INVESTIGATION OF INFLUENZA B VIRUS REPLICATION POTENTIAL IN SWINE PRIMARY RESPIRATORY EPITHELIAL CELLS AND PHYLODYNAMIC ANALYSIS OF EQUINE INFLUENZA A H3N8 VIRUSES

BY

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ABBREVIATIONS

cRNA: Complementary RNA

DMEM: Dulbecco’s Modified Eagle Medium

EIV: Equine Influenza virus

FBS: Fetal bovine serum

HA: Hemagglutinin

HAI: Hemagglutination-inhibition

HEF: Hemagglutinin-Esterase Fusion

IAV: Influenza A virus

IBV: Influenza B virus

ICV: Influenza C virus

IDV: Influenza D virus

M1: Matrix protein

M2: Ion channel

MDCK: Madin-Darby canine kidney cell

MOI: Multiplicities of infection

mRNA: Messenger RNA

NA: Neuraminidase

NEP: Nuclear export protein

Neu5,9Ac2: N-acetyl-9-O-acetylneuraminic acid

Neu5Ac: N-acetylneuraminic acid

NP: Nucleocapsid protein
NS1: Non-structural protein 1
NS2: Non-structural protein 2
PA: Polymerase acidic protein
PB1: Polymerase basic protein 1
PB2: Polymerase basic protein 2
qRT-PCR: Quantitative reverse transcription polymerase chain reaction
RBCs: Red blood cells
SAα2,3-Gal: sialic acid α2,3-galactose
SAα2,6-Gal: sialic acid α2,6-galactose
TNF-a: Tumour Necrosis Factor alpha
IFN-a: Interferon alpha
vRNA: Viral RNA
vRNPs: Viral ribonucleoproteins
TPCK: Tolylsulfonyl phenylalanyl chloromethyl ketone
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Influenza viruses are respiratory pathogens that cause significant mortality worldwide. The subtype of influenza A virus currently affecting worldwide equine populations is H3N8, leading to epidemics and transboundary pandemics. The individual gene segments of an isolate named A/equine/Montana/9564-1/2015 were phylogenetically characterized. BLASTn search revealed that the polymerase basic protein 1 (PB1), polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), and matrix (M) segments of this H3N8 isolate shared the highest percentage identity to A/equine/Tennessee/29A/2014 (H3N8) and the polymerase basic protein 2 (PB2), neuraminidase (NA), and non-structural protein (NS) segments to A/equine/Malaysia/M201/2015 (H3N8). Maximum likelihood phylogenetic trees constructed using H3N8 viral genomes of both equine and canine origin, established that A/equine/Montana/9564-1/2015 belonged to the Florida Clade 1 viruses.

A review of the current progress in influenza B virus (IBV) research includes the peer-reviewed articles that have been published on five IBV proteins. Understanding the biology of the proteins encoded in the IBV genome, and their differences from IAV, is crucial in for vaccine research. The current understanding of the evolutionary dynamics and epidemiology of IBV is discussed which helps inform vaccine strategies and prevent...
IBV infections. Although IBV is thought to primarily infect humans, there is evidence of sporadic infections occurring in other species. These studies of natural and experimental infections of IBV are discussed, followed by a summary of the current literature on the studies done in ferrets.

Swine are susceptible to infection by IBV, indicating that IBV could be a swine pathogen and natural reservoir for IBV. The second study looked at the replication kinetics of Victoria and Yamagata lineages of IBV in swine primary nasal turbinate, trachea and lung epithelial cells. Productive replication of B/Brisbane/60/2008 (BR08); B/Florida/04/06 (FL06); B/Hong Kong/286/2017 (HK17) and B/Utah/09/2014 (UT14) was seen at 0.01 and 0.1 multiplicities of infection. Peak titers at 33°C were greater than or equal to titers at 37°C for most of the experiments. HK17 (Victoria lineage) grew to the highest titers in nasal turbinate and lung cells. Lineage-specific differences in replication could not be observed in this study. Molecular analysis of will shed more light on the role of swine in the pathogenesis of IBV.
Chapter 1 Introduction and Objectives

1.1 Classification of Influenza Viruses

Influenza viruses belong to the family Orthomyxoviridae. Viruses that are a part of the Orthomyxoviridae family contain virions that are spherical or pleomorphic in shape. Influenza virions measure approximately 80-120 nm in diameter. They are enveloped viruses that contain a segmented negative sense RNA genome. Having diversified through evolution, influenza viruses are classified into types A, B and C. This classification is based on the antigenic differences in nucleocapsid (NP) and matrix (M1) proteins.

Influenza A (IAV) and B (IBV) viruses are predominantly responsible for annual influenza in humans. Characterized by symptoms such as cough, fever, muscle and joint pain, sore throat and a runny nose, influenza is estimated to be responsible for 290,000 to 650,000 global deaths annually. This respiratory disease can spread rapidly, impacting children (less than 59 months old) and the elderly (older than 65 years of age) to a greater degree (W. Paul Glezen et al., 1980; Van Voris, Belshe, & Shaffer, 1982). Both IAV and IBV are included in the seasonal flu vaccine for humans.

IAV is divided into subtypes based on the hemagglutinin (HA) and the neuraminidase (NA) proteins. 18 different HA subtypes and 11 different NA subtypes have been discovered. Isolated for the first time in 1940, Influenza B virus comprises of a single group of HA and NA antigens (Francis, 1940; Nerome et al., 1998). Unlike IAV which has been isolated from several wild and domestic animals such as swine, equine, seals, whales, mink and birds. IBV has been primarily associated with human infections (Cauldwell, Long, Moncorge, & Barclay, 2014). Despite humans being considered the
predominant host, there is evidence to show that other species such as pheasants, horses, dogs and seals can harbor IBV or possess antibodies specific to IBV (Bodewes et al., 2013; Chang CP, 1976; Kawano J, 1978).

Unlike IAV and IBV which have eight genome segments, Influenza C virus (ICV) has seven genome segments. The Hemagglutinin Esterase Fusion (HEF) protein is the only glycoprotein present at the surface of the ICV virion. It combines the receptor binding activity (mediated by HA in IAV and IBV), receptor destroying activity (mediated by NA in IAV and IBV) and fusion activity in ICV (Georg Herrler & Klenk, 1991). There are reports of ICVs in pigs, feral dogs and dromedary camels, but ICV is primarily known to cause a mild respiratory disease in humans (Guo, Jin, Wang, Wang, & Zhu, 1983; Ohwada et al., 1987).

Influenza D virus (IDV) was discovered in 2011 in a pig that showed influenza-like symptoms in Oklahoma. IDV shares approximately 50% homology to ICV, but differences in the receptor binding pocket of HE protein have modelled (Hause et al., 2013). While IDV’s prevalence is highest in cattle with this agricultural animal as a primary reservoir, antibodies to IDV have been found in pigs, goats and sheep (Quast et al., 2015). Not much is known about the potential zoonotic transmission of IDV to humans, but increased transmission in animal reservoirs indicates that IDV may pose a public health concern (Hause et al., 2013).

Influenza viruses have a wide host range, with some animal species being susceptible to infection by all four types of viruses. The segmented nature of influenza viruses enables reassortment when cells are infected simultaneously with different human and animal viruses. This is known as antigenic shift. Antigenic shift, which occurs in IAV can create
novel viruses to which humans are susceptible as they have no preexisting immunity. For example, the Hong Kong flu of 1968 which was caused by an antigenic shift of H2N2 strain to H3N2 strain, caused the death of approximately one million people worldwide. Antigenic drift is the accumulation of mutations in genes of influenza viruses that code for antibody-binding sites. These mutations allow the virus to escape preexisting immunity and cause widespread mortality. The outbreaks due to antigenic drifts are usually less severe than the pandemics associated with antigenic shifts. These periodic changes in the antigenic properties of influenza viruses cause its epidemic nature. Thus, an understanding of the biology, epidemiology and transmission of influenza viruses in various species will aid in the development of antiviral therapies to fight infections.

1.2 Structure and Genome Organization of Influenza Viruses

The influenza virion contains helical ribonucleoprotein complexes (also called vRNPs). vRNPs are a central part of the viral life cycle. The virion has a viral envelope that contains a core. Three transmembrane proteins are a part of the viral envelope: HA (Hemagglutinin), NA (Neuraminidase) and M2 (Ion channel) are present on the outside; M1 (matrix protein) is present beneath the membrane. HA is the major protein forming the viral envelope. It contains binding sites for neutralizing antibodies as well as the sialic acid receptor. Cleavage of HA into HA1 and HA2 is essential for virus infection. NA, which forms tetrameric spikes, is responsible for removing the sialic acid receptor enabling release of infectious viral particles which causes spread of the infection. M2, the minor component of the viral envelope, functions as an ion channel. When influenza viruses are budding from infected cells, the outer lipid layer is taken from the host’s
plasma membrane. M2 protein is instrumental in the release and exit of the vRNPs from the M1 matrix (Nayak, Balogun, Yamada, Zhou, & Barman, 2009).

The viral core has vRNPs that contain negative stranded vRNAs and nucleoprotein (NP). Nuclear export protein (NEP) is present in small amounts along with the three polymerase proteins named PB1, PB2 and PA. This forms the viral RNA polymerase complex (Wu & Voth, 2003).

IAVs encode 10 polypeptides that are derived from 8 viral RNA segments (Figure 1.1). In IAV and IBV, segments 1, 3, 4 and 5 encode one protein per segment: PB2, PA, HA and NP proteins. Polymerase subunit 1 (PB1) is encoded on segment 2 in all influenza viruses. Some strains of IAV also have a PB1-F2 protein which contains 87 amino acids, in a +1 alternate reading frame. PB1- F2 has been implicated in killing host cells (W. Chen et al., 2001). While segment 6 encodes only the NA protein (Bouvier & Palese, 2008) in IAV, in IBV it encodes the NB protein too in a -1 reading frame. NB protein corresponds to the M2 protein in IAV. IAV expresses the M2 ion channel from segment 7, while IBV encodes its BM2 protein in a +2 alternate reading frame. NS1 interferon-antagonist protein is expressed from segment 8 in both IAV and IBV. The NEP/NS2 protein is expressed by mRNA splicing from this segment (Bouvier & Palese, 2008).

ICV is similar in genome organization to IAV and IBV; it consists of only seven segments as HEF combines the functions of HA and NA. ICV uses Neu5,9Ac2 (N-acetyl-9-O-acetylneuraminic acid), while IAV and IBV utilize Neu5Ac (N-acetylneuraminic acid). While human IAV preferentially binds to α2-6 linkage, avian IAV binds to α2-3 linkage, and swine IAV can binds to both α2-6 and α2-3 linkages. ICV’s HEF recognizes Neu5,9Ac2 receptor regardless of its linkage to the next sugar. The HEF also has esterase
activity which cleaves acetyl from the C9 position of sialic acid-containing glycans (G. Herrler et al., 1985).

**Figure 1.1** Diagrammatic illustration of the genome organization of influenza A virion (Horimoto & Kawaoka, 2005)

### 1.3 Replication of Influenza Viruses

A diagrammatic illustration of the replication of influenza viruses is shown below (Figure 1.2). The first step of virus replication is the virus entry. The HA protein undergoes cleavage into HA1 and HA2 by serine proteases. HA1 contains the receptor binding and antigenic sites, whereas HA2 mediates fusion of the viral envelope with the cell membrane. Viruses often undergo amino acid changes at antigenic sites, and these minor changes can accumulate in a process known as antigenic drift. After the virus is endocytosed, a low pH environment in the endosome facilitates a conformational change in the HA, and the fusion peptide is exposed. The viral envelope is then merged with the
endosomal membrane through the fusion peptide. After endocytosis, the M2 protein ion channel causes the flow of ions from the endosome to the interior of the virus. This causes a disruption in the protein-protein interactions within the cell allowing the release of the ribonucleoprotein structure from the membrane (M1) protein (Helenius, 1992). This allows RNPs to be released from the matrix of the virus into the cytoplasm of the cell.

Viral proteins and RNPs are imported into the nucleus where the influenza viral RNA synthesis occurs. vRNA is the template to synthesize mRNA templates for viral protein synthesis. At the same time, vRNA is used as the template to make complementary RNA (cRNA) intermediates from which the RNA polymerase can transcribe more vRNA. The poly(A) tail is encoded in a negative sense vRNA, which is then transcribed into the positive sense mRNA. mRNA capping occurs through a process called “cap snatching”. PB1 and PB2 proteins are involved in taking 5’ capped primers from pre-mRNA transcripts of the host cell, to initiate viral mRNA synthesis.

Viral mRNA is then exported and translated into viral proteins in a similar fashion to the host mRNA. vRNA is exported to the cytosol with the help of M1 and NEP/NS2 proteins. Within the RNA complex, vRNA and NP interact with M1. NEP also interacts with M1 to mediate the export of the M1-RNP complex via nucleoporins in the cytoplasm. HA, NA and M2 envelope proteins are synthesized from the mRNA of viral origin on the ribosomes. They are moved into the endoplasmic reticulum, where folding occurs. Following folding, the mRNA is trafficked into the Golgi apparatus for post-translational modifications.
In the next step, the viral RNA is packaged and assembled. There is evidence that packaging signals exist on vRNA segments which helps the full genome incorporate into the viral particles efficiently. After assembly, influenza virus budding occurs. Influenza HA protein is a driving force for virus to pinch-off from the cell surface of infected cells with the help of influenza M2 protein. Virus particles are released through the sialidase activity of NA protein. Through NA’s activity of breaking down mucins in the respiratory tract, it aids in virus infectivity.

Figure 1.2 Replication of influenza viruses
(Arias et al., 2009)

1.4 Epidemiology and evolution of EIV and IBV

Influenza A viruses infect animals and humans, whereas influenza B viruses mainly infect humans. Influenza A virus is considered to be the ancestor of all other influenza viruses. H7N7 and H3N8 are the subtypes that are related to equine influenza. H3N8 is
the only subtype that currently circulates in equines. Isolated for the first time in 1963, A/eq/Miami/63 is considered the prototype EIV isolate (Waddell, Teigland, & Sigel, 1963).

The oldest H3N8 EIV isolates, dated from 1963 to 1988, were grouped under the pre-divergence lineage. Differences in the sequence analysis of the HA gene led to the classification of EIV into the Eurasian and American lineages. The American lineage evolved into the Argentinian, Kentucky and Florida sub lineages (Lai et al., 2001). The Florida sub lineages can be further classified into Clade 1 and Clade 2 viruses. Clade 1 viruses include representative strains such as A/eq/South Africa/04/2003-like and A/eq/Ohio/2003-like viruses, and has caused outbreaks in Mongolia, India and China. Clade 2 viruses are representative of strains like A/eq/Richmond/1/2007-like viruses. Interestingly, some isolates from Europe have changes in their amino acid sequences which makes them similar to Clade 2 lineages viruses (Bryant et al., 2009).

Studies on the phylogeny of EIV from different parts of the world have demonstrated the evolution of EIV. Surveillance studies in North America detected the presence of EIV in Canada and West Indies. A 56.6% morbidity rate of EIV amongst equine respiratory outbreaks was seen in Ontario, Canada during five different outbreaks (Diaz-Mendez et al., 2010). EIV antibodies have also been reported from horses and donkeys in the Leeward Islands of West Indies (Bolfa et al., 2017). A high prevalence of antibodies against EIV has also been reported in Brazil, with movement and aggregation of animals possibly causing high rates of transmission (Favaro et al., 2018).

The genetic evolution EIV in France showed that American and Eurasian lineages prevailed till 2003, but after 2005 the Florida sub lineage Clade 2 was dominant
EIV isolates were detected in Ireland, Sweden and the UK as well (Back et al., 2016; Gildea, Quinlivan, Arkins, & Cullinane, 2012). Several of these studies highlight the possibility of reassortment as a mechanism for EIV evolution.

Asia had EIV infections in several countries such as China, Mongolia, India and Japan (Qi et al., 2010). Amino acid changes in the antigenic sites were seen in Asian strains of Florida sub lineage clade 2. Differences between the isolates and the vaccine strain were due to amino acid substitutions in A, B and C antigenic regions (Qi et al., 2010). Thus, the emergence of newer strains due to mutations is an issue worldwide. The high number of circulating strains and lack of cross-protection highlights the importance of regular surveillance and evolutionary profiling of this respiratory disease.

Unlike IAV which is divided into several subtypes based on the antigenicities of hemagglutinin (HA) and the neuraminidase (NA) proteins, IBV does not have any subtypes. In the 1980s, IBV diverged into two genetically and antigenically distinct lineages named Victoria and Yamagata after B/Yamagata/16/88 and B/Victoria/2/87, which were the original isolates (Rota et al., 1990). The Victoria lineage viruses belong to the B/Brisbane/60/2008 genetic clade (Group 1), whereas the Yamagata lineage viruses are classified into two clades. Group 2 in the Yamagata lineage is represented by B/Brisbane/3/2007, and Group 3 is represented by B/Bangladesh/3333/2007. In seasons where IBV was predominant or co-circulating alongside influenza A virus (IAV), Victoria lineage predominated 64% of seasons, whereas Yamagata lineage predominated 36% of seasons (Caini et al., 2015)
Victoria-like viruses were prevalent worldwide in the 1980s, but the Yamagata emerged worldwide in the 1990s. In some parts of Asia, Victoria-like viruses circulated in the late 1990s, but this lineage re-emerged globally in 2001. Phylogenetic trees constructed from the HA genes showed that the Victoria lineage comprised of a single tree-trunk that had several side branches which circulated for short periods of time indicating that it undergoes greater positive selection, whereas Yamagata lineage has multiple clades circulating at the same time (Vijaykrishna et al., 2015). Additionally, Yamagata viruses bind predominantly to α-2,6-linked sialic acid host receptors whereas Victoria viruses bind to both α-2,3 and α-2,6 binding capacities.

Molecular analysis of viral genes provides an insight into the epidemic potential of different strains. Phylogenetic analysis, in particular, enables the identification of circulating strains belonging to different lineages (Lindstrom et al., 1999; Rota et al., 1990). performing a molecular analysis of viral genes provides an insight into the epidemic potential of different strains by allowing the identification of amino acid residues that contribute to antigenic drift. Phylogenetic analysis also enables the identification of circulating strains belonging to different lineages (Lindstrom et al., 1999; Rota et al., 1990).

Epidemiological studies from several European countries like Belgium, Spain, Finland and Israel highlight the reassortment of circulating strains. Phylogenetic analysis of circulating strains reveal an evolutionary pattern of IBV that has alternatingly dominant lineages and their co-circulation during a particular season.

1.5 Objectives
The aim of the study in chapter 2 was to characterize an equine H3N8 virus isolate. This isolate was obtained from a 3-year-old unvaccinated horse having respiratory disease, from Montana, USA, in 2015. Phylogenetically distinct clades/lineages of EIV have been co-circulating globally, posing a challenge in the selection of vaccine strains. Understanding the evolutionary profile of EIV helps estimate the phylogenetic diversity and distribution of equine influenza viruses. Phylogenetic characterization of all the eight gene segments of this isolate was done using currently available sequences of both canine and equine H3N8 subtypes in the influenza virus resource database.

Current progress in IBV research is discussed in Chapter 3. Findings on IBV proteins such as the polymerase proteins, nucleoprotein, non-structural protein 1, matrix protein and hemagglutinin proteins are discussed in brief. The epidemiology of Victoria and Yamagata lineages of IBV are discussed, and the evolutionary dynamics of IBV. Non-human IBV infections are discussed: both experimental and natural.

The ability of IBV to infect swine primary respiratory epithelial cells derived from the nasal turbinates, trachea and lung cells of a day-old gnotobiotic piglet was investigated in chapter 4. The effect of different multiplicities of infection and temperature on IBV replication was also looked at in this study. The replication of B/Brisbane/60/2008 (BR08) and B/Hong Kong/286/2017 (HK17) belonging to the Victoria lineage, and B/Florida/04/06 (FL06) and B/Utah/09/2014 (UT14) belonging to the Yamagata lineage was studied at 33°C and 37°C. The three primary cells, and MDCK cells which were used as a positive control, were infected with the four strains of IBV at 0.01 and 0.1 multiplicities of infection. This study aids in a greater understanding of the pathogenesis of IBV in swine.
Chapter 2  Phylogenetic Analysis of a Sporadic Isolate of Equine Influenza A H3N8 from an Unvaccinated Horse in 2015

2.1 Abstract

Equine influenza, caused by the H3N8 subtype, is a highly contagious respiratory disease affecting equid populations worldwide and has led to serious epidemics and transboundary pandemics. This study describes the phylogenetic characterization of a recently isolated H3N8 virus from a nasal swab obtained from a sporadic case of natural infection in an unvaccinated horse from Montana, USA. BLASTn search revealed that the polymerase basic protein 1 (PB1), polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), and matrix (M) segments of this H3N8 isolate shared the highest percentage identity to A/equine/Tennessee/29A/2014 (H3N8) and the polymerase basic protein 2 (PB2), neuraminidase (NA), and non-structural protein (NS) segments to A/equine/Malaysia/M201/2015 (H3N8). Phylogenetic characterization of individual gene segments, using currently available H3N8 viral genomes, of both equine and canine origin, further established that A/equine/Montana/9564-1/2015 belonged to the Florida Clade 1 viruses. This study provided us insights about the evolutionary relationship and in vitro cross-species infectivity of A/equine/Montana/9564-1/2015 (H3N8) virus.
2.1 Introduction

Equine influenza epizootics, which affect horses, zebra, mules, and donkeys all over the world, are characterized by an acute dry cough, high body temperature, mucopurulent nasal discharge, lethargy and anorexia (Cardwell, Newton, Wood, Geraghty, & Ellis, 2000; Lai et al., 2001; Mumford et al., 1998; Timoney, 1996). Vaccination failure, the mobility of unvaccinated horses and insufficient quarantine measures are major predisposing factors for equine influenza occurrence in places where EIV is endemic (Cullinane & Newton, 2013). Equine influenza viruses are type A viruses and the major subtypes affecting horses include H7N7 and H3N8 which were previously designated as equine 1 and equine 2 viruses. However, currently, H3N8 (Kitchen, Kehler, & Henthorne, 1963) is the only subtype affecting worldwide equine populations. A1/equine/Prague/56 was the first H7N7 prototype isolated from Prague, Czech Republic, which affected horses in Sweden and Eastern Europe around 1955–1956 (Scholtens, Steele, Dowdle, Yarbrough, & Robinson, 1964; Sovinova, Tumova, Pouska, & Nemec, 1958; Webster, 1993). Equine H3N8 prototype, A2-equine/Miami/63 was originally isolated from Miami, Florida in 1963, as an upper respiratory disease manifestation in imported animals from Argentina (Kitchen et al., 1963; Waddell et al., 1963; Wilson, Bryans, & Doll, 1965). Since 1979, only H3N8 variants have been found in circulation, even though both H7N7 and H3N8 co-circulated in the equine populations in earlier times (Cullinane & Newton, 2013; Daly et al., 1996; Webster, 1993). It is believed that homo/intra and hetero/inter-subtype reassortments have played a crucial
role in the evolution dynamics of equine influenza virus (EIV), where intra-subtype reassortments enhanced virulence in EIV H3N8, and inter-subtype reassortments led to the extinction of EIV H7N7 (Murcia, Wood, & Holmes, 2011). Compared to H7N7, H3N8 affects horses of all ages, irrespective of vaccination coverage. Previous studies on the phylogeny of EIV from different parts of the world have clearly demonstrated the evolution of equine influenza viruses, particularly the H3N8 subtype, which is the prominent subtype in circulation (Alves Beuttemmuller et al., 2016; Bountouri, Fragkiadaki, Ntafis, Kanellos, & Xylouri, 2011; Fougerolle et al., 2017; Olguin Perglione, Golemba, Torres, & Barrandeguy, 2016; Perglione et al., 2016; Rash et al., 2017). EIV H3N8 has been causing major transcontinental pandemics across the world and has crossed over species to successfully establish in the canine host since 2004 (Crispe, Finlaison, Hurt, & Kirkland, 2011).

The equine H3N8 subtype diverged genetically into American and Eurasian lineages around the 1980s, however American lineages have been circulating in Europe and vice-versa (Bryant et al., 2009; Daly et al., 1996). No cases of Eurasian lineages have been reported since 1994 (Murcia et al., 2011). Around 1990, the American lineage diverged antigenically and genetically into Kentucky, South America and Florida sub-lineages with the Florida sub-lineage circulating predominantly (Lai et al., 2001). Around the early 2000s, the Florida sub-lineage diverged into two clades: Clade 1 (FC1) and Clade 2 (FC2) (Bryant et al., 2009; Xie, Anderson, Daramragchaaa, Chuluunbaatar, & Gray, 2016; Yamanaka et al., 2016). Even though the clade separation was not so pronounced for the H3N8 isolates in 2002–2003, the divergence became indisputable for strains isolated after 2005 (Paillot, 2014). Since 2007, all Asian and European isolates have been found to
belong to FC2, whereas North American isolates belong to FC1, but the reverse can also occur, owing to the increased international mobility of the animals for races and exhibition. Such activities can lead to loose transmission bottle-neck and mixed infections, which may contribute to vaccination failures (Hughes et al., 2012; Murcia et al., 2011).

Cross-species transmission of EIV in canines, characterized by the complete genome transfer of equine H3N8, occurred initially in the United States, followed by the UK and Australia in the early 2000s (Gonzalez et al., 2014; Kirkland, Finlaison, Crispe, & Hurt, 2010). Canine influenza, caused by H3N8, has been associated with fever, cough, suppurative pneumonia and per-acute death (Crawford et al., 2005; Daly et al., 2008; Kirkland et al., 2010; Murcia & Wood, 2011). Both cats and calves have been experimentally infected with EIVs and the infected animals showed clinical symptoms, virus shedding and demonstrated contact transmission (Lin et al., 2010; Su et al., 2014; Tu et al., 2009). Interestingly, two H3N8 strains of equine origin, with close relation to the European H3N8 EIVs, were isolated from pigs in China during 2004–2006 (Tu et al., 2009). Experimental infection of humans with EIV H3N8 was demonstrated more than 50 years ago by the National Institute of Health and it was found that humans are susceptible to H3N8 with clinical manifestations (Kasel, Alford, Knight, Waddell, & Sigel, 1965; Minuse, McQueen, Davenport, & Francis, 1965; Morens & Taubenberger, 2010). Further, serological evidence of EIV H3N8 has been reported in humans with occupational exposure indicating its zoonotic potential (Larson et al., 2015). These evidence of cross-species transmission and broad host spectrum raise the possibility of zoonosis and thus could have a serious implication on public health.
Previous outbreaks in vaccinated and unvaccinated horses in Europe in 1979 and 1989 showed that EIV can undergo a rapid rate of antigenic drift and cause vaccination failures (Van Oirschot, Bruin, de Boer-Luytze, & Smolders, 1991; van Oirschot, Masurel, Huffels, & Anker, 1981). The Office International des Epizooties (OIE) vaccine recommendations remain unchanged since 2010, and mandates to include both clades—FC1 represented by A/equine/South Africa/04/2003-like or A/equine/Ohio/2003-like viruses and FC2 represented by A/equine/Richmond/1/2007-like viruses. The OIE also recommends periodical update of the vaccine strains, based on the epidemiological survey results (Paillot et al., 2016). While, vaccination is the only solution to effectively control and prevent EIV outbreaks, the continuous antigenic drift between the strains poses a serious threat for necessary protection, especially for horses involved in cross country events (Elton & Cullinane, 2013). Continued surveillance and reporting of EIV from different countries is of utmost importance, to ensure the effective coverage of circulating strains by the current vaccine strains and thereby boosts herd immunity.

The aim of this study was to phylogenetically characterize all eight segments of an equine H3N8 virus isolate, obtained from a 3-year-old unvaccinated gelding showing respiratory disease, from Montana, USA, in 2015. Phylogenetically distinct clades/lineages of EIV have been co-circulating globally, undergoing gene reassortments, thereby posing a serious challenge in the selection of vaccine strains. Hence, understanding the evolutionary profile of EIV is imperative to estimate the phylogenetic diversity and distribution of equine influenza viruses. Our phylogenetic analysis inferred that the polymerase basic protein 1 (PB1), polymerase acidic (PA), and nucleoprotein (NP) segments of A/equine/Montana/9564-1/2015 clustered with
A/equine/Tennessee/29A/2014 and hemagglutinin (HA), polymerase basic protein 2 (PB2), neuraminidase (NA), matrix (M), and non-structural (NS) segments clustered with A/equine/Malaysia/M201/2015 and A/equine/Tennessee/29A/2014, both belong to Clade 1 (FC1) viruses of Florida sub-lineage (Bao et al., 2008).

2.2 Materials and Methods

A BLASTn search analysis, optimized for highly similar sequences (megablast), was conducted for all the eight gene segments of A/equine/Montana/9564-1/2015 (H3N8) (Y. Chen, Ye, Zhang, & Xu, 2015). Both canine and equine nucleotide sequences of H3N8 subtype were acquired from the influenza virus resource (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database, accessed 5 January 2018) and phylogenetic analyses were performed using MEGA 7.0. Nucleotide sequences were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) and the evolutionary history of each segment was inferred by constructing maximum likelihood trees, using the best nucleotide substitution models, suggested by the ‘test for best DNA/ protein fitness’ in MEGA 7.0 (Bao et al., 2008; Edgar, 2004). The best nucleotide substitution models inferred for maximum likelihood trees for the gene segments were general time-reversible with gamma distributed with invariant sites (GTR+G+I) for PB2, PB1, NP; Tamurai–Nei with gamma distributed with invariant sites (TN93+G+I) for PA, HA; general time-reversible with gamma distributed (GTR+G) for NA; Hasegawa–Kishino–Yano with gamma distributed with invariant sites (HKY+G+I) for M; Hasegawa–Kishino–Yano with gamma distributed (HKY+G) for NS (Hasegawa, Kishino, & Yano,
1985; Rodriguez, Oliver, Marin, & Medina, 1990; Tamura & Nei, 1993). All nucleotide positions containing gaps and missing data were partially deleted and very strong branch filters were applied to run the analysis. For each taxon, the bootstrap value was determined from 1000 replicates to verify the tree topology.
Figure 2.1 Phylogenetic analysis of the HA sequence. The evolutionary history of hemagglutinin (HA) nucleotide sequence of A/equine/Montana/9564-1/2015 was inferred using the maximum likelihood method by MEGA 7.0, with a bootstrapping of 1000 replicates. The analysis involved 161 sequences of both canine and equine origin, and canine H3N8 strains were shown as a collapsed branch. Different phylogenetic groups of equine influenza virus (EIV) were color-coded and marked. Orange filled square = pre-
Figure 2.2 Phylogenetic analysis of the NA sequence. The evolutionary history of neuraminidase (NA) nucleotide sequences of A/equine/Montana/9564-1/2015 was inferred using the maximum likelihood method by MEGA 7.0, with a bootstrapping of 1000 replicates. The analysis involved 161 sequences of both canine and equine origin, and canine H3N8 strains were shown as a collapsed branch. Different phylogenetic groups of EIV were color-coded and marked. Orange filled square = pre-divergence; Green filled circle = Eurasian; pink filled diamond = American lineage (Kentucky + Argentine). Bootstrap values are shown at each node and A/equine/Montana/9564-1/2015 is highlighted in red.
Argentina); Blue filled triangle = Florida sub-lineage Clade 2; Red Open square = Florida sub-lineage Clade 1. Bootstrap values are shown at each node and A/equine/Montana/9564-1/2015 is highlighted in red.

Figure 2.3 Phylogenetic trees of PB2, PB1, NP, NA, M and NS sequences. Phylogenetical analyses of nucleotide sequences of PB2, PB1, PA, NP, M, and NS segments of A/equine/Montana/9564-1/2015 were inferred using the maximum likelihood method by MEGA 7.0 [48], with a bootstrapping of 1000 replicates. The analysis involved EIV sequences of both canine and equine origin, and canine H3N8 strains were shown as a collapsed branch. Subtrees, involving Florida sub-lineage Clade 1 (Red open square) and Florida Clade 2 (Blue filled triangle) viruses, are shown. Bootstrap values are shown at each node and A/equine/Montana/9564-1/2015 is highlighted in red.
2.3.1 BLAST Analysis

The sequences of the eight segments—polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M1 and CM2), and non-structural proteins (NS1, NS2)—were obtained in full length, and sizes were 2341, 2274, 2151, 1762, 1572, 1410, 982, and 838 nucleotides (nt) respectively. Only the NA segment was nearly complete, with 20 and 30 nt missing at the 5′ and 3′ terminal regions, respectively. The sequences were submitted to GenBank and accession numbers were assigned: MG198996 (PB2), MG198997 (PB1), MG198998 (PA), MG198999 (HA), MG199000 (NP), MG199001 (NA), MG199003 (M), and MG199003 (NS). The BLASTn search analysis, optimized for highly similar sequences (megablast) for all the eight segments of A/equine/Montana/9564-1/2015, demonstrated the highest percentage identity to the H3N8 strains of equine origin and not to any of the H3N8 strains of canine origin, indicating the absence of any species cross over and intra-subtype genetic exchange. A/equine/Montana/9564-1/2015 demonstrated equally high percentage identity to six H3N8 strains isolated from Tennessee in 2014 (A/equine/Tennessee/4A/2014, A/equine/Tennessee/27A/2014, A/equine/Tennessee/28A/2014, A/equine/Tennessee/28B/2014, A/equine/Tennessee/29A/2014, A/equine/Tennessee/30A/2014) and A/equine/Malaysia/M201/2015. Considering the sequence similarity between these highly homologous strains, we used A/equine/Tennessee/29A/2014 as a representative strain for BLASTn results and phylogenetic analysis. A/equine/Montana/9564-1/2015 showed a percent identity score of
99.78 (PB1), 99.81 (PA), 99.54 (HA), 99.87 (NP), and 99.8 (M) to 
A/equine/Tennessee/29A/2014, while the PB2, NA and NS segments demonstrated 
percent identities of 99.83, 99.93 and 100 respectively, to 
A/equine/Malaysia/M201/2015. The HA segment of A/equine/Montana/9564-1/2015 also 
showed percent identity scores of 99.23% and 99.18% with two strains from Malaysia, 
A/equine/Malaysia/M201-2/2015 and A/equine/Malaysia/M201-1/2015. Overall, 
A/equine/Montana/9564-1/2015 shared the highest percent identity with two equine 
H3N8 viruses of Florida Clade 1 sub lineage, A/equine/Tennessee/29A/2014 and 
A/equine/Malaysia/M201/2015.

2.3.2. Phylogenetic Analysis

To estimate the evolutionary history of A/equine/Montana/9564-1/2015, we performed 
phylogenetic analyses of all the eight gene segments, with the currently available 
sequences of both canine and equine H3N8 subtypes in the influenza virus resource 
database (Bao et al., 2008; Chang et al., 2007). The EIV sequences we analyzed, included 
the sequences from pre-divergence, Eurasian and American lineages (Kentucky and 
South American sub-lineages) and Clades 1 and 2 of the Florida sub-lineage. The total 
number of canine and equine sequences of H3N8 subtype, used to construct the 
phylogenetic trees for each segment 
were PB2 (124), PB1 (130), PA (120), HA (161), NP (122), NA (161), M (121) 
and NS (121). For the phylogenetic analyses, we used a fixed number of nucleotides for 
each gene
segment: PB2 (2280), PB1 (2274), PA (2151), HA (1695), NP (1497), NA (1410), M (982), and NS (838).

The evolutionary history of the eight viral gene segments obtained by the phylogenetic analyses was in complete agreement with the percent identity score obtained by BLASTn, clustering with equine influenza A H3N8 viruses and not with canine H3N8 viruses. The evolutionary history of HA and NA segments of A/equine/Montana/9564-1/2015, was shown as complete trees, in which the different phylogenetic groups of EIV were color-coded and grouped to describe the pre-divergence, Eurasian and American lineages (strains from Kentucky and Argentina/South American sub-lineages), the Florida sub-lineage and Clade 1 and Clade 2 viruses (Figure 2.1 and Figure 2.2). The phylogenetic tree of the NA segment of our isolate, clustered with A/equine/Malaysia/M201/2015 and A/equine/Tennessee/29A/2014 (Figure 2.2). A similar cluster can be seen in the phylogenetic tree of HA. The HA segment of A/equine/Malaysia/M201/2015 was not available in the database; however, the HA segment of A/equine/Montana/9564-1/2015 clustered with another strain from Malaysia, i.e., A/equine/Malaysia/M201-1/2015 along with A/equine/Tennessee/29A/2014 (Figure 2.1). The oldest H3N8 EIV isolates, dated from 1963 to 1988, were grouped under the pre-divergence lineage and the isolates from Europe and Asia from 1989 to 1994 were grouped under the Eurasian lineage (Fougerolle et al., 2017). Isolates, dated from 1990 to 2001, that belonged to the Kentucky and Argentina/South American sub-lineages, were grouped under American lineage, along with Florida sub-lineage Clade 1 and Clade 2 viruses, isolated from 2005 to present (Bryant et al., 2009; Lai, Rogers, Glaser, Tudor, & Chambers, 2004; Paillot, 2014).
2.4 Discussion

EIV H3N8 epidemics have been reported worldwide, on a large scale, since the first reported case of H3N8 occurred in Florida in 1963. Just like any other influenza epidemic in the past, EIV epidemics occur in vaccinated and immunologically naïve populations, in particular, H3N8 EIV epidemics can happen in horses of all ages, regardless of vaccination status (Alves Beuttemuller et al., 2016; Guthrie, 2006; Paillot & El-Hage, 2016; Pusterla et al., 2015). Vaccination breakdowns have been associated with EIV outbreaks in the past, as documented in thoroughbred yearlings in Kentucky, and several other parts of the world, including France and Ireland and horses imported into South Arabia and Japan (Pusterla et al., 2015).

According to the OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition, the year 2015 witnessed an increased activity in H3N8 EIV cases, reported from 46 premises over 23 states in the USA (Office International des Epizooties (OIE) Conclusions and Recommendations, 2017). In 2016, 30 confirmed EIV cases were reported from 16 states (Office International des Epizooties (OIE) Conclusions and Recommendations, 2017). Unfortunately, there is no vaccination data available on these outbreaks from the USA. In 2015–2016, EIV outbreaks were reported in other parts of the world, such as Ireland, Sweden, and the UK. The outbreaks reported in the UK occurred in unvaccinated animals. As per the report, no cases have been reported from Asia and South America during this period (Office International des Epizooties (OIE) Conclusions and Recommendations, 2017). The OIE report also concluded that EIV
H3N8 viruses isolated from the USA in 2016 were homologous to the isolates from 2015 and belonged to the Florida sub-lineage Clade 1 (FC1). On the other hand, viruses detected from the UK in 2015–2016 were Florida sub-lineage Clade 2 (FC2) viruses. The data we obtained from phylogenetic analysis were in complete agreement with this observation.

The BLASTn analysis of all viral genome segments, except PB2, NA, and NS, showed the highest percentage identity to six H3N8 EIV strains isolated from Tennessee in 2014. Considering the percentage identity between these six highly homologous strains, we used A/equine/Tennessee/29A/2014 as a representative strain for our phylogenetic analysis. It was very difficult to conclude that this sporadic infection occurred from the contaminated premises, as there were no other horses on the premises reported to have EIV infection, according to available information to us. We also checked the influenza virus database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database, accessed 5 January 2018) to gather information on the EIV isolates reported from North America from 2000 to 2017. It is noteworthy that, only 1/69 isolates (A/equine/Montana/9233/2007(H3N8)) have been reported from Montana during the past 17 years. According to the report from the OIE Expert Surveillance Panel, EIV cases occurred in the USA in 2015–2016 (Office International des Epizooties (OIE) Conclusions and Recommendations, 2017). However, we could not find any documentation of new recent EIV isolates from North America during 2015–2017, which indicates either the new isolates are homologous to the old known strains or lack of reporting of the new isolates. The phylogenetic analysis, using the maximum likelihood algorithm, used all the available full-length sequences of H3N8 currently available in the
database, of both equine and canine origins. The phylogenetic reconstruction of the individual genes was in complete agreement with the BLAST results and A/equine/Montana/9564-1/2015 clustered with FC1 viruses, originated in the USA. The fact that PB2, NA, M, NS and HA segments clustered with A/equine/Malaysia/M201/2015 (H3N8) is interesting, because Malaysia has been EIV free since 1977 and this outbreak in 2015, which started as a sporadic case occurred in a time frame, concurrent with the import of four racehorses (http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=19160, accessed 5 January 2018). Historically, EIV originating from Asia and Europe, usually clusters with Florida Clade 2 viruses. On the contrary, A/equine/Malaysia/M201/2015 clustered with Florida Clade 1 viruses, together with A/equine/Tennessee/29A/2014. It is difficult to trace the time and means of introduction of A/equine/Malaysia/M201/2015 in the USA. It is worth noting that the sequences currently available in the database for A/equine/Malaysia/M201/2015 are only for four segments, i.e., PB2, NA, M, and NS. Only HA sequence is available for A/equine/Malaysia/M201-1/2015. The BLASTn percent identity score of M segment showed 99.8% and 99.59% to A/equine/Tennessee/29A/2014 and A/equine/Malaysia/M201/2015, respectively. Had there been all eight segments available, A/equine/Montana/9564-1/2015 might have shown a higher sequence identity to A/equine/Malaysia/M201/2015 than Tennessee 2014 strains. Overall, A/equine/Montana/9564-1/2015 viral segments clustered with Florida Clade 1 viruses, A/equine/Tennessee/29A/2014 and A/equine/Malaysia/M201/2015 and hence, belong to the Florida Clade 1 group of equine influenza viruses.
Overall, this study provided us insights about the evolutionary relationship of A/equine/Montana/9564-1/2015 (H3N8) virus. A comprehensive genome-scale analysis of new isolates is essential to understand the molecular evolution and phylodynamics of EIV, which in turn would help in the strategic selection of vaccine strains, effective surveillance, and control. Antigenic and genetic variations caused by evolutionary processes play a critical role in determining the dynamics of host range and tropism of influenza viruses. In vivo studies are needed to evaluate the cross-species transmissibility of EIV H3N8 and its ability to cause infections and respiratory diseases in other mammalian hosts, including humans.
Chapter 3  Current Progress in Influenza B Virus Research

3.1 Introduction

Influenza viruses belonging to the *Orthomyxoviridae* family, are important viral pathogens that can cause significant respiratory disease. Influenza A virus (IAV) and influenza B virus (IBV) are the two types of influenza viruses that co-circulate in humans.

IBV has a genome chain length of 14,639 nucleotides. Understanding the biology of the 11 encoded proteins of IBV encoded in this genome, and their differences from IAV, is crucial in for vaccine research. This review discusses the peer-reviewed articles that have been published on five IBV proteins in brief. Two lineages of IBV co-circulate in most regions around the world, prompting the usage of the quadrivalent vaccine containing two IBV strains every flu season to prevent vaccine mismatch. This review summarizes the current understanding of the evolutionary dynamics and epidemiology of IBV which help inform vaccine strategies and prevent IBV infections.

Isolated for the first time in 1940, IBV is thought to be primarily a human pathogen. However, there is evidence that other non-human species are susceptible to IBV infections as well. This review discusses these studies of natural and experimental infections of IBV, followed by a summary of the current literature available on the studies done in ferrets.
3.2 IBV Proteins

The eight segments of the IBV genome encode 11 proteins [4]. There are three RNA-dependent RNA polymerase subunits (PB1, PB2 and PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein (M1) and two nonstructural proteins (NS1 and NS2). IBV also has two unique proteins NB and BM2, which are encoded by segment 6 and segment 7, respectively (Hatta, Goto, & Kawaoka, 2004). This review discusses the polymerase proteins, NP, NS-1, M1 and HA proteins in brief.  

3.2.1 Polymerase Proteins of Influenza B virus (PB1, PB2 and PA) 

PB1, PB2 and PA proteins are trimeric complexes that, along with the nucleoprotein (NP), are essential for expression of the viral genome (Jambrina, Barcena, Uez, & Portela, 1997). These viral RNA-dependent RNA polymerases play a role in the transcription and replication of the IBV genome. Each polymerase protein has a nuclear localization function. While PA and PB1 associate with the cytoplasm and the nucleus, the PB2 protein locates exclusively to the nucleus (Deng et al., 2011). Understanding the assembly and nuclear transport for the polymerase complex would provide new avenues to develop therapeutics to fight IBV infections. An RNA synthesis assay was developed that could be adapted to screen for polymerase inhibitors (Reich, Guilligay, & Cusack, 2017). Small molecule compounds have also been identified that inhibit the growth of IBV by inhibiting the binary complexes formed between the polymerase units (Muratore et al., 2012). During the initiation of viral mRNA synthesis, the RNA polymerase protein produces capped RNA fragments by cleaving the host mRNAs (Wakai, Iwama, Mizumoto, & Nagata, 2011). Studies done between the polymerase proteins of influenza A virus and
IBV showed that there is an incompatibility between the subunits of both types of viruses for RNA transcription and replication (Crescenzo-Chaigne, Naffakh, & van der Werf, 1999; Iwatsuki-Horimoto et al., 2008; Wunderlich et al., 2010). Analysis of the crystal structure of IBV’s PB2cap shows affinity for m7GDP and GDP, unlike IAV’s PB2cap which prefers m7GDP (Liu et al., 2015). In the IBV PB2cap.m7 GTP complex, the methyl group is missing, and the guanine and ribose moieties are inverted around the long axis of the base. These properties allow IBV to bind to a wider range of cap binding structures compared to IAV (L. L. Xie et al., 2016).

Phylogenetic analysis of 20 IBV sequences revealed lineage-specific amino acid substitutions in the three polymerase proteins. The three segments have maintained separate lineages, which helps preserve the viral fitness (Dudas, Bedford, Lycett, & Rambaut, 2014). Additionally, a functional association seems to exist between PA, NP and M proteins as well as the PB1 and PB2 proteins (Hiromoto et al., 2000). Differences in the topology of the PA gene phylogenetic tree showed that the polymerase genes don’t co-evolve as a unit; there is significant reassortment between the viruses (Hiromoto et al., 2000).

3.2.2 Nucleoprotein (NP)

NP, which is a critical component of the ribonucleoprotein complex of IBV, plays a role in the transcription and replication of the viral genome. This basic protein consists of 560 amino acids and has a molecular mass of 62kDa. Containing a head, body and tail loop, the RNA-binding groove is found at the end of the tetrameric structure (Ng et al., 2012). Several structural differences exist between the nucleoprotein of IBV and IAV. The N-terminus of IBV’s NP, which is longer than IAV by 50 amino acids is not critical for the
viral RNA’s transcription or replication. It was shown that the nuclear protein of IBV could not be replaced by the NP of IAV. N-terminal deletions of amino acid residues in the positions 51 and 69 in B/Ann Arbor/1/66 virus NP did not have any impact on the nuclear accumulation of the protein. (Stevens & Barclay, 1998). The nucleoprotein of IBV inhibits the replication and polymerase activity of IAV in a dose dependent manner. A study conducted later looked at the role of the NP protein during viral infection instead of protein expression like the previous studies. Contradicting results were seen, showing that virus attenuation followed mutations in the viral NP protein. It was also seen that mutation in the N-terminal region had an effect of the transcription and replication of the virus during infection (Sherry, Smith, Davidson, & Jackson, 2014).

3.2.3 Non-Structural Protein 1 (NS1)

IBV induces the ISG15 protein during infection, which blocks the conjugation of ISG15 protein. The RNA-binding domain of the NS1 protein is responsible for this inhibition (Yuan & Krug, 2001). When an embryonated chicken egg was infected with a mutant virus that lacked the NS1 gene, viral growth was not supported. The mutant NS1-IBV induced IFN-β in human lung epithelial cells in higher levels than the wild-type virus, indicating that NS1 protein has a function as a viral IFN antagonist (Dauber, Heins, & Wolff, 2004). IBV’s NS1 protein can complement IAV and result in infection when IAV’s NS1 is deleted. In the absence of other viral proteins, it was seen that IRF-3 nuclear translocation and IFN-β promoter was still activated by the N terminal and C terminal independently (Donelan et al., 2004). dsRNA-binding regions exist on IBV NS1 protein that form the binding epitope, which consists of conserved tracks of basic and hydrophilic residues (Yin et al., 2007). An important study showed that the limited host
range of IBV is caused in part due to the species-specific binding of the NS1 protein. ISG15 protein, which is ubiquitin-like, is induced by the alpha/beta-interferon, and plays an important role in antiviral activity against influenza. NS1 of IBV binds to human and non-primate ISG15, but not mouse or canine ISG15. This shows the possibility of IBV infecting non-human primates, but not mice or canines without adaptive mutations (Sridharan, Zhao, & Krug, 2010). Further studies in other animals to show the role of NS1 in ISGylation would provide further insight in the biology of IBV and help in the development of more accurate models (Versteeg et al., 2010).

The crystal structure of the N-terminal region of the NS1 protein in conjugation with human ISG15 showed that this region interacts with a pair of ISG15 molecules (Guan et al., 2011). A high affinity binding site is formed around the ISG15 protein’s N-terminal domain and NS1 protein as a result of the low affinity binding between them (Li et al., 2011). A novel RNA-binding site was discovered in the C-terminal domain of IBV that is not present in IAV (Ma et al., 2016). 22 human proteins were such as DHX9, ILF3 etc., were found to interact with two RNA-binding domains of IBV NS1 protein. SNRNP200 was identified as a positive regulator caused by host IFN responses. ILF3 exhibited dual roles in the induction of IFN and the replication of IBV (Patzina, Botting, Garcia-Sastre, Randall, & Hale, 2017).

3.2.4 Influenza B Matrix Protein 2 (BM2)

Encoded by the 7th RNA genome segment, the BM2 protein is translated via a termination-dependent initiation from a bi-cistronic mRNA in an open reading frame that is +2 nucleotides from the matrix (M1 protein). BM2 protein is made up of a 7-residue ectodomain, a 19-residue transmembrane domain, and an 82-residue cytoplasmic tail.
Key residues in the transmembrane domain such as His19, Trp23 and His27 play a role in BM2 activity and opening the channel (Otomo, Toyama, Miura, & Takeuchi, 2009; Rouse, Carpenter, Stansfeld, & Sansom, 2009). The BM2 initiation codon overlaps with the M1 termination codon, and this unique stop-start mechanism can be utilized to express multiple proteins from one mRNA (Hatta, Kohlmeier, Hatta, Ozawa, & Kawaoka, 2009). BM2 forms a tetrameric structure where the channel pore is surrounded by four protein bundles (Balannik, Lamb, & Pinto, 2008).

Several studies showed that BM2 is an integral membrane protein that closely associates with cellular proteins (Paterson, Takeda, Ohigashi, Pinto, & Lamb, 2003; Watanabe, Imai, Ohara, & Odagiri, 2003). Similar to the M2 protein in IAV, the BM2 protein can transform the pH of the trans-golgi network (Betakova & Hay, 2009). Its function as a proton-selective ion channel was thought to help in uncoating the virus in virus-infected cells. When transmembrane (TM) domain residues Ser9, Ser12, Phe13, Ser16, His19, and Trp23 in the BM2 ion channel were mutated to cysteine, the greatest disruption in ion channel function was observed (Taylor, Udo, Lamb, & Pinto, 2005). NMR spectroscopy studies provided detailed structural information about the BM2 TM peptide which contains a histidine in the tetrameric transmembrane (Williams, Shcherbakov, Wang, & Hong, 2017; Williams, Tietze, Lee, Wang, & Hong, 2016).

To investigate BM2’s function, BM2 knockout viruses were generated using reverse genetics. Initial studies showed that BM2 is essential for IBV replication and infectivity, playing a role in the assembly of the viral ribonucleoprotein complex into the virion (Hatta et al., 2004; Imai, Watanabe, Ninomiya, Obuchi, & Odagiri, 2004; Jackson, Zurcher, & Barclay, 2004). The cytoplasmic domain of BM2 has been found to play a
role in aiding the association of the M1 protein with lipid membranes and virion morphology (Imai, Kawasaki, & Odagiri, 2008).

The growth of IBV and BM2 activity are not hindered by the antiviral drug amantadine. The ion channel’s pore is lined with polar serine residues and not hydrophobic amino acids, and this confers drug resistance to the BM2 protein. The use of more polar compounds is essential in inhibiting BM2 and disrupting viral infectivity (Pinto & Lamb, 2006; Taylor et al., 2005). The BM2 proton channel is a good antiviral target: a monoclonal antibody targeting the ectodomain of BM2 proton channel inhibited viral replication (Y. J. Wang et al., 2010). DNA enzymes containing the 10-23 catalytic motif against the RNA of IBV BM2 can effectively inhibit viral RNA replication. This makes the DNA enzymes a good candidate to develop a therapeutic agent against IBV (Kumar et al., 2013). It has also been shown that BM2 can replace IAV’s M2 protein to support viral replication (Wanitchang, Wongthida, & Jongkaewwattana, 2016).

3.2.5 Hemagglutinin (HA)

The HA protein is a surface protein that binds to the sialic acid of the host cell in the early stages of influenza infection. This receptor binding specificity of HA plays an important role in determining the host range of the influenza virus.

It was shown that in cells of CV-1 origin carrying the SV40 genetic material, HA and Neuraminidase (NA) proteins accumulate in the trans-golgi network, enabling HA to get desialidated before the proteins migrate to the cell surface. In Madin-Darby Canine Kidney cells, de-sialidation occurs on the cell surface (Luo, Nobusawa, & Nakajima, 2002). The NA protein removes sialic acid from the HA protein as well as cellular glycolipids or glycoprotein, thereby preventing the aggregation of virus particles and
permitting the release of virus from host cell receptors (Palese et al., 1974; Shibata et al., 1993).

HA is a homotrimer, with each monomer HA0 composed of a globular head and stem. The native HA0 is cleaved by proteases HA1 contains the residues that form the receptor binding pocket in the head. They facilitate interaction with the host cell. The stem contains the fusion peptide that is necessary for cytosolic release. It is encoded by both HA1 and HA2. HA2 is also conserved across IAV and IBV.

Homology modelling of the HA1 structure of Influenza B virus showed that two amino acid types could be present at the position 269: Residue 269 is Pro in the Yamagata sub-lineage, unchanged since B/Lee/40, but Ser in all viruses of the Victoria sub-lineage. This provides the signature for the two lineages into which the influenza B virus HA bifurcated in 1978 (Hay et al., 2001). The cytoplasmic tail of Influenza B virus regulates fusion depending upon the amino acid sequence. The hydrophobicity of a single amino acid was important in syncytium formation. It was seen that Leucine was not essential for HA-mediated fusion (Ujike, Nakajima, & Nobusawa, 2006).

The HA0 sequence has a dual functional constraint, i.e., to remain a suitable substrate for host-encoded proteases and to maintain a functional fusion domain. Since HA0 cleavage occurs extracellularly in all human influenza viruses, it is an appropriate target for antibody response. Because of its functional constraints, the epitope is extremely well conserved, providing the vaccine with broad specificity against all circulating influenza B viruses. Three amino acids were found to be conserved, particularly site 197 in the B/Victoria viruses that were analyzed (Pechirra et al., 2005).
It was seen that amino acid variation, while highest at the surface positions, had no relationship to the degree of variability in the HA loop. Compensatory changes are made to neutralize charges in a loop (Tung, Goodman, Lu, & Macken, 2004). Acylation was not significant in membrane fusion by, but the pore formation and pore dilation were appreciably affected by the amino acid sequence of the cytoplasmic tail and the existence of a single acylation site in cytoplasmic tail residue 578.

### 3.3 Epidemiology of the Victoria and Yamagata lineages

In a comprehensive epidemiological study conducted across 20 countries worldwide, influenza B (IBV) data was analyzed for the seasons between 2000-2013. 20% of all influenza cases reported in these countries were due to IBV, highlighting the importance of IBV when studying the epidemiology of influenza infections (Caini et al., 2015). Another study done for the Asia-Pacific region identified IBV to be the cause of 0% to 92% of laboratory-confirmed cases of influenza in a given season/year (Jennings et al., 2018). Despite clear indications that IBV has a serious impact on human health, there is a scarcity in literature about the epidemiology of this virus. Greater number of local studies are required to better understand IBV’s burden (Tafalla, Buijssen, Geets, & Noordegraaf-Schouten, 2016).

Since 1983, two distinct evolutionary pathways have existed, changing the landscape of IBV’s epidemiology (Rota et al., 1990). The B/Yamagata/16/88 and B/Victoria/2/87 lineages are genetically and antigenically distinct (Biere, Bauer, & Schweiger, 2010). While Victoria-like viruses were prevalent worldwide in the 1980s, the Yamagata lineage took over in the 1990s. In some parts of Asia, Victoria-like viruses circulated in the late
1990s, but this lineage re-emerged globally in 2001. Co-circulation of both lineages was seen during certain years. In seasons where IBV was predominant or co-circulating alongside influenza A virus (IAV), Victoria lineage predominated 64% of seasons, whereas Yamagata lineage predominated 36% of seasons (Caini et al., 2015). Epidemiological dynamics of the Victoria and Yamagata lineages vary significantly. Phylogenetic trees constructed from the HA genes showed that the Victoria lineage comprised of a single tree-trunk that had several side branches which circulated for short periods of time. The Victoria lineage undergoes greater positive selection pressure, which makes the virus more likely to undergo antigenic drift. The Yamagata lineage is more conserved than the Victoria lineage, with multiple clades circulating for longer periods of time (Vijaykrishna et al., 2015).

Studies have shown the Yamagata viruses bind predominantly to α-2,6-linked sialic acid host receptors whereas Victoria viruses bind to both α-2,3 and α-2,6 binding capacities. This correlates to the different age distributions of humans that are susceptible to infections by the two lineages, since children have a greater number of α-2,3 receptors and a lower level of α-2,6 receptors compared to adults. Thus, differences in the hemagglutinin binding preferences influence the phylodynamic patterns of both lineages, with the Victoria lineage being more susceptible to seasonal bottlenecks and antigenic selection. The Yamagata lineage is more likely to infect older people and has lower transmission rates.
3.4 Evolutionary dynamics of IBV

Understanding the evolutionary dynamics of influenza is essential for the prevention and management of viral infection. Historically, emerging epidemic strains were classified by antigenic analysis. Hemagglutination Inhibition assay may not always provide information regarding evolutionary relationships as viruses from different lineages have different amino acid substitutions at key positions which is not always reflected by changes in the HI assay in terms of antigenic properties. Thus, performing a molecular analysis of viral genes provides an insight into the epidemic potential of different strains by allowing the identification of amino acid residues that contribute to antigenic drift. Phylogenetic analysis also enables the identification of circulating strains belonging to different lineages (Lindstrom et al., 1999; Rota et al., 1990).

Molecular analysis of circulating IBV strains in the 1998-2001 influenza seasons in Quebec, Canada revealed distinct evolutionary patterns in the HA1, NS1 and NA protein sequences. Isolates from 1998-1999 period were not closely related to the vaccine strain (B/Harbin/7/94) of that year when a comparison of HA1 and NS1 sequences was done, but similarities existed in the NA sequences (Abed, Coulthart, Li, & Boivin, 2003).

Analysis of isolates from 2001-2002 winter season in European countries such as Belgium, Spain, Finland and Israel revealed the categorization of IBVs into the B/Yamagata/16/88 or B/Victoria/2/87 lineages. The appearance of B/Victoria/2/87 lineage viruses marks the spread of this lineage from Asia to Europe, with Israel being a possible geographic route. Progression of the flu season led to a dominance of B/Hong Kong/1351/02-like viruses in these countries aided by the advantages of reassortment (Chi, Bolar, Zhao, Rappaport, & Cheng, 2003). The Yamagata lineage, on the other
hand, dominated Beijing, China in 2013-2014. While HA segments were derived from the Yamagata lineage, NA segments were derived from the Victoria lineage, showing the reassortment had an important role in the evolution of IBVs in this region as well (Fang et al., 2015).

Phylogenetic analysis of IBV isolates from the years 2004-2012 in Taiwan provided insight into the evolutionary processes that cause a persistent virus to become predominant. HA sequences from 2007 to 2012 were used to track epidemiological changes in TW08-I which caused an epidemic in the 2011-2012 influenza season in Taiwan. Drift mutations were seen in several gene segments of Yamagata lineage viruses derived from TW08-I (Yang et al., 2012).

Surveillance of IBV strains from 1996-2012 in Sao Paulo, Brazil was conducted. Some years had either Victoria or Yamagata lineages viruses dominating, whereas other years like 2005, 2006 and 2008 saw a cocirculation of both lineages. This alternating pattern could be the cause of decrease in vaccine match that is observed, highlighting the need for quadrivalent vaccines containing strains from both lineages of IBV (Paiva et al., 2013). Similar studies in Riyadh, Saudi Arabia during 2010-2011 and Italy during 2010-2015 point to co-circulation of the Victoria and Yamagata lineages. Variations in the amino acid sequences of HA and NA proteins of the circulating strains point to the need for increased surveillance to discover newly emerging strains (Ali, Amer, & Almajhdi, 2014; Tramuto et al., 2016). Analysis of IBV strains between 1995-2008 in Malaysia pointed at a dominant lineage (Victoria or Yamagata) that alternated every 1-3 years. Specific amino acid substitutions in the HA segment and frequent introductions of both
lineages into Malaysia was thought to contribute to this evolutionary pattern (Sam et al., 2015).

Thus, distinct evolutionary patterns of IBV can be observed based on epidemiological studies conducted in Europe, Asia and South America. The alternating dominance of both lineages and their co-circulation in an influenza season are common. Reassortment between circulating strains and drift mutations that affect the antigenicity of the viruses contribute to IBV evolution. Gaining a better understanding of IBV’s epidemiology can inform antiviral therapies and vaccine formulation to combat its infection.

### 3.5 Natural Occurrence of Influenza B in Non-Human Species

The first evidence of influenza B infection in pigs was observed in 1966 in Budapest, Hungary, where influenza B outbreaks in humans resulted in cough, serous nasal discharge and conjunctivitis in pigs. Analysis of eight infected farms was done by collecting 924 swine serum samples, and in four farms 8 to 24 percent of pigs had a HI antibody titer to IBV. However, direct reproduction of IBV in the respiratory tract was not observed (Takatsy & Farkas, 1969). Earlier this year, a study done in Taiwan showed the first evidence of natural IBV-infected swine in a clinical setting. Genetic characterization of the IBVs showed that they belonged to the B/Brisbane/60/2008 genetic clade of Victoria. This was considered to be a sporadic case of IBV transmission from humans to pigs (Tsai & Tsai, 2019). There is also a report of IBV isolation from horses as mentioned by Kawano and group, but the article could not be accessed (Kawano J, 1978).
In 1971, IBV that resembled B/Victoria/98926/70 was isolated from a 2-month-old Dachshund in Taipei city. This was the first Influenza B virus isolate of canine origin obtained under natural conditions (Chang CP, 1976). Failure to re-isolate the virus or obtain a HI antibody titer one month later caused Chang CP et. al. to conclude that the virus failed to produce an antigenic response in the dog or that the dog might have contracted the virus from infected humans whom it shared a close contact with. Zoo and migratory birds in Hungary had an outbreak due to IBV infection. 4.1% of the animals tested had IBV antibodies, demonstrating the susceptibility of avian species to IBV (Romvary, Meszaros, & Barb, 1980).

In the spring of 1999, 12 juvenile harbor seals (Phoca vitulina) that were stranded near the Dutch coast had respiratory problems. Growth in cell culture, electron microscope analysis and subsequent reverse transcriptase polymerase chain reaction confirmed the presence of IBV in these animals. Lower viral shedding and delayed IgG antibody response to HA and NA was observed. Phylogenetic analysis indicated a lack of genetic or antigenic drift of IBV in seals. This influenza B/Seal/Netherlands/1/99 virus was similar to strains that circulated in humans 4 or 5 years earlier (Fouchier, Bestebroer, Martina, Rimmelzwaan, & Osterhaus, 2001). This was the first evidence of IBV in seal populations, highlighting the possibility of seals being an animal reservoir that new IBVs can emerge from. Virus attachment was seen in the epithelial cells of the respiratory tract in seals, consistent with the previously mentioned study (Ramis, van Riel, van de Bildt, Osterhaus, & Kuiken, 2012).
3.6 Seroprevalence studies of IBV in Non-Human Species

A study done in Japan in 1978 indicated that antibodies to Influenza B virus were present in 16 out of 504 horse sera and 1 out of 1030 swine sera (0.1%) that were tested. All the positive horses were born in 1976, which was the same year that a human outbreak of influenza B occurred. It was therefore suggested that these horses might be infected from man (Kawano J, 1978). In 2000, sera collected from 971 seals showed a prevalence of antibodies to Influenza B virus in 2% of the animals after 1995, but none before 1995. Wild pigs in Croatia were positive for hemagglutination-inhibition (HAI) antibodies against virus B/Beijing/184/93-like strain in 68 (67.32%) samples in a study conducted in the same year (Zupancic et al., 2000). Two years later, antibodies to Influenza B virus were found in serum samples collected from Caspian seals (Phoca Caspica). 14% of samples collected in 1997 and 10% of samples collected in 2000 were detected positive by ELISA (Ohishi et al., 2002). HAI titers to Influenza B virus was observed in cervid sera from Croatia. 31% of free-ranging and 54% of captive cervids that were studied had an antibody titer of >1:20 (Zupancic et al., 2002).

Indirect evidence of Influenza B virus was also seen in South American fur seal (Arctocephalus australis) samples in Uruguay. This was the first evidence of IBV in this species (Blanc et al., 2009). In 2013, serum samples from harbor seals and gray seals living in Dutch coastal waters during 2002-2012 were analyzed. It was shown that 9 out of 21 samples collected in 2010 were positive, whereas only 1 out of 150 samples was positive in 2011. This study showed that seals were infected by a virus closely resembling B/Yamanashi/166/98, but antigenically different from B/Seal/Netherlands/1/1999 (Bodewes et al., 2013).
3.7 Cell cultures utilized in the study of IBV

Cell cultures are widely used for the production of influenza vaccines. Vaccine manufacturers use several continuous cell lines, amongst which Madin-Darby Canine Kidney (MDCK) cells are the most extensively used. Guinea pig serum was seen to inhibit the infectivity of IBV grown in chick embryo or MDCK cells (Yamamoto et al., 1987). It was seen that viral envelope of IBV was disrupted, degrading the M1 protein and genomic RNA (Goshima et al., 1988). However, cell cultures derived from the organs of animals, known as primary cell cultures, offer several advantages. Although they have limited growth in vitro, they represent an environment closest to the in vivo condition. Use of primary cell cultures can help inform in vivo studies or eliminate the need for them depending on the study’s requirements. Primary cells from several non-human species have been used to study IBVs.

Primary ferret kidney cell cultures exhibited cytopathic effects when infected with four different strains of IBV in 1970 (Johnson & Schieble, 1970). The metabolic activity of seven different IBV strains was measured using hamster tracheal organ cultures (Gurevitz, Schulze, Swierkosz, Arens, & Schwarz, 1987). Ferret airway cells inoculated with IBV and IAV from different lineages also supported efficient replication with little variation between the strains. Lower titers were observed in the cells infected with IBV compared to IAV (Elderfield et al., 2015).

Mink lung epithelial cells contain alpha-2,6 and alph-2,3 receptors, which are essential for the entry of influenza virus, at levels that are similar to MDCK cells. A mink lung epithelial cell line was created which supported IBV infection (Schultz-Cherry, Dybdahl-
Sissoko, McGregor, & Hinshaw, 1998). Chick embryo brain cultures supported the replication of influenza B viruses when enriched with neurons or astroglial cells (Parker, Spence, & O'Callaghan, 1997).

3.8 Use of Ferrets and Other Non-Human Species to Study IBV

The ferret is a widely used animal to study the replication, transmission and pathogenesis of Influenza A viruses. Several clinical symptoms and laboratory signs are similar when ferrets and humans are infected with human or avian IAV. Unlike mice which require attenuation, ferrets are naturally infected with IBV. The sialic acid receptors in the respiratory tract of both species are comparable, making ferrets a useful animal to model human or avian influenza infection. Additionally, transmission and seroconversion of IAV has been detected in ferrets.

In contrast, experimental infections of IBV in ferrets have been few. Ferrets were used to study the metabolic changes occurring during convalescence from IBV infection. One study indicated an alteration in the lipid metabolism 24 hours after dissemination of the virus during convalescence (Kang, Galloway, Bean, Cook, & Olson, 1991). Another study looked at the hepatic lipid disturbances and ferrets as a possible model to study the steatosis of Reye’s syndrome (Kang et al., 1991).

Ferrets have also been used to study the effect of antiviral agents of IBV infection. Single-dose treatments of 2’-FluorodGuo and 2’-FluorodDAP in ferrets infected with B/Singapore/222/79 inhibited viral replication in the upper respiratory tract, although the response was reduced in comparison to ferrets infected with IAV (Jakeman, Tisdale, Russell, Leone, & Sweet, 1994). The ability of peramivir, an influenza neuraminidase
(NA) inhibitor, to block IBV infection was tested in ferrets. A single intravenous infection of peramivir was seen to lower viral titers and symptoms in ferrets following infection with IBV (Kitano et al., 2011). The fitness of neuraminidase resistant IBVs was studied in ferrets to obtain a better understanding of the public health threat that they could pose (Pascua et al., 2016). In order predict the amino acid substitutions that may arise when IBV strains are under prolonged exposure to zanamivir, a neuraminidase inhibitor, IBV was passaged in vitro under zanamivir pressure. A NA mutation E117D was identified that conferred resistance, but caused decreased viral replication in ferrets (Oh et al., 2018).

Studies comparing IAV and IBV infections in ferrets showed that lower pathogenesis was seen in IBV-infected ferrets compared to IAV. Milder clinical symptoms were thought to be the result of lower viral replication in the lungs. This reduced viral replication was also implicated in the lower induction of inflammatory cytokines TNF-a and IFN-a (Y. H. Kim, Kim, Cho, & Seo, 2009). Another study used an outbred ferret model to model the genetic diversity in humans. Infection of these outbred ferrets with multiple IAV and IBV strains showed that the mildest clinical symptoms were observed in IBV strains. The disease severity was correlated to the antibody response in the upper respiratory tract of ferrets. (Huang et al., 2011).

The expression of immune molecules such as cytokines, chemokines, and immune mediators was studied in the upper respiratory tract (URT) of ferrets infected with IAV (H1N1 pdm09 and H3N2) and IBV. Interleukin-1, alpha interferon, and tumor necrosis factor alpha mRNAs levels were correlated to a peak in the viral titers. This study
demonstrated the similarities in the localized immune responses in ferrets when infected with IAV or IBV (Carolan et al., 2016).

Experimental infection of ferrets with two B/Victoria (B/Brisbane/60/2008 and B/Bolivia/1526/2010) and two B/Yamagata (B/Florida/04/2006 and B/Wisconsin/01/2010) was done to study clinical and pathological profiles. The clinical illness caused by B/Brisbane/60/2008 was the most severe. B/Brisbane/60/2008 also caused higher viral burden compared to viruses belonging to the B/Yamagata lineage and established an infection in the lower respiratory tracts of the ferrets. (Huang et al., 2014). B/Florida/04/2006 virus was serially passaged in mice until very virulent, and then infected in ferrets. The mouse-adapted virus replicated in the upper and lower respiratory tracts of ferrets. This was the first study to show successful ferret-to-ferret transmission of IBV in contact and droplet conditions (E. H. Kim et al., 2015). Transmission of IBV between ferrets was not affected by the absence of the NB membrane protein, showing that the NB protein is not responsible for replication or transmission of IBV via respiratory droplet exposure in the in vivo ferret model (Elderfield et al., 2016). Some studies using ferrets were done to aid in vaccine research (Sun et al., 2019). 5-6 months old ferrets were used to study whether the live attenuated influenza vaccine (LAIV), which contains IBV components, would offer cross-protection against the different lineages. Ferrets vaccinated with monovalent LAIV had a lower level of challenge virus compared to the unvaccinated ferrets (Kiseleva et al., 2018).

3.9 Experimental IBV infections in other species

The susceptibility of domestic pigs to Influenza B virus was demonstrated in 1966 when piglets that were intranasally injected with B/Budapest/10/65 demonstrated antibodies
and infected naïve animals by contact transmission (Takatsy & Farkas, 1969). In 2015, an experimental challenge in pigs showed that B/Brisbane/60/2008 (Victoria lineage) and B/Yamagata/16/1988 (Yamagata lineage) caused symptoms and influenza-like lesions. B/Brisbane/60/2008 transmitted successfully to sentinel animals.

An experimental infection involving monkeys and cats showed that these animals are susceptible to Influenza B virus infection by intranasal instillation, but evidence of contact transmission was not clearly established. The dogs included in this study were not susceptible to IBV infection (Paniker & Nair, 1972). SPF hamsters were infected with different strains of IBV to study the differences between wild type and cold adapted strains (Reeve, Pibermann, & Gerendas, 1981).

Guinea pigs were infected intranasally with IBV and an adjuvant. High levels of hemagglutination-inhibiting antibodies and neutralizing antibodies demonstrated the utility of this scheme in setting up immune sera for in vitro diagnosis (Havlickova, Prokesova, Zanvit, Tacner, & Limberkova, 2006). Influenza B viruses belonging to both B/Victoria-like and B/Yamagata-like lineages were shown to replicate in the respiratory tract of guinea pigs in an experiment. Injection showed and mental infection. Aerosol/droplet transmission was observed, with higher efficiency at 5°C compared to 20°C (Pica, Chou, Bouvier, & Palese, 2012). Guinea pigs were vaccinated with neuraminidase intramuscularly and intranasally to study the ability of recombinant neuraminidase vaccination to prevent viral infection. The vaccinated animals were susceptible to infection via contact and air borne transmission routes, although the viral titers were lowered. Intranasal vaccination prevented virus transmission to naïve animals (McMahon et al., 2019).
Report of lack of attachment of influenza B virus to trachea and bronchi of cetaceans is consistent with absence of reported influenza B virus infections in these species (Zupancic et al., 2002).
Chapter 4 Replication Kinetics of IBV in Swine Primary Respiratory Epithelial Cells

4.1 Abstract

Influenza B virus (IBV) is a respiratory pathogen that poses a significant burden to human health. Humans are the sole reservoir of IBV, however there is evidence to show that swine are susceptible to IBV. In this study we infected swine primary respiratory epithelial cells with B/Brisbane/60/2008 (BR08), B/Florida/04/2006 (FL06), B/Hong Kong/286/2017 (HK17) and B/Utah/09/2014 (UT14) at 0.01 and 0.1 multiplicities of infection (MOI). Experiments were conducted at 33°C and 37°C, representing the temperatures of the upper and lower respiratory tract, respectively.

4.2 Introduction

Influenza B virus (IBV) is a respiratory pathogen belonging to the Orthomyxoviridae family. While IBV’s closest relative influenza A virus (IAV) predominates globally in terms of disease prevalence and severity, IBV also has a significant impact on the overall burden of influenza disease (W. P. Glezen, Schmier, Kuehn, Ryan, & Oxford, 2013). 37% of the total healthcare costs due to influenza illness for the seasons 2001/2002 to 2008/2009 was attributed to IBV (Yan, Weycker, & Sokolowski, 2017).

IBV diverged into two separate lineages in the 1980s. The antigenically distinct lineages were named Victoria and Yamagata after B/Yamagata/16/88 and B/Victoria/2/87, which were the original isolates (Rota et al., 1990). While the Victoria lineage viruses only have one genetic clade, the B/Brisbane/60/2008 which is viewed as Group 1 of IBV, the Yamagata lineage viruses are divided into two clades. Group 2 in the Yamagata lineage is
represented by B/Brisbane/3/2007, and Group 3 is represented by B/Bangladesh/3333/2007. One lineage usually predominates in any given year, but viruses of both lineages often co-circulate during a flu season (Caini et al., 2015). Victoria-like viruses dominated in the late 1980s, and Yamagata lineage was predominant in the 1990s. The Victoria lineage re-emerged worldwide in 2001 (R. B. Chen & Holmes, 2008). Specific nucleotide substitutions in the hemagglutinin (HA) of IBV characterize the specific lineage and groups within lineages (Arvia, Corcioli, Pierucci, & Azzi, 2014). Reassortment between and within the Victoria and Yamagata lineages can generate viruses having genetic variation that may affect viral antigenic properties (R. B. Chen & Holmes, 2008).

Although IBV has established itself primarily as a human pathogen, there is serological evidence of IBV in several other mammalian species. IBVs have been isolated in dogs, pheasants, horses, and pigs although in several studies the criteria to establish infection were not met (Chang CP, 1976; Kawano J, 1978; Romvary et al., 1980; Zupancic et al., 2000; Zupancic et al., 2002). IBV was shown to cause an infection and circulate in harbor seals (Phoca vitulina) and gray seals (Halichoerus grypus) (Bodewes et al., 2013).

Serving as a natural host for influenza viruses, pigs can be infected with both avian and human influenza viruses. The porcine respiratory tract possesses both sialic acid α2,3-galactose (SAα2,3-Gal) linked receptors and α2,6-galactose (SAα2,6-Gal) linked receptors (Ito et al., 1998). Swine, avian and human influenza viruses can undergo reassortment in pigs, resulting in novel influenza viruses. These novel viruses may contain mutations that allow transmission to humans thereby establishing a human
infection. They may otherwise maintain infection in pigs, making them a reservoir for human infection. Pigs thus play an important role in the ecology of influenza viruses. Antibodies to IBV in pigs have been detected in some studies, but the susceptibility of domestic pigs to Influenza B virus was demonstrated in 1968 when piglets that were intranasally injected with B/Budapest/10/65 demonstrated antibodies and infected naïve animals by contact transmission. Little or no clinical illness was observed (Takatsy & Farkas, 1969). In 2015, an experimental study was done which showed that IBV from both lineages can infect and replicate in pigs. IBV transmission was observed in pigs that were infected with viruses belonging to the Victoria lineage (Ran et al., 2015).

Cell cultures form an important platform for vaccine production for influenza viruses. Factors such as the sialic acid receptors distribution and cellular environment affect the ability of different cell lines to replicate in influenza viruses (Rott, 1992). Continuous cell lines like Madin-Darby Canine Kidney (MDCK) cells have been used extensively for influenza studies. Insufficient antiviral defense in MDCK cells promotes efficient replication of IBV (Frensing et al., 2011). African green monkey kidney (Vero) cell line and HeLa cell lines (immortal cell line derived from cervical cancer cells) are other continuous cell lines that provide an alternate host system to study IBV pathogenesis and infection biology (Govorkova, Murti, Meignier, deTaisne, & Webster, 1996; Yamanaka, Shirasawa, & Yoshizawa, 1995).

Primary cells closely resemble the in vivo characteristics of the susceptible host they are derived from. Exhibiting mitotic activity in the first 2-4 weeks, they have a limited growth capacity in vitro compared to transformed cells (Petursson & Fogh, 1963). Primary epithelial cells from several species have been used to gain a better
understanding of influenza-host interactions (Castleman & Hancock, 2014; Fischer, Sidhaye, King, Lane, & Pekosz, 2010; Newby, Rowe, & Pekosz, 2006; H. Y. Wang et al., 2018; Zeng et al., 2019; Zeng et al., 2013; Zhao, Liang, & Ji, 2011). Moreover, primary epithelial cells of swine origin are used to study the infection models of various diseases including influenza (Allen C Bateman et al., 2010; Guseva, Knight, Whittimore, & Wyrick, 2003; Kasza, Bohl, & Jones, 1960; Schierack et al., 2006).

Mink lung epithelial cells actively supported the replication of IBV to titers comparable to MDCK cells (Schultz-Cherry et al., 1998). Chick embryo brain cultures too supported the replication of influenza B viruses when enriched with neurons or astroglial cells (Parker et al., 1997). It was also seen that ferret tracheal epithelial cells when infected with IBV showed productive replication (Elderfield et al., 2015). Our lab previously demonstrated that swine primary respiratory epithelial cells derived from a day-old gnotobiotic piglet could be infected with four types of influenza viruses (Sreenivasan et al., 2019).

This study looked the replication kinetics of IBV in swine primary respiratory epithelial cells derived from the nasal turbinate, trachea and lung cells of a day-old gnotobiotic piglet at different multiplicities of infection of the virus. The primary cells were infected with IBV strains belonging to both Victoria and Yamagata lineages. Temperature dependent replication of IBV was seen at both 33°C and 37°C. B/Hong Kong/286/2017 (Victoria lineage) grew to the highest titers in nasal turbinate and lung primary epithelial cells.
4.2 Materials and Methods

Swine primary nasal turbinate, trachea and lung cells were isolated from a day old gnotobiotic piglet. These cell types were developed and characterized as described by Sreenivasan et. al (Sreenivasan et al., 2019). The primary cells were grown in Dulbecco’s Modified Eagle Medium (DMEM/F-12), 10% FBS (PAA Laboratories Inc., Dartmouth, MA, USA) and penicillin-streptomycin (100 U/mL) (Life Technologies, Carlsbad, CA, USA), Mouse epidermal growth factor (EGF) (Sigma (5 ng/mL)), insulin (5 µg/mL) transferrin (5 µg/mL) and selenium (5 ng/mL) (Sigma). All cell cultures were maintained in a humidified incubator at 37°C or 33°C with 5% CO₂. The primary cells of passages 2 to 10 were used for this study.

4.2.1 Culture of Influenza B viruses

The Influenza B viruses used in this study were B/Brisbane/60/2008 (BR08), B/Florida/04/2006 (FL06), B/Hong Kong/286/2017 (HK17) and B/Utah/09/2014 (UT14).

For this study, MDCK cells were maintained in Dulbecco’s Modified Eagle 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). MDCK cells were cultured in the T-25 flask, inoculated with the virus inoculum at 0.01 multiplicity of infection (MOI) and incubated at 37°C in 5% CO₂ for 1 h. After infection, the virus growth medium, containing fresh DMEM with 0.3% bovine serum albumin (BSA) made from 6% stock, 1 µg/mL tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, Saint Louis, MO, USA) and penicillin-streptomycin (100 U/mL) (Life Technologies, Carlsbad, CA, USA), was added for further incubation at 37°C in 5% CO₂ for 72 h. The infected cell cultures were freeze-
thawed. For removal of cellular debris, the supernatant was spun at 500× g for 10 min at 4°C. The viruses were stored at -80°C until further use. The Reed and Muench method was applied to determine the virus titers (50% cell culture infectious dose (TCID\textsubscript{50}) endpoint dilution assay) in MDCK cells (Reed & Muench, 1938a).

4.2.2 Replication Kinetics

Approximately 2 × 10\textsuperscript{5} cells/well were seeded on 24 well plates. The cells were respectively infected with B/Brisbane/60/2008 (BR08) or B/Florida/04/06 (FL06) or B/Hong Kong/286/2017 (HK17) or B/Utah/09/2014 (UT14). Virus growth medium for primary cells included Opti-MEM (Thermofisher Scientific, Waltham, MA), 0.001% (0.01 µg/ml) TPCK Trypsin (Pierce, Thermofisher Scientific, Waltham, MA), and 1% antibiotic-antimycotic (Thermofisher Scientific, Waltham, MA). MDCK cells were infected at the same MOI, with DMEM, 0.1% (1 µg/ml) TPCK Trypsin (Pierce, Thermofisher Scientific, Waltham, MA), and 1% antibiotic-antimycotic (Thermofisher Scientific, Waltham, MA). Samples were collected at 24 h intervals until 120 h post infection.

Approximately, 1 × 10\textsuperscript{4} MDCK cells were seeded on flat bottom 96 well plate (Greiner bio-one, NC, USA) and incubated overnight. Serial ten-fold dilutions of the samples were prepared in virus infection media and were inoculated on pre-seeded MDCK 96-well cell culture plates. The inoculated plates were incubated for 5 days at respective temperatures. The infectivity of the virus was determined by hemagglutination assay using 1% Turkey red blood cells (Lampire Biological Laboratories, Pipersville, PA, USA). Titers were then calculated using Reed and Muench formula (REED & MUENCH, 1938b).
4.3 Results

Figure 4.1 Morphology of porcine primary respiratory epithelial cells. Panel (a) shows uninfected nasal turbinate, trachea and lung primary cells under a phase contrast microscope (20x). Panel (b) shows nasal turbinate, trachea and lung primary cells infected with B/Hong Kong/286/2017 (72h post-infection) under a phase contrast microscope (10x).
Figure 4.2 Swine primary nasal turbinate epithelial cells were infected with (a) 0.01 MOI or (b) 0.1 MOI of B/Brisbane/60/2008 (BR08) or B/Florida/04/2006 (FL06) or B/Hong Kong/286/2017 (HK17) or B/Utah/09/2014 (UT14) at 33°C and 37°C. Virus titers were expressed in log10TCID50/ml. Dotted line represents the replication kinetics at 33°C. Data shown here are representative of two independent experiments performed in triplicates ± SE.
Figure 4.3 Swine primary trachea epithelial cells were infected with (a) 0.01 MOI or (b) 0.1 MOI of B/Brisbane/60/2008 (BR08) or B/Florida/04/2006 (FL06) or B/Hong Kong/286/2017 (HK17) or B/Utah/09/2014 (UT14) at 33°C and 37°C. Virus titers were expressed in log_{10} TCID_{50}/ml. Dotted line represents the replication kinetics at 37°C, while the bold line represents the kinetics at 33°C. Data shown here are representative of two independent experiments performed in triplicates ± SE.
Figure 4.4 Swine primary lung epithelial cells were infected with (a) 0.01 MOI or (b) 0.1 MOI of B/Brisbane/60/2008 (BR08) or B/Florida/04/2006 (FL06) or B/Hong Kong/286/2017 (HK17) or B/Utah/09/2014 (UT14) at 33°C and 37°C. Virus titers were expressed in log_{10}TCID_{50}/ml. Dotted line represents the replication kinetics at 37°C, while the bold line represents the kinetics at 33°C. Data shown here are representative of two independent experiments performed in triplicates ± SE.
Figure 4.5 MDCK cells were infected with (a) 0.01 MOI or (b) 0.1 MOI of B/Brisbane/60/2008 (BR08) or B/Florida/04/2006 (FL06) or B/Hong Kong/286/2017 (HK17) or B/Utah/09/2014 (UT14) at 33°C and 37°C. Virus titers were expressed in log10TCID50/ml. Dotted line represents the replication kinetics at 33°C, while the bold line represents the kinetics at 33°C. Data shown here are representative of two independent experiments performed in triplicates ± SE.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Max. Titer Log_{10}TCID_{50}/ml (33˚C )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR08</td>
<td>MDCK (5.36) &gt; Trachea (3.43) &gt; NT (3.35) &gt; Lung (2.43)</td>
</tr>
<tr>
<td>FL06</td>
<td>MDCK (3.94) &gt; Trachea (3.45) &gt; NT (3.37) &gt; Lung (2.9)</td>
</tr>
<tr>
<td>HK17</td>
<td>MDCK (4.51) &gt; NT &amp; Trachea (4.36) &gt; Lung (4.33)</td>
</tr>
<tr>
<td>UT14</td>
<td>Trachea (4.39) &gt; MDCK (4.34) &gt; Lung (3.4) &gt; NT (3.29)</td>
</tr>
</tbody>
</table>

Table 4.1 shows the peak viral titers (log_{10}TCID_{50}/ml) B/Brisbane/60/2008, B/Florida/04/06, B/Hong Kong/286/2017 and B/Utah/09/2014 grew to in nasal turbinate, trachea, lung and MDCK cells in decreasing order of titers at 33˚C and 37˚C at 0.01 multiplicity of infection.
Virus Peak Titer Log$_{10}$TCID$_{50}$/ml (33°C)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Peak Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR08</td>
<td>MDCK (5.35) &gt; Lung (4.46) &gt; Trachea (4.14) &gt; NT (3.84)</td>
</tr>
<tr>
<td>FL06</td>
<td>MDCK (5.57) &gt; Trachea (5.38) &gt; NT &amp; Lung (4.25)</td>
</tr>
<tr>
<td>HK17</td>
<td>Lung (4.47) &gt; MDCK (4.45) &gt; NT (4.31) &gt; Trachea (3.42)</td>
</tr>
<tr>
<td>UT14</td>
<td>MDCK (5.40) &gt; Trachea and Lung (4.34) &gt; NT (3.99)</td>
</tr>
</tbody>
</table>

Virus Peak Titer Log$_{10}$TCID$_{50}$/ml (37°C)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Peak Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR08</td>
<td>MDCK (4.61) &gt; Trachea (3.16) &gt; NT (3.04) &gt; Lung (2.85)</td>
</tr>
<tr>
<td>FL06</td>
<td>MDCK (5.24) &gt; NT (3.7) &gt; Trachea (3.6) &gt; Lung (3.46)</td>
</tr>
<tr>
<td>HK17</td>
<td>NT (4.65) &gt; Lung (4.49) &gt; MDCK (4.45) &gt; Trachea (3.3)</td>
</tr>
<tr>
<td>UT14</td>
<td>MDCK (5.44) &gt; NT (4.3) &gt; Trachea (3.38) &gt; Lung (3.35)</td>
</tr>
</tbody>
</table>

Table 4.2 shows the peak viral titers (log$_{10}$TCID$_{50}$/ml) B/Brisbane/60/2008 (BR08), B/Florida/04/06 (FL06), B/Hong Kong/286/2017 (HK17) and B/Utah/09/2014 (UT14) grew to in nasal turbinate (NT), trachea (T), lung (L) and MDCK cells in decreasing order of titers at 33°C and 37°C at 0.1 multiplicity of infection.

<table>
<thead>
<tr>
<th>Temperature &amp; MOI</th>
<th>NT</th>
<th>Trachea</th>
<th>Lung</th>
<th>MDCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>33°C and 0.01 MOI</td>
<td>HK17 (4.36)</td>
<td>UT14 (4.49)</td>
<td>HK17 (4.33)</td>
<td>BR08 (5.36)</td>
</tr>
<tr>
<td>33°C and 0.1 MOI</td>
<td>HK17 (4.31)</td>
<td>FL06 (5.38)</td>
<td>HK17 (4.47)</td>
<td>FL06 (5.57)</td>
</tr>
<tr>
<td>37°C and 0.01 MOI</td>
<td>HK17 (3.58)</td>
<td>FL06 (3.53)</td>
<td>HK17 (3.51)</td>
<td>BR08 (5.32)</td>
</tr>
<tr>
<td>37°C and 0.1 MOI</td>
<td>HK17 (4.65)</td>
<td>FL06 (3.66)</td>
<td>HK17 (4.49)</td>
<td>UT14 (5.44)</td>
</tr>
</tbody>
</table>

Table 4.3 shows the peak titers B/Brisbane/60/2008 (BR08), B/Florida/04/06 (FL06), B/Hong Kong/286/2017 (HK17) and B/Utah/09/2014 (UT14) replicated to in swine primary nasal turbinate (NT), trachea (T), lung (L) and MDCK cells at different temperatures and MOIs.

Swine primary respiratory epithelial cells derived from nasal turbinate, trachea and lungs were infected with four IBV strains (Figure 4.1). The viruses belonging to the Victoria
lineage were B/Brisbane/60/2008 (BR08) and B/Hong Kong/286/2017 (HK17); Yamagata lineage were B/Florida/04/2006 (FL06) and B/Utah/09/2014 (UT14).

Replication kinetics in the primary cells were compared to MDCK cells. The TCID$_{50}$ titers of the stock viruses were $3.16 \times 10^6$ TCID$_{50}$/ml and $2.67 \times 10^6$ TCID$_{50}$/ml for BR08, $2.37 \times 10^6$ TCID$_{50}$/ml for FL04; $1.58 \times 10^6$ TCID$_{50}$/ml and $1.9 \times 10^6$ TCID$_{50}$/ml for HK17; $3 \times 10^7$ TCID$_{50}$/ml for UT14.

Infection experiments were done at 0.01 MOI and 0.1 MOI. TPCK trypsin was used at a concentration of 0.01 µg/ml (0.001%) on the primary epithelial cells to cause minimum cell toxicity, whereas 1µg/ml (0.1%) was used for infecting MDCK cells.

4.3.1 Replication Kinetics in Nasal Turbinate Cells at 0.01 MOI

All four viruses replicated productively in nasal turbinate cells at 33°C (Figure 4.2a). FL06 reached a peak of 3.37 logs by 48 hours post infection (hpi), whereas BR08, HK17 and UT14 reached their maximum titers of 3.35 logs, 4.36 logs and 3.29 logs by 72 hpi. All four viruses followed a similar trend to MDCK cells at 33°C; reaching their peak titers at the same time points in nasal turbinate cells as in MDCK cells.

Replication of the four IBVs at 37°C was less productive than the replication at 33°C. While HK17 grew best to a titer of 3.58 logs, FL06 and UT14 grew to titers 2.56 logs and 2.44 logs respectively. Interestingly, BR08 had a low titer of 1.77 logs by 72 hpi at 37°C.

MDCK cells supported all the viruses better than nasal turbinate cells at 37°C.

4.3.2 Replication Kinetics in Nasal Turbinate Cells at 0.1 MOI

By 24h post infection, BR08 replicated to a titer of 3.84 logs at 33°C and 3.04 logs at 37°C, showing no significant difference in titers between the two temperatures. Similarly, HK17 grew to titers of 4.31 logs at 33°C and 4.65 logs at 37°C by 72 hpi while UT14
grew to 3.99 logs at 33˚C and 4.34 logs at 37˚C. Contrastingly, FL06 grew better at 33˚C, reaching a peak titer of 4.25 logs at 33˚C (Figure 4.2b).

4.3.3 Replication Kinetics in Trachea Cells at 0.01 MOI

Trachea cells supported the replication of the four IBVs when infected at 33˚C (Figure 4.3a). At this temperature, BR08 and HK17 had no titer by 24 hpi, whereas FL06 and UT14 began replicating by 24 hours post-infection. BR08, HK17 and UT14 showed higher peak viral titers at 33˚C when compared to 37˚C. The titers obtained were 3.43 logs for BR08, 4.36 logs for HK17 and 4.49 logs for UT14. FL06 replicated equally well at both temperatures reaching 3.53 logs at 37˚C. Interestingly, all the viruses had no titer by 120 hpi in the trachea cells.

4.3.4 Replication Kinetics in Trachea Cells at 0.1 MOI

FL06 replicated the best in trachea cells reaching a titer of 5.38 logs by 48 hours post-infection. BR08 and UT14 followed similar patterns of replication, reaching peak titers of 4.14 logs and 4.34 logs respectively by 72 hpi. HK17 exhibited the lowest titers at 33˚C, reaching 3.42 logs by 96 hpi. While HK17 grew equally well at 33˚C and 37˚C, BR08, FL06 and UT14 were supported better by trachea cells at 33˚C. The pattern of replication varied as well, with BR08, FL06 and HK17 reaching peak titers earlier at 37˚C. By 120 hpi, BR08 and FL06 were not replicating (Figure 4.3b).

4.3.5 Replication Kinetics in Lung Cells at 0.01 MOI

Lung cells support the replication of all four viruses, with HK17 growing most efficiently to a peak titer of 4.33 logs at 72 hpi (Figure 4.4a). BR08 and UT14 replicated to higher titers at 33˚C, reaching peak titers of 2.43 logs at 48 hpi and 3.40 logs at 48 hpi.
respectively. FL06 replicated more efficiently at 37°C with a peak titer of 3.38 logs by 24 hpi. This was similar to the pattern of replication in MDCK cells at 0.01 MOI, where FL06 grew better at 37°C, reaching a peak titer of 4.36 logs. By 120 hpi only FL06 had a titer.

4.3.6 Replication Kinetics in Lung Cells at 0.1 MOI

BR08, FL06 and UT14 reached peak titers of 4.46 logs at 48 hpi, 4.25 logs at 72 hpi and 4.34 logs at 48 hpi respectively in the 33°C experiment (Figure 4.4b). These three viruses replicated less efficiently in at 37°C, reaching peak titers of 2.85 logs at 72 hpi (BR08), 3.46 logs at 24 hpi (FL06) and 3.35 logs at 48 hpi (UT14). HK17 displayed similar growth curves and peak viral titers growing of 4.45 logs at 48 hpi at both temperatures.

4.3.7 Replication Kinetics of MDCK Cells at 0.01 MOI

All four viruses reached high titers in MDCK cells at 33°C and 37°C (Figure 4.5a). No significant differences in titers were seen between the two temperatures for BR08, HK17, and UT14. BR08 grew the best at 33°C, reaching 5.36 logs, whereas HK17 and UT14 grew to peak titers of 4.51 logs and 4.34 logs respectively. BR08 grew to a high titer of 5.32 logs by 72 hpi at 37°C, while HK17 and UT14 replicated to titers of 4.42 logs at 48 hpi and 4.40 logs at 72 hpi respectively. FL06 replicated slightly better at 37°C reaching a titer of 4.36 logs by 48 hpi, whereas it only grew to 3.94 logs at 48 hpi in the 33°C experiment.

4.3.8 Replication Kinetics of MDCK Cell at 0.1 MOI

When MDCK cells were infected with one of the four viruses at 0.1 MOI, the titers obtained at 37°C were lesser than or equal to the titers obtained at 33°C. BR08 showed a
significant difference in titers between the two temperatures, growing to 5.35 logs by 48 hpi at 33°C, whereas at 37°C it grew to a peak titer of 4.61 logs by 48 hpi. At 33°C FL06, HK17 and UT14 grew to titers of 5.57 logs at 48 hpi, 4.45 logs at 48 hpi and 5.40 logs at 48 hpi respectively. Similar peak titers of 5.24 logs at 24 hpi, 4.45 logs at 48 hpi and 5.44 logs at 48 hpi were seen for these three viruses at 37°C (Figure 4.5b).

BR08 showed productive replication at 33°C in all three primary cells and MDCK cells. In the 33°C experiments, the highest titers were seen in MDCK cells (5.3 logs) followed by lung cells (4.46 logs). Higher titers were observed when all cells were infected with BR08 at 0.1 MOI compared to 0.01 MOI (Table 4.1 and 4.2). In the 37°C experiments, replication of BR08 was restricted to less than 2 logs in the primary cells at 0.01 MOI. Interestingly, MDCK cells yielded a titer of 5.3 logs when infected with BR08 at 37°C and 0.01 MOI.

FL06 showed the highest titer of 5.57 logs in MDCK cells when infected at 33°C and 0.1 MOI. Unlike BR08, in the 0.01 MOI experiments, comparable titers were seen at both temperatures. Amongst the primary cells, the highest titer was observed in trachea cells (5.38 logs) at 33°C and 0.1 MOI. It was seen that in the 33°C experiments, peak titers of FL06 were reached at 48h in all cell types except lung cells at 0.1 MOI which showed the peak titer at 72h.

HK17 showed productive replication at both temperatures and MOIs. No significant differences were seen in titers between 33°C and 37°C in the 0.1 MOI experiment, whereas at 0.01 MOI, the titers at 33°C were a log higher than titers at 37°C in the primary cells. The highest titer for HK17 (4.65 logs) was seen in nasal turbinate cells at 37°C in the 0.1 MOI experiment (Table 4.3).
UT14 grew best in MDCK cells at 0.1 MOI and 37°C, reaching a peak titer of 5.44 logs by 48 hpi. At 0.01 MOI, nasal turbinate and trachea cells exhibited a higher titer at 33°C compared to 37°C. No significant differences in titers were seen in lung and MDCK cells between both temperatures. At 0.1 MOI, UT14 grew best in MDCK cells reaching a peak titer of 5.44 logs, followed by nasal turbinate cells in which it replicated to 4.34 logs at 72 hpi.

4.4 Discussion

This study looked at the utility of a swine isogenous cell culture system in studying the replication kinetics of influenza B virus (IBV) at ambient temperatures that correspond to the upper and lower respiratory tract of swine. The nasal turbinate trachea and lung swine primary respiratory epithelial cells represent the upper, middle and lower respiratory tract of swine, respectively. The replication of B/Brisbane/60/2008 (BR08) and B/Hong Kong/286/2017 (HK17) belonging to the Victoria lineage, and B/Florida/04/06 (FL06) and B/Utah/09/2014 (UT14) belonging to the Yamagata lineage was studied at 33°C and 37°C. The three primary cells, and MDCK cells which were used as a positive control, were infected with the four strains of IBV at 0.01 and 0.1 multiplicities of infection.

Though humans are accepted to be the only reservoir for IBV, there is evidence of IBV infections in non-human species. Antibodies to IBV detected in swine in the 1960s and in 2015 indicate the exposure of swine to this virus (Ran et al., 2015; Takatsy & Farkas, 1969). Furthermore, animal studies conducted have shown that IBV is capable of replicating in swine (Takatsy & Farkas, 1969). The natural infection of IBV in pigs in Taiwan, although sporadic, is a public health concern as it can lead to human-swine-
human transmission in the future (Tsai & Tsai, 2019). Reassortment of IBV with other influenza viruses in pigs can also create the potential for new zoonotic viruses. This study demonstrates that IBV is capable of replicating in the in vitro model of the swine respiratory tract. Our previous study showed that BR08 and FL06 replicated in the nasal turbinate, trachea and lung primary epithelial cells when infected with 0.1 MOI at 33°C and 37°C (Sreenivasan et al., 2019). Here we show that appreciable viral titers can be observed at a lower multiplicity of infection (0.01 MOI) as well. In addition, successful replication of contemporary strains HK17 and UT14 is observed in these primary cells.

IBV replicated in a temperature dependent manner in all three primary cells. Peak titers at 33°C were greater than or equal to titers at 37°C for most of the experiments. Exceptions include HK17 which when infected at 0.1 MOI in the nasal turbinate cells grew to 0.3 logs higher at 37°C. Infection of MDCK cells did not result in significant differences in titer between the two temperatures.

The growth of BR08, FL06 and UT17 was supported best by MDCK cells, which lack the anti-influenza activity of canine Mx protein (Frensing et al., 2011). Interestingly, HK17 (Victoria lineage) grew to the highest titers in nasal turbinate and lung cells. This is in line with the swine seroprevalence study done in 2015, which showed that higher HI antibody titers were seen in viruses belonging to the Victoria lineage when compared to the Yamagata lineage (Ran et al., 2015). The growth of BR08, FL06 and UT14 was sub-optimal in the primary cells, although close to that in MDCK cells at certain conditions.
Notably, lineage-specific differences in replication could not be observed in this study. Further investigation into the molecular basis for the differences in replication between the older BR08 and contemporary HK17 will shed more light on the role of swine in the pathogenesis of IBV.

The submerged monolayer of swine primary cells used in this study does not accurately mimic the three-dimensional architecture of the swine respiratory tract. Experiments performed in 3D cell culture systems or air liquid interface systems would provide additional information at a cellular level when studying the tropism and infectivity of influenza viruses (Allen C. Bateman, Karasin, & Olsen, 2013; Elderfield et al., 2015; H. Y. Wang et al., 2018).
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