other viral proteins such as PB2 and NS1 have also been involved in host range restriction and host innate immune response [41]. In the case of humans, zoonotic infections are seen in two ways. One could be isolated, dead-end infections which fail to establish and adapt as in the case of Ebola and hantaviruses. The

second one happens when the virus adapts and establishes as intermediate or secondary hosts, and also sustain horizontal transmission and also reverse zoonosis. [42] Such stable host switch events lead to strong adaptations which can resist the evolutionary pressures or the antagonistic environment posed by the novel hosts. The novel human-adapted IAVs such as H5N1 and H9N2 are examples for this type of establishment of seasonal viruses [43-45].

Influenza viruses undergo antigenic drift, acquiring mutations in HA and NA frequently which enables the virions to sustain the existing immunity and thereby causing seasonal epizootics. Influenza virus can undergo gene reassortments via antigenic shift and cause pandemics. Antigenic shifts in the hemagglutinin and neuraminidase glycoproteins of influenza A have led to several new influenza subtypes giving rise to high pathogenic and low pathogenic strains affecting several hosts mainly waterfowls.

IAV and IBV have 8 negative-sense, single-stranded RNA segments, whereas ICV and IDV have only 7 segments. IAV proteins include 5 structural proteins, HA (hemagglutinin), NA, M1, M2, and NP (ribonucleoprotein); 3 subunits of the RNA polymerase complex, polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA); and 3 nonstructural proteins, NS1, NS2 (nuclear export protein [46]), and PB1-F2 [47]. Recent studies have suggested that NS2 and (probably) NS1 of IAV are structural proteins that can be detected in virions [48]. IBV has 6 structural proteins, HA, NA, NB, M2, M1, and NP; 3 subunits of RNA polymerase complex, PA, PB1, and PB2; and 2 non-structural proteins, NS1 and NS2. ICV has 4 structural proteins, M2, M1, NP, and the hemagglutinin-esterase fusion (HEF) protein that replaces the HA and NA of IAV or IBV; 3 subunits of RNA polymerase complex, P3, PB1,

and PB2; and 2 nonstructural proteins, NS1 and NS2 (Figure. 2.1). ICV has hemagglutinin esterase fusion protein that combines the function of HA and NA, to perform the receptor binding, receptor destroying and membrane fusion. ICV has two IAV has several subtypes depending on the HA and NA proteins and causes severe epidemics and pandemics affecting humans. So far there is 18 HA and 11 NA types, of which H1 to H16 and N1 to N9 have been isolated from birds. H17, H18, N10, N11 have been identified in bats. Out of these, only 3 subtypes (H1, H2, H3) and two NA (N1, N2) subtypes have been reported to have caused human epidemics and capable of transmission. IAV exists in multiple mammalian species, whereas IBV and ICV primarily infect humans. IBV has no subtypes but possesses two lineages causing localized epidemics and affecting mainly humans and, to some extent, seals [49]. The IBV genome was also recently detected in domestic pigs, indicating that the virus may infect this agricultural animal [47]. Compared to the IAV and IBV, ICV infections cause mild disease and were found to have coexisted with IAV and IBV infections in humans [50]. A striking feature of IDV is that it has multiple mammalian hosts similar to IAV, although we consider bovines as the natural reservoir [51].
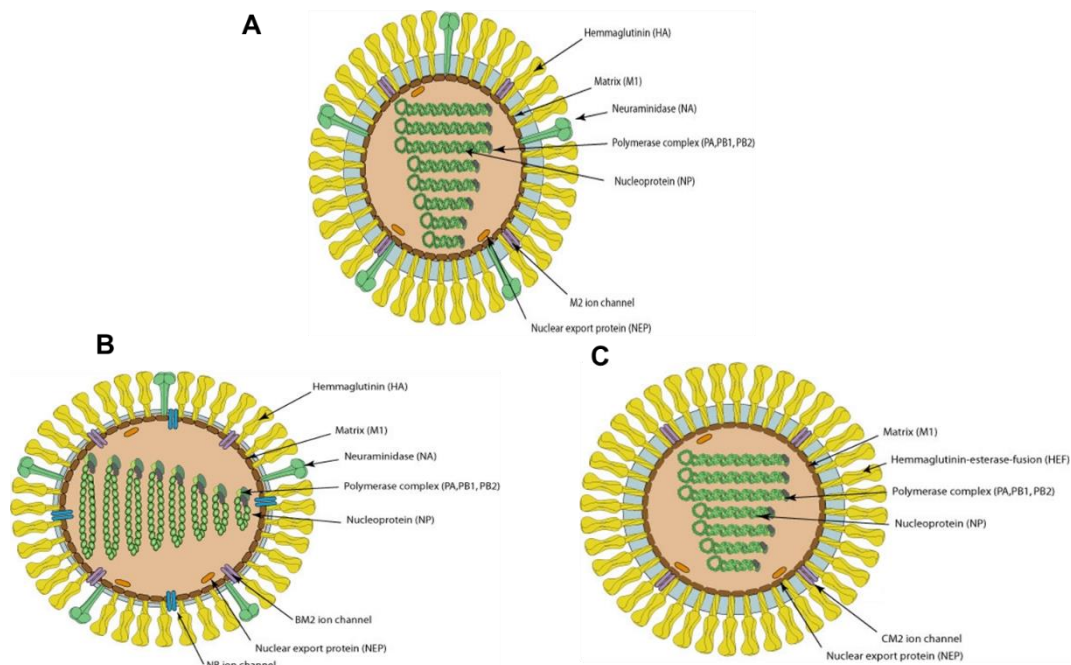
Figure. 2.1 Influenza types and structure. Structure of influenza type A (A), type B (B), and type C and D (C). Adapted from https://viralzone.expasy.org/6?outline=all_by_species, accessed May 16, 2018.

Swine respiratory system harbors various forms of sialic acids with different linkages or modifications that make this agricultural animal species susceptible to infections by all four types of influenza viruses. In addition to influenza A virus (IAV), influenza C (ICV) and D (IDV) have been isolated from swine (1). Our recent investigation also revealed the presence of influenza B virus in U. S. pig farms that were previously infected with porcine reproductive and respiratory syndrome virus (PRRSV) (3). There has been no documentation of any incidence of influenza in bovines until 2011-2013, during which the serological evidence against newly isolated influenza C like virus (later designated as influenza D virus) of swine origin, was detected in bovine herds of the USA. A schematic diagram showing different types of influenza and their potential host spectra are given in
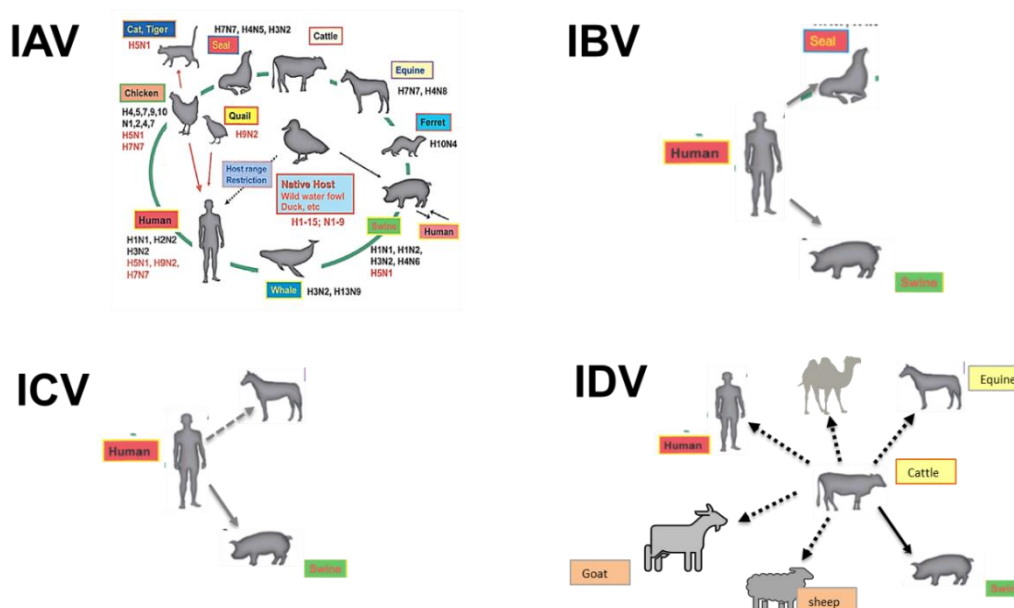
Figure 2.2. Host spectra of the four types of influenza

2.1.1 Influenza A virus (IAV)

Swine influenza in North American pig populations, particularly in Midwest U.S. dates back to 1930 when H1N1 was initially isolated in pigs [52]. This classic lineage derived from 1918 Spanish pandemic flu, is still a robust virus and is floating in the swine and human population for more than 80 years. Besides, other reassortant viruses like H3N2 which shares the gene segments NP, M, NS from classical swine virus; PB1, HA, NA from H3N2 human seasonal IAV; PB2, PA from avian IAV [53, 54] and H1N2 viruses have been evolved and well-established in swine populations. Numerous variants of second generation reassortants between human pandemic H1N1 2009 and pig endemic H1N1 viruses have been reported worldwide [55]. The mixed sialic acid receptor distribution in the swine respiratory tract, consisting of both α-2,3 and α-2,6 linkages will make them extremely susceptible to both swine and human influenza types and thus are excellent mixing vessels for the different subtypes of IAV.

2.1.2 Influenza B (IBV)

Though humans are the primary reservoir of influenza B, reports on the susceptibility of domestic piglets to influenza B virus have been reported early back in 1966 [56]. Serosurveillance studies conducted by our group on the midwest US swine herds from 2010 -2012 found that 38.5% (20/52) of sampled farms and 7.3% (41/560) of tested swine sera were positive. It is not clear whether animals, especially swine could act as a primary reservoir, or is it the reverse zoonotic potential of the IBV that is causing the disease. Further studies in 2014, demonstrated high seroprevalence rate against IBV in porcine reproductive and respiratory syndrome virus (PRRSV) positive swine herds, which indicates a strong association between these respiratory viruses. Experimental infection of pigs with two antigenic and genetic lineages of IBV: B/Brisbane/60/2008 (Victoria lineage) and B/Yamagata/16/1988 (Yamagata lineage) showed influenza-like symptoms and successful transmission with seroconversion among pigs [29]. Further studies are needed to know whether swine can also act as a potential reservoir for human IBV.

2.1.3 Influenza C (ICV)

ICV is a ubiquitous pathogen affecting humans and antibodies to ICV are prevalent in all humans. Natural infection of ICV has also been reported in swine [57]. ICV has been isolated from pigs in China in 1981[31]. Genetic analysis showed that Chinese swine ICV share high similarities to human ICV, suggestive of interspecies transmission. Serological screening in Japan and Great Britain has shown incidences of ICV in pigs in the 1990s [58, 59]. While the interspecies transmission of ICV is from human to pigs or pigs to human is still under debate, further investigation is warranted to determine the host spectrum.

2.1.4 Influenza D (IDV)

A new influenza virus type has recently emerged in U.S. cattle and pig population, lately officially designated as influenza D (IDV), as this particular virus is different from the previously established IAV, IBV and ICV viruses [2]. IDV was originally isolated from a piglet showing influenza-like symptoms in Oklahoma in 2011[4, 60]. Further serological screening among cattle and small ruminants showed antibodies against IDV. There are two distinct antigenic and genetic lineages for IDV and these two lineages co-circulate in the U.S swine and bovine populations[47]. IDV has been isolated from other parts of the world such as France, China, Italy which shows its intercontinental transmission[13, 15, 61, 62]. Although cattle are considered to be a principal reservoir for IDV, the role of other animals cannot be dismissed.

2.2 Bovine Influenza: connecting the past and present

The notable respiratory tract infections of cattle of the past included bovine parainfluenza 3 (the Late 1950s), bovine rhinotracheitis virus (1966), bovine viral diarrhea virus (1964), bovine respiratory syncytial virus (1950s). The first report on bovine influenza dates back to 1949 from Japan [63]. There are several reports of influenza infections and diseases reported from the cattle in Russia, Germany, and Poland, but the reports are not accessible currently as the publications were in their native languages and not in English. For some, there are English abstracts, which we have made use of in this review [64-66].

2.2.1 Host restriction factors in bovines

It is interesting to note that the host repertoire of Influenza A viruses expanded over time to include swine, equines, canines, seals, whales, ferrets and poultry since its initial

emergence in human, and avian population, but there was only very few evidence for influenza A occurrences in bovines in the past. Despite, the growing host range of influenza over the last century, the remote incidence of influenza in the bovine species could be due to some host restriction factors present in the bovine respiratory tract that is hindering the influenza virus adaptation. Humans have a close association with swine and both zoonoses and reverse zoonoses phenomenon have been documented. Pigs are considered as the mixing vessels for influenza viruses. Interestingly, human association with bovines is equally old and closer, however, there was no influenza occurrence in bovine species. As such, the influenza A and B viruses can replicate in the in-vitro bovine systems [67-74].So there must be an in-vivo host factor causing the interference, which could be receptors or host innate immune response. Bovine lactoferrin (bLf) is one such protein that was shown to have anti-influenza activity [75-87]. Lactoferrin is a 76kDa glycoprotein with a single polypeptide chain of 689 amino acid residues, present in the biological fluids and the specific granules of polymorphonuclear leukocytes and is involved in immunomodulation, iron absorption, and pathogen inhibition. Bovine lactoferrin was also found to have inhibiting property against other enveloped viruses [88]. At the molecular level, bovine lactoferrin binds to the influenza virus hemagglutinin and is found to have inhibition against H1N1 and H3N2 influenza viruses. Pietrantoni et al. demonstrated that bLf interferes with caspase 3 function and inhibit the nuclear export of the viral ribonucleoproteins to the cytoplasm and maintains this function in its desialylated, deglycosylated, apo and ion-saturated forms [79, 82]. BLf is a protein with two symmetrical and globular lobes: C and N lobe each with two sub-domains, I and II with an interdomain cleft that binds to an iron atom. C lobe binds with the HA stem region which

includes HA2 and some important aa residues of the HA1 region spanning the universal conserved epitope, which explains the.broad spectrum anti-influenza activity of the bLf C-lobe[88]. Bovine lactoferrins have also been known for anti-infective, anti-cancer and anti-inflammatory effects. Oral administration of the bovine lactoferrins reduced the lung consolidation score and the leucocyte infiltration in the bronchoalveolar lavage fluid in mice [89].

2.2 Natural cases of Bovine influenza

First recorded evidence of influenza connected to cattle dates back to 1949 in Japan, where 160,000 cattle were infected in the western and middle part of Japan [63]. This particular incidence of cattle influenza had a short course with recovery in 2-3 days and the documented symptoms include high temperature (40-42 ^{O}C), blepharitis, nasal discharge, anorexia, tympanitis, pneumonia, joint problems, a decrease in lactation etc. Saito also mentioned some previous major cattle influenza outbreaks in Japan that occurred in the Fall of 1889 and 1893 and some minor outbreaks in 1914-16 in this report [63].

The first report on the isolation of influenza virus from animals was documented from Hungary in 1962 [90]. Further, there were reports on cattle influenza from several countries especially from the old USSR, with publications written in their native language, occasionally with English abstract and keywords. Among which, the earliest report was on the seroprevalence of influenza in domestic species of animals in 1969 [65]. During the period 1970-1980, isolates of influenza A have been reported in cattle from Russia, concurrent to the time of the Hong Kong H3N2 outbreak in humans. In 1973, there were reports on the isolation and identification of the A-Hong Kong (H3N2) virus from cattle suffering from respiratory diseases from Russia [91]. A/calf/Duschambe/55/71 was

isolated, from a terminally ailing calf with respiratory illness using embryonated chicken eggs [91]. In 1973, there were other outbreaks reported from Russia (old USSR) among cattle and another study on the antigenic characteristics of the influenza viruses isolated from the birds and animals of the USSR, both of which are not available in English [92, 93]. A preliminary report on the isolation of influenza A virus strains has been reported from the cattle in 1974 [94]. Further, in 1976 and 1977, there were studies from Russia, describing cattle influenza and the examination of the cattle [95, 96]. In 1977, a study was conducted where the calves were experimentally inoculated with 3 strains of Hong Kong H3N2 viruses from humans, such as strains A/Michigan/l/72 and A/England/42/72 and A/Aichi/2/68 along with the calf influenza A strain, A/calf/Duschambe/55/71. None of the human strains caused respiratory disease in calves, except for the A/calf/Duschambe/55/71. Clinical symptoms include nasal discharge, cough, and mild rhinitis. There was virus shedding for A/Aichi/2/68 and A/calf/Duschambe/55/71 for five and seven days respectively. A/calf/Duschambe/55/71 was considered as a host range variant of Hong Kong/68 strains isolated from humans [97]. In 1978, Wagner et al. described influenza and enzootic bronchopneumonia in cattle from Germany [98].

In the late 20th century, an idiopathic condition manifested with a sporadic drop in milk production occurred in dairy cows [99]. Brown et al. reported seroconversion against influenza A virus in cattle associated with reduced milk yield and respiratory disease from Great Britain. The virus isolation from these seroconverted animals was negative. The animals seroconverted to influenza A virus did not have antibodies against the bovine viral diarrhea (BVD), infectious bovine rhinotracheitis (IBR), parainfluenza-3 (PI3), and bovine respiratory syncytial virus (BRSV) [100, 101]. Further, in 1999, Gunning et al. reported

that the cases of influenza in milking cows kept increasing with an annual incidence rate of 10-20% in some herds. The clinical symptoms include a sudden drop in the milk yield, mild pyrexia, anorexia, occasionally respiratory signs such as nasal discharge and increased respiratory rate. High levels of neutrophils and haptoglobin in the blood can be seen in most cases. Paired sera from five herds were tested against IBR, PI3, BRSV, Adenovirus, M.bovis, H. somnus, C. psittaci, C. brunetti, P. hemolytica, P. trehalosi, Treponemes by the appropriate serological test to check the antibody responses. BRSV and PI3 were present in all the herds; BVD and IBR were detected in some herds. The cattle sera tested showed significantly high antibody titer to the two human influenza A viruses: 60% for A/England/333/80 (H1N1) and 65% for A/England/427/88 (H3N2) and only 5% of the cows were seronegative against both these viruses [102].

A report on finding the influenza genes in the cattle, citing the work done at Veterinary Laboratories Agency near Weybridge, the UK by Dr. Ian Brown and his colleagues was published online in Nature International weekly journal of science on January 9, 2002 (https://www.nature.com/news/1998/020107/full/news020107-4.html. Accessed 3/31/2018). However, we could not find out any further published information later. In 2002, another study from Northern Ireland, involving 84 pairs of paired acute and convalescent cattle sera collected in 1998 and 1999 from 17 outbreaks of the respiratory disease combined with diarrhea and milk drop syndrome were tested against A/Eng/333/80 (HIN1) and A/Eng/427/88 (H3N2). About 56.5 and 58.8 % of the convalescent sera showed seroconversion against H1N1 and H3N2 respectively, with H3N2 antibody titer remained high compared to H1N1. The study also described the antibody response of the cattle sera against the human and porcine strains, with the highest number of positive sera

against human H3N2 strains, but the virus isolation in specific pathogen-free chicken embryos was unsuccessful from the 142 cattle with similar clinical history [103]. In 2007, an experimental inoculation of calves with highly pathogenic avian influenza virus (HPAIV) A/cat/Germany/R606/2006 (H5N1) demonstrated 100 % seroconversion with neutralizing antibodies against the homologous strain. This study also reported very low shedding of the virus as determined by the nasal swab fluid inoculation into embryonated chicken eggs and MDCK cells from the inoculated animals. Virus neutralization and the ELISA test conducted at 3 months post inoculation demonstrated seroconversion in all inoculated calves and one contact animal, providing evidence for contact transmission between calves [104].

Another study by Crawshaw et al. in 2008, also demonstrated rising antibody titers against the A/England/333/80 (H1N1) and human influenza A/Eng/427/88 (H3N2) from a Holstein Friesian herd suffering from acute fall in the milk production and found no association with other infectious diseases such as BRSV, BVD, IBR and PI3. The loss of milk production was measured as 159.9 L, which is the difference in the mean of the milk production by the controls and affected animals, which comes to about 2% of the lactation yield per cow in a herd [105]. A study to find the infectious etiology of the fatal cases of calf pneumonia in 48 calves from 27 herds did not detect influenza A virus. The study also demonstrated that the severe lung pathology is due to Mannheimia-Pasteurella in 36/40 (90%) cases; *Arcanobacterium pyogenes* in 16/40 (40%) cases; *Mycoplasma bovis* in 12/40 (30%) cases; bovine respiratory syncytial virus in 4/40 (10%) cases; *Histophilus somni* was detected in 2/40 (5%) cases, while bovine herpesvirus-1, bovine viral diarrhoea virus and parainfluenza virus-3 were 1/40 (2.5%) [106].

2.2.3 Seroprevalence studies in bovine species

Naturally occurring antibodies against the H3N2 viruses have been detected in the crossbred yak, cattle, and water buffaloes in Kathmandu, Nepal and goats and cattle in West Bengal, India as demonstrated by the single radial immunodiffusion tests [46]. An experimental inoculation of seronegative yak with A/Hong Kong/1/68 (H3N2), A/England/42/72 (H3N2) and A/Prague/1/56 (Heq1 Neq1) resulted in a serological response against all these strains [46]. This study has been the second documented reference that was reported from South West Asia, after the natural infection that happened in Japan in 1949. Precipitation and complement fixation tests to study the incidence A/Port Chalmers/73 (H3N2) of the A2/Hong Kong/1/68 and /PR8 in 14 animal species from Ottawa area in Canada revealed that only six species such as dog, cat, rabbit, goat, chipmunk, and sheep showed seropositivity [107]. In Great Britain, a serosurveillance study was conducted on swine and bovine sera from a period between September 1973 and July 1977, against A/Swine/Wisconsin/66 (HSwlN1), A/Swine/1976/30 (HSwlN1), A/Hong Kong/1/68 (human H3N2), A/Port Chalmers/73 (human H3N2). This study showed seropositivity in swine samples towards human influenza H3N2 viruses with 4.5% in 1974, 1.7% in 1975 and 2.3% in 1976 against A/Port Chalmers/73 showing the evidence for reverse zoonosis. None of the bovine sera was positive against the tested swine (H1N1) and human (H3N2) influenza viruses [108]. Another study to determine the seroprevalence of influenza B and C viruses in animals has been described from Japan in 1978 [109]. Seroprevalence study conducted in animal sera from different species of animals involving cattle, horses, pigs, dogs, cats, minks and rats over the period 1975-1977 in Japan, demonstrated antibodies against 15 subtypes, out of the 16 subtypes of influenza A viruses

tested [110]. In the case of cattle, out of 728 serum samples, only 1.5% and 1% were seropositive against H0 and H3Aichi respectively and were seronegative against other subtypes [110].

In Northern Ireland, around 200 sera from crossbred and indigenous sheep breeds were collected to test the seroprevalence against influenza A and other viruses such as parainfluenza types 1,2,3, respiratory syncytial virus, bovine adenovirus Maedi-visna virus and bovine virus diarrhea virus and demonstrated no antibodies against influenza A, Maedi-visna and parainfluenza virus 1 and 2 [111].

A review by Lopez and Woods, based on the published pathological and serological studies described the seroprevalence of influenza A and B viruses in ruminants, detected by the complement fixation and hemagglutination inhibition tests from different parts of the world such as USA, Italy, Rumania, USSR, Nepal, India, and Hungary. A/calf/Duschambe/55/71 (H3N2) is a cattle influenza A strain from Russia. Both strains of H1N1 and H3N2 have been isolated from cattle such as Sw/Shope (H1N1) from Hungary, several H3N2 strains from USSR, and two viruses with an unidentified hemagglutinin and a type 2 neuraminidase in Hungary and the USSR [112]. A serological study conducted in sera from 5 calves inoculated with swine influenza virus showed a significant association between the mean diameter of the hemolysis zone obtained by the single radial hemolysis (SRH) test and the geometric mean of the hemagglutination inhibition (HI) titer after periodate treatment and receptor-destroying enzyme [113]. Another serological survey involving 177 paired calf sera from the years 1978-1981 showed that 3.4% of the calves seropositive to swine influenza virus [114]. Intranasal inoculation of live swine influenza virus (SIV) A/sw/IL/75 (H1N1) in calves caused respiratory tract disease, with virus

shedding and also caused contact transmission to sentinel animals. Seroconversion was observed at 9 days post-infection and virus neutralization antibodies were demonstrated at 14 and 21 days post-infection (dpi) [115].

Even though there has been a serosurveillance study of Influenza A (H1N1) antibodies in calves, which showed twenty-seven percent of positive and 31% low positive, there was no evidence of clinical infection in cattle [116]. In Egypt, seroprevalence in different species has been demonstrated in serum samples collected from different mammalian species such as human, goat, cattle, buffaloes, sheep, horses, swine, donkey, sewage rats and stray dogs and cats [117]. Calf and human fetal kidney cells were utilized to demonstrate the plaque formation by influenza B viruses [118]. Madin-darby bovine kidney cells have been utilized in characterization studies to grow many influenza viruses including WSN strain of influenza A [70, 119, 120].

2.2.4 Experimental infection in cattle

In 1956, an experimental infection demonstrating direct inoculation of the influenza PR8 strain compared to the new castle disease virus into the lactiferous sinus of the goat mammary glands resulted in the production of neutralizing antibodies in the milk and blood. The authors also found that even after surgical removal of the mammary gland, the neutralizing antibody level in the blood phased out slowly compared to the new castle disease virus [121].

Several studies have been previously reported from cattle in the 20th century, and some of the reports are in non-English languages [64-66]. Experimental infection in different mammalian species including calves and lambs to characterize the antibody responses showed appreciable HI titers in calves after primary infection, leading to an

anamnestic response after challenge with homologous antigen [122]. Another study by Campbell et al. described an experimental infection of A/calf/Duschanbe/55, a Hong Kong type A strain isolated from calves along with human strains A/Michigan/l/72 and A/England/42/72 in calves and found shedding of A/Michigan/l/72 and A/calf/Duschanbe/55, without any clinical symptoms [97]. A study on recombinant vaccinia virus expressing HA of swine influenza A virus A/NJ/11/76 developed antibodies when inoculated in cattle, sheep, and poultry, while the wild-type virus did not cause any antibody response. There was no contact transmission reported by the wild-type or recombinant virus in these species [123]. To find out the role of the cattle egrets in H5N1 outbreaks in Vietnam, these birds were intranasally challenged with highly pathogenic avian influenza (AI) A/duck/Vietnam/40D/04 (H5N1). The egrets contracted the infection and some were dead in a week, however, there was no contact transmission happened to the co-housed chickens [124].

2.2.5 Influenza Virus structure and use of bovine cell cultures

Earlier studies about the genome structure and segmented genome have been demonstrated in 1962 [125]. Several studies on the nucleic acid structure of the influenza virus have been demonstrated by sucrose gradient centrifugation in the late 1940s to 1970s, where the virus has been derived from the infectious allantoic fluid, chorioallantoic membranes, and fibroblasts of the chicken embryos [126-142].

The first and foremost demonstration of propagating high yield of infectious influenza virus was done in a continuous cell line from bovine kidney (MDBK cells) by Choppin, P. W. in 1969 [143]. Since then MDBK cells have been utilized extensively to grow the virus stocks of influenza especially WSN to study the ribonucleic acid genome

and time course of polypeptide chain synthesis and its assembly into virions at different stages of the virus life cycle [73, 144-149]. The WSN strain of Influenza A virus was grown on MDBK cells and purified virions have been separated by gradient centrifugation and electrophoretically analyzed to determine seven polypeptides [144, 150]. Simultaneously, pig and canine kidney continuous cell lines were also used to investigate the persistent infection of influenza and also to study the nuclear protein and two non-structural proteins NS1 and NS2 [146, 147, 151]. The existence and characterization of RNA -dependent RNA nucleotidyl transferase (RNA -polymerase) have been initially reported in 1966 [152]. RNA polymerase enzyme has also been demonstrated in chicken fibroblasts upon fowl plague infection compared to uninfected cells and the susceptibility of the enzyme to actinomycin D and Dextran sulfate in contrast to the new castle disease virus-induced RNA polymerase [153, 154]. The complementary strand synthesis of the parental viral RNA by the RNA polymerase has also been studied in-vitro [155, 156]. The isolation of Ribonuclease protein (RNP) with RNA polymerase activity by discontinuous sucrose gradient fractionation of influenza-infected cells also used the BHK-21F cells and MDBK cells have been described [157].

The concept of Von Magnus particles and defective interfering (DI) influenza viruses has been studied using MDBK cells, and HeLa cells and found that DI viruses produced during persistent infection were in good correlation with the ability of the host cell species to produce the infectious virions [69, 158, 159]. Host-dependent variation in the relative amount of the cleaved and uncleaved hemagglutinin polypeptide during influenza infection has been reported by comparing the amount of HA produced by the WSN strain of influenza Ao and the RI/5-strain of influenza A2 in primary monkey kidney

cells, MDBK, BHK21-F and chicken embryos and also found that uncleaved HA is more in the early growth cycle of influenza virus than the later stage when the cytopathic effects are more pronounced [160]. Studies on the HA polypeptide cleavage by the action of plasmin produced by the host cell plasminogen activators have also been done in MDBK cells [161]. Plaque formation by influenza type A and B viruses has been extensively studied in calf kidney and specific pathogen-free chicken kidney cells and found the linear relationship between plaque number and virus concentration [162].

MDBK cells have been widely used to study the biological properties of the mutant influenza virus, produced by the host cell-mediated selection pressure. Influenza A, WSN strain grown on the chicken embryo fibroblasts caused fuzzy and clear plaque morphology when grown in MDBK cells. The parental fuzzy virus and mutant clear viruses demonstrated different binding affinities. The clear viruses produced a high yield with large amounts of mRNA, hemagglutination in the presence of calf serum components and remained cell associated when transferred from 0 ℃ to 37 ℃, unlike the fuzzy viruses [163]. Another interesting feature about the influenza viruses grown on different host cell systems is about the amount of the carbohydrate added to the viral hemagglutinin protein which in turn determines the host binding property. A study conducted using the WSN-F strain of influenza A in chicken embryo fibroblasts and MDBK cells in the presence of tunicamycin showed that HA generated by the MDBK cells, contained 4000 daltons of carbohydrate in excess than the virus grown in the chicken embryo fibroblasts, which reduces the receptor binding affinity of the virus. This study also demonstrated that MDBK derived influenza viruses have more highly branched and complex asparagine-linked oligosaccharides and galactose-containing bisected oligosaccharides compared to HA

subunits from chicken embryo fibroblasts which is different to the carbohydrate profile of the host cells of origin [164]. Another study was conducted to determine the role of the host cell-specific glycosylation of the HA1 subunits particularly at residue 129, at the tip of the HA and residue at 184, close to the receptor binding pocket in receptor binding properties of three influenza variants in MDBK cells. The study demonstrated that glycosylation is site-specific in all the variants that are grown in MDBK cells and the reduction in the receptor binding properties of influenza viruses associated with MDBK cells is due to the cumulative effect of having large complex glycans at 129 and His to Asn substitution at residue 184 [165].

The first study to use temperature-sensitive mutants in man was developed by co-infection of wild-type influenza A/ Hong Kong/1968 (H3N2) virus and influenza A/Great Lakes/1965 (H2N2) on primary bovine kidney cells [166]. This study shed some light in the direction of live attenuated vaccine by demonstrating the development of serum and nasal neutralizing antibodies and resistance to a subsequent challenge by a wild-type virus. Several other studies using temperature-sensitive mutants of influenza have also utilized MDBK cells for virus propagation [167, 168].

Bovine cell cultures have been widely used for the antigenicity and pathogenicity studies of influenza A and B types during 1960-70s [73, 169-172]. A virus propagation system based on the roller cultures along with the use of the maintenance medium of improved composition has been demonstrated using bovine embryo kidney cells and MDBK cells [173, 174]. WSN-infected MDBK cells have been used for the quantitative measurement of the plus- strand and minus-strand RNAs synthesized during the early and late replication cycle and their differential regulation of the transport [175]. MDBK cells

can produce M and NS gene segments in full length, without virus production, when infected by a mutant A/WSN virus for several passages [67].

Tracheal and lung organ cultures of the cow embryo has been used for influenza A2 virus propagation along with new-born guinea pigs, rabbits, chickens and pig embryos and demonstrated that tracheal organ cultures were more sensitive and supported viruses [176]. Bovine and porcine tracheas have been used as explant cultures to see the interaction between Mycoplasma and influenza, where the tracheas of both animal origin were infected with *Mycoplasma hyorhinis* and superinfected with influenza and vice-versa. The swine trachea showed a synergistic pathologic effect with a complete loss of the tracheal ciliated epithelium, when infected with *Mycoplasma hyorhinis* on day 0 and Swine influenza at day 2 of the experiment while the bovine trachea did not show such effect under similar conditions. On the other hand, swine trachea infected with influenza and superinfected with Mycoplasma clearly demonstrated enhancement of the growth of the Mycoplasma by influenza compared to the controls [177]. Cow embryo tracheal organ cultures and kidney tissue cultures were used to study the reactogenic and immunogenic changes of influenza A/Hong Kong/1/68 (H3N2) virus over serial passages of 24 and 48 h intervals and found that 24 h passages reached attenuation rapidly and retained immunogenic potency better than 48 h passages [178]. Virus propagation of WSN in bovine, human and chicken embryo cell culture showed that airborne stability of the WSN virus varied between the virus propagated in cell culture versus embryonic eggs and the maximum stability was obtained at low relative humidity [179]. Infectivity of equine H3N8 influenza A virus has been studied in calves and bovine cells [180]

2.2.6 Sensitivity of influenza virus to bovine serum factors

Influenza virus hemagglutination has been inactivated by cow's milk [181]. Further, there have been some publications showing the ability of the different factors in the biological fluids of the ruminants such as bovine amniotic fluid, mucoprotein from bovine submaxillary glands [182, 183]. The sensitivity of influenza viruses to periodate resistant inhibitors in the normal bovine serum has been studied earlier [184]. Bovine sera, especially from fetal calf or normal calf, induce the formation of hemagglutination inhibitors upon treatment with potassium periodate, similar to the phenomenon observed with treated sera from human, rabbits, rats, guinea pig, horses, goat, chicken, and monkey. Only the mouse and hamster sera did not show any increase in the hemagglutination inhibition titer after periodate treatment [185]. Further, a quantitative estimation of the non-specific hemagglutinin inhibitors of influenza virus present in the sera of 27 species involving laboratory, domestic, wild animals and birds, showed that sera of sheep, goats, and cattle belong to a separate group based on the physicochemical properties of antiviral inhibitors such as its sensitivity to heating, potassium periodate, trypsin, 2-Mercaptoethanol, and rivanol. This study also demonstrated that non-specific inhibitors present in bovine sera are heterogenous *ie*. both thermolabile and thermostable types [186]. The non-Ig inhibitors, also called as beta inhibitors in the normal bovine and mouse sera, are mannose-binding lectins which bind to the carbohydrate on the hemagglutinin, blocking the receptor binding sites and thus inhibits the infectivity and hemagglutinating activity of the H1 and H3 influenza A viruses. The serum inhibitor in bovine serum resembled conglutinin, which is a Ca(2+) dependent N-acetylglucosamine and mannose-binding lectin and the hemagglutination inhibition property was abrogated by the polyclonal and monoclonal anticonglutinin antibodies [187-189]. These conglutinins also

act as opsonins for the phagocytosis of influenza A viruses [190]. A recombinant bovine conglutinin demonstrated sugar binding, hemagglutination inhibition, conglutination and neutralization activity against influenza A virus, like the native conglutinin [191]. Bovine serum proteins such as conglutinin, collectin-43 (CL-43) and collectin-46 (CL-46) are C-type lectins, of which conglutinin and CL-43 exhibit antiviral properties against influenza A and rotaviruses. Conglutinin in the serum of the dairy cows is dependent on the season, breeding, stage of the reproductive cycle and infection [192, 193]. Aprotinin is a natural 58 amino acid protease inhibitor from bovine lungs, already intended to use in humans for pancreatitis and bleeding has the potential to suppress the cleavage of the pandemic H1N1 influenza virus in different host systems such as human tracheobronchial epithelium, human intestinal Caco-2 cells and chicken embryonated eggs [194].

Influenza virus infection in calf kidney cells demonstrated the production of cell-free, but tissue and virus-specific RNA synthetase compared to non-infected cells which can be inhibited by the species-specific interferon. This study used the calf kidney cells, chicken embryo fibroblasts upon infection with influenza and New castle disease virus respectively demonstrated a direct correlation of antiviral protective effect of the interferons on the infected cells [195]. A consumable low-molecular-weight fraction (CLMWF) of immunoglobulin-depleted bovine colostrum whey, can help in the antibacterial (Streptococcus) and antiviral (influenza) immune defense in vivo, by the maturation of the antigen presenting cells [196]. Anti-influenza activity of the two human/bovine chimeric Mx proteins was studied by substituting the GTPase effector domain (GED) of the human with bovine and vice-versa and showed that bovine Mx1 proteins have a higher activity against the influenza virus particularly the motifs located in

its N-terminal portion which is responsible for the interaction with the cellular and viral factors [77]. Bovine pulmonary Surfactant (R), is an efficacious antigen vehicle for intranasal vaccination and is widely used in newborns for the acute respiratory distress syndrome. A synthetic analog for this compound, SF-10 has been prepared to avoid the risk of BSE. HA combined with SF-10 stimulated higher levels of anti-HA-specific s-IgA in nasal-wash and serum IgG than induced by HA-poly (I:C), a mucosal vaccine used for protection [197].

2.2.7 Vaccination studies

In the late 20th century, a vaccination study on two important viral diseases affecting livestock, such as foot and mouth disease and influenza, was conducted in sheep and cattle. Interestingly, both these viruses exist in multiple serotypes. In case of influenza, 100 % of animals seroconverted against the homologous serotype influenza (A/PR/8/34) and 18.7 % of animals seroconverted to each of the heterologous serotypes (A/Shanghai and A/Leningrad) in calves. Similarly, 100 % seroconverted against homologous serotype influenza (A/PR/8/34) and 10% and 17.5% showed a response to the heterologous serotypes A/Leningrad and A/Shanghai respectively in lambs. Only 1/32 and 1/40 responded to both heterologous serotypes in calves and sheep respectively [198]. Bovine adenovector based booster for H5 HA for Human adeno vector immunization of naïve or adenovirus primed mice ensured full protection from a potentially lethal challenge with A/Hong Kong/483/97 in mice [199]. Oral administration of the bovine late colostrum has been found to have augmented the local and systemic cellular immunity and also the activation of cellular immunity in mice by increasing the NK cell activity, together with high levels of IL-12 and IFN-γ [80].

2.2.8 Antibody reactions with influenza

Antigen-antibody reactions to influenza viruses have been studied as early as in the 1970s [200]. A modified technique for HI test has been demonstrated by incorporating a species-specific IgG fraction into the virus-test sera mixture suggested its utility in detecting low concentrations of viral antibodies and also in determining the common antigenic relationships between virus strains, along with appropriate controls with different reagents used in the assay [201].

2.3 Epidemiology of influenza D

Global current epidemiology of the influenza D virus showed seroprevalence in ruminants that include dairy cattle, buffaloes, sheep, and goats (Figure 2 3). Two influenza D lineages have been characterized based on their distinct genetic and antigenic properties, represented by the D/OK (D/swine/Oklahoma/1334/2011 and D/660 (D/bovine/Oklahoma/660/2013). A third lineage has also been reported from Japan [3]. D/660 lineage is the most common lineage in North America. Lately, there are more swine IDV viruses that have been isolated from different parts of Eurasia [13, 15, 202]. Table 2.1 shows the year-wise seroprevalence observed for different species from North America and Eurasia [11, 13, 15, 61, 202]. Recently a very high seroprevalence rate of 99-100% have been found in camelids for the bovine IDV [12]. Nationwide distribution of bovine and swine influenza D occurrence/seroprevalence were shown in Figure 2.3. Global scenario of IDV is shown in Figure 2.4. Amino acid alignment analyses performed on currently available twenty-six full sequences (26/50 sequences in NCBI) of HEF protein by MUSCLE and visualized by Jalview 2.10.4 was shown in Figure 2.5 [203, 204]
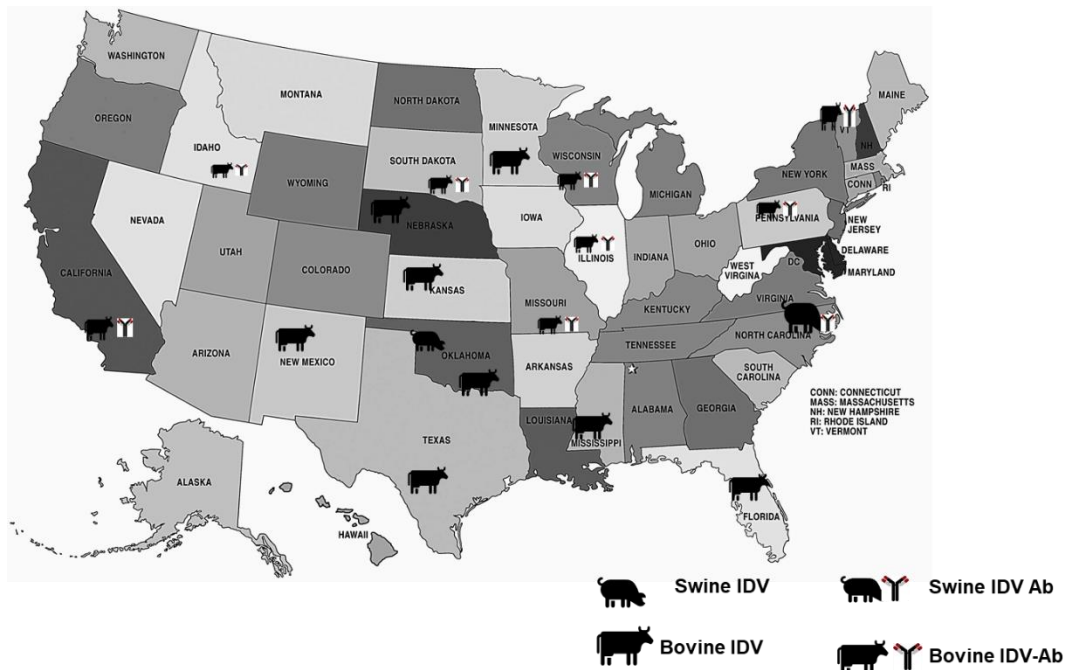
Figure.2.3 National distribution of influenza D virus isolation and the seroprevalence shown with an antibody icon.
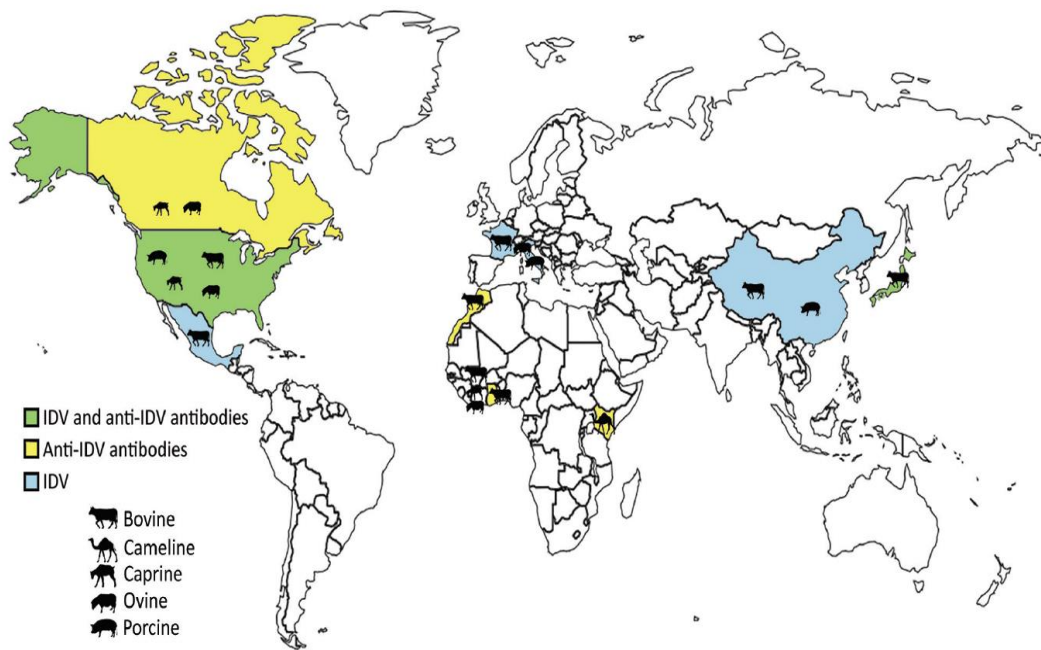
Figure.2.4 Global scenario of influenza D. Adapted from [12].
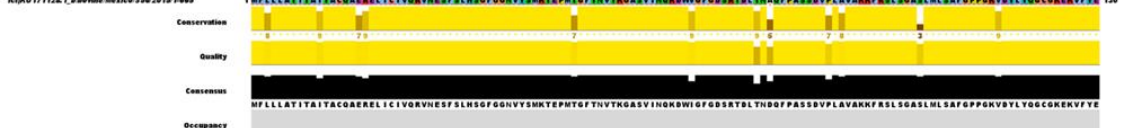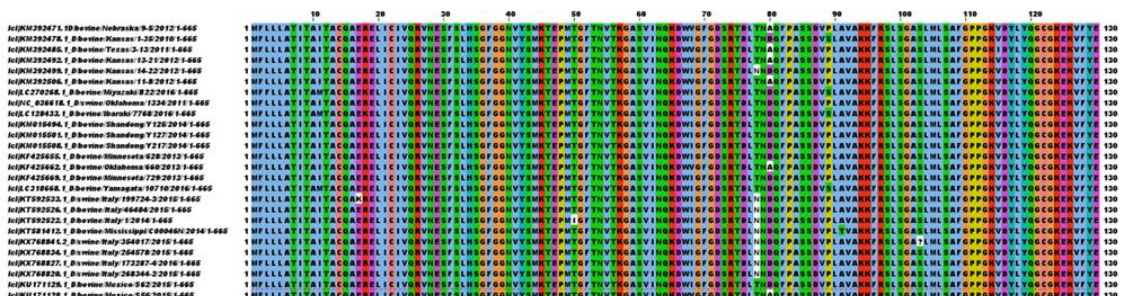
Table 2.1. Incidence and Seroprevalence of influenza D (in percentages) in different mammalian species.

Country		Year	Buffalo	Dairy cattle	Sheep	Goats	Swine	Equines	Camelids	General	Occupational	Pre-dominant lineage
USA		2013					9.5			1.3		
		2016									94-95	Bovine
		2015			5.2	8.8						
		2017						11				Bovine swine
China		2017	5.9		7.8	33.8						Swine
France		2014										Bovine
Japan		2016		30.5								Bovine
Africa	Togo			21								
	Morocco	2017		42								Bovine
	Kenya								99-100			
Italy		2016					11.7					Swine
Ireland		2015										Bovine
Mexico		2015										Bovine
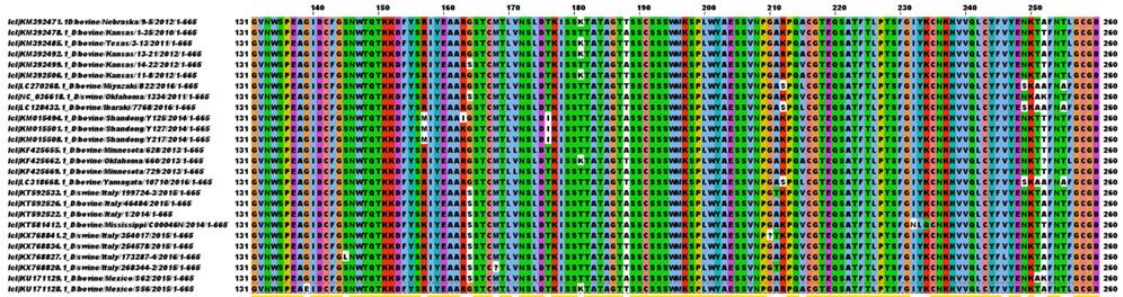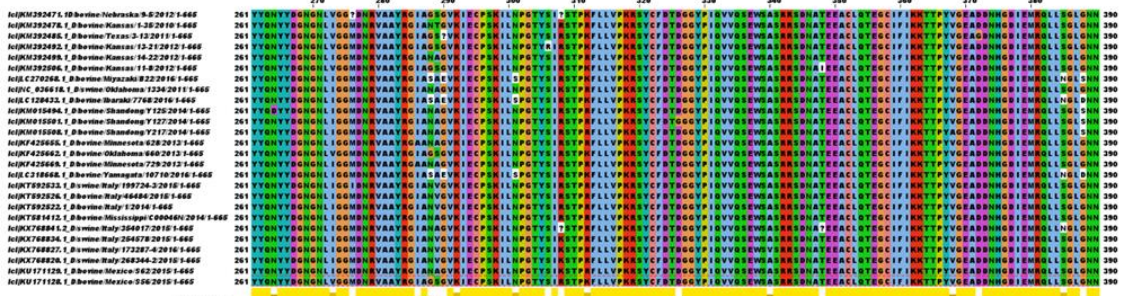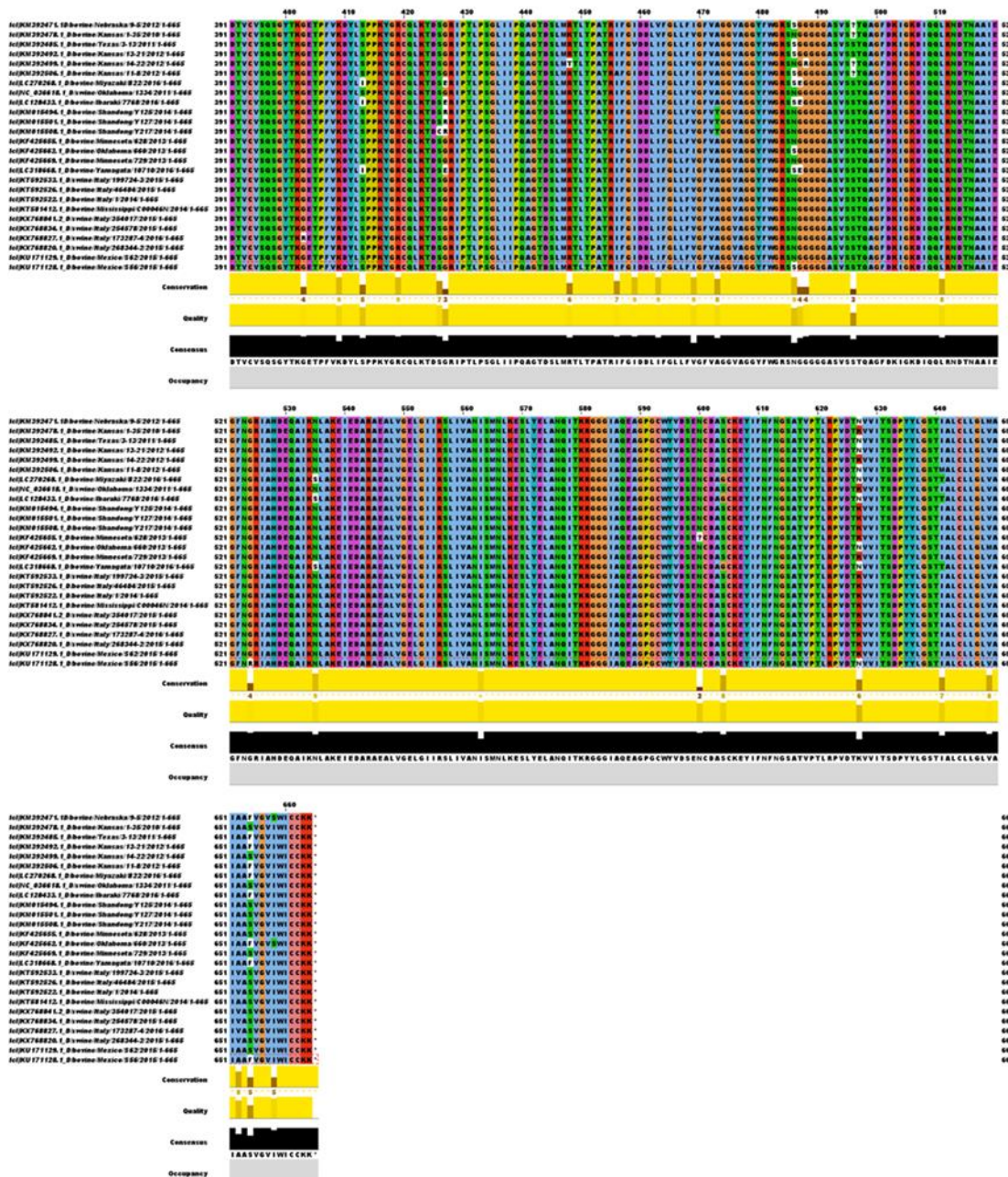
Figure 2.5 Amino acid sequence alignment of twenty-six full sequences of Hemagglutinin esterase fusion protein of the swine and bovine isolates available in the database. HEF amino acid sequences were aligned using MUSCLE and then visualized using JalView. Non-conserved amino acids were shown in white boxes.

2.4 Animal models of Influenza

Various animal models such as mice, ferrets, guinea pigs, pigs and non-human primates were used to study the pathogenicity and transmissibility of influenza viruses and also to evaluate the immunogenicity of vaccines [205-208]. Transmission experiments of influenza A and B types in various animal models also showed higher efficiency in contact route compared to aerosol [6]. Swine IDV was also reported to transmit by contact in pigs and ferrets [4]. The airborne transmission experiments largely depend on environmental factors such as temperature, humidity, airflow of the experiment room and infectious dose used. Among these factors, the rate of change and direction of air currents in the facility is an important variable, especially in aerosol transmission experiments. Previous experiments using influenza A and B type viruses, in guinea pigs conducted in environmental test chambers demonstrated efficient transmission lasting for a longer period at 5°C, 20% relative humidity (RH) than at 20°C, 20% RH and also stated that high temperatures and high humidity significantly reduced the transmission efficiency [209]. Large droplets of size >5-10 um facilitates only short-range transmission, while small droplets <5 um are responsible for long-range transmission [210].

2.5 In-vitro models: Use of Primary cell culture

Primary cells have been widely used to study the physiological, biological and pathological mechanisms of the mammalian body. The earliest literature available in the primary cell culture was about the development and cultivation of the primary human amnion cells in 1957 [211]. The morphological evaluation of the human amnion cells in primary culture and its transformed variant (Strain FL) in continuous cell culture showed that transformed cell line showed multivesicular bodies and membrane limited particles.

These morphological structures observed in the younger passages of the FL cells is assumed to be related to the transformation process [212] The morphological and genetic studies showed that the transformed amnion cells exhibited malignant properties, while the primary cells showed non-malignant characteristics [213, 214] Primary cells mimic the physiological properties in-vivo and hence is the best in-vitro model to study the mechanistic details of the normal or diseased conditions of the body. Primary cells have a limited growth in-vitro and show considerable mitotic activity in the first 2-4 weeks with the mitotic index as high as 1.8%. [214]. A comparative study of the primary and transformed human cells in-vitro have shown that the nature of the growth, nutritional characteristics and metabolic profile of the primary and transformed cells vary [215]

Epithelial surfaces of our body are equipped with a highly sophisticated machinery with several different types of proteins molecules that play a crucial role in maintaining the homeostasis and cell polarity with each tissue. Among these, tight junction proteins are macromolecular complexes consisting of several membrane proteins, that are important for the cell-cell interactions and cell-extracellular matrix interactions and also for transcellular and paracellular transport and permeability. There are several types of proteins in the cell membrane that helps in the integrity of the epithelium of which three types of proteins form the junctional complex in cell junctions which includes tight junction, adherens junction, and desmosomes. Tight junction proteins are transmembrane proteins and are located in the apical-most of the junctional complex [216] Tight junctions formed by the cell act as a semi-permeable barrier to the paracellular movement of cargo and act as a fence that connects the apical and basolateral domains of the plasma membrane. Tight junction proteins act as a multifunctional complex, critical for the epithelial and endothelial layers

to form its distinct compartments in the body, of which most important is the regulation of several signaling and trafficking molecules required for the cell differentiation, polarity, and proliferation.

Figure 2.6. Apical junctional complex and tight junctions. (A) Schematic diagram of the junctional complex. (B) Schematic structure of tight junction strands. (C) Functions of tight junctions. (D) Molecular components of tight junctions. Three families of tight junction transmembrane proteins, such as occludin, claudins, and JAMs, as well as some scaffold proteins and polarity proteins are shown, adapted from [216].

Primary cell cultures were widely used for studying various animal and human viruses since the 1960s [217-221]. Species-specific primary cell cultures have been developed and used over the years [222-226] Primary cell cultures of swine-origin have been used for normal physiological and pathological studies of several infectious diseases [217, 227-231]. Primary swine respiratory epithelial cells have been used to study the immunological and pathophysiological aspects of several respiratory diseases including influenza [231]

Primary cell cultures have been an excellent in-vitro system to study the virulence and pathogenetic characteristics of the influenza viruses. Influenza studies using primary cell culture from humans and swine has been utilized for studying the virulence, and receptor binding specificities of the viruses from different host origin [21-25]. Several studies have been conducted on swine tracheal/bronchial/lung epithelial cells to study the pathogenesis and anti-viral responses at the transcriptional and translational level as the swine species share the most anatomical and physiological characteristics to humans [26-28] Pigs are the mixing vessels of influenza viruses and harbor receptors for both the avian and human influenza viruses. Recently, it was found that domestic pigs are susceptible to influenza B, and C viruses [29-31]. Further, influenza D has been initially isolated from the swine in different parts of North America and Eurasia [4, 13, 16].

2.6 Virus- Receptor interactions

Influenza A viruses of avian and equine origin bind to Sia α 2-3 Gal receptor (MAL-II), while the human viruses bind to Sia α 2-6 Gal receptor (SNA) [25]. A study on selected 8 influenza B viruses isolated from 1940-1990 showed that these viruses have binding preferences towards ganglioside, carrying lacto-series type I and II sugar chains with the

Neu5Acα2–6Gal linkage, however, B/Gifu/2/73 strain bound to lacto-series gangliosides containing Neu5Acα2–6Gal and Neu5Acα2–3Gal linkages [232]. Glycan array to characterize the receptor binding specificities of influenza B viruses have shown that the Yamagata-like strains predominantly bound to α-2,6-linkage glycans while Victoria-like strains preferentially bound to both α-2,3- and α-2,6-linkage glycans and also explained a third group of viruses that bound to sulfated glycans, which are Victoria-like strains [233, 234]. All equine and avian viruses, which are known to recognize N-acetyl and N-glycolyl sialic acid linked to galactose by the alpha2,3 linkage (NeuAc alpha2,3Gal and NeuGc alpha2,3Gal), agglutinated erythrocytes from chickens, ducks, guinea pigs, humans, sheep, horses, and cows. However, the human viruses, that preferentially bind to NeuAc alpha2,6Gal, agglutinated all but the horse and cow erythrocytes. It was also observed that cow and horse erythrocytes contain a large amount of SA alpha2,3Gal-, and no SA2,6Gal-specific lectin-reactive oligosaccharides on the cell surface, while human and chicken erythrocytes contained both types of oligosaccharides [235]. So, agglutination patterns exhibited by different erythrocytes can be used to characterize the receptor specificity of different influenza viruses. Bovine coronaviruses and influenza C viruses share same receptor, 9-O-acetyl N-acetyl neuraminic acid. [236, 237]. IDV also uses 9-O-acetylated sialic acids for the receptor-mediated endocytosis but has an open receptor cavity that can hold diverse glycan moieties thus facilitating a broad host tropism of IDV compared to ICV [238].
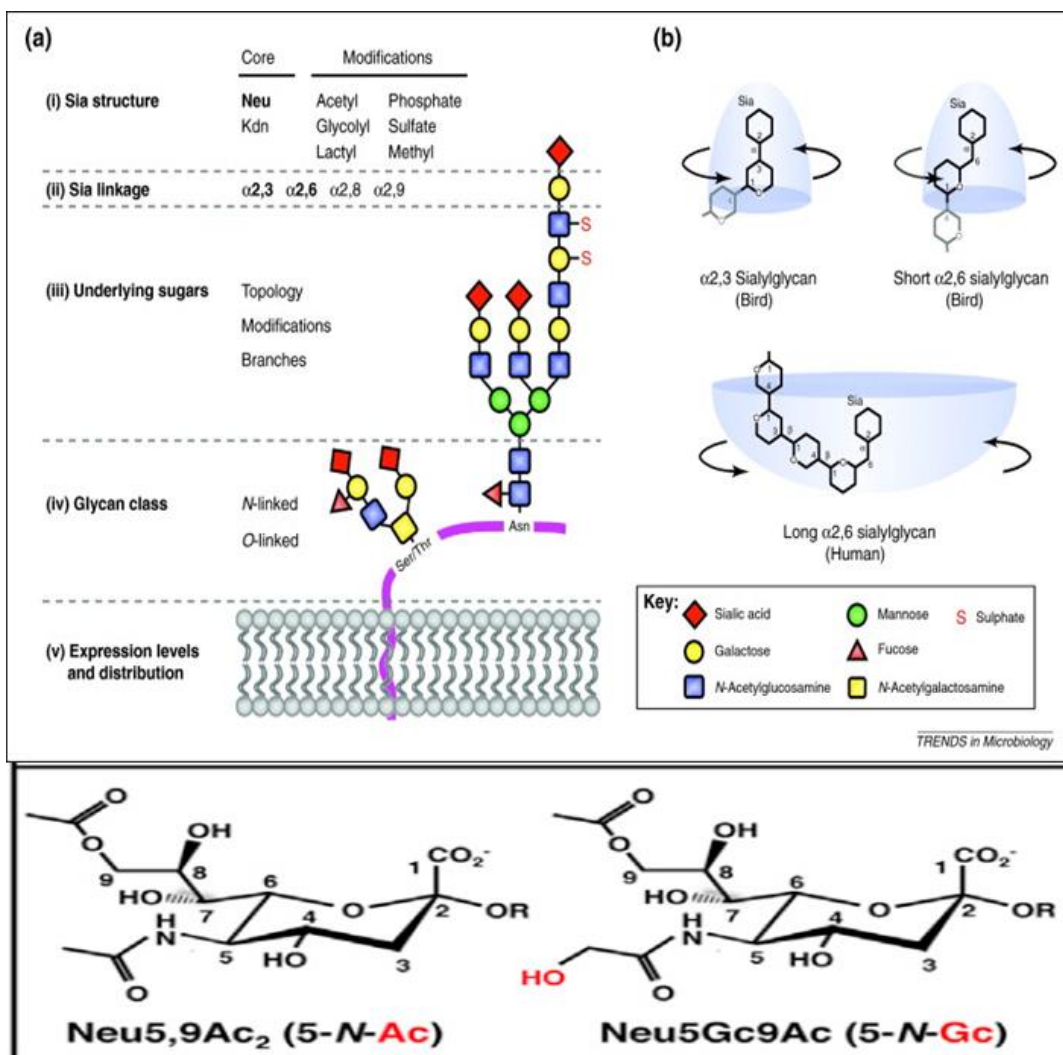
Figure 2.7. Sialic acid classification and 9-O acetyl sialic acids. The structure of proposed receptors of IDV, Neu5,9 Ac2 and Neu5Gc9Ac is also shown.

Chapter 3. Replication and Transmission of the Novel Bovine Influenza D Virus in a Guinea Pig Model

Abstract

Influenza D virus (IDV) is a novel influenza virus that infects cattle and swine. The goal of this study was to investigate the replication and transmission of bovine IDV in guinea pigs. Following direct intranasal inoculation of animals, the virus was detected in nasal washes of infected animals during the first 7 days postinfection. High viral titers were obtained from nasal turbinates and lung tissues of directly inoculated animals. Further, bovine IDV was able to transmit from the infected guinea pigs to sentinel animals by means of contact and not by aerosol dissemination under the experimental conditions tested in this study. Despite exhibiting no clinical signs, infected guinea pigs developed seroconversion and the viral antigen was detected in lungs of animals by immunohistochemistry. The observation that bovine IDV replicated in the respiratory tract of guinea pigs was similar to observations described previously in studies of gnotobiotic calves and pigs experimentally infected with bovine IDV but different from those described previously in experimental infections in ferrets and swine with a swine IDV, which supported virus replication only in the upper respiratory tract and not in the lower respiratory tract, including lung. Our study established that guinea pigs could be used as an animal model for studying this newly emerging influenza virus.

3.1 Introduction

Influenza viruses are negative-sense, single-stranded RNA viruses classified in the *Orthomyxoviridae* family. There are three recognized genera of influenza viruses, designated influenza A virus (IAV), influenza B virus (IBV), and influenza C virus (ICV). IAV and IBV have 8 negative-sense, single-stranded genome RNA segments, whereas C type has only 7 segments. IAV viral proteins include 5 structural proteins- HA, NA, M1, M2 and NP (ribonucleoprotein); 3 subunits of RNA polymerase complex- polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA); and 3 non-structural proteins- NS1, NS2 (nuclear export protein, NEP) and PB1-F2 [239]. Recent studies have suggested that NS2 and probably NS1 of IAV are a structural protein that can be detected in virions [48]. IBV has 6 structural proteins- HA, NA, NB, M2, M1, and NP; 3 subunits of RNA polymerase complex- PA, PB1, and PB2; and 2 non-structural proteins- NS1 and NS2. ICV has 4 structural proteins- M2, M1, NP and Hemagglutinin esterase fusion (HEF) protein that replaces the HA and NA of IAV or IBV; 3 subunits of RNA polymerase complex- P3, PB1, and PB2; and 2 nonstructural proteins-NS1 and NS2. Depending on the HA and NA proteins, IAV has several subtypes and causes severe epidemics and pandemics affecting humans. It also infects various other species of mammals and birds across the world, which can result in more widespread of IAV infection and more lethal outcomes especially in poultry than those seen in humans. IBV has no subtypes but possesses two lineages, causing localized epidemics and affecting mainly humans, and to some extent seals [240]. IBV genome was also recently detected in domestic pigs indicating that the virus may infect this agricultural animal [29]. Compared

to A and B types, ICV causes mild disease and was found to have co-existed with IAV and IBV infections in humans [50, 241].

In 2011, a new Influenza virus was isolated from Oklahoma in a 15-week old swine showing influenza-like symptoms. Electron microscopic studies have shown features similar to orthomyxoviruses. Further studies revealed that this virus was negative for neuraminidase and positive for O-acetyl esterase activity, which is a characteristic of ICV. Genera-specific real-time RT-PCR failed to detect the virus. However, the new virus showed 50 % homology to human ICV [4]. Deep RNA sequencing showed that the HEF protein of the new virus has a conserved enzymatic site, but a divergent receptor binding site compared to ICV. The virus also exhibited broader cellular tropism than ICV. A serological survey showed that the virus is widespread in swine and bovine herds of the United States [2]. In addition to a swine isolate, several bovine strains were also isolated from diseased bovines located in different farms of the USA Midwest region. Bovines with respiratory disease complex on the west coast were also found to harbor IDV [47, 242]. The new group of viruses did not cross-react with ICV antibodies by HI and AGID assays and also failed to undergo reassortment with ICV in vitro. Phylogenetic analysis also failed to identify reassortment between ICV and IDV in field isolates. Because these newly discovered viruses shared unique biologic, genetic, and antigenic properties different from ICV, a proposal was made to classify these viruses in a new genus- influenza virus D or IDV [2].

The bovine and swine IDVs shared more than 96% homology in all the segments. Most divergent segments were HEF and P42/M with 96.7-99.0% and 96.9-99.2% identity respectively. The segments with the highest homology were PB1 and NS with 98.9-99.1%

and 98.8-99.2% respectively [2]. Apart from the strains isolated from different parts of U.S., bovine IDV was also identified in Shandong Province in China and France [61, 243]. The strains from China shared 94-99% homology to the strains in the U.S. [243]. The geographical distribution of Bovine IDV shows that virus is prevalent in the bovine population and is at least circulating in North America, Europe, and Asia.

Swine IDV was found to infect ferrets and pigs and can also be transmitted to naïve animals by contact. Swine IDV was able to replicate in nasal turbinates and shed detectable levels of virus in nasal washes. However, swine IDV was not detected in trachea and lungs in either ferrets or pigs indicating its inability to replicate in the lower respiratory tract. The animals seroconverted but neither ferrets nor pigs developed clinical symptoms and lesions typical of Influenza [4]. Ferrets have been used as surrogates for human influenza infection and pathogenesis. The ability of swine IDV to replicate in the upper respiratory tract of ferrets may indicate the zoonotic potential of these viruses.

The objective of this study was to examine viral replication, transmission, and virulence properties of the novel bovine IDV in guinea pigs. Our study was based on the following considerations. First, guinea pig has been used extensively as an alternate mammalian animal model for studying the pathogenesis of many influenza viruses including IBV [5-7]. Second, this animal model was susceptible to infections by Influenza A virus subtypes and demonstrated virus replication in lungs. Third, guinea pigs were able to transmit human influenza viruses from one animal to another [7]. Fourth, they are easier to handle and house, and less expensive compared to ferrets. Importantly, guinea pigs share similar airway hyperresponsiveness and have bronchus-associated lymphoid tissue as humans [8, 9]. Finally, despite being highly similar to a swine IDV strain used previously

for ferret and pig studies, the bovine IDV selected for this study is a representative strain of another antigenic lineage different from swine IDV, possessing some distinct variations in the HEF protein, a major mediator of host range and viral tropism [10].

In this study, guinea pigs were divided into three groups – a) direct inoculated group, b) contact transmission group and c) aerosol transmission group. Our results showed that bovine IDV efficiently replicated in the lower and upper respiratory tracts of guinea pigs. Detection of IDV in lungs of infected animals is novel because our previous studies in ferrets and pigs revealed a lack of virus replication in lungs. We also found that naïve guinea pigs acquired infection from the co-caged infected guinea pigs indicating that bovine IDV can be transmitted by direct contact. Taken together, results of our experiments demonstrated that guinea pigs, being a good mammalian host, and widely used in Influenza research, could be further explored for mechanistic studies of IDV infection and pathogenesis.

3.2 Materials and methods

3.2.1 Cells and virus

Human rectal tumor (HRT-18G) cells and Madin-Darby canine kidney (MDCK) cells maintained in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories Inc., Dartmouth, MA, USA) and penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) (100 U/ml) were used in this study for cell culture. Influenza D/bovine/Oklahoma/660/2013 virus was originally isolated from the bovine herds of Oklahoma and has been designated a representative strain of a genetic and antigenic lineage different from the swine FLUDV strain that was used for previous ferret and pig studies (8). The virus was grown on human rectal tumor (HRT-18G) cells at

a multiplicity of infection (MOI) of 0.1. The cells were allowed to reach only 60% to 70% confluence at the time of infection. The virus was suspended in 2 ml Dulbecco's modified Eagle's medium (DMEM) and incubated at 37°C in 5% CO_2 for 1 h. Following infection, fresh DMEM with 0.5 μg/ml tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, Saint Louis, MO, USA) was added for further incubation at 37°C in 5% CO_2 for 5 days. After 5 days, the infected cell cultures were frozen and thawed. The supernatant was spun at 500 × g for 10 min at 4°C to remove the cellular debris. Determination of virus titers in MDCK cells was done according to the method of Reed and Muench (19). Virus loads in nasal washes and tissue homogenates were titrated using MDCK cells by indirect immunofluorescence assay (IFA). DMEM supplemented with penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) (200 U/ml) and TPCK-treated trypsin (Sigma, Saint Louis, MO, USA) (0.5 μg/ml) was used as the virus growth and maintenance medium.

3.2.2 Animals

Specific-pathogen-free (SPF), viral-antibody-free (SPF/VAF), 30-day-old guinea pigs of the Dunkin-Hartley strain (Elm Hill Labs, MA, USA) weighing 300 to 350 g were used for the study. The animals were ear-tagged for identification purposes. The duration of the experiment was 3 weeks, which included a 1-week acclimatization period. Animals were provided with food and water ad libitum and kept on a 12-h light/dark cycle. The temperature and relative humidity (RH) of the animal housing ranged from 72 to 75°F and 25% to 33%, respectively. Control animals were housed in a separate room away from the room housing the infected animals and were processed before the inoculated animals. Strict precautionary measures were followed to prevent cross-contamination between animals in

different cages. Sentinel animals were sampled from inoculated animals in transmission experiments. Gloves were changed between cages during cleaning and handling, and masks and surfaces were disinfected to prevent any possible cross-contamination.

3.2.3 Experimental design

Guinea pigs were divided into three experimental groups for testing growth kinetics of the virus, direct contact transmission, and aerosol transmission. The group for studying virus kinetics consisted of 10 infected animals and 3 mock-infected animals. The animals were infected intranasally with 3×10^5 50% tissue culture infective doses ($TCID_{50}$)/300 μl of influenza D/bovine/Oklahoma/660/2013 virus (bovine IDV). Half (150 μl) of the virus inoculum was delivered in each nostril. The 3 control animals were mock infected with equal volumes of phosphate-buffered saline (PBS). The body weights of all of the animals were recorded before the challenge. Guinea pigs were briefly anesthetized using isoflurane prior to infection.

For the contact and aerosol transmission experiments, 6 animals were inoculated intranasally with 3×10^5 $TCID_{50}$/300 μl of influenza D/bovine/Oklahoma/660/2013 virus (C/660) under conditions of isoflurane anesthesia and the remaining 6 animals were left uninfected. To test contact transmission, 3 infected animals were kept in a cage. At 24 h postinfection (hpi), three sentinel animals were added to the same cage. For studying aerosol transmission, 3 infected animals were housed in a cage with a double-walled wire partition that permitted airflow but prevented direct contact. Three sentinel animals were added at 24 h postinfection. The cages were kept away from the vents to minimize the dilution of aerosols by room ventilation and to provide a conductive environment for virus transmission. The animal experiments were approved by the Institutional Animal Care and

Use Committee of South Dakota State University (IACUC no. 14-011A) and were conducted under biosafety level 2 conditions.

3.2.4 Monitoring and sample collection.

Body weight and temperature were monitored daily starting from 2 days before challenge. Prior to challenge, blood was collected from all of the animals from the jugular vein/cranial vena cava under conditions of isoflurane anesthesia. Animals were monitored on a daily basis after the virus challenge for clinical signs, and body temperature and body weight were recorded. Nasal washes were collected from all the infected animals in the directly inoculated group and from three control animals at 1, 3, 5, 7, and 9 days postinfection (dpi). Additionally, two infected animals were euthanized using CO_2 at 1, 3, 5, 7, and 9 dpi. Blood, nasal wash, nasal turbinate, trachea, and lung samples were collected. The numbers of infected animals assessed each day from group I were as follows: day 1, n = 10; days 2 and 3, n = 8; days 4 and 5, n = 6; days 6 and 7, n = 4; days 8 and 9, n = 2.

For contact and aerosol transmission experiments, the animals were monitored for weight loss, temperature changes, and other clinical signs on a daily basis. The nasal wash samples were collected from all the animals at 48-h intervals (2, 4, 6, 8, 10, 12, and 14 dpi) from the time of challenge under conditions of isoflurane anesthesia. These animals were euthanized at 14 dpi, and blood, nasal wash, nasal turbinate, trachea, and lung samples were collected.

3.2.5 Collection of nasal washes

The nasal washes were collected by instilling 1 ml of PBS using a sterile 25-to-28-gauge cannula into the nostrils and collecting the washes by draining to sterile containers

or Petri dishes. Animals were anesthetized using isoflurane, and alternate nostrils were used for sample collection on alternate days. Nasal washes collected in the Petri dishes were transferred to 1.5-ml tubes and then centrifuged at $500 \times g$ for 6 min at 4°C to remove any debris. The supernatants were stored at −80°C until analysis.

3.2.6 Estimation of virus load in nasal washes and tissues

Lung, trachea, and nasal turbinates from guinea pigs were collected and stored at −80°C. One gram of tissue was homogenized using DMEM supplemented with penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) (200 U/ml) and a Stomacher circulator at high speed for 2 min. The homogenized tissue fluid was clarified by spinning at $500 \times g$ for 8 min at 4°C and stored at −80°C. For trachea and nasal turbinate analyses, DMEM with penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) (200 U/ml) was added and homogenized tissue fluid was collected and stored at −80°C until titration.

For virus isolation, MDCK cells were used for determining the virus titer present in nasal washes and tissue homogenates. 7×10^3 cells were seeded on 96 well tissue culture plates and allowed to grow overnight. When the cells reached 60-70 % confluent, serial tenfold dilutions of the sample were inoculated on cell culture plates after the plates were washed with sterile PBS. The inoculated cell culture plates were incubated for 5 days at 37°C. After 5 days, the infected cell culture plates were washed with PBS, fixed in 80% acetone and allowed to dry. The plates were stained for the determination of virus titer in nasal washes and tissue homogenates by indirect immunofluorescence assay. Virus titration was determined using Reed and Meunch formula to find the fifty percent endpoints [244].

3.2.7 Indirect Immunofluorescent assay

Determinations of virus titers in nasal washes and tissue homogenates were performed by indirect immunofluorescence assay (IFA). The fixed infected cell culture plates were moistened with PBS for 10 min at room temperature before staining. A 100-µl volume of rabbit polyclonal primary antibody (IgG) against bovine IDV was added at a 1:200 dilution to each of the wells. The plates were incubated at 37°C for 45 min. Following three washes with PBS, 50 µl of affinity-purified fluorescein-labeled goat anti-rabbit IgG secondary antibody (KPL, Gaithersburg, MD, USA) was added at a 1:1,000 dilution. After the secondary antibody was added, the plates were incubated at 37°C for 45 min.

3.2.8 Hemagglutination Inhibition Assay

The pre and post infection sera were treated with receptor-destroying enzyme (Denka Seiken, Chuo-ku, Tokyo, Japan) before doing HI assay. RDE treatment was done according to manufacturer's protocol and HI assay was done according to WHO manual [245]. HI assay was performed using 1% turkey RBCs (Lampire Biological Laboratories, Pipersville, PA, USA).

3.2.9 Virus genome sequencing and analysis

To determine the HEF sequence of viruses isolated from infected animals, we employed HRT- 18 G cells for the cultivation of the infected lung homogenates, which was followed by deep RNA sequencing. Briefly, cells were allowed to reach 60-70% confluency and then infected with bovine IDV at 0.1 MOI in 1 ml of DMEM. After 1 h infection, fresh virus infection medium, i.e. DMEM supplemented with 200 U/ml penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA), and 0.5 µg/ml TPCK

Trypsin (Sigma, Saint Louis, MO, USA), was added for further incubation at 37ºC for 5 days. The virus grown from the infected lung homogenates was sequenced using an Illumina MiSeq as described previously [4].

The nucleotide sequences of HEF obtained from the inoculum, and the lung homogenates of guinea pigs were analyzed against all the homologous sequences in the NCBI database using standard nucleotide BLAST[246]. The nucleotide sequences were translated to amino acid sequences using ExPASy tool [247]. Pairwise protein alignments were performed using multiple sequence alignment tool from Clustal omega [248]

3.2.10 Gross pathology, histology, and immunohistochemistry (IHC)

Following experimental bovine IDV infection of guinea pigs, euthanasia was performed at prescribed time points. A complete necropsy was performed to look for any macroscopic lesion in all the organs. Lung, trachea, and nasal turbinate samples were collected in 10% neutral buffered formalin and embedded in paraffin wax. Sections (5 μm thick) were then cut and stained with hematoxylin and eosin for histopathological examination.

Immunohistochemistry was performed on lung tissue sections stained with a primary rabbit polyclonal antibody developed against purified bovine IDV. Sections were deparaffinized, rehydrated, and immersed in 3% H_2O_2–distilled water for 30 min to block endogenous peroxidase. After washing with Tris-buffered saline (TBS) buffer was performed, the sections were treated with 5% goat serum (Life Technologies, Carlsbad, CA, USA)–TBS buffer for 1 h. The lung sections were then stained in a 1:1,500 dilution of polyclonal rabbit antibody against purified bovine IDV for 1 h at room temperature. After rinsing with TBS was performed, the sections were stained with 100 μl of horseradish

peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Dako EnVision+ System-HRP [DAB], Carpinteria, CA, USA) for 30 min, followed by counterstaining with hematoxylin. Lung sections stained with PBS and isotype antibody instead of specific primary antibody served as negative controls.

3.3 Results

3.3.1 Growth kinetics of bovine IDV in the upper and lower respiratory tracts of guinea pigs.

To determine the growth kinetics of bovine IDV, ten animals were intranasally inoculated with $3x10^5$ TCID$_{50}$/300 µl of bovine IDV and three animals were mock-infected with PBS. Clinical significance of bovine IDV in guinea pigs was assessed by regular monitoring of the change in body weight and temperature post infection over a period of 9 days, while viral replication in the lower and upper respiratory tract of the guinea pigs was evaluated by determining the amount of virus shed in nasal washes and tissue homogenates such as lungs, nasal turbinates over 9 days period. Overall, there were no obvious clinical signs of influenza infection (body weight and temperature) and the directly infected animals behaved similarly to mock-infected animals. For each animal, the change in body weight post infection was calculated by comparing the post-infection body weight to the average of body weight recorded two days prior to the infection. The differences were calculated as percentage body weight change for each animal plotted for each day. The directly inoculated animals did not show any significant change in body weight compared to the mock-infected animals. An increase in body weight in mock-infected animals compared to directly inoculated animals from 5dpi to 9 dpi was noted, but this change was insignificant (Figure.1A). Similarly, the difference of post-infection body temperature was

compared to the average pre-infection temperature for each animal and the average of the differences was plotted for each day (Figure. 1B). Despite the observation of an increased trend in the temperature of infected animals on days 0, 1, and 2 post-infection compared to mock-infected animals, the changes in temperature were not significantly different from the mock-infected animals. In the remaining days of the experiment, infected animals showed little or no change in the temperature. These results indicated that bovine IDV infection had no effect on the body weight and temperature changes during the 9 days period in guinea pigs.

Next, we determined whether virus replicated in the upper and lower respiratory tract of infected animals by focusing on the quantitative analysis of virus loads in nasal washes, nasal turbinates, and lungs by titrating the samples on MDCK cells. Nasal washes were collected from the directly inoculated and mock-infected animals at 48 h intervals starting from 1 dpi through 9 dpi. To determine the tissue tropism of the virus, two random animals were selected and euthanized on 1,3,5,7,9 dpi and lungs, and nasal turbinates were collected. Results of our experiments showed that IDV shedding in the nasal washes started at low levels and then reached the highest titer of 5.75 $\log_{10}\text{TCID}_{50}$/ml on 3dpi (Figure.1C). The virus shedding in nasal washes reduced to undetectable levels on 9 dpi. This clearly showed that bovine IDV can replicate in the upper respiratory tract. Virus growth from nasal turbinates ranging from 4.10- 6.0 $\log_{10}\text{TCID}_{50}$/g on 1, 3, 5, and 7 dpi, and further confirmed that bovine IDV could successfully replicate in the upper respiratory tract of guinea pigs (Figure. 1D). Robust bovine IDV replication was also demonstrated in the lung homogenates of guinea pigs. We noticed that there was a substantial increase in virus titers in lung homogenates of directly inoculated animals starting from 1 dpi through 7 dpi, with

a peak of 8 $\log_{10}TCID_{50}/g$ on 5 dpi. By day 9, the virus was cleared from the lungs (Figure.1E). The presence of virus in the lungs as well as in nasal washes and nasal turbinates until 7 dpi indicated that virus was actively replicating in the respiratory tract of the guinea pigs. Previous studies in pigs and ferrets have shown that IDV isolated from swine (swine IDV) was detected only in the upper respiratory tract, not in the lungs of infected animals.
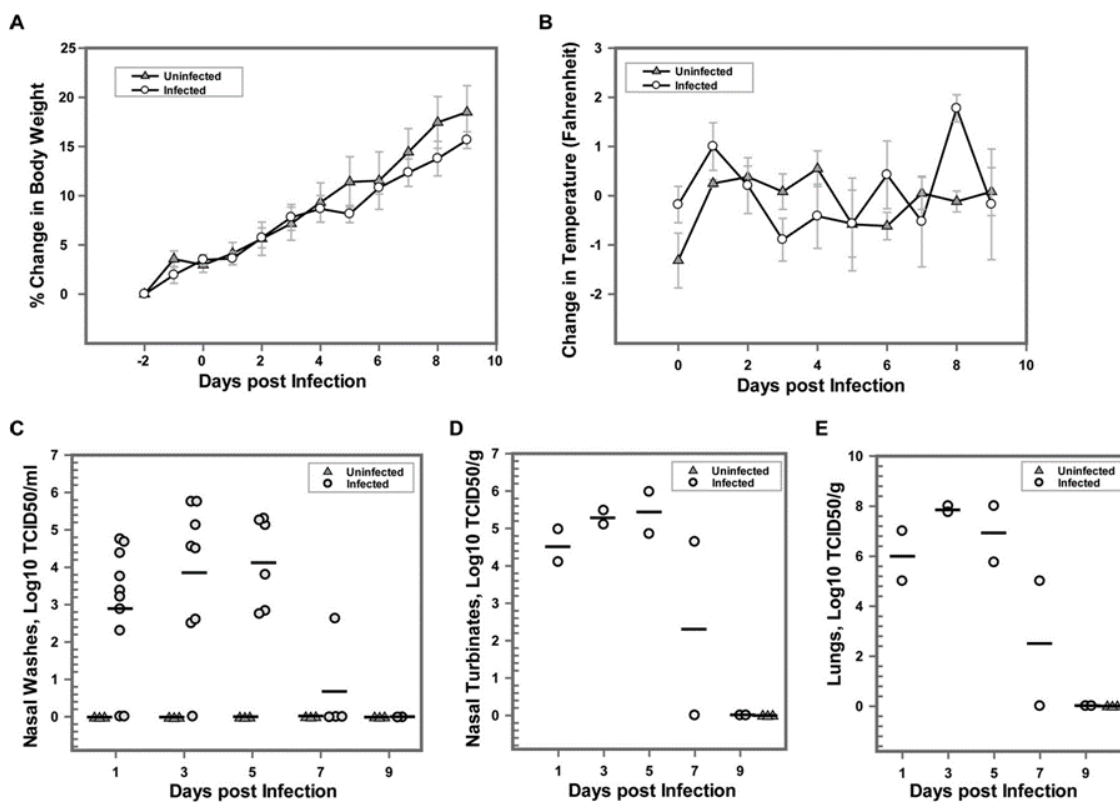
Figure 3.1. Body weight and temperature changes and virus titers in nasal washes, lungs, and nasal turbinates in guinea pigs after intranasal inoculation of bovine IDV in the directly inoculated group. A total of 10 guinea pigs were intranasally inoculated with 3×105 TCID50/300 μl of bovine IDV (shown as "Infected") and 3 guinea pigs were mock inoculated with PBS (shown as "Uninfected"). Body weight and temperature were measured daily from 2 days before infection until 14 dpi. (A and B) Percentage changes in

body weight (A) and changes in body temperature (B) expressed as means ± standard errors (SE). (C to E) Nasal washes were collected at 1, 3, 5, 7, and 9 dpi. Two inoculated animals were randomly euthanized on each of those days to assess virus load in lungs and nasal turbinates. All mock-inoculated animals were euthanized at 9 dpi. Virus titers in nasal washes are expressed as log10 TCID50 per milliliter (C); virus titers in nasal turbinates (D) and lung (E) are expressed as log10 TCID50 per gram. For panels C, D, and E, each shape represents an individual animal. Horizontal bars show the mean viral titers for each time point.

3.3.2 Seroconversion in directly inoculated guinea pigs

Pre- and post-infection serum samples were tested for the presence of virus-specific antibodies against bovine IDV by the hemagglutination inhibition (HI) assay. All the guinea pigs were seronegative for bovine IDV prior to infection as demonstrated by the absence of virus-specific antibodies. The antibody response to bovine IDV infection was measured on days 1, 3, 5, 7, and 9 dpi in the directly inoculated group. In the directly inoculated group, virus-specific antibodies were detected by HI at 7 and 9 dpi. One of the 2 infected animals seroconverted on 7 dpi (HI titer, 40), and 2 of the 2 infected animals seroconverted with titers of 20 and 80 on 9 dpi (Table 1). The presence of detectable levels of antibody showed that guinea pigs could be a good model to study the replication and kinetics of bovine IDV.

Table 3.1. Seroconversion of guinea pigs after bovine IDV infection

Groups	Control	Infected animals			Sentinels
	9 dpi	7 dpi	9 dpi	14 dpi	14 dpi
Direct inoculated	0/3	1/2 (40)	2/2 (20,80)		
Contact transmission				3/3 (80,40,80)	1/3 (40)
Aerosol transmission				3/3 (80,80,20)	0/3

Virus-specific antibody titers determined by HI are given in parentheses.

3.3.3 Transmission of bovine IDV in co-caged guinea pigs

To test whether bovine IDV can transmit through contact, 3 directly inoculated guinea pigs at 1 dpi were allowed to co-cage with three sentinel animals. The experimental setup for contact transmission facilitated not only direct but also indirect contact (droplets and aerosols). The potential transmission of bovine IDV in guinea pigs through contact was investigated by analysis of the changes in the body weight and temperature and also in the virus load in the nasal washes, nasal turbinates, and lungs from the infected and sentinel animals over a period of 14 days. Nasal washes were collected at 48-h intervals from 2 dpi to 14 dpi, and all of the animals were euthanized at 14 dpi. There were no significant differences in body weight and temperature between infected and sentinel animals (Figure. 2A and B). The infected and sentinel animals did not show any observable clinical signs during the 14-day experiment. All 3 infected animals shed the virus in the nasal washes and cleared the virus by 8 dpi (Figure. 3A). Two of three sentinel animals acquired infection from cage mates, and bovine IDV was detected in their nasal washes. One sentinel animal shed virus in the nasal washes at 4.6 log10 TCID50/ml starting on 6 dpi and cleared the virus by 10 dpi, while the other sentinel animal started viral shedding on 8 dpi and continued to shed virus at low levels until 14 dpi. Intriguingly, one of the

directly inoculated guinea pigs that shed virus once (2 dpi) in the nasal washes and then tested negative until 12 dpi shed virus at 14 dpi with a low titer of 2.25 log10 TCID50/ml. This animal could have acquired infection from the co-caged sentinel guinea pig by contact.

Further, to confirm the presence of transmission, guinea pig sera from the contact transmission group were tested for the presence of virus-specific HI antibodies. All three directly infected animals in the contact group seroconverted with HI titers of 40 to 80, and one sentinel animal seroconverted with an HI titer of 40 at 14 dpi, thus confirming the occurrence of short-range transmission by contact (Table 1). The absence of serum HI antibody titer in the other contact-infected sentinel animal can be explained by the late commencement of infection (8 dpi), indicating that the animal did not have enough time to develop antibodies by 14 dpi. At 14 dpi, the lungs and nasal turbinates of all 3 infected animals revealed no detectable levels of virus, indicating that the guinea pigs had cleared the virus (Figure. 3B and D). Among the sentinel animals, the animal which acquired late infection without seroconversion showed very high titers of 8 log10 TCID50/g in the lungs and 5.0 log10 TCID50/g in the nasal turbinates at 14 dpi (Figure. 3B and D). Taking the data together, the presence of virus in the nasal washes, lungs, and nasal turbinates and the antibody seroconversion demonstrated that bovine IDV can be transmitted from infected guinea pigs to naive guinea pigs by contact.
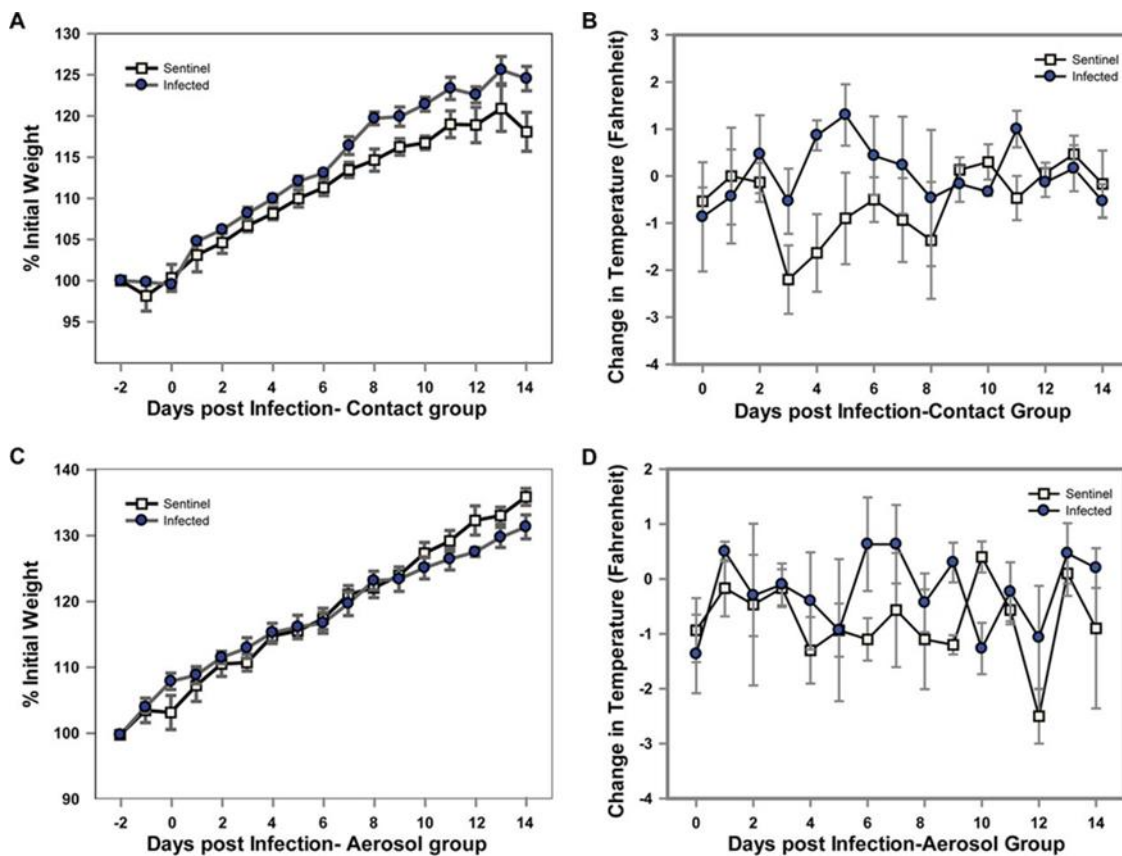
Figure 3.2 Changes in body weight and temperature of guinea pigs in the contact transmission group (A and B) and in the aerosol transmission group (C and D). Three guinea pigs were intranasally inoculated with 3×105 TCID50/300 μl of bovine IDV, and 3 uninfected guinea pigs were added to the cage as sentinels after 24 h. Body weights and temperatures were measured daily from 2 days prior to infection to 14 dpi. (A and B) Changes in percentages of body weights compared to pre-infection body weights (A) and changes in temperatures of infected animals compared to temperatures of sentinels (B) in contact transmission group. The data are expressed as means ± SE. (C and D) A similar experiment with the same parameters (body weight and temperature) measured was conducted for the aerosol transmission study.
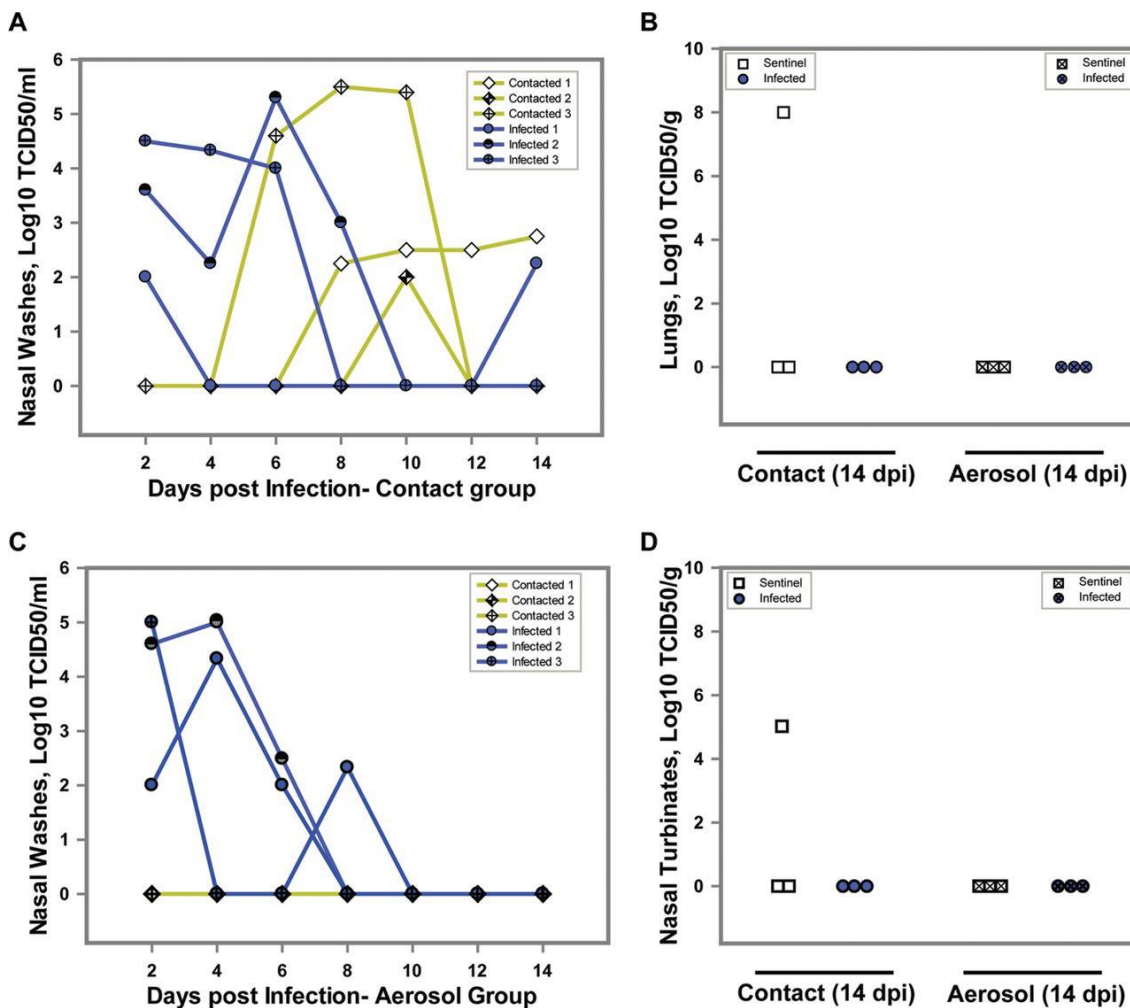
Figure 3.3 Virus titer in nasal washes, lungs, and nasal turbinates of guinea pigs exposed to bovine IDV by the aerosol or contact route. In both the contact transmission group and aerosol transmission group, 3 guinea pigs were intranasally inoculated with 3×10^5 TCID50/300 μl of bovine IDV. After 24 h of infection, 3 uninfected guinea pigs were added to each cage as sentinels. Nasal washes were collected at days 2, 4, 6, 8, 10, 12, and 14 dpi from all animals. All the guinea pigs were euthanized at 14 dpi. (A and C) Virus titers in nasal washes from the contact transmission group (A) and the aerosol transmission group (C) are expressed as log10 TCID50 per milliliter. (B and D) Virus titers in lungs (B) and nasal turbinates (D) of the infected and sentinel animals in the contact and aerosol

transmission groups at 14 dpi are expressed as log10 TCID50 per gram. For panels B and D, each shape represents an individual animal.

3.3.4 Aerosol transmission of bovine IDV

In order to test the transmissibility of bovine IDV via aerosol or droplets, three guinea pigs were inoculated intranasally with 3×105 TCID50/300 µl and three noncontact sentinel animals were placed in the same cages after 24 h. The infected and sentinel animals were separated with double-walled wire mesh, thus facilitating only airborne transmission and preventing all types of direct contact. The duration of the experiment and the collection of nasal washes were similar to the conditions used for the contact transmission experiment. Similarly, to the contact transmission group, the body weight and temperature changes between the infected and sentinel animals were not significant (Figure. 2C and D). In the aerosol transmission group, 3/3 directly infected animals showed detectable virus replication and shed the virus in their nasal washes until 6 dpi, with a peak of 5.0 log10 TCID50/ml on 2 and 4 dpi (Figure. 3C). None of the three sentinel animals shed any detectable level of virus in nasal washes or had virus present in lungs and nasal turbinates during the study. This suggested that bovine IDV cannot be transmitted by the aerosol route. At 14 dpi, none of three infected animals had detectable virus in the lungs or nasal turbinates, indicating that the virus had cleared by day 14 (Figure. 3B and D). In the HI assay, all three infected animals in the aerosol transmission group had seroconverted with HI titers of 20 to 80 at 14 dpi (Table 1). In marked contrast, neither virus replication nor virus-specific HI antibodies were found in three sentinel animals. Our data suggested that bovine IDV was incapable of transmitting infection in guinea pigs by the aerosol route

under the experimental conditions used in this study. A future study is needed to draw a conclusion regarding whether it can be transmitted by the aerosol route in guinea pigs.

3.3.5 Pathology of bovine IDV in the respiratory tract of guinea pigs.

Neither experimentally infected guinea pigs nor contact-infected guinea pigs showed any clinical signs of disease after bovine IDV infection. However, mild to moderate macroscopic changes were observed in the lungs of the directly inoculated animals and contact-infected animals during necropsy. No other lesions were observed in any other organs. On necropsy, primary gross lung lesions showed areas of congestion and hemorrhage on 1 dpi that progressed to areas of pulmonary consolidation at 3, 5, and 7 dpi. Lungs from an uninfected animal did not show any macroscopic lesions; however, lungs from the sentinel animal infected by contact showed similar areas of pulmonary consolidation comparable to the macroscopic lung lesions of directly inoculated animals on 5 and 7 dpi (Figure. 4A1, B1, and C1). Histopathological examination of lungs showed minimal to mild inflammation in the trachea with infiltration of lymphocytes and plasma cells, mild hyperplasia of tracheobronchial lymph nodes (not shown), mild to moderate atelectasis in lungs, vascular congestion, bronchitis with desquamation of bronchial epithelium with exudate, and peribronchial and perivascular cuffing by lymphocytes and plasma cells in all of the directly inoculated animals at 3, 5, and 7 dpi. In the directly inoculated animals, alveolar septa were thickened by neutrophils and RBCs, and bronchopneumonia with an accumulation of luminal exudate was also observed (Figure.4A1 to A3). The sentinel animal that acquired the infection from a co-caged infected animal by contact developed similar inflammatory changes with thickened alveolar septa, bronchitis, peribronchial and perivascular lymphocyte and plasma cell

infiltration, and mild neutrophilic bronchopneumonia at 14 dpi (Figure. 4B1 to B3). One of the three uninfected control animals showed the presence of foreign material possibly derived from the bedding material and demonstrated mild sloughing of pulmonary epithelium and suppurative inflammation within the examined lung.
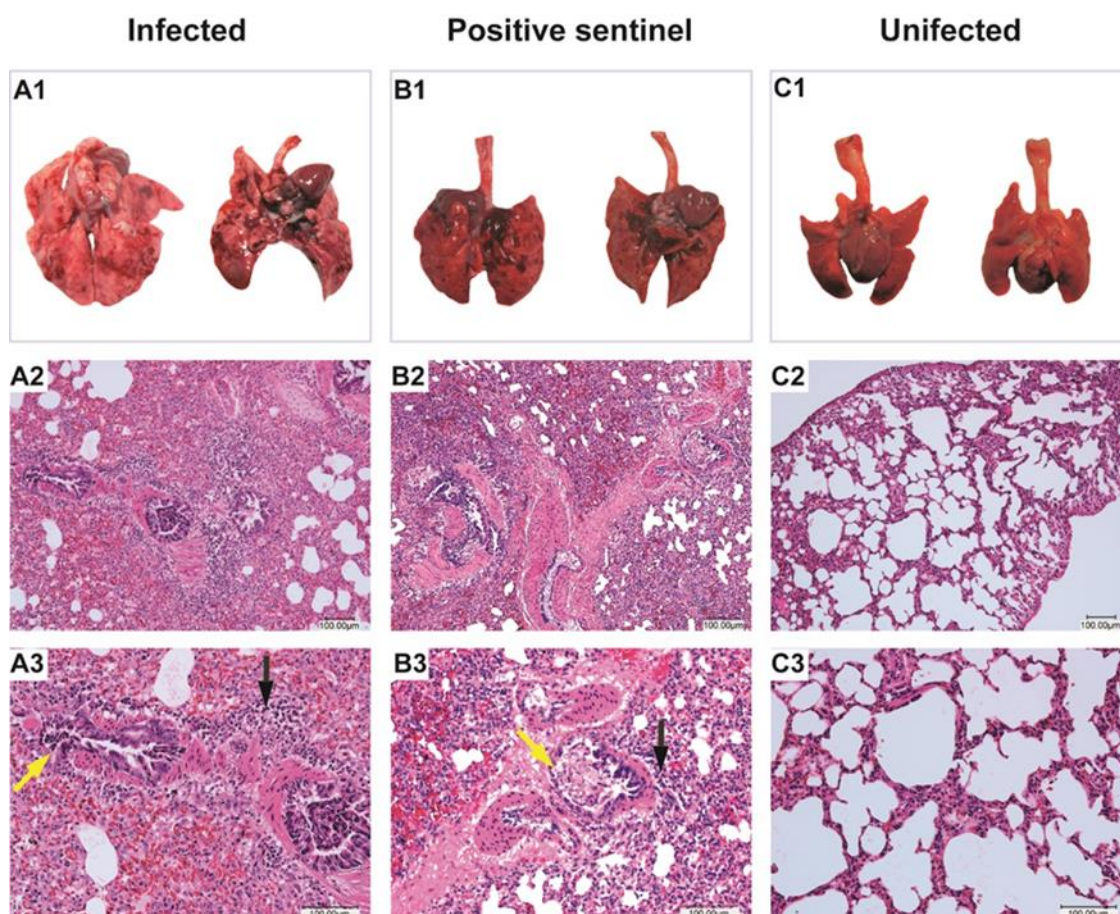
Figure 3.4 Pathological changes of the lungs in infected, positive-testing sentinel guinea pigs and uninfected guinea pigs after bovine IDV infection. Macroscopic lung lesions as indicated as follows: A1, a directly inoculated animal at 7 dpi; B1, a positive sentinel infected by contact at 14 dpi; C1, an uninfected animal at 9 dpi. Panels A1 and B1 showed multifocal areas of pulmonary consolidation in directly inoculated and contact-infected sentinel animals. Microscopic lung lesions are indicated as follows: detailed histology

results are shown at magnifications of ×100 for the second row (A2, B2, and C2) and ×200 for the third row (A3, B3, and C3). Hematoxylin and eosin (H&E) staining of lung sections from a directly inoculated animal at 7 dpi (A2 and A3) and from a positive-testing sentinel infected by contact at 14 dpi (B2 and B3) showed multifocal areas of alveolar inflammation with infiltration of lymphocytes, plasma cells (black arrows), and RBCs in the lung parenchyma. Bronchiolar inflammation with desquamation of the epithelial cells (yellow arrows) and peribronchial infiltration of lymphocytes were also seen. (C2 and C3) Uninfected lung sections showed clear alveolar spaces without any inflammatory cell infiltration. Bars, 100 μm.

Immunohistochemistry of the lung sections demonstrated the presence of bovine IDV-specific antigen in the lungs of directly inoculated and infected sentinel animals (Figure. 5 and 6). The presence of the viral antigen in lungs correlated with the severity of lung macroscopic pathology. Bovine IDV antigens were present in larger amounts within alveolar septum and within bronchi and the bronchiolar epithelium in the lungs of directly inoculated animals at 3, 5, and 7 dpi compared to 1 dpi (Figure. 5). At 14 dpi, the amount of viral antigen in the lungs of a sentinel animal which acquired the infection by contact was comparable to the amount of viral antigen present in those of the directly inoculated animals (Figure. 6A1 to A3), which further confirmed that bovine IDV transmission had occurred by contact. No viral antigen was detected from the lungs of the uninfected control animals (Figure. 6B1 to B3). Further, an isotype antibody from a directly inoculated bovine

IDV-positive lung showed no staining, confirming the specificity of the primary antibody used for IHC staining (Figure. 6C1 to C3).
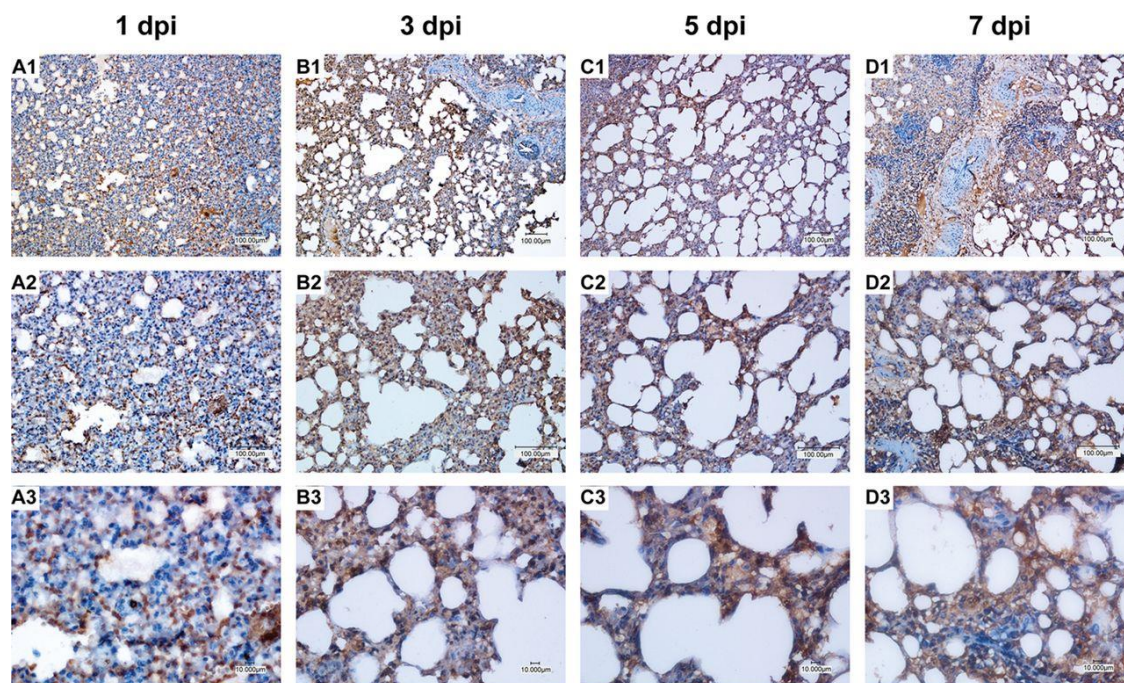
Figure 3.5 Bovine IDV antigen in the lungs of directly inoculated animals. Brown staining indicates cells positive for bovine IDV antigen in the infected lung tissue. Magnifications are ×100 for the first row (panels A1, B1, C1, and D1), ×200 for the second row (panels A2, B2, C2, and D2), and ×400 for the third row (panels A3, B3, C3, and D3). Viral antigens were present in the alveolar and bronchial epithelial cells. Viral antigens present in the infected lung tissue at 1 dpi (A1, A2, and A3), 3 dpi (B1, B2, and B3), 5 dpi (C1, C2, and C3), and 7 dpi (D1, D2, and D3) are shown. Bars, 100 μm and 10 μm.
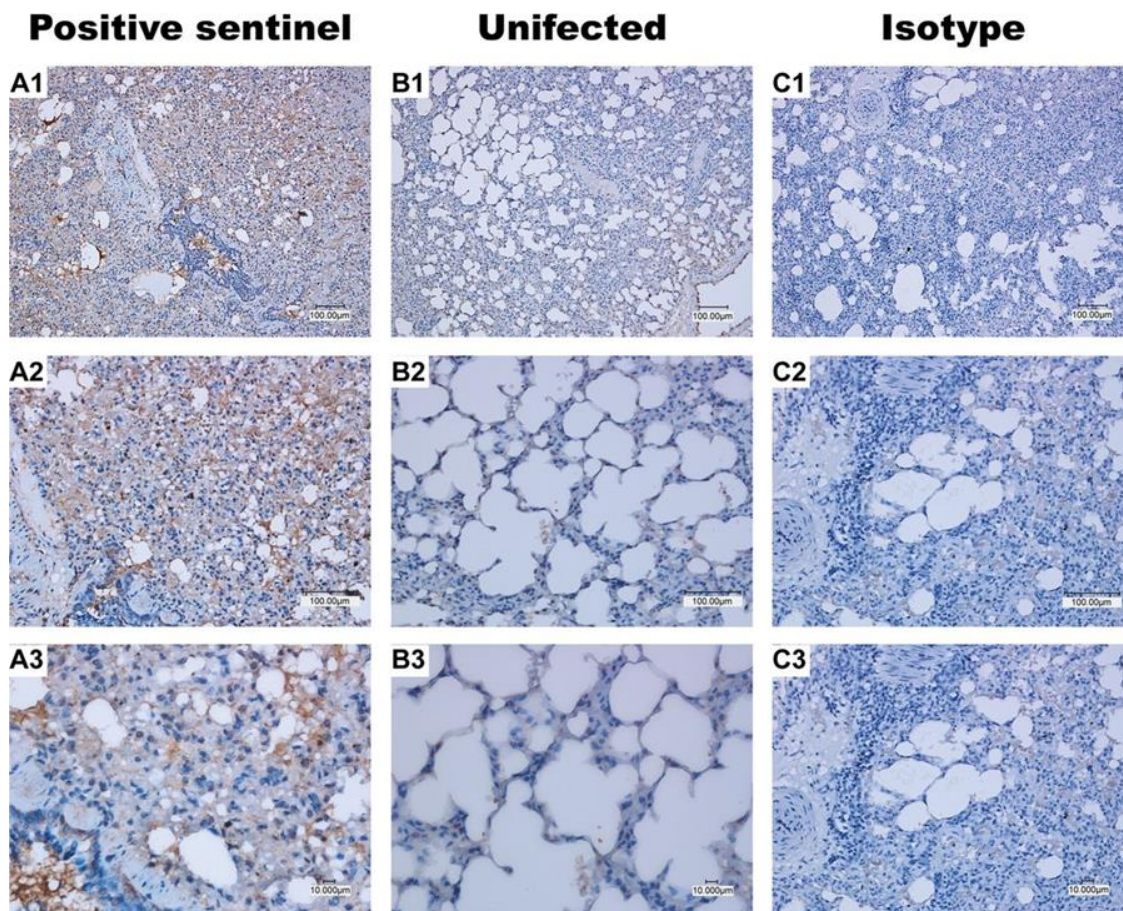
Figure 3.6 Bovine IDV antigen in the lung tissue of the sentinel animal infected through contact. Panels A1, A2, and A3 represent lungs from the sentinel animal infected by contact. Brown staining shows cells positive for bovine IDV antigen. (B1 to B3) Uninfected animals did not show any brown stained infected cells for bovine IDV. (C1 to C3) Isotype antibody staining did not show any brown staining, confirming the specificity of the primary antibody used for immunohistochemistry. Magnifications are ×100 for the first row (panels A1, B1, and C1), ×200 for the second row (panels A2, B2, and C2), and ×400 for the third row (panels A3, B3, and C3). Bars, 100 μm and 10 μm.

3.3.6 Full-length-genome analysis of viruses isolated from the lungs of directly inoculated and co-caged sentinel guinea pigs

To determine whether bovine IDV had evolved some interesting mutations that promoted viral adaption to guinea pigs, we determined the full-length genome of viruses isolated from the lungs of directly inoculated and co-caged sentinel animals. The sequence analysis was conducted to examine and determine the similarities and differences of the full genome of the parent inoculum and the full genomes of the viruses propagated from lung homogenates of directly inoculated or sentinel animals. For deep RNA sequencing, viral RNA was extracted from the parent inoculum and also from the viruses recovered from lung homogenates of directly inoculated and co-caged sentinel guinea pigs. HRT-18G cells were used for the virus propagation.

Full-genome-sequence analysis showed that both of the recovered virus isolates from guinea pig lungs exhibited mutations in HEF, P42, and NP proteins compared to the parent inoculum. HEF protein sequence analysis of the parent inoculum and of virus from the lung of sentinel animal which acquired the infection by contact revealed 1 mutation, A746G, with an amino acid change of N249S. Compared to the inoculum, HEF protein sequence analysis of the bovine IDV that originated from the directly inoculated guinea pig revealed one mutation, C755T, with an amino acid change of A252V. While protein PB1 mutated only in a contact-infected sentinel-derived virus with D232N, PB2 mutated only in a directly inoculated animal with E44D and N668K mutations (Table 2). In contrast to proteins HEF, PB1, and PB2 lacking uniform mutations, the other proteins exhibited mutations common to the two virus samples (derived from directly infected and sentinel animals). These mutations consisted of E41R and L316F in P42 and E247D in NP. No

mutations were observed in NS protein (Table 2). Interestingly, we found that most of the observed amino acid changes had occurred at frequencies of approximately 93% to 99% in our RNA-seq reads. There were two changes (HEF A252V and PB1 D232N) occurring at around 50% frequency, indicating strong polymorphism at these two positions.

Table 3.2. Viral genome and protein sequence changes in directly inoculated and sentinel contact-infected animal lung isolates compared to parent inoculum of IDV.

Virus source	HEF		P42		NP		P3		PB1		PB2	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	Aa	nt	aa
Direct inoculated animal	C755T	A252V	G121A	E41R	G741C	E247D	A297G		T18G		G146C	E44D
			A122G		T760C		A804G		T21C		C2018G	N668K
			C960T	L316F	T768C							
					A792G							
Sentinel infected by contact	A764G	N249S	G121A	E41R	G741C	E247D	A297G		T18G			
			A122G		T760C		A804G		T21C			
			C960T	L316F	T768C				G719A	D232N		
					A792G							

3.4 Discussion

Bovine and swine FLUD viruses are prevalent in cattle and swine populations as demonstrated by the serological studies conducted earlier [4, 10, 243]. Since this is a novel virus, different from the existing influenza genera, a laboratory animal model would help to understand the viral and host factors responsible for the virulence, pathogenesis, and transmissibility of this virus. In this study, we evaluated the virulence of the unadapted bovine IDV in guinea pigs by testing its ability to replicate and transmit by means of aerosol spread and contact. Generally, Influenza viruses need to be adapted to the host species

before they can replicate to high titer and induce disease in a model host [205]. Adaptation of the virus *in vivo* results in mutations that can facilitate better receptor binding and promote viral fitness, thereby increasing replication efficiency and virulence of the virus. These adapted viruses could be different from the parent strains. Previous studies in guinea pig and ferret models have shown exceptions and demonstrated high titers of influenza A virus in the upper respiratory tract and high transmissibility to co-caged animals without any adaptation [5, 7].

Selection of an infectious dose $3x10^5$ $TCID_{50}/300$ µl for guinea pig infection experiment was based on the earlier study of swine IDV in pigs and ferrets [4]. Guinea pigs after intra-nasal inoculation of bovine IDV showed no clinical signs and behaved normally. However, detection of virus replication in the nasal washes from 1-7 dpi indicated that guinea pigs were susceptible to bovine IDV infection. Intra-nasal inoculation of 10^6 $TCID_{50}/ml$ swine IDV infection in pigs and ferrets also demonstrated no clinical symptoms and shed virus in nasal washes from 3 dpi [4]. Following intranasal inoculation of bovine IDV, all the infected guinea pigs seroconverted with HI antibody titers ranging from 20-80 within a period of 14 days. Taken together, these findings are similar to the previously reported studies of influenza A viruses in guinea pigs, indicating guinea pigs can act as a natural susceptible animal model for studying the antigenicity and pathogenicity of bovine IDV.

Earlier studies of influenza A viruses in guinea pig models also showed that virus replication largely occurred in the upper respiratory tract in high viral titers and showed no or low detectable levels of virus in lungs [5, 7, 249]. Surprisingly, bovine IDV replicated to high titers of 7.75 and 8 $log_{10}TCID_{50}/g$ virus in the lungs of directly inoculated guinea

pigs at 3 and 5 dpi respectively and hence showed that guinea pig can support IDV replication in the lower respiratory tract in a very efficient manner. The sentinel animal that acquired the infection from the infected cage mate by contact transmission also showed 8 $\log_{10}TCID_{50}$/g virus in the lungs. Similar pathogenicity study of bovine IDV using an isolate designated D/Zoetis in gnotobiotic calves also showed 7 $\log_{10}TCID_{50}$/ml virus in the lungs with no observable clinical signs [250]. Another study to evaluate the pathogenicity and virulence of bovine IDV in eight-week-old pigs, demonstrated viral replication in both upper and lower respiratory tract including lung, the presence of seroconversion in all inoculated pigs and also transmitted the infection by contact [250]. Our findings in guinea pigs are in a good agreement with these findings obtained in pigs and gnotobiotic calves with bovine IDV, further strengthening its position as an animal model to study this newly emerging influenza virus.

Intriguingly, bovine IDV, as shown here and reported elsewhere, replicated in both lower and upper respiratory tract of animals (guinea pigs or pigs or calves), while swine IDV replicated only in the upper respiratory tract, not in lower respiratory tract and lung, of ferrets and pigs. These contrasting results indicate that bovine and swine viruses appear to differ in lung tropism of animals. This observation is also consistent with our clinical data that bovine IDV is often isolated from lungs of diseased calves, while swine virus is only isolated from the upper respiratory tract of diseased pigs (data not shown). We reasoned the difference in lung tropism between swine D/OK and two bovine viruses (D/660 and D/Zoetis) might be a result of differences in the receptor recognition of the HEF protein. Further experimental verification is required to test this prediction.

The distribution of the receptors in the mammalian host tissue is an important factor determining the host susceptibility and tissue tropism of Influenza virus. Earlier studies in guinea pigs to determine the distribution of α2,6 and α2,3 sialylated glycoproteins, which are the main receptors of Influenza A viruses have been reported. While α2,6 and α2,3 sialylated glycoproteins were both present in nasal epithelia and trachea, only α2,3 sialylated glycoproteins were present in the lung [251, 252]. Similar to human influenza C virus, swine and bovine IDV utilizes 9-O-acetyl-N-acetylneuraminic acid (Neu5,9Ac2) as the cellular receptor to trigger an infection and encodes sialate-O-acetylesterase to cleave the 9-O-acetyl group to release the virions [4]. However, it is not clear whether IDV can bind to Neu5,9Ac2 with both linkages as a similar efficiency. It can be argued that bovine viruses such as D/660 and D/Zoetis bind to Neu5,9Ac2 receptor well regardless of the specific linkage, while swine D/OK only preferably binds to Neu5,9Ac2 with α2,6 linkage, not that with α2,3 linkage present in the lungs of various animals. This hypothesis should be tested in future investigation.

The transmissibility of influenza viruses was studied using various animal models such as mice, ferrets, guinea pigs, pigs and non-human primates [205]. The presence of bovine IDV in the bovine population of U.S, France, and China could be a potential threat to public health. To determine the transmissibility of bovine IDV, we tested two different models: contact and aerosol/respiratory droplet. A high level (2/3 animals) of bovine IDV transmission occurred from infected guinea pigs to sentinel animals by contact as demonstrated by the seroconversion (1/3 animals) and high titer of virus in the lungs in sentinels (1/3 animals), while the aerosol route failed to cause infection. The airborne transmission experiments largely depend on environmental factors such as temperature,

humidity, airflow of the experiment room and infectious dose used. Among these factors, the rate of change and direction of air currents in the facility is an important variable, especially in aerosol transmission experiments. Previous experiments using influenza A and B type viruses, in guinea pigs conducted in environmental test chambers demonstrated efficient transmission lasting for a longer period at 5°C, 20% relative humidity (RH) than at 20°C, 20% RH and also stated that high temperatures and high humidity significantly reduced the transmission efficiency [209]. Large droplets of size >5-10 um facilitates only short-range transmission, while small droplets <5 um are responsible for long-range transmission [210]. The temperature and humidity levels of our facility varied from 22-24°C and 21-30% respectively during the study. Further, we kept the cage away from the direction of airflow of the room and did not use any controlled air chambers for aerosol transmission study. The absence of infection in the sentinels in the aerosol transmission group can be attributed to the above factors. Similar aerosol transmission study done in guinea pigs using a pandemic H1N1 2009 influenza A virus demonstrated the incidence of virus shedding between days 2 and 4 dpi and also reported no transmission upon reversal of the cage positions relative to airflow, showing that efficiency of infection increased when the orientation of infected to uninfected cages was in the direction of air flow [7]. The absence of persistent infection and productive replication in the sentinel animals in the aerosol group can thus be justified under the given experimental conditions. Low aerosol transmission ability of IDV, in general, has puzzled us when we initially studied IDV in pigs. RT-PCR screening approximately 3000 nasal swabs of pigs with influenza-like symptoms only resulted in 4 positive samples (data not shown). The low aerosol transmission issue has been observed also in our ferret study where IDV was not detected

in the aerosol group; despite 1/3 sentinel ferrets exhibited seroconversion [4]. It should be noted that the inefficient transmission observed in pigs, ferrets, and guinea pigs is in a marked contrast with that observed in cattle in that approximately 15% nasal swabs of cattle manifested influenza-like symptoms were tested positive for IDV [2]. So, our current hypothesis is that IDV is unique among influenza viruses in that it transmits through aerosol efficiently only in its primary host cattle, not in other tested animal species including guinea pigs. This working model will be tested in our future experiment.

Transmission experiments of influenza A and B types in various animal models also showed higher efficiency in contact route compared to aerosol [6]. Swine IDV was also reported to transmit by contact in pigs and ferrets [4]. In our study, two sentinel animals acquired the infection by contact as demonstrated by the serum hemagglutination inhibition (HAI) titer of one of the sentinel animal (HAI titer: 80) and high titer of virus present in the lung and nasal washes in the other. Interestingly, the sentinel guinea pig infected by contact acquired a strong infection enough to spread to the co-caged seropositive direct inoculated animal causing to start a second round of virus shedding in nasal washes on 14 dpi (nasal wash titer $2.25\log_{10}TCID_{50}$/ml on 14 dpi), however there was no detectable amount of virus in the lungs at 14 dpi. A serum HAI titer of 40 or above is considered to be the gold standard for protection by reducing the pathogenesis of influenza viruses in humans. Our data showed that a serum HAI titer of 80 in that co-caged seropositive animal definitely has alleviated the pathogenesis caused by virus reactivation by reducing the amount of virus shedding in nasal washes ($2.25 \log_{10}TCID_{50}$/ml) and also prevented the virus replication in lungs at 14 dpi. Earlier studies in guinea pigs and ferrets using potent monoclonal IgG antibody administered intramuscularly against

A/California/04/2009 (H1N1) virus hemagglutinin failed to protect the animals from the airborne transmission. However, the same monoclonal IgG antibody when administered intranasally and also as recombinant IgA administered intramuscularly in a dose-dependent manner significantly reduced the virus shedding in nasal washes and prevented virus replication in lungs [249]. This explains the incidence of the low level of virus shedding in the seropositive animal and also underlines the role of mucosal immune responses over systemic immune responses in the pathogenesis and prevention of the influenza virus infection.

Since we described this new influenza virus in cattle, designated influenza D virus (IDV) in 2013, there have been published reports on the detection of this virus in diseased calves in China and France. Also, a recent study observed that IDV was frequently detected in calves with acute respiratory disease and was not identified in clinically healthy animals. In addition, recognized viral etiological pathogens commonly associated with bovine respiratory disease, bovine viral diarrhea virus, bovine coronavirus, bovine herpesvirus 1 and bovine respiratory syncytial virus, were not detected. These findings suggest an important role of IDV in the bovine respiratory disease (BRD) complex and challenge our understanding of BRD. Despite the progress, it is not yet clear whether IDV can spread from bovines to humans. The zoonotic potential of bovine IDV has not yet been reported and is an area worth study. A serum survey conducted in humans earlier to swine IDV showed seroprevalence only in 1.3% of the human samples tested [4]. Considering the pathogenicity of the bovine IDV and host tropism in ferrets, guinea pigs, pigs and calves, the zoonotic potential of these viruses cannot be dismissed. Further serological studies have to be done to study the prevalence of these viruses in personnel and occupational workers,

closely associated with animals and animal facilities such as farmers, veterinarians, animal technicians, laboratory staff, and animal keepers.

Overall, guinea pigs can act as a good feasible model for studying the pathogenesis of influenza D viruses for the following reasons: 1) virus was able to replicate in the nasal turbinates and virus shed through nasal washes; 2) unlike other influenza types, bovine IDV replicated to a high titer in lungs and therefore can productively replicate in the lower respiratory tract; 3) bovine IDV transmitted from the infected guinea pigs to naïve animals by contact; 4) No adaptation required *in vivo* for pathogenicity experiments; and 5) All the direct inoculated and contact infected guinea pigs seroconverted. The data we presented here show that guinea pigs are naturally susceptible to IDV and therefore can be an excellent animal model to study the host-virus interactions and would help in devising future strategies for the in-depth study of pathogenicity, antigenicity, and immunogenicity of this novel influenza virus.

Chapter 4. Comparative study of virulence, pathogenesis and tropism of swine and bovine influenza D viruses to human influenza C virus in guinea pig model

Abstract

Influenza D virus (IDV) is an emerging pathogen with cattle as its primary host species. Influenza D virus isolated from the cattle and swine populations from North America and Eurasia shares 50% homology to the human influenza C virus. Previous research by our group has successfully established guinea pig as a suitable animal model to study the virulence and transmission of IDV and also demonstrated productive replication of this virus in upper and lower respiratory tract. The goal of this study was to investigate the replication kinetics and virulence of bovine and swine influenza D isolates (96-98% homology), in comparison to human influenza C in guinea pigs. Despite the similarity, both bovine and swine IDVs differ antigenically and genetically and belong to two different lineages. Guinea pigs upon intranasal inoculation of D/bovine/660/Oklahoma/2013 (bovine IDV), D/swine/1334/Oklahoma/2011 (swine IDV) and C/Victoria/2/2012 (human ICV) did not exhibit any clinical signs. However, all the infected animals seroconverted at 7 days post-infection (dpi). Guinea pigs infected with ICV did not shed the virus in nasal washes at 1 dpi and only 2/8 shed virus at 3 dpi. In contrast, in bovine IDV infected group, 9/10 animals shed the virus in nasal washes at 1 dpi, while the swine IDV group (8/8) began to shed the virus only at 3 dpi. Hence, the disparity in the virus-shedding pattern of swine IDV could be an adaptation lag due to the subtle difference in receptor binding specificity and virus tropism. Deep RNA sequencing of viral genomes in the nasal washes, receptor binding preference, and structural modeling of receptor binding domain of hemagglutinin-esterase fusion protein are currently

underway to identify the key factors and mechanisms involved in the differential replication kinetics, viral tropism, pathogenesis of the bovine and swine influenza D viruses.

4.1 Introduction

Influenza viruses are negative-sense, single-stranded RNA viruses classified in the Orthomyxoviridae family. There are four recognized genera of influenza viruses, designated influenza A (IAV), influenza B (IBV), influenza C (ICV) and influenza D (IDV) (https://www.cdc.gov/flu/about/viruses/types.htm). IAV and IBV have 8 negative-sense, single-stranded RNA segments, whereas ICV and IDV have only 7 segments. IAV has several subtypes depending on the HA and NA proteins and causes severe epidemics and pandemics affecting humans. So far there is 18 HA and 11 NA types, of which H1 to H16 and N1 to N9 have been isolated from birds. H17, H18, N10, N11 have been identified in the bats. IAV exists in multiple mammalian species, whereas IBV and ICV primarily infect humans. IBV has no subtypes but possesses two lineages causing localized epidemics and affecting mainly humans and, to some extent, seals [49]. The IBV genome was also recently detected in domestic pigs, indicating that the virus may infect this agricultural animal [29]. Compared to the IAV and IBV, ICV infections cause mild disease and were found to have coexisted with IAV and IBV infections in humans [50]. A striking feature of IDV is that it has multiple mammalian hosts similar to IAV, although we consider bovines as the natural reservoir [51]

Influenza D virus which is recently emerged and initially isolated from the USA has been found to have a transboundary existence and has been reported from North America, Eurasia and Africa [3, 12, 15, 16, 61]. Influenza D virus originally isolated from

the swine and bovines share 50 % amino acid identity to influenza C virus, which is primarily a human pathogen [2, 4]. Influenza C has been isolated from natural infections of the pigs and dogs in the past and also have been used for experimental infections [31, 253, 254]. Previous studies have shown that several human ICV strains were antigenically closely related to the swine ICV strains, which implicates the interspecies transmission of ICV, but the directionality of the transmission is inconclusive [30, 255]. Despite, similar genetic organizational makeup, influenza D virus cannot undergo gene reassortment with ICV and hence genetically and antigenically distinct from ICV which led to the classification of a new genus in the Orthomyxoviridae family [2, 60]. Genetic analyses have shown that there were only two lineages of IDV represented by D/swine/Oklahoma/1334/2011 (D/OK) and D/bovine/Oklahoma/660/2013 (D660), but recently a third lineage (D/bovine/Ibaraki/7768/2016) was reported from Japan [3, 47, 60].

The virus ecology of influenza D has been expanding since it was discovered in 2011-2013. Serological evidence of IDV has been found in small ruminants (goats and sheep), buffaloes, equines, and camelids [11-13]. Influenza D antibodies have also been detected from the occupational workers which implicate the public health importance of this newly emerged virus [14]. Even though the majority of IDVs isolated from North America belong to D660 lineage, it is interesting to note that recent isolates of IDVs of Eurasian origin are mostly of D/OK lineage [3, 13, 15, 16]. As stated earlier, humans are the primary host for ICV, but ICV has also been isolated from pigs and the directionality of transmission still needs to be addressed. Like ICV, IDV also uses the HEF protein, for the virus entry and exit, and these proteins share a conserved enzymatic site and divergent receptor binding sites. IDV exhibits broad host tropism, and IDV HEF has exceptional acid

and thermal stability compared to ICV [17]. Based on these phenotypic characteristics, we wanted to identify the molecular factors/ determinants responsible for the broad host tropism of IDV compared to ICV. The second thing is, HEF of the IDVs belonging to two lineages may have genetic and antigenic differences. Here we wanted to investigate the differences in the virulence, and pathogenicity of these two lineages in the guinea pig model, which can support IDV as evidenced from our past study [256]. The results of our study demonstrated that swine IDV (sIDV) has a differential replication kinetics in the tissues and nasal washes of the guinea pigs, compared to bovine IDV (bIDV), but both IDVs replicated in upper and lower respiratory tract. On the contrary, ICVs replicated in the upper respiratory tract and not in the lower respiratory tract.

4.2 Materials and methods

4.2.1 Cells, viruses, and Animals

Specific-pathogen-free (SPF), viral-antibody-free (SPF/VAF), 30-day-old guinea pigs of the Dunkin-Hartley strain (Elm Hill Labs, MA, USA) weighing 300 to 350 g were used for the study. The animals were ear-tagged for identification purposes. The duration of the experiment was 3 weeks, which included a 1-week acclimatization period. Animals were provided with food and water ad libitum and kept on a 12-h light/dark cycle. The temperature and relative humidity (RH) of the animal housing ranged from 72 to 75°F and 25% to 33%, respectively. Control animals were housed in a separate room away from the room housing the infected animals and were processed before the inoculated animals. Strict precautionary measures were followed to prevent cross-contamination between animals in different cages Gloves were changed between cages during cleaning and handling, and masks and surfaces were disinfected to prevent any possible cross-contamination.

4.2.2 Experimental design

Guinea pigs were divided into experimental groups for testing growth kinetics of the virus. Three virus-infected groups included 1) influenza D/bovine/Oklahoma/660/2013 2) influenza D/swine/Oklahoma/1334/2011 3) C/Victoria/2/2012 The group for studying virus kinetics consisted of 10 infected animals and 5 mock-infected animals. The animals were infected intranasally with 3×10^5 50% tissue culture infective doses Half (150 µl) of the virus inoculum was delivered in each nostril. The 5 control animals were mock infected with equal volumes of phosphate-buffered saline (PBS). The body weights of all the animals were recorded before the challenge. Guinea pigs were briefly anesthetized using isoflurane prior to infection. The animal experiments were approved by the Institutional Animal Care and Use Committee of South Dakota State University (IACUC no. 15-017A) and were conducted under biosafety level 2 conditions.

4.2.3 Monitoring and sample collection.

Body weight and temperature were monitored daily starting from 2 days before challenge. Prior to challenge, blood was collected from all of the animals from the jugular vein/cranial vena cava under conditions of isoflurane anesthesia. Animals were monitored on a daily basis after the virus challenge for clinical signs, and body temperature and body weight were recorded. Nasal washes were collected from all the infected animals in the directly inoculated group and from three control animals at 1, 3, 5, and 7, days post-infection (dpi). Additionally, two infected animals were euthanized using CO_2 at 1, 3, 5, and 7 dpi. Blood, nasal wash, nasal turbinate, soft palate, trachea, and lung samples were collected. The numbers of infected animals assessed each day from group I were as follows: day 1, n = 10; days 2 and 3, n = 8; days 4 and 5, n = 6; days 6 and 7, n = 4; days 8 -14, n =

2.Two animals from each group was kept for antibody development .These animals were euthanized at 14 dpi, and blood, nasal wash, nasal turbinate, soft palate, trachea, and lung samples were collected.

4.2.4 Collection of nasal washes

The nasal washes were collected by instilling 1 ml of PBS using a sterile 25-to-28-gauge cannula into the nostrils and collecting the washes by draining to sterile containers or Petri dishes. Animals were anesthetized using isoflurane, and alternate nostrils were used for sample collection on alternate days. Nasal washes collected in the Petri dishes were transferred to 1.5-ml tubes and then centrifuged at $500 \times$ g for 6 min at 4°C to remove any debris. The supernatants were stored at −80°C until analysis.

4.2.5 Estimation of virus load in nasal washes and tissues

Nasal turbinates, soft palate, trachea, and Lung, from guinea pigs were collected and stored at −80°C. One gram of tissue was homogenized using DMEM supplemented with penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) (200 U/ml) and a Stomacher circulator at high speed for 2 min. The homogenized tissue fluid was clarified by spinning at $500 \times$ g for 8 min at 4°C and stored at −80°C. For trachea and nasal turbinate analyses, DMEM with penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) (200 U/ml) was added and homogenized tissue fluid was collected and stored at -80°C until titration.

For virus isolation, MDCK cells were used for determining the virus titer present in nasal washes and tissue homogenates. 7×10^3 cells were seeded on 96 well tissue culture plates and allowed to grow overnight. When the cells reached 60-70 % confluent, serial tenfold dilutions of the sample were inoculated on cell culture plates after the plates were

washed with sterile PBS. The inoculated cell culture plates were incubated for 5 days at 37ºC. After 5 days, hemagglutination was carried out on the infected cell culture plates using 1% turkey RBCs, for the determination of virus titer in nasal washes and tissues. Virus titration was determined using Reed and Muench formula to find the fifty percent endpoints [244]

4.2.6 Hemagglutination Inhibition Assay

The pre and post infection sera were treated with receptor-destroying enzyme (Denka Seiken, Chuo-ku, Tokyo, Japan) before doing HI assay. RDE treatment was done according to manufacturer's protocol and HI assay was done according to WHO manual [245]. HI, assay was performed using 1% turkey RBCs (Lampire Biological Laboratories, Pipersville, PA, USA).

4.2.7 Virus genome sequencing and analysis

The infected lung homogenates of selected animals from each time point were sequenced using an Illumina MiSeq as described previously [4]. The nucleotide sequences of the seven viral genome segments obtained from the inoculum and the nasal washes of guinea pigs were analyzed in the NCBI database using standard nucleotide BLAST[246]. The nucleotide sequences were translated into amino acid sequences using ExPASy tool [247]. Pairwise protein alignments were performed using multiple sequence alignment tool from Clustal omega [248]

4.2.8 Gross Pathology and Fluorescent in-situ hybridization test

Following experimental infection of guinea pigs, euthanasia was performed at prescribed time points. A complete necropsy was performed to look for any macroscopic lesion in all the organs. Nasal turbinate, soft palate, trachea, and lung samples were

collected in 10% neutral buffered formalin and embedded in paraffin wax. Sections (5 μm thick) were then cut and stained with hematoxylin and eosin for histopathological examination. IDV in respiratory tract tissues was detected using in situ hybridization (ISH) with radioactive isotopes of sulfur (35 181 S) labeled negative-sense RNA probes of HEF and nucleoprotein (NP) as described [Wan, 2018 #2206].

4.2.9 High-performance liquid chromatography

Nasal turbinates, soft palate, and lungs were collected, washed with PBS and snap frozen before it is processed for High-performance liquid chromatography. The tissues were homogenized in homogenization buffer (0.5 M sucrose, 50 mM sodium maleate, pH 6.5. The homogenate was spun at 650 g for 10 min. The supernatant was collected and resuspended in homogenization buffer and spun again for 650 g for 5 min, Collected the supernatant and pooled the fractions. Diisopropylfluorophosphate (1mM) was added as a protease inhibitor and incubated on ice for 30 min. Samples were diluted 1:20 with ice-cold water, adjusted to 1mM EDTA and spun at 100,000xg for 30 min. After centrifugation, the pellet was resuspended in 1 M NaCl and spun again at 100,000xg for 30 min. Resuspended the pellet in 50 mM Tris-HCl, pH 6.5 and re-pelleted the membranes. Peptide-N-Glycosidase F The pellet is dried and the lipids were removed by Chloroform/Methanol/water and then treated with Neuraminidase (NEB) to release the sialic acids. Exchange chromatography is carried out by BioRad AG50 1x8 (hydrogen form) and then BioRad AG3 x4a (formate form) and then DMB derivatization 7.8mM DMB (4,5-Methylenedioxy-1,2-phenylenediamine dihydrochloride)

in a solution containing: β-mercaptoethanol, sodium hydrosulfite, and acetic acid followed by separation with reverse phase HPLC, by doing isocratic Elution at a flow rate of

0.5mL/min using Ascentis® C18 HPLC Column 5 μm particle size, L × I.D. 15 cm × 4.6 mm (Sigma-Aldrich, cat. No. 581324-U SUPELCO), and 9:7:84 ACN: MeOH : H_2O as eluent.

4.3. Results

4.3.1 Clinical signs, changes in body weight and temperature

To determine the differences in the virulence and pathogenesis of influenza D viruses compared to C viruses, we inoculated guinea pigs intranasally with 3×10^{5} $TCID_{50}$/300 ul of the respective viruses. The body weight and temperature were monitored from two days before the inoculation till the end of the experiment. The guinea pigs after challenge with three different viruses did not develop any clinical signs and the directly inoculated animals behaved in a similar way as the mock-infected animals. For each animal, the change in body weight post-infection was calculated by comparing the post-infection body weight to the average of the body weights recorded two days prior to the infection. The differences were calculated as the percentage of body weight change for each animal plotted for each day. The directly inoculated animals did not show any significant change in body weight compared to the mock-infected animals (Figure. 4.1A). Similarly, the difference between the post-infection body temperature and the average pre-infection temperature was determined for each animal, and the averages of the differences were plotted for each day (Figure. 4.1B). In the remaining days of the experiment, infected animals showed little or no change in body temperature. These results indicated that bovine

IDV infection had no effect on the body weight and temperature changes during the 14-days period in guinea pigs.

Figure 4.1. Body weight and temperature changes in guinea pigs after intranasal
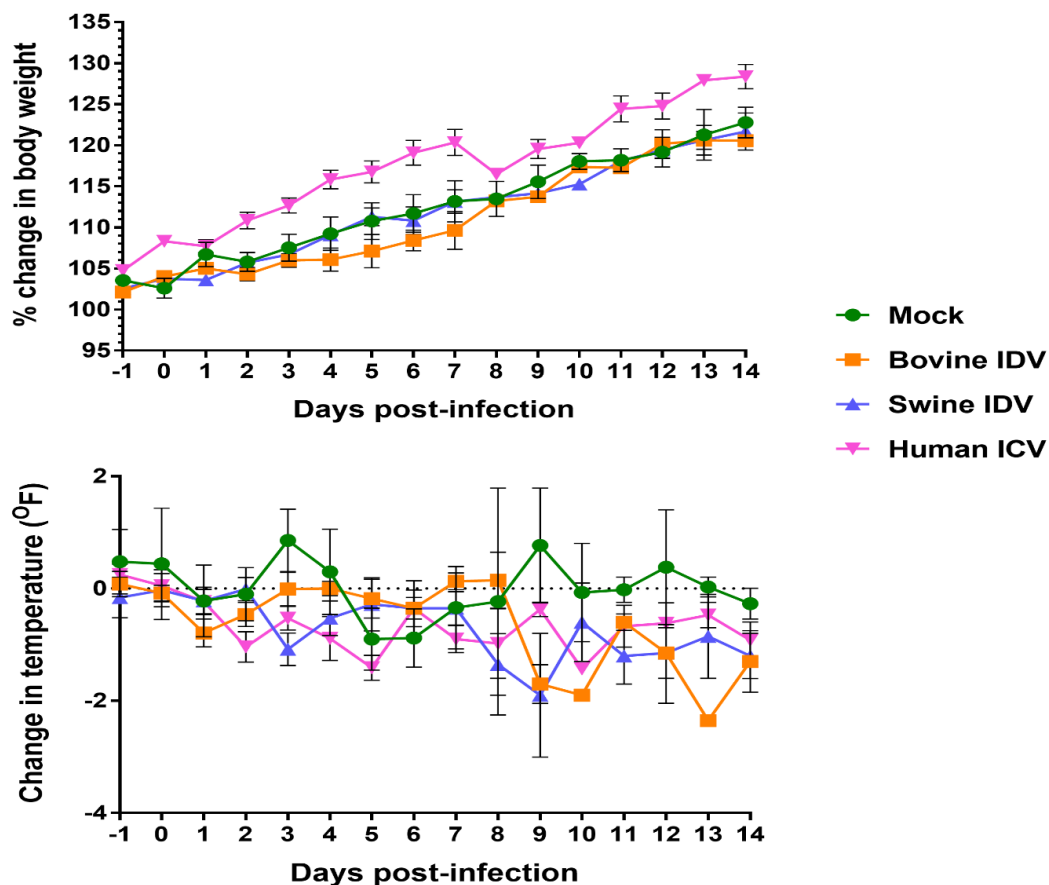
inoculation with bovine IDV, swine IDV, and human ICV. A total of 10 guinea pigs were intranasally inoculated with 3×10^5 TCID$_{50}$/300 μl for each virus group and 5 guinea pigs were mock inoculated with PBS. Body weight and temperature were measured daily for 2 days before infection until 14 dpi. (A and B) Percentage changes in body weight (A) and changes in body temperature (B) expressed as means ± standard errors (SE).

4.3.2 Replication kinetics and tissue tropism

To determine the viral replication kinetics of the three viruses, in the respiratory tract of the infected animals, a quantitative analysis of the virus loads in the nasal washes were conducted by titrating the samples on MDCK cells. Nasal washes were collected from the direct inoculated and mock-infected animals at 48-h intervals from 1 dpi to 7 dpi. The results of our experiment showed that nasal shedding was present in the bIDV infected animals from 1 dpi to 7 dpi. In the bIDV group, the number of animals which demonstrated the nasal shedding was 9/10, 8/8, 6/6 and 2/4 on 1, 3, 5, 7 dpi respectively. In the case of swine IDV, none of the animals had the virus in the nasal washes on 1 dpi, but 8/8, 6/6 and 4/4 animals shed s-IDV on 3, -5, and 7 dpi. This lag in the virus shedding shown by the sIDV group is the striking feature. Only two animals in the human ICV infected group demonstrated virus shedding. In the human ICV group, 0/10, 2/8, 2/6 and 1/4 animals shed the virus on 1, 3,5, 7 dpi respectively.
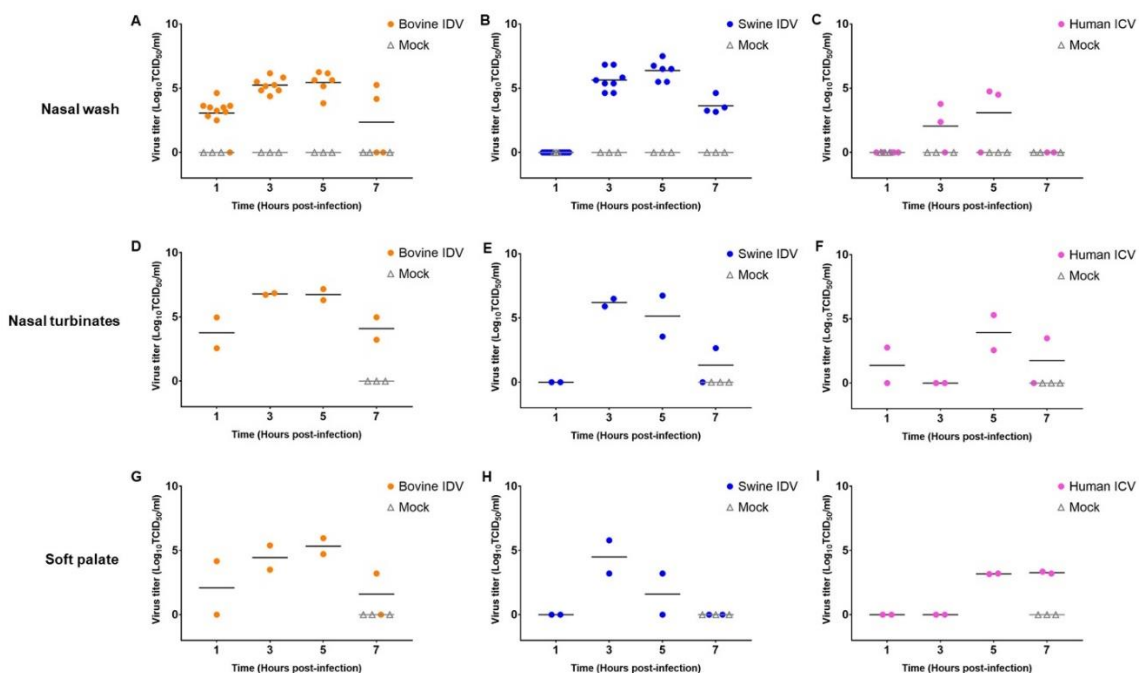
Figure 4.2. Replication kinetics of the different viruses in nasal washes, and upper respiratory tract (nasal turbinates and soft palate). Nasal washes were collected from the three virus groups on 1, 3, 5, and 7 dpi. Two inoculated animals per group were randomly euthanized on each of those days to assess virus load in nasal turbinates, soft palate. Three mock-inoculated animals were euthanized at 7 dpi. Virus titers in nasal washes are expressed as $\log_{10}$ $TCID_{50}$ per mL and in nasal turbinates, and soft palate was expressed as $\log_{10}$ $TCID_{50}$ per gram. For all the panels, each shape represents an individual animal and horizontal bars show the mean viral titers for each time point.
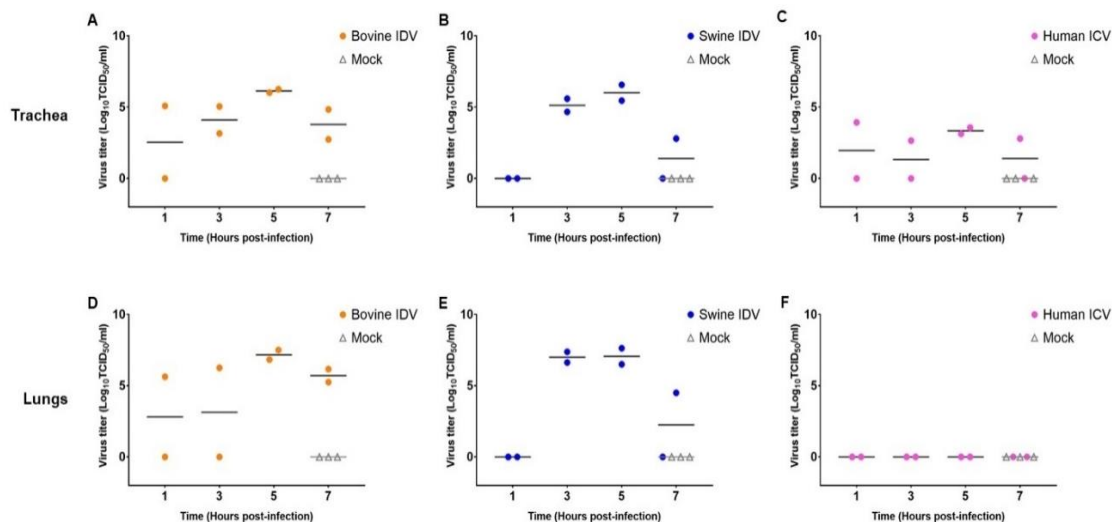
Figure. 4.3. Replication kinetics of the different viruses in the lower respiratory tract. Two inoculated animals per group were randomly euthanized on each of those days to assess virus load in trachea, and lungs. Three mock-inoculated animals were euthanized at 7 dpi. Virus titers in nasal turbinates, soft palate, trachea, and lungs are expressed as $\log_{10}$ $TCID_{50}$ per gram. For all the panels, each shape represents an individual animal and horizontal bars show the mean viral titers for each time point.

To determine the tissue tropism of the virus, two random animals were selected and euthanized on 1, 3, 5, and 7 dpi from each group and nasal turbinates, soft palate, trachea, and lung, were collected. Three animals from the mock-infected group were also euthanized at 7 dpi. In all the groups, the tissue homogenates from nasal turbinates, soft palate, trachea, and lungs showed appreciable viral titer from 1 dpi through 7 dpi, except for lungs in ICV, where there was no virus isolated at any time points (Figure 4.2 and Figure 4.3). In the bIDV group, the virus load in tissue homogenates for the different time points (given in bold letters): nasal turbinates-**1**-2/2, **3**-2/2, **5**-2/2,**7**-2/2; Soft palate-**1**-1/2, **3**-2/2, **5**-2/2,**7**-1/2 (Figure 4.2); trachea-**1**-1/2, **3**-2/2, **5**-2/2,**7**-2/2; lungs-**1**-2/2, **3**-2/2, **5**-2/2,**7**-2/2 were noted. Mean viral titer of bIDV was lowest in the soft palate compared to the other three tissues at 1 dpi. The peak viral titer for bIDV in nasal turbinates, soft palate, trachea, and lungs were noticed at 5 dpi. In the case of sIDV, , the virus load in tissue homogenates for the different time points: nasal turbinates-**1**-2/2, **3**-2/2, **5**-2/2,**7**-1/2; Soft palate-**1**-0/2, **3**-2/2, **5**-2/2,**7**-0/2; trachea-**1**-0/2, **3**-2/2, **5**-2/2,**7**-1/2; lungs-**1**-0/2, **3**-2/2, **5**-2/2,**7**-1/2 were noted. In the ICV group, the virus load in tissue homogenates for the different time points: nasal turbinates-**1**-1/2, **3**-0/2, **5**-2/2,**7**-1/2; Soft palate-**1**-0/2, **3**-0/2, **5**-2/2,**7**-2/2; trachea-**1**-1/2, **3**-1/2, **5**-2/2,**7**-1/2; lungs-**1**-0/2, **3**-0/2, **5**-0/2,**7**-0/2 were noted

4.3.3 Tissue tropism- the presence of vRNA in tissues

To confirm the tissue tropism, we also conducted Fluorescent in-situ hybridization (FISH) to stain the viral RNA in the different tissues. The results obtained from FISH correlated with the virus isolation. FISH staining revealed IDV RNAs of both bovine and swine origin distributed in the lining epithelial cells of the mid nasal septum, soft palate, trachea and the bronchioles of the lung. Similar to the virus isolation from the tissues,

tissues from 5 dpi showed the highest localization of the vRNAs in both IDV groups (Figure. 4.4, 4.5). On the contrary, only a few samples from the ICV group showed vRNAs, but only in the nasal septum and soft palate (Figure. 4.6). We could not find any ICV RNAs in the trachea, which is contrary to what we observed in virus isolation. No ICV RNA was detected in the lungs of the ICV infected group by VI and FISH.

4.3.4 Gross Lesions and Histopathology

Despite any clinical symptoms, the guinea pigs showed mild to moderate macroscopic changes in the lungs, characterized by areas of congestion and hemorrhage which eventually progressed to areas of pulmonary consolidation on 3, 5, and 7 dpi (data not shown). Lungs from mock-infected animals did not show any macroscopic lesions. Histopathological examination of the lungs showed minimal to mild inflammation in the trachea, mild to moderate atelectasis, bronchitis, and bronchiolitis with denudation of the epithelium, severe infiltration of inflammatory cells such as neutrophils, lymphocytes, and RBCs in the lung parenchyma with a thickened alveolar septum. Peribronchial and perivascular cuffing by lymphocytes and plasma cells and bronchopneumonia with luminal exudate were also observed in the lungs of animals infected by all the three viruses on 1, 3, 5, and 7 dpi (Figure. 4.7).
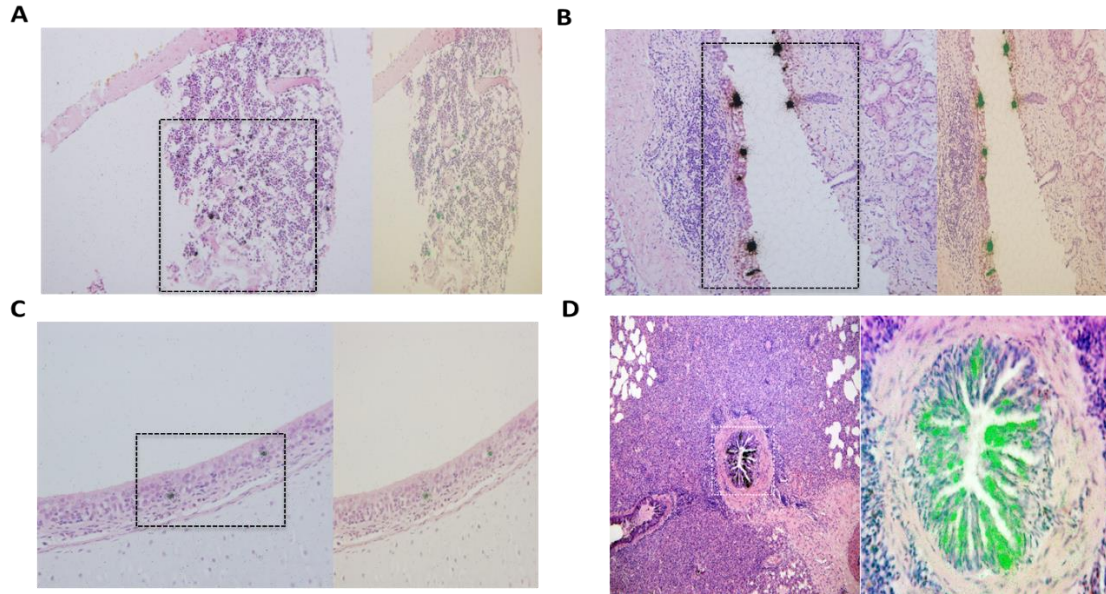
Figure. 4.4. Bovine IDV RNA positive cells in the guinea pig tissues detected by FISH. Representative images of mid nasal septum (A), soft palate (B), trachea (C), and lungs (D) from 5 dpi demonstrated bIDV RNA positive cells as black silver grains in radio autographs (green deposits under the epipolarized light in the inset).
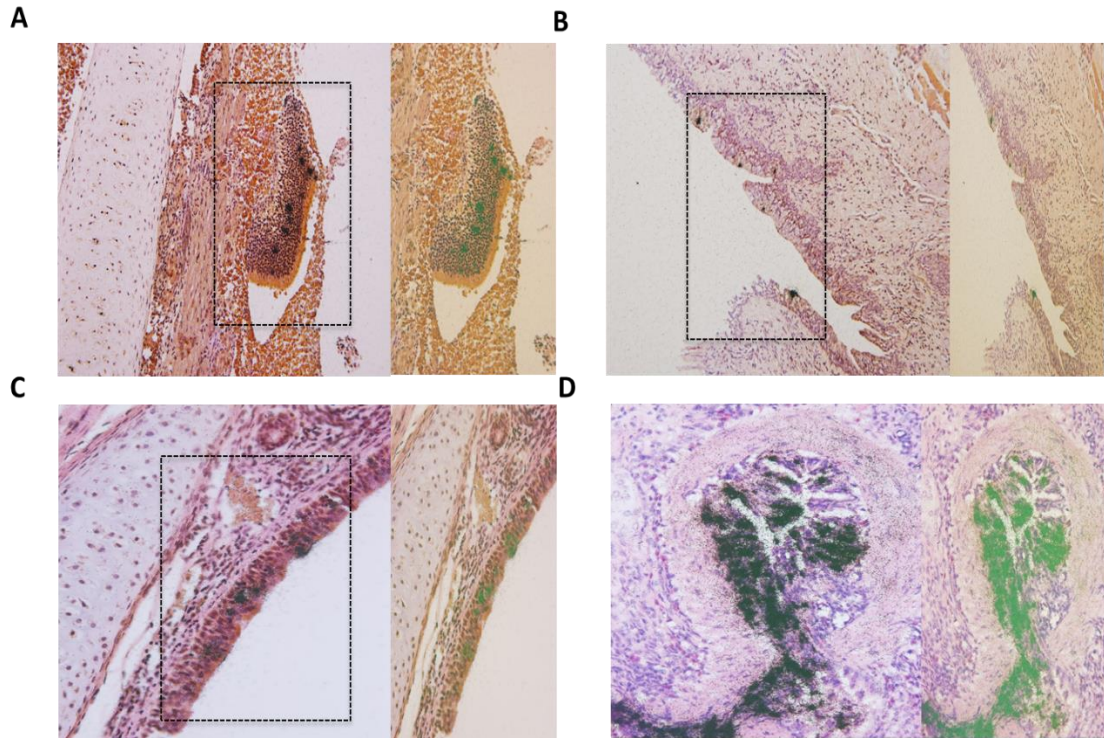
Figure. 4.5. Swine IDV RNA positive cells in the tissues detected by ISH. Representative images of mid nasal septum (A), soft palate (B) trachea (C), and lungs (D) from 5 dpi showed bIDV RNA positive cells as black silver grains in radioautographs (green deposits under the epipolarized light in the inset).
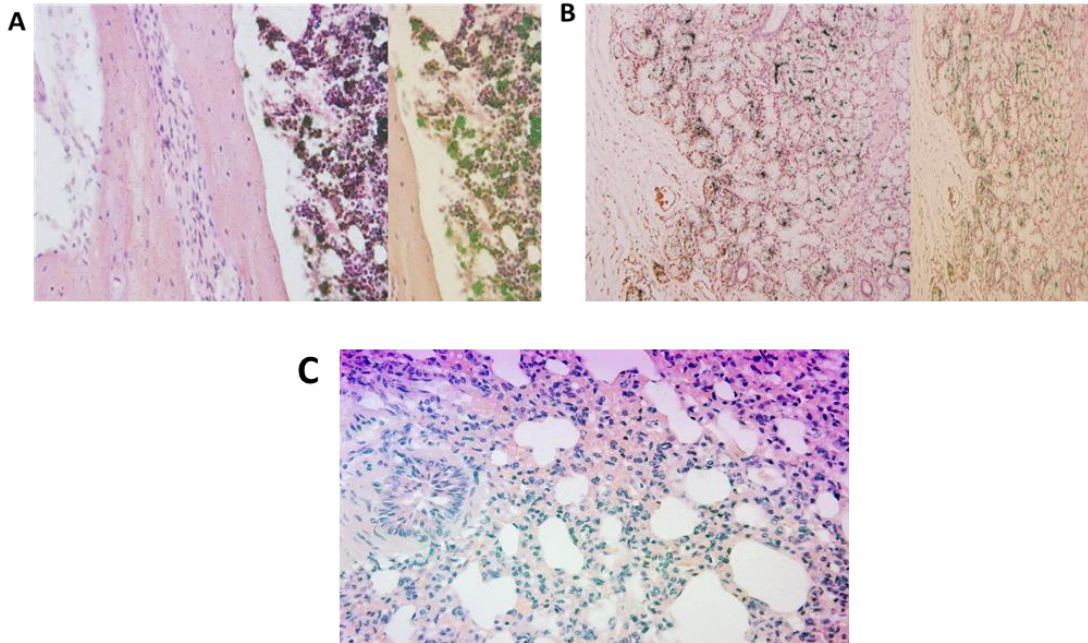
Figure.4.6. ICV RNA positive cells in the tissues detected by ISH. Representative images of mid nasal septum and soft palate 5 dpi (A, B) showed bIDV RNA positive cells as black silver grains in radioautographs (green deposits under the epipolarized light in the inset). Mock lung tissue without any vRNA staining is shown in C.
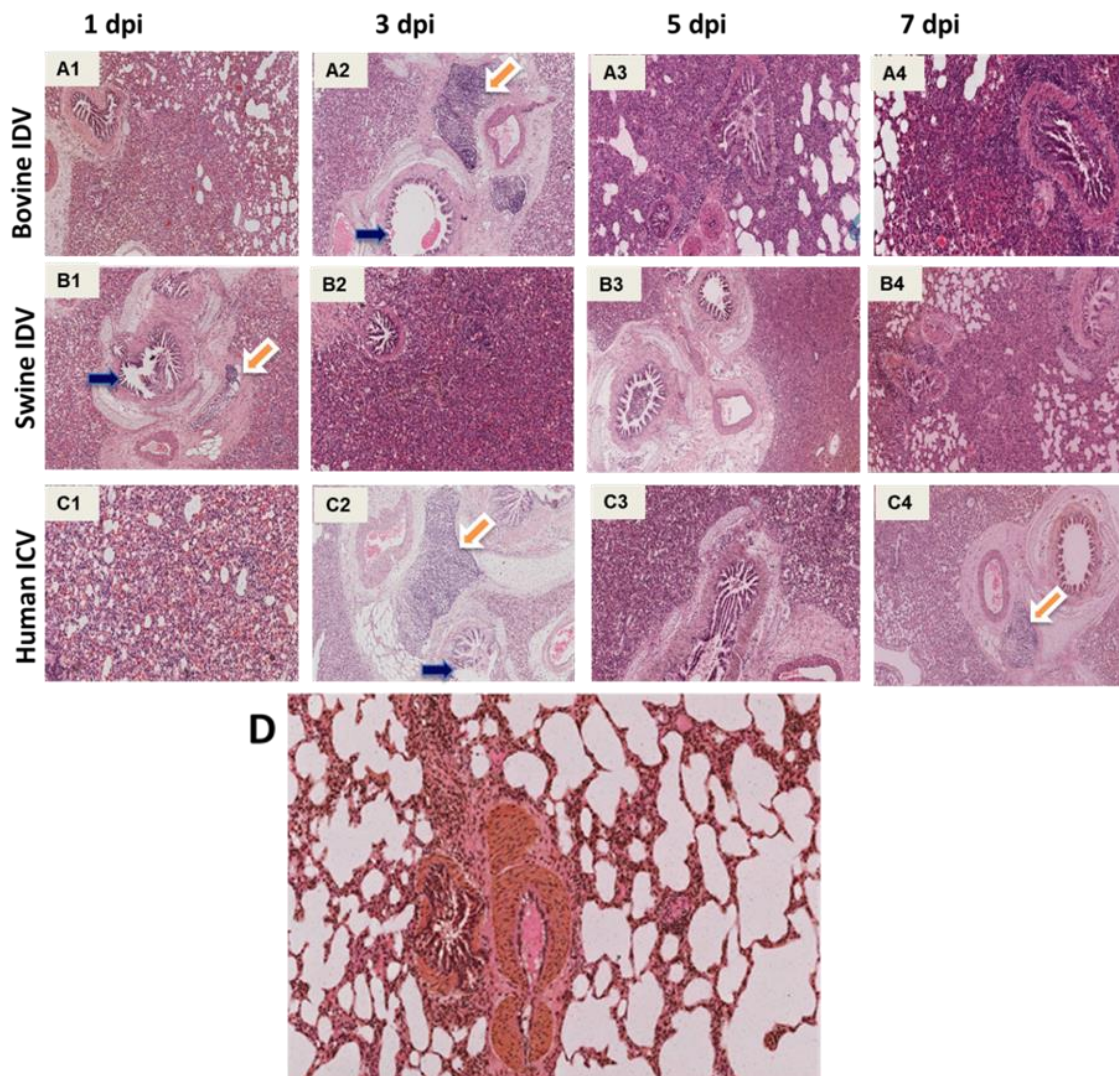
Figure. 4.7 Pathological changes in the lungs of guinea pigs infected with bIDV, sIDV and ICV. Representative images of histopathological lesions in the lungs from animals on 1,3,5, and 7 dpi for bIDV (A1-A4); sIDV (B1-B4); ICV(C1-C4). Histologically, the lung tissue showed multifocal areas of alveolar inflammation with infiltration of lymphocytes, plasma cells (yellow arrows), and RBCs in the lung parenchyma. Bronchiolar inflammation with desquamation of the epithelial cells (blue arrows) and peribronchial infiltration of lymphocytes were also seen. Lungs from the mock animal on 7dpi showed no such lesions.

4.3.5 Seroconversion

Two intranasally inoculated animals from each group were maintained till 14 dpi for the seroconversion. All the guinea pigs were seronegative for all the three viruses we used. Pre- and post-infection sera from all the animals in the bIDV, sIDV and ICV groups (days 1, 3, 5, 7, 14 post-infection) and mock animals were tested for the presence of heterologous and homologous antibody titer against bIDV, sIDV, and hICV. Antibody responses were detected in animals from 7 dpi (10-20) and reached higher titer by 14 dpi. bIDV sera showed a homologous high titer of 320 and 80 and heterologous titer of 80 by 14 dpi. sIDV sera demonstrated a homologous high titer of 1280 and 640 with a very low heterologous titer of 10 by 14 dpi. ICV sera showed no cross-reactivity with both the IDV viruses. It is also noted that only 1/2 animals showed a homologous titer of 80 in the ICV group.
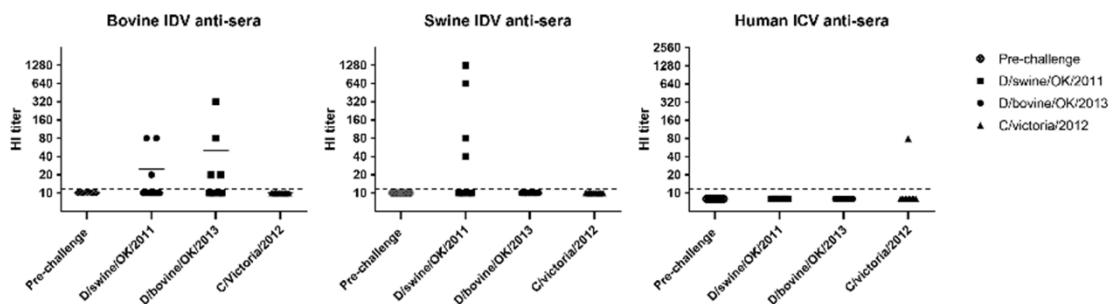
Figure. 4.8. Seroconversion and cross-reactivity of the guinea pigs after infection with bIDV, sIDV, and ICV by HI assay. Homologous and heterologous antibody titers of the respective antisera against different viruses used in the study were given in A-C. Each shape represents an individual animal. Mean of the HI titers is represented by a line.

4.3.6 Sialic acid estimation in guinea pig tissues by HPLC

Based on the tissue tropism demonstrated by the IDV and ICV, cryopreserved tissues of nasal turbinates, soft palate, and the lunges were analyzed to quantify the amount and type of the 9-O acetyl sialic acids by the HPLC. HPLC analyses have shown that nasal turbinates, soft palate, and the lungs demonstrated a high amount of Neu5Ac, along with the reasonably good amount of Neu5Gc. Different concentrations of the sialic acid expressed in pmol to that of the total sialic acid in each tissue is given in Figure 4.9. Further, 9-O acetyl sialic acids were present in all the three tissues, further confirming the association of IDV with 9-O acetyl sialic acids demonstrated by the glycan binding experiments were done earlier. Besides Neu5Ac and Neu5Gc, guinea pig respiratory tract also possesses Neu5,9Ac2 (Sialic acid which has acetyl group at C5 and C9), Neu5Gc9Ac (Acetyl position at C9 and glycolyl group at C5), which are the two predominant receptors that play a role in the virus entry.
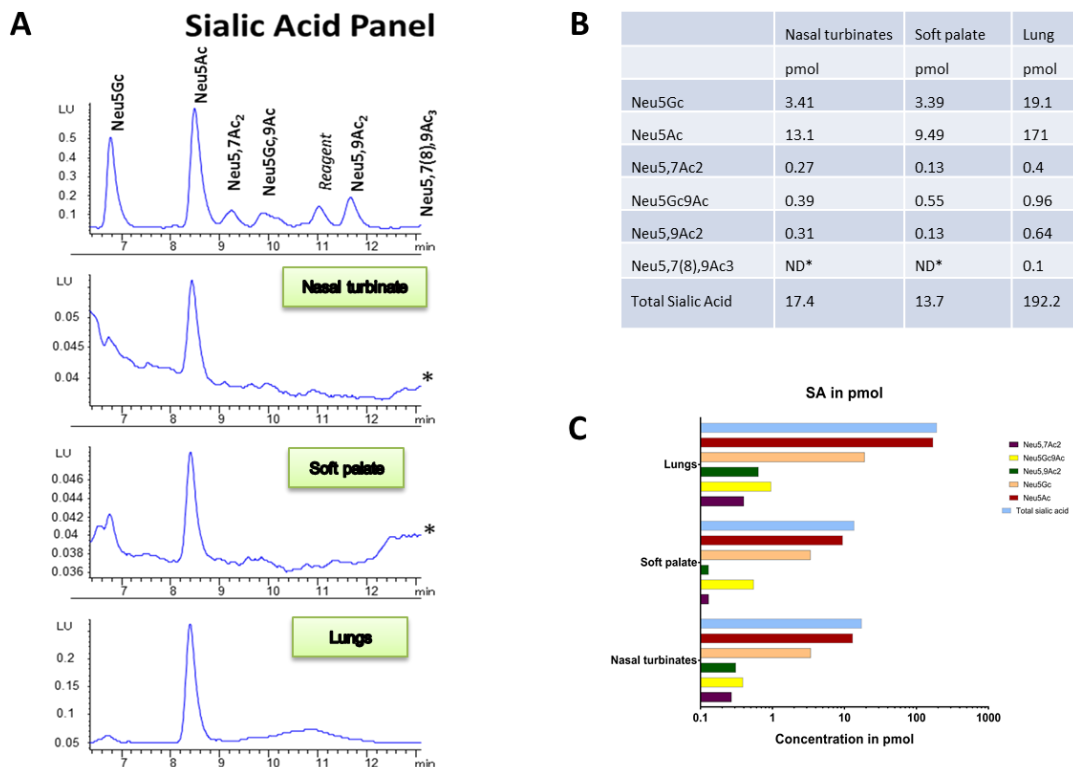
Figure 4.9. Estimation of sialic acids in guinea pig respiratory tissues by HPLC. Concentrations of different types of sialic acids to total sialic acid present in nasal turbinates, trachea, and lungs expressed in pmol concentration were shown in B and C.

4.3.7 Deep RNA sequencing analyses of nasal washes

From our data, it was evident that the swine and bovine isolates of IDV demonstrated differential pattern of replication and virus shedding in guinea pigs. The bovine isolate of IDV productively replicated in the upper and lower respiratory tract and caused nasal shedding from 1dpi, while the swine isolate demonstrated a lag in adaptation for 1-2 days but ended up showing a high titer on 3 dpi. This peculiar replication pattern was observed in the nasal washes and tissue homogenates, which prompted us to determine whether there is the presence of any possible mutations in sIDV that facilitated to evolve

like bIDV and mediate its viral adaptation and virulence to promote high viral titer, as that of bIDV on 3 dpi. We conducted deep RNA sequencing of the inoculum we used for the challenge and the nasal wash samples from the 3 dpi and 5 dpi of all the three virus groups. Between the inoculum sequences of sIDV and bIDV, there were 5,4,3,23,7,14, and 4 amino acid changes in PB2, PB1, P3, HEF, NP, P42 and M segments respectively. Further, we did deep RNA sequencing of the nasal washes from the animals euthanized on 3 dpi and 5 dpi and compared to the viral genome of the respective inoculum sequences. Deep RNA sequencing analyses of the nasal washes from swine and bovine IDVs demonstrated no mutations in the HEF gene compared to the respective inoculum sequence in bIDV group, while sIDV infected animals showed some non-significant point nucleotide mutations in the HEF protein. In the case of sIDV, only PB2 (6ST) and P3 showed amino acid changes in the viral genome derived from the nasal washes on 3 and 5 dpi, while PB1, HEF, NP, and NS showed some random nucleotide changes without any amino acid mutations compared to parent inoculum. In the bIDV group, PB2 gene showed amino acid mutations (E44D and N668K) in all the animals from 3 dpi and 5 dpi compared to the bIDV inoculum. The polymerase proteins such as PB2, PB1, and NP showed some non-significant point mutations, which were not observed in all the animals. P42 is the only segment in the sIDV group, while HEF, P42, NS, P3 were the segments in the bIDV group that did not show any mutations.

N-glycosylation sites of HEF protein, the main receptor binding protein play any role in the host adaptation and transmission. The predicted N-glycosylation sites of HEF, for all the three inoculum sequences determined using NetNGlyc 1.0 server [257] were given in Figure 4.10 and Table 4.6. The parent inoculum of sIDV used in the study revealed

6 glycosylation sites, compared to 7 in the bIDV. It is interesting to note that Italian swine and bovine IDV isolates, both belonging to D/OK lineage possess seven N-glycosylation sites, the same number as bovine IDV (D660) (Table 4.6).

Table 4.1 Aminoacid sequence analyses of the sIDV and bIDV inoculum. Changes in aminoacid residues between the inoculum sequences were given as swine amino acid: site no: bovine aminoacid for each viral segment.

Protein	Variable sites	Swine aminoacid:site:bovine aminoacid						
PB2	5	S6T	K119R	A293S	M352I	V521I		
PB1	4	K374R	A384V	D388N	R663K			
P3	3	K69R	L266M	T322I				
HEF	23	I68V	D80A	S164G	T181K	A188T	K212R	V215A	A251T
		K252A	F256L	I273V	N288G	A289S	G290R	K308R	R312K
		V469I	N486S	G524E	K627N	V649M	S654F	I658S	
NP	7	P74L	S132T	E247D	K381E	A462T	M569V	I574V	
P42/M	14	V14I	M26L	K27R	K37R	E38D	K41R	C91S	N93S
		I256M	P257S	G290S	L316F	D359E	P366L		
NS	4	N90S	G119E	F122L	D278A				

Table 4. 2 Viral genome and protein sequence changes in the PB1 and NP of the nasal wash samples of two animals from 3 dpi and 5 dpi compared to parent inoculum of bIDV, N represents gap.

bIDV	PB1								NP			
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Inoculum	G649		G652	E217	A709		T931	W311	G1243		A1251	
D3-S1	G649 C		G652 T	E217 Q	N		T931	W311	G1243		A1251 G	
D5-S1	G649		G652	E217	A709		T931	W311	G1243		A1251	
D3-S2	G649		G652	E217	A709		T931	W311	G1243		A1251	
D5-S2	G649		G652	E217	A709 G		T931 A	W311 R	G1243 C		A1251 G	

Table 4.3 Viral genome and protein sequence changes in the PB2 and PB1 segments of the nasal wash samples of two animals from 3 dpi and 5 dpi compared to parent inoculum of sIDV. Consistent nucleotide and aminoacid sequence changes compared to sIDV inoculum are highlighted 'N' represents gap.

sIDV	PB2										PB1			
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Inoculum	T16	6S	C660		A776		A832		T835		N		A553	
D3-S1	T16 A	6S T	C660		A776		A832		T835		N		A553	
D5-S1	T16 A	6S T	C660		A776		A832 G		T835 C		A55 2		A553 G	
D3-S2	T16 A	6S T	C660		A776 G		A832		T835		N		A553	
D5-S2	T16 A	6S T	C660 T		N		A832		T835		A55 2		A553	

Table 4.4 Viral genome and protein sequence changes in the P3 segment of the nasal wash samples of two animals from 3 dpi and 5 dpi compared to parent inoculum of sIDV. Consistent nucleotide and aminoacid sequence changes compared to sIDV inoculum are highlighted 'N' represents gap.

DOK	P3													
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Inoculum	A471		C615		A618	I206	G877	E293	G939		G1327		C1528	
D3-S1	A471		C615T		A618G	I206M	G877	E293	G939		G1327		C1528	
D5-S1	A471G		C615		A618	I206	G877	E293	G939		G1327		C1528	
D3-S2	A471		C615T		A618G	I206M	G877C	E293Q	G939		G1327		C1528	
D5-S2	A471		C615T		A618G	I206M	G877	E293	N		G1327C	*	C1528T	*

Table 4.5 Viral genome and protein sequence changes in the HEF, NP, and NS segments of the nasal wash samples of two animals from 3 dpi and 5 dpi compared to parent inoculum of sIDV. Consistent nucleotide and aminoacid sequence changes compared to sIDV inoculum are highlighted 'N' represents gap.

DOK	HEF						NP				NS	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Inoculum	T885		C905	P302	C1024		N		A1250		A833	
D3-S1	T885		N		C1024		C1242		A1250G		A833	
D5-S1	T885		C905	P302	C1024T		C1242		A1250G		A833C	
D3-S2	T885G		C905G	P302R	N		C1242		A1250G		A833C	
D5-S2	N		N		C1024		C1242		A1250G		A833C	

Hemagglutinin-esterase Fusion [Influenza D virus (D/bovine/Oklahoma/660/2013)]

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Name:   D660_HEF        Length:   664
MFLLLATITAITACQAERELICIVQRVNESFSLHSGFGGNVYSMKTEPMTGFTNVTKGASVINQKDWVGFGDSRTDLTNA     80
QFPASSDVPLAVAKKFRSLSGASLMLSAFGPPGKVDYLYQGCGKEKVFYEGVNWSPEAGIDCFGSNWTQTKKDFYSRIYE    160
AARGSTCMTLVNSLDTKISSKTATAGTTSSCSSSWMKSPLWYAESSVNPGARPQACGTEQSATFTLPTSFGIYKCNKHVV    240
QLCYFVYENKTAFNTLGCGDYYQNYYDGNGNLVGGMDNRVAAYRGIAGSRVKIECPSKILNPGTYSIRSTPKFLLVPKRS    320
YCFDTDGGYPIQVVQSEWSASRRSDNATEEACLQTEGCIFIKKTTPYVGEADDNHGDIEMRQLLSGLGNNDTVCVSQSGY    400
TKGETPFVKDYLSPPKYGRCQLKTDSGRIPTLPSGLIIPQAGTDSLMRTLTPATRIFGIDDLIFGLLFIGFVAGGVAGGY    480
FWGRSSGGGGGASVSSTQAGFDKIGKDIQQLRNDTNAAIEGFNERIAHDEQAIKNLAKEIEDARAEALVGELGIIRSLIV    560
ANISMNLKESLYELANQITKRGGGIAQEAGPGCWYVDSENCDASCKEYIFNFNGSATVPTLRPVDTNVVITSDPYYLGST    640
IALCLLGLMAIAAFVGVSWICCKK

..........................N.........................N..........................     80
..........................................................N...................    160
..............................................................................    240
........N.....................................................................    320
..............................N...............................................    400
..............................................................................    480
..................................N...........................................    560
..........................................................N...................    640
......................                                                            720
```

Hemagglutinin-esterase Fusion [Influenza D virus (D/swine/Oklahoma/1334/2011)]

```
Name:   HEF_DOK  Length:   664
MFLLLATITAITACQAERELICIVQRVNESFSLHSGFGGNVYSMKTEPMTGFTNVTKGASVINQKDWIGFGDSRTDLTND    80
QFPASSDVPLAVAKKFRSLSGASLMLSAFGPPGKVDYLYQGCGKEKVFYEGVNWSPEAGIDCFGSNWTQTKKDFYSRIYE   160
AARSSTCMTLVNSLDTKISSTTATAGTASSCSSSWMKSPLWYAESSVNPGAKPQVCGTEQSATFTLPTSFGIYKCNKHVV   240
QLCYFVYENKAKFNTFGCGDYYQNYYDGNGNLIGGMDNRVAAYRGIANAGVKIECPSKILNPGTYSIKSTPRFLLVPKRS   320
YCFDTDGGYPIQVVQSEWSASRRSDNATEEACLQTEGCIFIKKTTPYVGEADDNHGDIEMRQLLSGLGNNDTVCVSQSGY   400
TKGETPFVKDYLSPPKYGRCQLKTDSGRIPTLPSGLIIPQAGTDSLMRTLTPATRIFGIDDLIFGLLFVGFVAGGVAGGY   480
FWGRSNGGGGGASVSSTQAGFDKIGKDIQQLRNDTNAAIEGFNGRIAHDEQAIKNLAKEIEDARAEALVGELGIIRSLIV   560
ANISMNLKESLYELANQITKRGGGIAQEAGPGCWYVDSENCDASCKEYIFNFNGSATVPTLRPVDTKVVITSDPYYLGST   640
IALCLLGLVAIAASVGVIWICCKK

..........................N.........................N..........................    80
..........................................................N...................   160
..............................................................................   240
..............................................................................   320
..............................N...............................................   400
..............................................................................   480
..................................N...........................................   560
..........................................................N...................   640
......................                                                           720
```

Hemagglutinin-esterase Fusion [Influenza D virus (C/Victoria/2/2012)]

```
Name:   C_Vic_HEF       Length:   655
MFFSLLLVLGLAEAEKIKICLQKQVNSSFSLHNGFGGNLYATEEKRMFELVKPKAGASVLNQSTWIGFGDSRTDKTNSAF    80
PRSADVSAKTADKFRSLSGGSLMLSMFGPPGKVDYLYQGCGKHKVFYEGVNWSPHAAIDCYRKNWTDIKLNFQKNIYELA   160
SKSHCMSLVNALDKTIPLHATAGTAKNCNNSFLKNPALYTQEVKPSEEKCGKENLAFFTLPTQFGTYECKLHLVASCYFI   240
YDSKEVYNKRGCDNYFQVIYDSSGKVVGGLDNRVSPYTGNSGDTPTMQCDMIQLKPGRYSVRSSPRFLLMPERSYCFDMK   320
EKGPVTAVQSVWGKGRQSDYAVDQACLSTPGCMLIQKQKPYIGEADDHHGDKEMRDLLSGLDYEARCVSQSGWVNDTSPF   400
AEEYLLPPKFGRCPLAAKEESIPKIPDGLLIPTSGTDTTVTKPKSRIFGIDDLIIGLLFVAIVEAGIGGYLLGSRKESGG   480
GVTKESAEKGFEKIGNDIQILRSSTNIAIEKLNDRISHDEQAIRDLTLEIENARSEALLGELGIIRALLVGNISIGLQES   560
LWELASEITNRAGDLAVEVSPGCWIIDDNICDQSCQNFIFKFNETAPVPTIPPLDTKIDLQSDPFYWGSSLGLAITAAIS   640
LAALVISGIAICRTK

..........................N......................N.....................         80
..............................................................N.................   160
..............................................................................   240
..............................................................................   320
........................................................................N.....   400
..............................................................................   480
......................................................N........               560
..............................................................................   640
..............                                                                 720
```

Figure 4.10 Glycosylation sites and the positions of the inoculum sequences of bIDV, sIDV, and ICV.as determined by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) Asn-Xaa-Ser/Thr sequons in the sequence output below are highlighted in blue. Asparagines predicted to be N-glycosylated (Threshold= 0.5) are highlighted in red.

Table 4.6 Predicted N-glycosylation sites of HEF protein of the parent inoculum of IDV and ICV used in the study, along with the sIDV and bIDV isolates from Italy.

Virus	No. of sites	Glycosylation sites						
D/bovine/ Oklahoma/ 660/2013	7	28 NESF	54 NVTK	146 NWTQ	249 NKTA	346 NATE	513 NDTN	613 NGSA
D/swine/O klahoma/1 334/2011	6	28 NESF	54 NVTK	146 NWTQ	346 NATE	513 NDTN	613 NGSA	
D/swine/Ita ly/199724- 3/2015	7	28 NESF	54 NVTK	146 NWTQ	249 NKTA	346 NATE	513 NDTN	613 NGSA
D/bovine/It aly/1/2014	7	28 NESF	54 NVTK	146 NWTQ	249 NKTA	346 NATE	513 NDTN	613 NGSA
C/Victoria/ 2/2012	5	26 NSSF	61 NQST	144 NWTD	395 NDTS	552 NISI		

4.4. Discussion

Previous studies have shown that IDV is common in bovines with respiratory disease and demonstrated that at least two genetic and antigenically distinct clades cocirculate [47]. Further, the metagenomic study of the virome revealed the etiological role of influenza D in the bovine respiratory disease complex of cattle [258]. Our previous study using the bovine IDV demonstrated that guinea pigs can be a good model to study the virulence, pathogenesis, and transmission of IDV [256]. The growing serological evidence of IDV of both lineages in other mammalian species such as goats, sheep, camel, horses warrants further investigation to differentiate any molecular and structural differences and to study the host-pathogen interactions exhibited by these two lineages. Moreover, serologic evidence of influenza D has been reported from the occupational workers [14]. We used human ICV as comparative strain because of its genetic similarity to IDV and its

ability to infect pigs. Previous studies using swine IDV in ferrets failed to isolate virus from the lower respiratory tract from pigs and ferrets, while our study with bovine IDV replicated productively in the upper and lower respiratory tract. Hence, it was important to understand the structural and molecular differences of these two different lineages in terms of virulence and pathogenicity. We used D/swine/OK/1334/2011 and D/bovine/OK/660/2013 as the representative IDV strains for our study.

Similar to our previous study, the guinea pigs did not show any clinical symptoms upon infection with any of the three viruses under study. No significant changes were observed in the body weight and temperature of the guinea pigs infected with bIDV, sIDV and ICV. However, there was virus shedding in the nasal washes exhibited by the animals in both IDV and ICV groups. The nasal shedding pattern demonstrated by the bIDV group was different from sIDV group. It is interesting to note that animals infected with sIDV neither shed any virus nor found in the tissue homogenates at 1 dpi. The results we obtained with FISH was consistent with our virus isolation data. The reason for the lag in virus shedding noticed for the animals infected with swine IDV in the nasal washes and the tissue homogenates from upper and lower respiratory tract warrants further investigation. We speculate that the key differences in the viral replication pattern demonstrated by the bovine IDV and swine IDV could be attributed to the differences in the receptor binding domains or due to any other viral genetic factors. To our knowledge, this is the first study to compare the sIDV with bIDV. An earlier study of sIDV using ferrets have shown nasal shedding at 3 dpi, but there was no nasal wash collection at 1 dpi. It is noteworthy that the swine and bovine IDV genomes share 96-99% homology in all the segments [2], yet the differential replication kinetics of sIDV demands further investigation. To understand the genetic and

molecular basis of these virus-related constraints, deep RNA sequencing of the nasal washes was carried out to find out any key differences in the viral genome that facilitated a faster adaptation and replication in the bIDV infected group compared to the sIDV group. Deep RNA sequencing data of the respective inoculum versus the nasal washes collected on 3 and 5 dpi from 2 animals in bIDV and sIDV groups were subjected to next-generation sequencing analyses. In the case of bIDV, there were no nucleotide changes in HEF, NS, P42, and P3 gene segments, while PB1, PB2, and NP showed some nucleotide changes occasionally without any amino acid changes. On the other hand, sIDV viruses in the nasal washes were mostly homologous to the inoculum sequence with some nucleotide point mutations in the HEF, PB1, NS, NP, P3, and PB2 gene segments, of which 6ST mutation in the PB2 (b-IDV like) needs to be addressed, whether this has any role in the host adaptation. It is noteworthy that no mutations were found in P42/M of both IDV groups.

In the case of ICV, only a few animals showed viral shedding. It is also a valid question whether guinea pigs can support ICV replication, as there were no previous studies of ICV done in guinea pigs. Guinea pigs have been used as animal models for studying several influenza A and B viruses but not for influenza C viruses [7, 209, 210]. Out of the 10 inoculated animals, only 3 demonstrated a productive replication-with 2 animals started shedding on 3 dpi (2.375, 3.775 logs) continued to 5 dpi (4.5, 4.75) and stopped, the third animal started virus shedding with 3.375 logs on 7dpi but could not track further shedding due to termination of that animal. ICV, being an upper respiratory pathogen, the replication kinetics of ICV was expected as it replicated in the nasal turbinate, soft palate and trachea of the guinea pigs. However, ICV did not replicate in the lower respiratory tract organ, lung possibly due to the intrinsic temperature sensitivity of

the ICV, HEF, and polymerase. At 37°C, it was demonstrated that HEF mediated membrane fusion decreases owing to the low efficiency of membrane pore formation, impaired oligomerization occurs which affects the trimerization of the influenza viral proteins from endoplasmic reticulum to plasma membrane via golgi and subsequently low HEF expression at the plasma membrane, which could be the reason why we could not find ICV replication in the lungs [259]. Another factor that is worth noting is that experimental infections of ICV in dogs where the animals were exposed to virus three times, also showed variations in the severity of infection among dogs and time course of antibody development also varied considerably from animal to animal [254]. The virus isolation was reported only in 4 out of 6 dogs after the second inoculation [254]. A recurring trend of virus excretion was observed in experimental infection of ICV in pigs where the directly inoculated pigs showed an intermittent virus excretion and contact transmission also occurred at 2 dpi [31]. Out of the two animals kept for seroconversion, only one animal showed an HI titer of 80, and the other animal with no titer never shed virus on any days, which shows that productive infection never happened in that animal.

FISH staining demonstrated the vRNA localized in the lining epithelia of mid nasal septum, soft palate, trachea, and lungs. Virus isolation (VI) data and FISH data almost matched, but there were a few differences which could be due to the difference in the location/parts of the tissue used for both assays. For example, the lung lobes used for the VI and FISH are different, nasal turbinates for VI versus mid nasal septum for the FISH because of the sample limitation. The pathology associated with both IDV s and ICV were similar and no significant differences were noted between the groups. Only very minimal microscopic lesions found in the upper respiratory tract compared to the lower respiratory

tract. Guinea pig lung showed typical lesions of influenza characterized by the pneumonic changes and the vast amount of alveolar infiltration with inflammatory cells. Histopathological lesions found in the lung on 3 and 5 dpi were more pronounced in bovine and swine IDV infected animals than that at 1 dpi for ICV, which shows the slow progression of ICV compared to IDV.

Seroconversion was noticed at 7 dpi in sIDV and bIDV groups and not in the ICV infected animals. This can be explained by the broad cell tropism exhibited by the IDV owing to the open receptor binding cavity of the hemagglutinin-esterase fusion glycoprotein which accommodates diverse glycan moieties and hence is responsible for the diverse host spectra [238]. The homologous titer of sIDV antisera was higher compared to bIDV antisera, however, the heterologous titer was vice-versa. It is often seen that bovine IDV antisera have a broad cross-protection, compared to swine IDV antisera. The explanation for this could be attributed to the difference in the amino-acid residues involved in the receptor binding domain that can affect the binding affinity and avidity with the glycan moieties, which renders bIDV with a broad receptor specificity and strong binding efficiency. Our glycan array experiments have shown that bovine IDV binds to more glycans than sIDV (unpublished data). Further, characterization of HEF mutants generated by the D/OK-RGS has demonstrated some key amino acid residues around the receptor binding pocket that play an important role in the antibody titer and cross-protection (unpublished data). In the case of ICV, the strain we used C/Victoria/2/2012 has not been studied in-vivo previously. An experimental study conducted in pigs infected with C/pig/Beijing/32/81 showed antibody responses in all the 6 animals which includes direct inoculated and contacted animals, whereas pigs infected with C/NJ/1/76 showed

seroconversion in only 2/6 animals which indicate the variability in antibody response between animals and between viruses [31]. C/Victoria/2/2012 is a human influenza C virus and so may exhibit a different viral ecology.

We analyzed the sialic acid distribution in the upper and lower respiratory tract of the guinea pig by HPLC, as IDV productively replicated in the upper and lower respiratory tract of the guinea pigs and also facilitated contact transmission with seroconversion in direct inoculated as well as contact sentinel animals [256]. Previous studies done by our lab (unpublished data) and others have shown that IDV, like ICV, uses the 9-O acetylated sialic acids as the receptor for the virus entry [238]. Our glycan array and in-vitro receptor binding specificity experiments have shown that IDV can bind to Neu5,9Ac2 as well as Neu5Gc9Ac in the same efficiency irrespective of α 2-3, or α 2-6 linkages, while ICV binds more efficiently to Neu5,9Ac2 (unpublished data). The results we obtained by HPLC of the guinea pig tissues, revealed that Neu5,9Ac2, as well as Neu5Gc9Ac, were present in the upper and lower respiratory tract. Taken together, the HPLC estimation of sialic acids, particularly the 9-O acetylated fraction of the sialic acids broadened our insights on IDV receptor biology.

Deep RNA sequencing of the nasal washes of sIDV revealed some amino acid mutations in the PB2 and NP segments, of which 6ST in PB2 is a bIDV like mutation. Whether these mutations are relevant to the adaptation of sIDV is yet to addressed. One important question, we need to explore is the disparity in the tissue tropism, particularly the lag in adaptation shown by the swine IDV group, as evidenced by the late shedding of the virus in the nasal washes and also in the tissue homogenates. It is interesting to note that HEF protein, mainly involved in the receptor binding, receptor destroying and

membrane fusion did not show any sequence changes in the guinea pigs compared to the respective inoculum. It is also said that NP and polymerase proteins also play an important role in the host adaptation. Structural modeling and comparison studies of these viral proteins from IDVs of two lineages is important as to what molecular determinants are really responsible for the differential phenotypic characteristics of these two lineages of influenza D viruses.

Overall, the comparative study of the IDVs with ICVs using the guinea pig model helped us to understand the differences in the replication kinetics of IDV compared to ICV. The lag adaptation exhibited by the sIDV is an interesting observation, whether this phenomenon is just found in guinea pigs or also in its natural host as in swine warrants further investigation. The unanswered questions need to be unraveled by planning new strategies to study the structural and molecular differences of HEF protein responsible for the receptor-mediated virus entry and other polymerase proteins responsible for the broader cell and host tropism. Several experiments are currently underway to fill the gaps and to would provide us new insights on the ecology and virus biology of these newly emerged influenza D viruses.

Chapter 5. Differential Replication of Four Influenza Virus Types in Porcine Respiratory Primary Epithelial Cells

Abstract

Influenza viruses are a group of respiratory pathogens that have evolved into four different types: A, B, C, and D. One common feature is that all four types are capable of replicating and transmitting among pigs. Human respiratory primary epithelial cell culture has been recently utilized to examine the replication and pathogenesis of influenza A viruses. However, little progress has been made in the development of the autologous cell culture system from swine to study influenza viruses. Here we describe the development of primary epithelial cells from swine nasal turbinates, trachea and lungs and determine their utility in the replication of four types of influenza viruses. Phenotypic characterization using immunocytochemistry coupled with flow cytometry analysis showed that cytokeratin was expressed at high levels in swine nasal turbinates, trachea, and lung cells, while the relatively low abundance of other epithelial cell markers (desmin, α-SMA, and vimentin) was detected. In addition, all three swine cells were found able to undergo polarization as measured by trans-epithelial electrical resistance (TEER) and expression of tight junction proteins including claudin-1, -3, Zona occludens protein -1 (ZO-1) and occludin-1. These results strongly suggest that the developed swine primary cells possess common characteristics of epithelial cells. Furthermore, sialic acid receptor profile analysis through lectin binding assay with Sambucus Nigra Lectin (SNA) and Maackia Amurensis Lectin II (MAL-II) demonstrated that three swine primary epithelial cells expressed higher levels of alpha 2,6 linkage sialic acid (SNA) than alpha 2,3 linkage sialic acid receptors (MAL-II). Finally, all three primary cells supported the replication of Influenza A, B, C and D viruses

to an appreciable level, but virus type-dependent replication kinetics were observed. Overall, these swine respiratory primary cells showed epithelial phenotype and are suitable for studying the comparative biology and pathobiology of four types of influenza viruses.

5.1 Introduction

The recurring changes in the evolution of influenza viruses demand close surveillance and more importantly, isolation and propagation of the virus from clinical samples to facilitate the host-pathogen interaction studies and to devise anti-viral therapeutics and prophylactic strategies. However, influenza virus propagation has been extensively supported by continuous cell lines for research as well as for commercial vaccine production. Several cell lines such as Madin Darby canine kidney cells (MDCK), Vero, MRC-5 and baby hamster kidney (BHK) cells, have been used extensively for influenza virus growth[18-20]. Primary cells have been utilized to study the influenza pathogenesis and virulence in the past [28, 260-263].

Primary cells have been widely used to study the physiological, biological and pathological mechanisms of the mammalian body. The earliest literature available in the primary cell culture was about the development and cultivation of the primary human amnion cells in 1957 [211]. The morphological evaluation of the human amnion cells in primary culture and its transformed variant (Strain FL) in continuous cell culture showed that transformed cell line showed multivesicular bodies and membrane limited particles. These morphological structures observed in the younger passages of the FL cells is assumed to be related to the transformation process [212] The morphological and genetic studies showed that the transformed amnion cells exhibited malignant properties, while the primary cells showed non-malignant characteristics [213, 214] Primary cells mimic the

physiological properties in-vivo and hence is the best in-vitro model to study the mechanistic details of the normal or diseased conditions of the body. Primary cells have a limited growth in-vitro and show considerable mitotic activity in the first 2-4 weeks with the mitotic index as high as 1.8%. [214]. A comparative study of the primary and transformed human cells in-vitro have shown that the nature of the growth, nutritional characteristics and metabolic profile of the primary and transformed cells vary [215]

Epithelial surfaces of our body are equipped with a highly sophisticated machinery with several different types of proteins molecules that play a crucial role in maintaining the homeostasis and cell polarity with each tissue. Among these, tight junction proteins are macromolecular complexes consisting of several membrane proteins, that are important for the cell-cell interactions and cell-extracellular matrix interactions and also for transcellular and paracellular transport and permeability.

Primary cell cultures were widely used for studying various animal and human viruses since the 1960s [217-221]. Species-specific primary cell cultures have been developed and used over the years [222-226] Primary cell cultures of swine-origin have been used for normal physiological and pathological studies of several infectious diseases [217, 227-231]. Primary swine respiratory epithelial cells have been used to study the immunological and pathophysiological aspects of several respiratory diseases including influenza [231]

Primary cell cultures had been an excellent in-vitro system to study the virulence and pathogenetic characteristics of the influenza viruses. Influenza studies using primary cell culture from humans and swine has been utilized for studying the virulence, and receptor binding specificities of the viruses from different host origin [21-25].Several

studies have been conducted on swine tracheal/bronchial/lung epithelial cells to study the pathogenesis and anti-viral responses at the transcriptional and translational level as the swine species share the most anatomical and physiological characteristics to humans [26-28] Pigs are the mixing vessels of influenza viruses and harbor receptors for both the avian and human influenza viruses. Recently, it was found that domestic pigs are susceptible to influenza B and C viruses [29-31]. Further influenza D has been initially isolated from the swine [4]. Here, we study the utility of the primary swine respiratory epithelial cells and the differential replication of four types of influenza compared to MDCK cells at $33^{o}C$ and $37^{o}C$. We found that A, B, C, and D type influenza viruses demonstrated a differential pattern of replication compared to the MDCK cells.

5.2 Materials & Methods

5.2.1 Isolation of primary respiratory epithelial cells

Swine nasal turbinates, trachea and lungs were collected from a day old gnotobiotic piglet. The tissues were washed with 1X PBS and cut into small pieces of $1mm^3$ and incubated with 800U of collagenase enzyme at $37^{o}C$ for 1.5 h. After incubation, the cells were strained through 70 um cell strainers and centrifuged at 500 g for 5 min. The cell pellet was washed two times with 1X PBS and then seeded on the collagenase coated flask. Cells were incubated at $37^{o}C$, 5% CO_2 and maintained using Dulbecco's Modified Eagle Medium (DMEM/F-12) (1:1) medium (Invitrogen, Grand Island, NY) supplemented with 5% FBS, 1% insulin-transferring selenium (ITS) supplement (Invitrogen, Grand Island, NY), 5 ng/ml mouse epidermal growth factor (EGF) (Invitrogen, Grand Island, NY), 100 U/ml Penicillin and 100 μg/ml Streptomycin.

5.2.2 Phenotyping of primary respiratory epithelial cells by Immunohistochemistry (IHC)

The primary respiratory epithelial cells were stained with antibodies against various epithelial, fibroblast and smooth muscle markers using the protocol as described previously [264]. Briefly, the cells were harvested from tissue culture flasks and washed with PBS. A cell suspension of approximately 10^6/ml was prepared and 100 µL of cell suspension was used for preparing cytospins (Cytospin 3; Thermo Shandon Inc.). Cytospins were air-dried, fixed in acetone for 7 min and stored at 4° C until staining. For staining, slides were equilibrated at room temperature and then rehydrated in PBS. The slides were blocked for non-specific protein binding with PBS containing 1 % goat serum. After PBS wash, cells were incubated in PBS containing 0.3 % hydrogen peroxide and 0.01% Sodium azide to block endogenous peroxidase activity. The presence of cytokeratin, vimentin, α-smooth muscle actin (ASMA) and desmin proteins was detected by immunohistochemical (IHC) staining using anti-cytokeratin mAb C6909 (IgG2a isotype), anti-vimentin mAb V5255 (IgM isotype), anti-ASMA mAb A2547 (IgG2a isotype) and anti-desmin mAb D1033 (IgG1). Monoclonal antibodies M9144 (IgG2a isotype), M9269 (IgG1 isotype) and M5170 (IgM isotype) were used as isotype-matched controls. Also, a negative control without primary antibody staining was also used. Cytospins were incubated with primary antibodies (Sigma-Aldrich, St. Louis, MO) for 1 h at 1 µg/ml concentration. After PBS wash, the slides were then incubated with 100 µl/slide of isotype-specific, biotinylated goat anti-mouse IgG2a, IgG1 or IgM antisera (1:2000 dilution; Caltag Laboratories) for 30 min and then by incubating with HRP–streptavidin solution for 30 min followed by the addition of Ready-to-use (RTU) diaminobenzene (DAB) substrate (Vector Laboratories). Cytospins

were counterstained with hematoxylin and examined under the light microscope. Images were taken at 20X magnification using an Olympus AX70 microscope.

5.2.3 Transepithelial electric resistance (TEER) and Indirect immunofluorescence assay (IFA)

Primary respiratory epithelial cells were differentiated and polarized on collagen-coated permeable supports in the cell culture media. About 1 million cells were seeded on a 24mm diameter (growth area 4.7 cm^2) collagen-coated transwell permeable filter (24 mm x 3 µm pore size). About 2 ml of the medium was added to the upper and lower chamber of the inserts in a six-well plate. The media was changed every other day and the polarization was measured by detecting the trans-epithelial electric resistance (TEER) by voltmeter in Ωs every 24 h. When the TEER stabilized between 2000-3000 Ω cm ^{2}, tight junction proteins were stained by IFA. For staining, washed and fixed the transwell filters with 4% paraformaldehyde, and permeabilized in 0.2% Triton X-100 in PBS. The filters were blocked using 5% normal goat serum, 0.2% Triton X-100, and the cells were incubated with rabbit polyclonal antibodies against claudin-1, -3, ZO-1, and occludin (5 µg/ml; Zymed), followed by secondary detection with goat anti-rabbit IgG-Alexa 488 (10 µg/ml) and counterstaining with propidium iodide. Normal rabbit IgG (5 µg/ml) was used as a negative control. Images were visualized using a confocal microscope.

5.2.4 Fluorescence-activated cell sorting (FACS)

Approximately, $5x10^5$ cells of swine primary respiratory epithelial cells were incubated with Biotinylated MAL-II specific for Sia2-3Gal and SNA (Vector laboratories) specific for Sia2-6Gal (final concentration 10 µg/ml) and the inhibitors n-acetyl neuraminic acid (NANA) and lactose for 1hr. After wash, cells were stained with Streptavidin-FITC

(1:200 dilution) for 30 min. Cells stained with only Streptavidin-FITC. served as negative control cells. Samples were analyzed using flow cytometry.

5.2.5 Viral replication kinetics

Approximately 2 X 10^5 cells/well were seeded on collagen coated 24 well plates and infected with 1) A/swine/Minnesota/2073/2008 (MN08) 2) A/swine/Iowa/0855/2007 (IA07) 3) A/California/04/2009 (CA04Pdm09) at 0.01 MOI; 4) B/Brisbane/60/2008 (BR08), 5) B/Florida/04/06 (FL06) at 0.1 MOI 6) D/Swine/Oklahoma/1334/2011, 7) D/bovine/Oklahoma/660/2013 (D660) 8) C/Johannesburg/1/1966 (C/JHB) at 1 MOI. Samples were collected at 24 h intervals until 120 h and were titrated on MDCK cells. Titers were calculated using Reed and Muench formula [265].

5.3 Results

5.3.1 Morphology and growth of swine primary respiratory epithelial cell

Swine primary respiratory epithelial cells derived from nasal turbinates, trachea, lungs formed small epithelial-like clusters by 18-24 h post-infection on tissue culture coated with type I collagen. The adhered cells appeared heterogeneous, however, 80 % of the cells attached were polygonal in shape with uniform dimensions with a cobblestone appearance. By 84h, the T-25 flasks reached 80-90% confluence. Swine primary respiratory cells can also grow on the normal tissue culture coated flasks but exhibit a lag time in reaching the confluence compared to the collagen I coated flask. The morphology of the cell monolayer was observed under the phase contrast microscope under 20 X (Figure 1). Ciliated cells can be seen in the primary cultures from nasal turbinates and trachea, which gradually disappears during subculture. There were around 5-10% fibroblast cells that appeared as spindle-shaped cells. The fibroblasts were removed by

treating the cell monolayer with 0.03% Trypsin for 3 min, every 48 h followed by PBS wash, and the addition of fresh media.

Primary respiratory epithelial cells were monitored daily till it reached confluence, with trypsin treatment, if needed to remove the fibroblasts. These respiratory primary cells are highly metabolic cells and hence the media gets exhausted and changes to acidic pH quickly. So, the media needs to be replenished daily at the stage of rapid cell growth. The cells can be sub-cultured in normal or collagen-coated tissue culture flasks. The sub-cultured cells attached to the to the plastic surface in 24 -48 h. The subcultures reached confluence in 5-7 days in a T-75 flask. At the later passages, some cells appeared irregularly sized, indicative of cell differentiation.
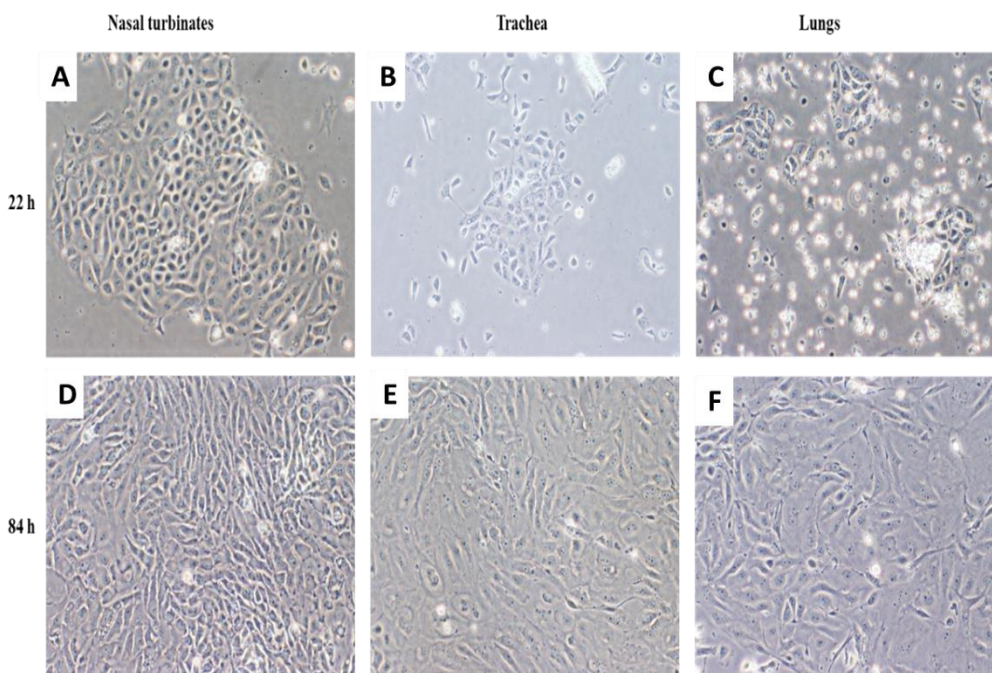
Figure 5.1. Morphology of the swine primary respiratory epithelial cells. Small clusters of epithelial-like cells appeared by 18-24h for nasal turbinates, trachea, and lungs (20X) (A-C). Confluent cell monolayer at 84h looked epithelial-like with cobblestone appearance with very less number of fibroblasts (D-F)

5.3.2 Primary swine respiratory epithelial cells are predominantly of the epithelial phenotype

To determine the phenotype, nasal turbinates, trachea and lung cells from passage 2, were stained with monoclonal antibodies targeting marker proteins such as cytokeratin (epithelial), vimentin (fibroblasts), desmin smooth and striated muscles), and α-smooth muscle actin (ASMA, smooth muscle), along with their isotype controls. About 95% of the nasal turbinate cells expressed cytokeratin indicative of their epithelial phenotype (Desmin, vimentin ASMA not shown). Less than 5% of the nasal turbinate cells expressed vimentin indicative of fibroblasts. (Figure 5.2). Similarly, 90 % of the tracheal and lung cells expressed cytokeratin, while 10% expressed vimentin in both the cells. Desmin and α-smooth muscle actin were not expressed in the earlier passages of these three types of cells.
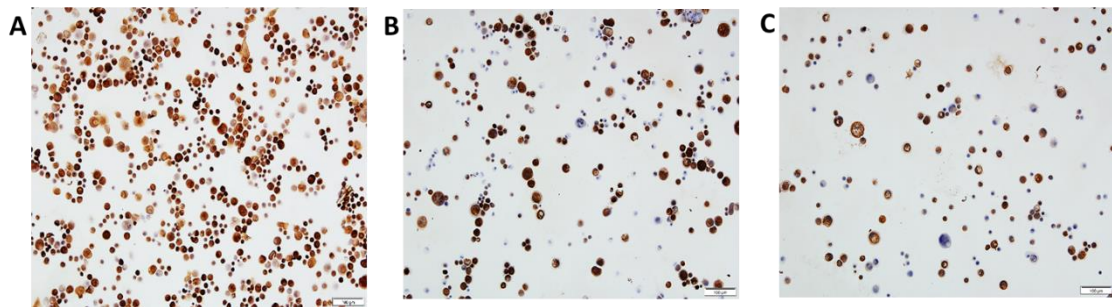
Figure 5.2. Immunocytochemical staining of the primary swine respiratory epithelial cells for cytokeratin. Nasal turbinates (A), trachea (B) and lungs (C) from cell passage no: 2 expressed brown stained cells positive for cytokeratin, indicative of their epithelial phenotype. Scale bars 100 µm.
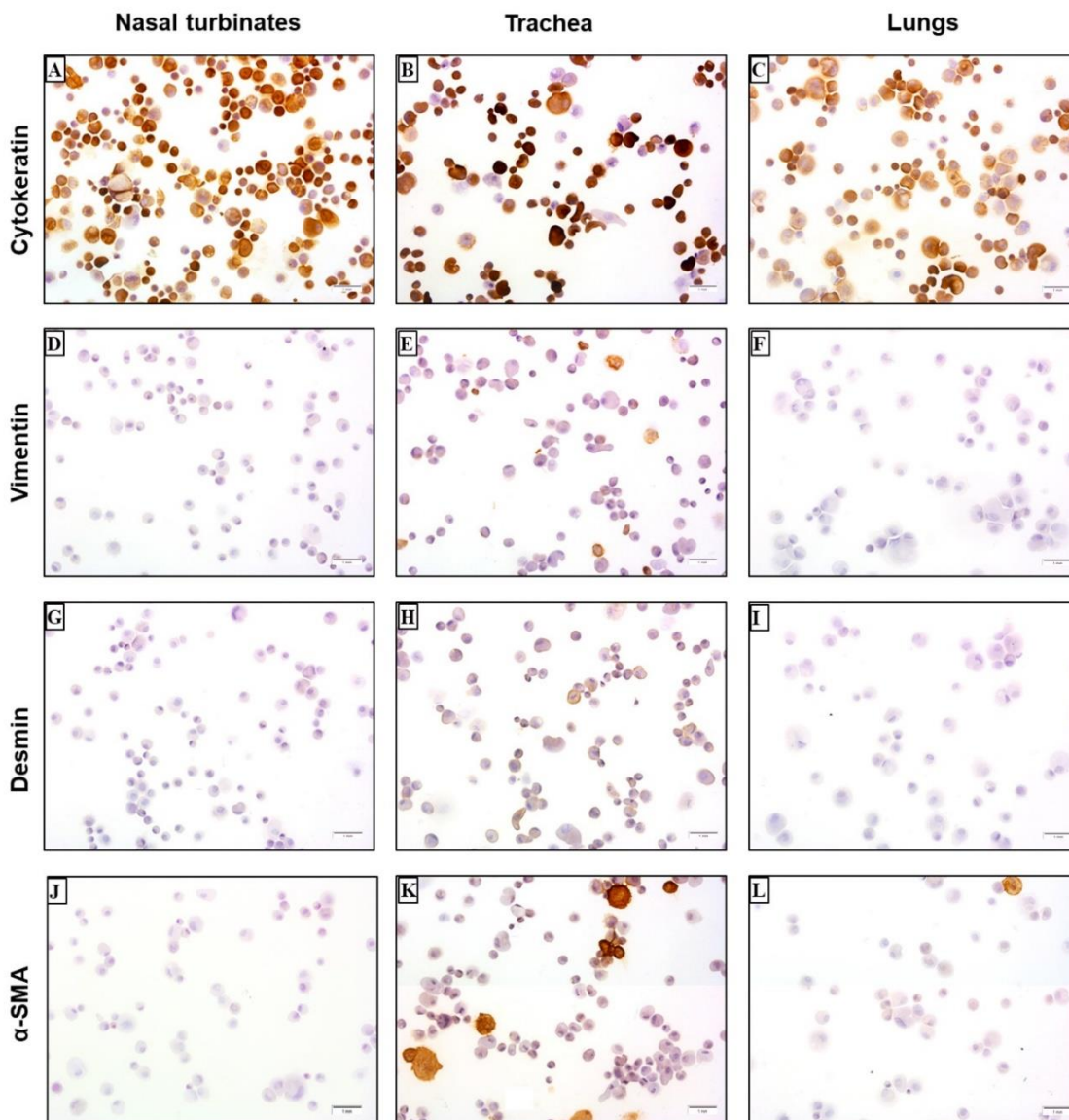
Figure 5.3 Immunocytochemical staining of primary swine respiratory epithelial cells at passage 7. Cytospins were prepared and stained with marker-specific monoclonal antibodies. Expression of cytokeratin (A-C), vimentin (D-F), desmin (G-I), and ASMA (J-L) for the nasal turbinate, trachea and lung cells. Brown colored cells indicate positive cells

All the three types of cells were serially passaged to determine the subculturing capacity, and immunocytochemical staining was done on alternate passages to know the phenotypic stability (Figure. 5.3). Nasal turbinate cells were able to grow to 18 passages,

while trachea and lung cells could grow until 20 passages. Nasal turbinate cells expressed cytokeratin and desmin at passage 17, and the intensity of staining for cytokeratin became faint in the late passages, showing that cell differentiation has occurred over time. Trachea cells expressed mainly cytokeratin, but there was some faint expression for ASMA. The major protein expressed by the lung cells was cytokeratin, however, there was a faint expression for vimentin, desmin and ASMA in cells from P17 (data not shown).

5.3.3 Swine primary respiratory epithelial cells can polarize, express tight junction proteins and produce TEER

Nasal turbinate, trachea and lung primary epithelial cells when grown on transwell inserts, expressed the tight junction proteins and polarized to form the apical and basolateral surfaces (Figure. 4B). The morphology of the cells at day 15 is shown in Figure. 4B. Tracheal primary epithelial cells polarized quickly to produce the maximum trans-epithelial electric resistance (TEER). Tracheal primary epithelial cells reached maximum TEER of 2600 Ω cm^2 by 12 days, and then decreased to 2500 Ω on day13. Lung cells showed a TEER with a maximum of 2240 Ω in 16 days and then decreased to 2040 Ω. Nasal turbinate cells polarized to reach the maximum TEER of 2030 Ωs by day 18 (Figure.4A). Swine intestinal epithelial cells (IPEC-1), another established cell line derived from the small intestine of a neonatal piglet was used as a positive control, along with a negative control (no cells on the transwell insert). Swine respiratory primary cells maintained a higher TEER than IPEC-1 cells after 5 days (Figure.5.4).
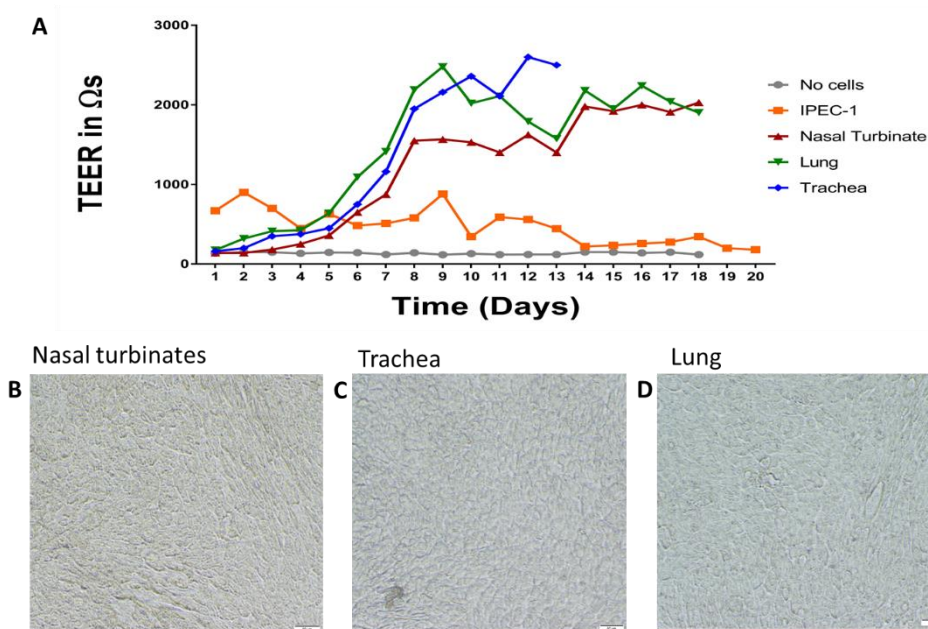
Figure. 5.4. Swine respiratory epithelial cells polarized to develop transepithelial electric resistance (TEER) which is measured in ohms and plotted against the function of time (4A); the morphology of the cells on the transwell filter inserts on day 15 shown in Figure (B-D).Scale bars 50 µm.

We tested the presence of the tight junction proteins by staining the polarized transwell filters by IFA. All the three types of cells were stained for tight junction proteins such as claudin-1, -3, occludin and zona occludens-1, along with the isotype antibody controls. Primary swine tracheal epithelial cells expressed claudin-1, -3, occludin, and zona occludens-1, with claudin-3 and ZO-1, showed some localization in the nucleus along with the cell-cell junctions. Primary nasal turbinate epithelial cells expressed tight junction proteins such as claudin 1, claudin-3, and occludin distributed mainly on the cell membrane/cell-cell junctions except for claudin-3 which showed some nuclear localization. Primary lung epithelial cells expressed only claudin 1 and claudin 3. Interestingly, claudin 3 was localized in the nuclei and cell membrane/junctions in the nasal

turbinate and tracheal cells and only seen in the cell membrane/junctions in the case of lung

cells (Figure.5.5)

	Nasal turbinates	Trachea	Lungs
Claudin 1	A	B	C
Claudin 3	D	E	F
Occludin	G	H	I
ZO-1	J	K	L
Isotype	M	N	O
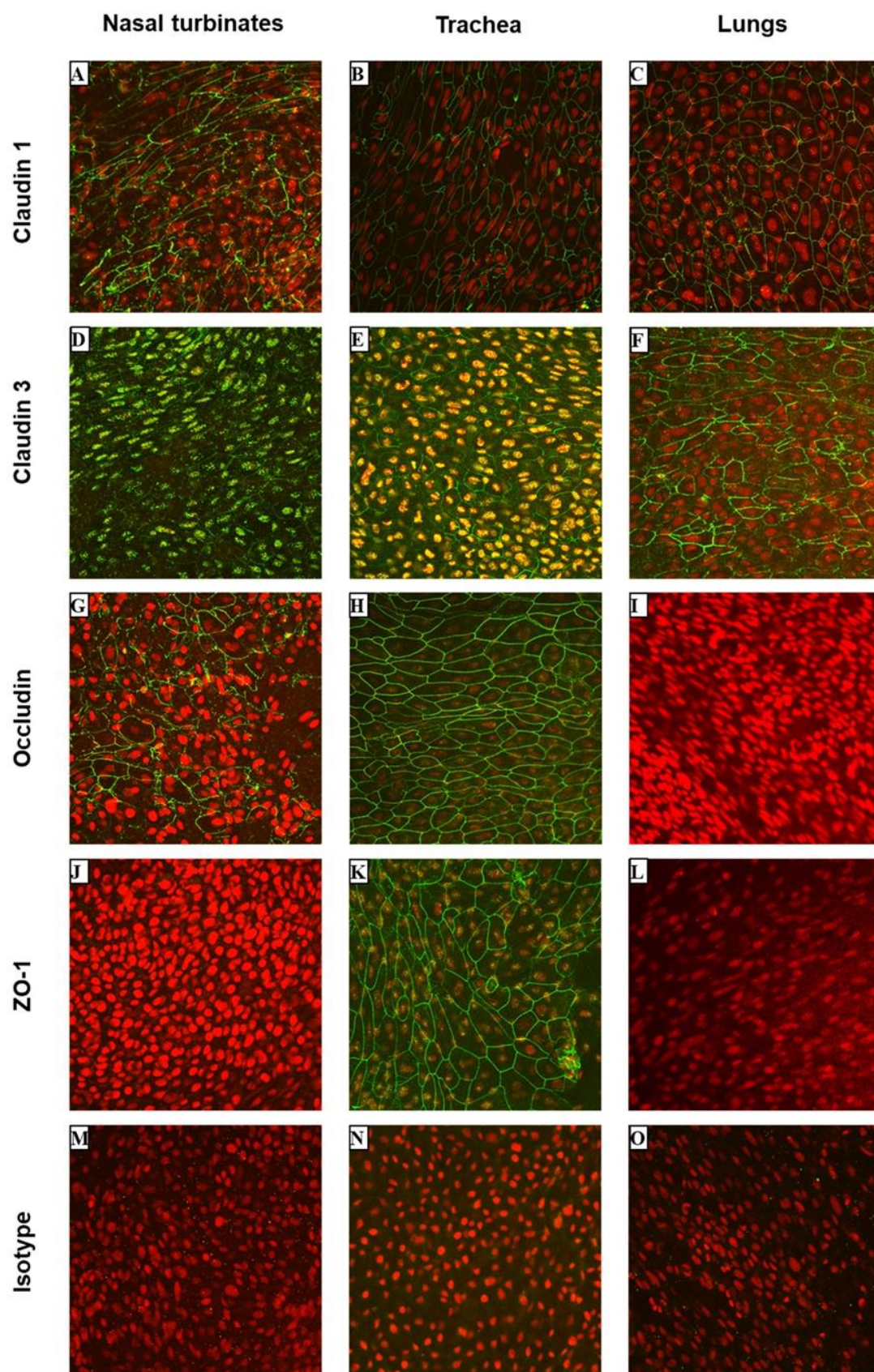
Figure. 5.5. Tight junction proteins expressed by the primary swine respiratory epithelial cells visualized by confocal microscopy. Polarized primary swine nasal turbinate, trachea and lung cells stained for tight junction proteins with Alexa Flour-488 tagged antibodies and nucleus counterstained by Propidium iodide (PI) were shown in the panels: claudin-1 (A-C), claudin-3 (D-F), occludin (G-I), ZO-1 (J-L) and isotype controls (M-O). Representative images were merged Z stack images.

5.3.4 Swine primary respiratory epithelial cells showed an increased expression of Sia2-6 galactose receptors

Biotinylated Sambucus nigra agglutinin (SNA) and Maackia amurensis lectin-2 (MAL-II) specific for Sia2-6Gal and Sia2-3Gal were used for staining the cells and the receptor specificity was measured by Fluorescent activated cell sorting. Swine primary respiratory epithelial cells derived from nasal turbinates, trachea, and lungs expressed Sia2-6Gal receptors than Sia2-3Gal receptors. Sia2-6Gal expression by nasal turbinates, trachea, and lungs was about 97.24, 83.73, and 78.14 % respectively. Sia2-3Gal receptors were comparatively very low showing 51.3, 9.1 and 4.3 % in nasal turbinates, trachea, and lungs respectively. The expression of the Sia2-6Gal and Sia2-3Gal decreased when the cells were treated with lactose (inhibitor for SNA) and n-acetylneuraminic acid (NANA-inhibitor for

MAL-II). The percentage of cells expressing Sia2-6Gal and Sia2-3Gal was given in Figure.5.6.
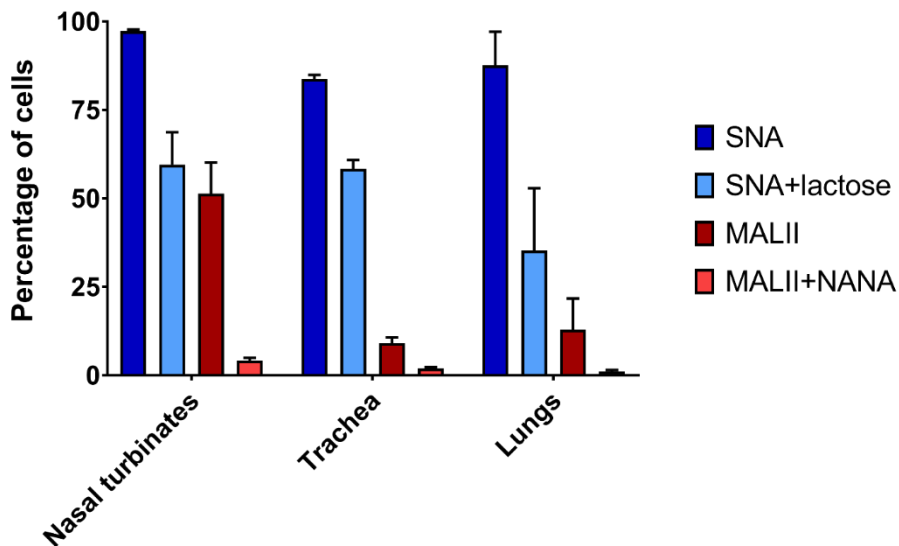
Figure.5.6. Expression of Sia2-6Gal and Sia2-3Gal in swine primary respiratory epithelial cells. Primary swine nasal turbinates, trachea and lungs were stained with SNA (Sia2-6Gal) and MAL-II (Sia2-3Gal). The percentage of cells showing expression of SNA and MAL II and their inhibition with lactose and NANA respectively were shown. Data shown here are representative of 2 independent experiments.

5.3.5 Primary swine respiratory epithelial cells support four types of influenza

Primary swine respiratory epithelial cells derived from nasal turbinates, trachea, and lungs were infected by all four types of influenza viruses affecting animal and human populations at 33°C and 37°C. We used IAV/MN08 (H1N1), IAV/IA07 (H3N2), IAV/CA04 Pdm09 (H1N1), IBV/FL06 and IBV/BR08, ICV/JHB1966, IDV/OK/2011 (swine-IDV) and IDV/660/2013 (bovine-IDV). MDCK cells were used as a positive control for each experiment.

Virus replication kinetics studies using these swine primary respiratory epithelial cells demonstrated that all the three influenza A viruses productively replicated in these primary cells at both 33°C and 37°C (Figure. 5.7). Swine IAV/MN08 (H1N1) replicated productively to give a better titer in tracheal and lung primary cells than the nasal turbinate cells. However, there is no significant difference in the titer for MN08 grown on these three different cells. Our data also showed that MN08 replicated more efficiently at 37°C compared to 33°C, again the difference is not significant. MDCK cells supported MN08 almost the same, with a slightly better virus titer at 33°C (Figure. 5.7).

On the contrary, swine IAV/IA07 H3N2 replicated in the nasal turbinates and lung cells better than tracheal cells and the temperature did not affect the virus kinetics, while the MDCK cells supported IA07 at both temperatures (Figure. 5.7) IAV/CA04 Pdm09 (H1N1) virus replicated to give a higher titer at 33°C than 37°C, in all the three types of the primary cells and in MDCK cells. Lung primary cells supported IAV/CA04 Pdm09 (H1N1) virus more than the nasal turbinates and tracheal cells (Figure. 5.7).

Influenza B virus kinetics on the three types of primary cells were also compared to MDCK cells (Figure. 5.7E-H). We used BR08 virus, which belonged to the Victoria lineage and FL06 which belonged to Yamagata lineage viruses. BR08 virus at 0.1 MOI infection on MDCK cells demonstrated the highest titer of 4.33 and 4.4 logs at 48 h at 33°C and 37°C respectively. Lung cells showed the highest titer of 4.96 logs at 72h at 33°C, while nasal turbinates and tracheal cells at both temperatures were lower (Figure. 7). The FL06 virus was supported in all the three primary cells. However, tracheal cells at 33°C at 48 h, showed the highest titer which is greater than the highest titer in MDCK cells.

The viral kinetics of ICV demonstrated similar kinetics shown by MDCK cells.

Among the influenza D viruses, both temperatures supported the virus replication in all the three different types of primary cells compared to MDCK cells, except D660 at 37ºC in nasal turbinate cells showed very low virus titer (Figure 5.7).

A Nasal turbinates at 33°C

B Nasal turbinates at 37°C

C Trachea at 33°C

D Trachea at 37°C

E Lungs at 33°C

F Lungs at 37°C

G MDCK at 33°C

H MDCK at 37°C

Legend:
IAV/CA04/H1N1pdm09
IAV/MN08/H1N1
IAV/IA07/H3N2
IBV/BR08
IBV/FL06
ICV/JHB1966
IDV/OK2011
IDV/OK2013
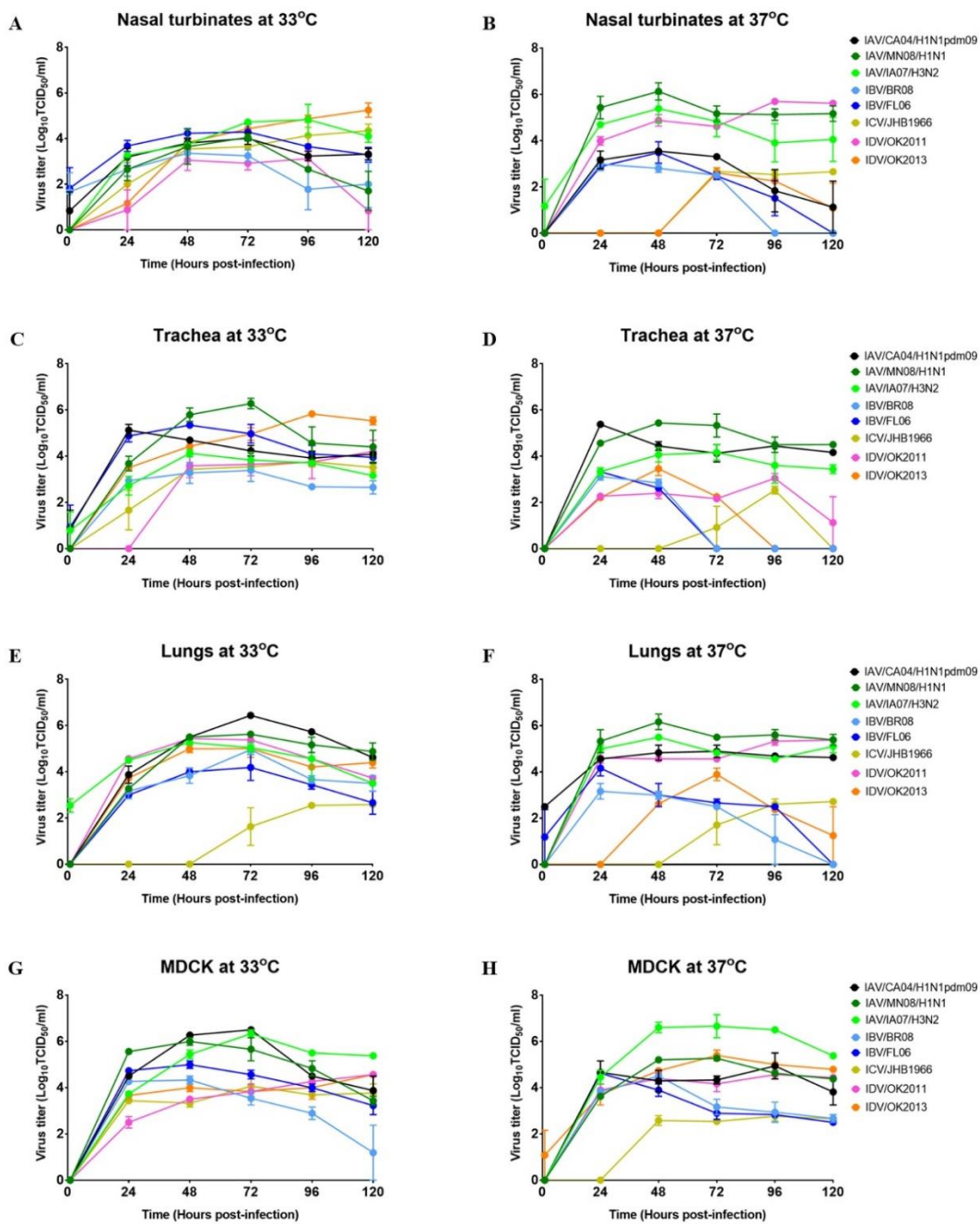
Figure. 5.7 Comparison of virus growth kinetics of primary swine respiratory epithelial cells to MDCK cells upon infection with IAV, IBV, ICV and IDV at 33°C and 37°C. IAV/CA04Pdm09/H1N1, IAV/MN08/H1N1, IAV/IA07/H3N2 were infected at 0.01 MOI; IBV/BR08 and IBV/FL06 were infected at 0.1 MOI; ICV/JHB1966, IDV/OK2011; IDV/OK2013 were infected at 1 MOI. Samples were taken at 24 h intervals and titrated on MDCK cells. Virus titers were expressed in $\log_{10}TCID_{50}$/ml.

5.4 Discussion

Epithelial surfaces of the body act as the first layer of defense, by providing anatomic, physiologic and immunologic barriers against potentially harmful pathogens and toxic substances. Digestive and respiratory tract epithelia are tuned to face a constant encounter with several bacteria/viral agents via ingestion/inhalation and can mount a comprehensive innate immune response. Several pathogens bind to the receptors of the epithelial cells for the cell entry. Hence, in-vitro epithelial cell culture models are key in studying the host-pathogen interactions. However, most of the continuous and transformed cell lines vary greatly in their genetical, phenotypical and physiological characteristics from their tissue origin and therefore the responses shown by these cells often comes with a limitation of reliability. Primary cell cultures closely mimic the physiologic and genetic conditions in-vivo and so are used to study host-pathogen interactions before in-vivo studies are attempted

Primary respiratory epithelial cell cultures from the pigs have been used widely to understand the interactions of epithelium with infectious agents. In this study, we developed primary cell cultures from the upper and lower respiratory tract such as nasal

turbinates, trachea, and lungs and characterized these cells to study its phenotype and to analyze its ability to polarize and express tight junction proteins. We also analyzed the sialic acid receptor expression of these cells to check its suitability to use in influenza studies.

Primary swine respiratory epithelial cells were heterogeneous cell populations when isolated from the respective tissues after collagenase digestion. Cells were derived from day-old gnotobiotic piglet, and hence assures a germ-free and healthy biological system, which can be used for studying a wide range of diagnostic and research applications. Primary swine respiratory epithelial cell population consisted of adherent polygonal-shaped cells with less than 5-10% of fibroblasts. Fibroblast cells were removed by treating the cells with 0.03% trypsin for 3-4 min. Immunocytochemical staining of these primary swine respiratory epithelial cells showed that these cells were predominantly of the epithelial phenotype. Immunocytochemistry was carried out on alternate passages to keep track of the phenotypic changes that could happen by cell differentiation during subculture. These primary swine respiratory epithelial cells derived from nasal turbinates, trachea, and lungs can be sub-cultured without much difference in their phenotype up to 18-22 passages before the cells become granular and detach from the surfaces.

Primary swine respiratory epithelial cells can undergo polarization and form functionally specialized apical, basolateral domains due to the specific distribution of the tight junction proteins. Polarization property has been displayed by epithelial cells, endothelial cells, leucocytes, neurons etc and therefore plays an important role in cell division, differentiation and growth, directional transport of molecules/cells and immune activation [266]. Primary swine respiratory cells grown on transwell inserts developed

multiple layers and contributed to the increase in TEER. All the three types of cells polarized and produced transepithelial electric resistance measured as high as 2000-3000 ohms (Ωs), more than the TEER measured for IPEC-1 cells.

We also investigated the tight junction protein expression of these primary epithelial cells. There are several types of proteins in the cell membrane that help in the integrity of the epithelium of which three types of proteins form the junctional complex in cell junctions which includes tight junction, adherens junction, and desmosomes. Tight junction proteins are transmembrane proteins and are located in the apical-most of the junctional complex [216] Tight junctions formed by the cell act as a semi-permeable barrier to the paracellular movement of cargo and act as a fence that connects the apical and basolateral domains of the plasma membrane. Tight junction proteins act as a multifunctional complex, critical for the epithelial and endothelial layers to form its distinct compartments in the body, of which most important is the regulation of several signaling and trafficking molecules required for the cell differentiation, polarity, and proliferation. We checked the expression of claudin-1, -3, occludin, zona occludens-1, E-cadherin, along with isotype antibody control to confirm the specificity. Our results showed that E-cadherin was not expressed in all the three-primary swine epithelial cells derived from nasal turbinates, trachea, and lungs. E-cadherin is a type of calcium-dependent adhesion molecule normally located on the basolateral side of the epithelial cells and forms adherens junctions [267]. The transmembrane tight junction proteins fall in 3 families: 1) single junctional adhesion molecule, (JAM); Crumbs protein homolog 3 (Crb3); coxsackievirus and adenovirus receptor (CAR) 2) triple {blood vessel epicardial substance (Bves), and 3) tetraspanning transmembrane proteins {Claudin, tight junction-associated MARVEL

proteins families, which include occludin, tricellulin, and MarvelD3. Of these, claudins are responsible for paracellular permeability [268]. Occludin is the first identified tight membrane protein with its C terminal region binding to ZO-1 which is associated with the actin cytoskeleton. There is site-specific variation in the expression of the tight junction proteins. Nasal turbinate and lung primary epithelial cells did not express ZO-1 while trachea expressed ZO-1 in abundance. Translocation of claudin 3 in the nucleus of the primary swine nasal turbinate cells was observed, while the tracheal cells expressed both in the nucleus and cell junctions and the lung cells showed claudin-3 only along the periphery/cell junctions. Claudin-1,-3 have been shown to have expressed in bronchioles and bronchi of mammals. Claudins have been detected in the distal lungs and claudin-3 was expressed predominantly by the alveolar epithelial cells, which completely agrees with our observation [268]. Claudin-3 was found to be expressed by the ciliated upper airway and type II alveolar epithelial cells [269, 270]. Claudin-3 is more expressed in type II alveolar epithelial cells than type I cells [271]. Although translocation of claudin from cytoplasm and nucleus has been demonstrated in several physiological conditions or cell lines, the exact mechanism has not been yet elucidated. Claudin-1,-2 has been expressed in the nucleus, without putative nuclear localization sequences. We speculate that the translocation of claudin-3 must have happened due to some interactions utilizing the PDZ domain [272]. The PDZ domain is formed by combining the first letters of three proteins postsynaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1).

Zona occludens-1 and occludin were usually found to have localized in the boundary of the apical and basolateral plasma membrane domains. Zona occludens-1 was

expressed in type II alveolar epithelial cells, when grown on transwell membranes after 4 days [273] Swine tissues were similar to humans anatomically and physiologically. Characterization studies in the human respiratory epithelium have been studied previously [274]. Human nasal epithelial cells in-vitro demonstrated mRNA expression for occludin, and, claudin 1 along with several other claudin proteins, while ZO-1 was expressed only in the human nasal mucosa in-vivo [274]. In our results, the primary swine nasal turbinate and lung cells did not express ZO-1. Just like human, ZO-1 was expressed in all non-human laryngeal epithelium such as mouse, rat, guinea pig, rabbit and pigs [275]. Larynx being the upper part of the trachea, may have the same tight junction/membrane protein composition Based on our results, primary swine tracheal epithelial cells showed ZO-1 expression, unlike respiratory epithelial cells derived from the upper and lower respiratory tract.

Influenza A viruses of avian and equine origin bind to Sia α 2-3 Gal receptor (MAL-II), while the human viruses bind to Sia α 2-6 Gal receptor (SNA) [25]. A study on eight selected influenza B viruses isolated from 1940-1990 showed that these viruses have binding preferences towards ganglioside, carrying lacto-series type I and II sugar chains with the Neu5Acα2–6Gal linkage, however, B/Gifu/2/73 strain bound to lacto-series gangliosides containing Neu5Acα2–6Gal and Neu5Acα2–3Gal linkages [232]. Glycan array to characterize the receptor binding specificities of influenza B viruses have shown that the Yamagata-like strains predominantly bound to α-2,6-linkage glycans while Victoria-like strains preferentially bound to both α-2,3- and α-2,6-linkage glycans and also explained a third group of viruses that bound to sulfated glycans, which are Victoria-like strains [233, 234]. Our results showed that all the three types of cells have more expression

for the Sia α 2-6 Gal receptor than Sia α 2-3 Gal receptor and hence supported both the FL06 (Yamagata) and BR08 (Victoria) lineage with slightly higher titer for the FL06 at both temperatures. Influenza C and D viruses bind to the 9-O-Ac-Neu5Ac-carrying oligosaccharide chains [276]. The lectin binding assay demonstrated that all the three types of the cells expressed more α 2-6 Gal receptors than α 2-3 Gal receptors. The expression of α 2-3 linkage was above 50% in nasal turbinates, while trachea and lung cells showed very low expression. It is already known that pig tracheal tissue expresses both Sia α 2-6 Gal and Sia α 2-3 Gal receptors [277]. Madin-Darby canine kidney cells have been widely used for influenza studies. Considering the SNA and MAL II expression of these primary cells derived from the swine respiratory tract, these cells can be an excellent platform to study the genetic and pathological aspects of influenza viruses, especially the swine-origin influenza viruses. Further, our viral replication kinetics data reflected the receptor distribution of these primary cells and demonstrated appreciable virus titers for all the four different types of influenza viruses at 33 and 37 ℃.

We compared the replication kinetics of IAV, IBV, ICV, and IDV in MDCK cells to that obtained in primary swine nasal turbinate, trachea and lung cells at both 33 and 37 ℃. Swine nasal epithelial cultures have been used for cystic fibrosis studies, but there was no literature available on influenza studies [231] Swine respiratory epithelial cells, especially of trachea and lung origin, have been used in influenza virus pathogenesis studies before [22-24, 26, 28] Another study that utilized a swine intestinal epithelial cell line, SD-PJEC for influenza production also described the peak titers obtained for different influenza A and B strains compared to MDCK [278]. This is the first study to our knowledge that describes the development of an autologous respiratory primary cell

cultures from both upper and lower respiratory tract and to test the utility to study the different types of influenza viruses. We used different MOIs for the different types of influenza viruses because 0.01 MOI failed to yield appreciable titers in these three types of primary respiratory epithelial cells used in the study. Previous studies have shown that pig respiratory tract possessed both Sia 2,6 and 2,3 linkage receptors, which is why they can support both avian and human influenza viruses.

The virus kinetics of IAV/CA04 Pdm09/H1N1 in the primary lung cells at 33°C is comparable to in MDCK at 33°C and the peak obtained is almost same for both the cells, however, it showed a lower titer in the nasal turbinate and trachea cells at 33°C. Overall, IAV/CA04 Pdm09/H1N1 grown at 37°C showed a lower peak and titer compared to that grown at 33°C except for the tracheal cells, where there is no significant difference noticed in the viral kinetics t 33°C and 37°C. IAV/CA04 Pdm09/H1N1 replicated to only 4 logs in SD-PJEC cells, which is a swine intestinal epithelial cell line [278] Similar to MDCK cells, IAV/MN08/H1N1 replicated to good titers in all the primary cells, except primary nasal turbinate cells at 33°C, with a peak observed between 48-72 h postinfection in all the primary cells. IAV/IA07/H3N2, the virus replicated to comparatively low titer in primary tracheal cells, while nasal turbinate and lung cells supported IA07/H3N2, to almost the same titers as MDCK cells at both temperatures. Influenza B viruses- FL06 replicated to an appreciable titer at all the time points, in all the primary cells at 33°C, while the virus titer was lost at 72 hpi in primary tracheal cells and at 120 hpi in nasal turbinates and lungs at 37°C.In the case of BR08, no replicable viruses were observed at 72 hpi in primary tracheal cells, while the nasal turbinates and lungs showed no titer at 96 hpi. Both BR08

and FL06 replicated to peak titer of above 4 logs, at 33 and at 37°C and showed an appreciable titer at all time points.

In the case of ICV, the replication was facilitated more at 33 °C, while a restricted growth was noticed, in all the three primary cells and MDCK cells at 37°C. ICV replication in primary nasal turbinate at 33°C, with a peak titer comparable to MDCK cells at 33°C, is justified considering ICV being a ubiquitous pathogen of the upper respiratory tract. At 33°C, ICV peak in tracheal cells is less than nasal turbinate and MDCK, but certainly better than lung cells. Among the two lineages used for the study, DOK, the swine lineage replicated well in all the three primary cells at both 33 and 37°C, with titers comparable to MDCK cells. However, the bovine lineage, D660 showed a restricted replication in the all the three primary cells with a low peak and lower titers at all the time points at 37°C, compared to the kinetics shown at 33°C. MDCK cells supported both the lineages at both temperatures in a similar fashion. In conclusion, we have characterized the primary swine respiratory epithelial cells, which are permissive to swine and human influenza viruses, in the same way as MDCK cells. These primary cells can be a suitable model to study all the four different types of influenza viruses which can help us in understanding more about the characterization and pathobiology of influenza viruses.

Chapter 6. Conclusions and future directives

Influenza D virus (IDV) is an emerging pathogen, initially isolated in 2011 from a swine with influenza-like illness, recently classified as a separate genus in the Orthomyxoviridae family. A little was known about the virulence, tropism, and pathogenesis of the IDV. So an animal model was required to study the genotypic and phenotypic characteristics of the virus. We chose to use guinea pig model as it was widely used mammalian model in the influenza studies, ease of handling, the susceptibility of the animals by contact transmission, and the physiological hyper-responsiveness of the guinea pig respiratory tract and anatomic similarity of bronchus-associated lymphoid tissue to humans [6, 209]

Our first study was proposed to meet the three objectives, which included the determination of the virus replication kinetics and also to check the transmission of IDV by contact and aerosol route. In the first study, we only used the bovine isolate of IDV (bIDV) for studying the viral replication kinetics, pathogenesis, and transmission. We found that bIDV replicated in the upper and lower respiratory tract of the guinea pig very productively shed the virus in nasal washes and were able to transmit the disease by contact. Further, the directly inoculated guinea pigs demonstrated seroconversion, confirming that guinea pigs can be a suitable model for studying the virulence and pathogenesis. We also conducted next-generation sequencing analyses on the viral populations derived from the lung homogenates and found some non-significant nucleotide mutations and some amino acid mutations.

Even though the first reports on IDV isolation came from swine, IDVs from bovine origin were the major strains isolated from the US and other parts of the world until 2017.

Further studies have shown that these two strains co-circulate and they differ in their genetic and antigenic characteristics, leading to the existence of two lineages of IDV. Preliminary characterization studies have shown that the IDV cannot cross-react with IAV, IBV or ICV antisera and can grow at 33°C, the temperature in the upper respiratory tract and at 37°C, which is the temperature of the lower respiratory tract. Hemagglutinin esterase fusion (HEF) protein of IDV shares 50% homology to ICV-HEF, which raises the question whether IDV can infect humans? Further IDV viruses can withstand exceptionally high temperature and low pH unlike other types of influenza viruses, which could be due to the peculiar characteristics of hemagglutinin esterase fusion protein compared to the hemagglutinin protein present in IAV and IBV. IDV HEF protein has a high-temperature tolerance as evidenced by the infectivity titer of 2 logs after treating the virus at 53°C for 2 h and resilience shown by IDV after exposure to pH as low as 3.0. Apart from this phenotypic characteristic, swine isolates of IDV have been increasingly isolated from Eurasia, particularly the recent isolates originated from the caprine and swine in China which set the stage for our second study.

The objective of the second study was aimed at determining any changes in the virulence and pathogenesis between the lineages as there is 97-99 % homology between the two lineages. Also, the structural and functional similarity of the ICV HEF with IDV HEF prompted us to include the ICV also in the comparative study. In the comparative study, we found that IDV of two lineages can replicate in the upper and lower respiratory tract also showed tropism towards the organs of the upper and lower respiratory tract including the soft palate which is located in between. On the contrary, ICV did not show any productive replication in the lower respiratory tract. However, ICV replicated in the

nasal turbinates, soft palate, and trachea, but not in the lungs. The inability of ICV to replicate in the lungs could be due to the intrinsic temperature sensitivity of the ICV HEF and polymerase. The second study provided us some insights about the pathogenesis and virulence of the IDV versus ICV and also the differential replication and shedding exhibited by the swine IDV compared to bovine IDV. Further, HPLC analysis of the guinea pig tissues and also the glycan array have shown that the 9-O acetylated sialic acids responsible for the IDV and ICV binding were present in the guinea pig upper and lower respiratory tract and soft palate, which agrees with our in-vitro studies.

Between the inoculum sequences of sIDV and bIDV, there were 5,4,3,23,7,14, and 4 amino acid changes in PB2, PB1, P3, HEF, NP, P42 and M segments respectively. To determine any possible adaptive mutations in sIDV vs bIDV , we did deep RNA sequencing of the nasal washes from the animals euthanized on 3 dpi and 5 dpi and compared to the viral genome of the respective inoculum sequences. Deep RNA sequencing analyses of the nasal washes from swine and bovine IDVs demonstrated no mutations in the HEF gene compared to the respective inoculum sequence in bIDV group, while sIDV infected animals showed some non-significant point nucleotide mutations in the HEF protein. In the case of sIDV, only PB2 (6ST) and P3 showed amino acid changes in the viral genome derived from the nasal washes on 3 and 5 dpi, while PB1, HEF, NP, and NS showed some random nucleotide changes without any amino acid mutations. In the bIDV group, PB2 gene showed amino acid mutations (E44D and N668K) in all the animals from 3 dpi and 5 dpi compared to the bIDV inoculum. The polymerase proteins such as PB2, PB1, and NP showed some non-significant point mutations, which were not observed in all the animals. P42 in the sIDV and HEF, P42, NS, P3 in the bIDV group, were the segments that did not

show any mutations. The second project gave us some valuable insights, but there are several gaps to be filled and so those areas will be pursued for future investigation. One such important question we have to study is the disparity in the tissue tropism, particularly the lag in adaptation shown by the swine IDV group, as evidenced by the late shedding of the virus in the nasal washes and also in the tissue homogenates. Structural modeling of the viral proteins belonging to two lineages is important as to what molecular determinants are responsible for the differential phenotypic characteristics of these two lineages of influenza D viruses.

In the third project, our objective was to establish an autologous primary cell culture system from the swine respiratory tract to test the different types of influenza, as pigs have been infected with all the four types of influenza either by natural or experimental infection. We successfully met the objective of establishing the cell culture system from nasal turbinate, trachea, lung and soft palate and have characterized the cells for the presence of cell markers such as cytokeratin, vimentin, desmin and α-smooth muscle actin. Influenza viruses bud through the apical domains of the epithelial cells, so we tested the ability of these cells to polarize and express tight junction proteins. All the three types of the cells originated from the nasal turbinate, trachea and lungs polarized to form the high TEER. The next objective was to determine the sialic acid receptors for its utility to study the influenza pathogenesis. Majority of the primary swine nasal turbinate, trachea and lung cells stained for SNA indicative of α2-6 sialic acids, while there was only a smaller population of the cells expressing MAL II (α 2-3 sialic acids). Our experiments have shown the utility of the cells to support all the four types of influenza with appreciable titers, so now we can take the study to next level by using these primary cells for some other

applications such as CRISPR-Cas 9 or transfection. Being non-transformed cell system, these cells hold a lot of potential to be used as a perfect in-vitro system to study the influenza pathogenesis as well as other respiratory tract infections. In future, we can use this system to develop air-liquid interface studies for studying various respiratory diseases affecting swine and humans. Another promising field is using these heterogeneous cell populations for developing three-dimensional organ culture. The phenotypic characteristics of these cells offer a great platform to study the host-pathogen interactions and also would help us to devise strategies for prophylactic and therapeutic measures.

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