Complete Genome Sequence and Pathogenicity of Two Swine Parainfluenzavirus Isolates from Pigs in the United States

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Complete Genome Sequence and Pathogenicity of Two Swine Parainfluenzaviruses Isolated from Pigs in the United States

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Abstract

Members of the family *Paramyxoviridae* are non-segmented, negative-strand RNA viruses. A large and diverse host species are infected by paramyxoviruses, including avian, porcine, canine, bovine, equine, ovine, reptiles, aquatic species and humans. In the last few decades, many novel paramyxoviruses have emerged causing catastrophic illnesses in different aquatic and terrestrial species of animals and some of them also made the species jump to humans. Two novel paramyxoviruses 81-19252 (Texas81) and 92-7783 (ISU92) were isolated in the 1980s and 1990s from the brain of pigs that experienced respiratory and central nervous system disease from South and North Central United States. To understand their importance as swine pathogens, molecular characterization and pathogenicity studies were undertaken. The complete genome of Texas81 virus was 15456 nucleotides (nt) and ISU92 was 15480 nt in length consisting of six non-overlapping genes coding for the nucleo- (N), phospho- (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large polymerase (L) proteins in the order 3’-N-P/C/V-M-F-HN-L-5’. The features related to virus replication and found to be conserved in most members of *Paramyxoviridae* were also found in swine viruses. These include: conserved and complementary 3’ leader and 5’ trailer regions, trinucleotide intergenic sequences, highly conserved gene start and gene stop signal sequences. The length of each gene of these two viruses was similar except for the F gene, in which ISU92 had an additional 24 nt
“U” rich 3’ untranslated region (UTR). The P gene of these viruses were predicted to express the P protein from the primary transcript and edit a portion of its mRNA to encode V and D proteins and the C protein was expected to be expressed from alternate translation initiation from the P gene as in Respiroviruses. Sequence specific features related to virus replication and host specific amino acid signatures in P, F, HN and L proteins indicated that these viruses probably originated from bovine parainfluenzavirus 3. Pairwise comparisons of deduced amino acid sequences of swine viral proteins with members of Paramyxoviridae and phylogenetic analysis based on individual genes as well as predicted amino acid sequences suggested that these viruses were novel members of the genus Respirovirus of the Paramyxovirinae subfamily and genotype A of bovine parainfluenzavirus type 3. The mild clinical signs and undetectable gross and microscopic lesions observed in swine parainfluenzavirus (sPIV3)-infected pigs indicate the inapparent nature of these viruses in pigs. Limited seroprevalence studies in serum samples collected from pig farms in Minnesota and Iowa in 2007-2008 by indirect ELISA revealed that sPIV3 are not circulating in these farms. The mild pathogenicity of sPIV3 can facilitate its development as a vaccine vector. The screening ELISA developed by us could be used to detect seroprevalence of sPIV3 in animal and human populations.
This thesis is dedicated to my family and friends. For the love, support and encouragement from my parents, Yonge Guo and Wei-min Qiao, and many friends.
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This thesis arose in part out of years of research that has been done since I came to Dr. Subbiah’s group. During that time, I have worked with a great number of people whose contribution in assorted ways helped in the research and made me obtain a great and unforgettable research experience.

In the first place I would like to record my gratitude to Dr. Elankumaran Subbiah for his supervision, advice, and guidance from the very early stage of my research as well as giving me an extraordinary experience throughout the work. Above all and the most needed, he provided me with patience, kindness, unflinching encouragement and support to keep me going through when I had hard times. His scientific passion and ideas to research always inspired me to be a good scientist in the future. I would also express my gratefulness to my committee members, Dr. X.J.Meng, Dr. Lijuan Yuan, Dr. Chris Roberts and Dr. Kevin Myles, who always gave valuable advice, critical comments on my project and helped me improve my work. I especially want to acknowledge Dr. Meng and Dr. Yuan who are more like mentors to me, giving me guidance, believing in me even when I didn’t believe in myself and always gave me their precious time when I turn to them for extra help in any aspects.

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General Conclusion
### Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>aMPV</td>
<td>Avian metapneumovirus</td>
</tr>
<tr>
<td>APHIS</td>
<td>Animal and Plant Health Inspection Service</td>
</tr>
<tr>
<td>aPMV2/6</td>
<td>Avian paramyxovirus type 2/6</td>
</tr>
<tr>
<td>ASPV</td>
<td>Atlantic salmon paramyxovirus</td>
</tr>
<tr>
<td>BeV</td>
<td>Beilong virus</td>
</tr>
<tr>
<td>bPIV3</td>
<td>Bovine parainfluenza virus type 3</td>
</tr>
<tr>
<td>bRSV</td>
<td>Bovine respiratory syncytial virus</td>
</tr>
<tr>
<td>CA</td>
<td>Croup associated</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDV</td>
<td>Canine distemper virus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>DMV</td>
<td>Dolphin morbillivirus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpi</td>
<td>Day(s) post inoculation</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double strand RNA</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>F</td>
<td>Fusion protein</td>
</tr>
<tr>
<td>FDLV</td>
<td>Fer-de-lance virus</td>
</tr>
<tr>
<td>GE</td>
<td>Gene-end</td>
</tr>
<tr>
<td>GS</td>
<td>Gene-start</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutination</td>
</tr>
<tr>
<td>HeV</td>
<td>Hendra virus</td>
</tr>
<tr>
<td>hMPV</td>
<td>Human metapneumovirus</td>
</tr>
<tr>
<td>HN</td>
<td>Hemagglutinin-neuraminidase</td>
</tr>
<tr>
<td>hpi</td>
<td>Hour(s) post inoculation</td>
</tr>
<tr>
<td>hPIV1/2/3</td>
<td>Human parainfluenza virus type 1/2/3</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>hRSV</td>
<td>Human respiratory syncytial virus</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee on the Taxonomy of Viruses</td>
</tr>
<tr>
<td>IFA</td>
<td>Indirect fluorescent antibody</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGS</td>
<td>Intergenic sequence</td>
</tr>
<tr>
<td>J-V</td>
<td>J-virus</td>
</tr>
<tr>
<td>kDA</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>L</td>
<td>Large polymerase protein</td>
</tr>
<tr>
<td>LPMV</td>
<td>La Piedad Michoacán paramyxovirus</td>
</tr>
<tr>
<td>M</td>
<td>Matrix protein</td>
</tr>
<tr>
<td>Mabs</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MenV</td>
<td>Menangle virus</td>
</tr>
<tr>
<td>MeV</td>
<td>Measles virus</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MoV</td>
<td>Mossman virus</td>
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<td>MPRV</td>
<td>Mapuera virus</td>
</tr>
<tr>
<td>MuV</td>
<td>Mumps virus</td>
</tr>
<tr>
<td>N</td>
<td>Nucleocapsid protein</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NDV</td>
<td>Newcastle disease virus</td>
</tr>
<tr>
<td>NiV</td>
<td>Nipah virus</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NNSV</td>
<td>Nonsegmented negative-strand RNA viruses</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>NVSL</td>
<td>National Veterinary Services Laboratory</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>P</td>
<td>Phosphoprotein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCV</td>
<td>Porcine circovirus</td>
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</table>
PEG  Polyethylene glycol
pfu  Plaque forming unit
PGI  Proliferative gill inflammation
PIV  Parainfluenza virus
PK15 Porcine kidney cell line
PoRV/LPMV Porcine rubulavirus
PPRV  *Peste-des-petits* ruminants virus
PRCV Porcine respiratory coronavirus
PRRSV Porcine reproductive and respiratory syndrome virus
PRV  Pseudorabies
RACE  Rapid amplification of cDNA ends
RBCs  Red blood cells
RDRP  RNA dependent RNA polymerase
RNA  Ribonucleic acid
RPV  Rinderpest virus
RT  Reverse transcription
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SeV  Sendai virus
SF  Shipping fever
SIV  Swine influenza virus
SPF  Specific pathogen free
sPIV  Swine parainfluenza virus
sPMV  Swine paramyxovirus
SV5  Simian parainfluenza virus 5
TCID50  50% Tissue culture infective dose
TioV  Tioman virus
TM  Transmembrane
TPMV  Tupaia paramyxovirus
TtPIV-1  *T. truncates* parainfluenza virus type 1
µl  Microliter
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>VN</td>
<td>virus neutralization</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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</table>
General Introduction

Members of the family *Paramyxoviridae* are non-segmented, negative-strand RNA viruses. Members of *Paramyxoviridae* have been isolated from many host species and this family includes viruses that are found associated with central nervous and respiratory system diseases. There are many important disease-causing viruses in this family, including animal pathogens that are economically important in poultry and livestock industry and human pathogens. Members in *Paramyxoviridae* family are not only important in economic and public health aspects, some of them are also extensively studied to be developed as a vaccine vector or for use as anticancer agents.

Paramyxoviruses are classified in the order *Mononegavirales*. Based on virus particle morphology, genome organization, and structure and sequence relatedness, family *Paramyxoviridae* can be divided into two subfamilies; *Paramyxovirinae* and *Pneumovirinae*. Currently, there are five genera within the subfamily *Paramyxovirinae*: *Rubulavirus*, *Avulavirus*, *Respirovirus*, *Morbillivirus*, and *Henipavirus*, and two genera within the subfamily *Pneumovirinae*: *Pneumovirus* and *Metapneumovirus*.

A large and diverse host species are infected by paramyxoviruses including avian, porcine, canine, bovine, equine, ovine, reptiles, aquatic species and humans. There are also extensive records of zoonotic paramyxoviruses derived from sick pigs in the recent decades all over the world.

The family *Paramyxoviridae* has a typical negative-stranded RNA virus structural pattern. The enveloped virus particle contains a left-handed, helically symmetrical
ribonucleoprotein core, called nucleocapsid, which comprises the non-segmented single-negative-stranded RNA genome surrounded by the nucleocapsid proteins, coated within a bilayer viral envelope. The genome length of family *Paramyxoviridae* ranges from 15,000 to 19,000 nucleotides (nt), encoding six to ten tandemly linked genes in the order 3'-N-P/C/V-M-F-HN-L-5'. Interestingly, the genome length of most members of *Paramyxoviridae* is divisible by six, known as “rule of six”.

Two swine paramyxoviruses (sPMV) (81-19252 (Texas81), and 92-7783 (ISU92)) were isolated from the pigs that experienced respiratory and central nervous system disease in the 1980s and 1990s from South and North Central United States, respectively. Texas81 virus was isolated from the brain of pigs that exhibited respiratory and neurological disease in Texas State, in 1981. Further information on the history of this virus is not available. ISU92 virus was isolated from a swine operation in the 1990s in north central United States. Affected pigs showed high fever and mild cough. Encephalitic signs were observed in several pigs with persistent squealing, head pressing, whole body tremors, and hind-limb ataxia leading to excessive mortality. Antigenic analysis by indirect fluorescent antibody assay at the National Veterinary Services Laboratory (NVSL), Ames, Iowa indicated that these two viruses were closely related to human parainfluenza virus (hPIV) type 1 and 3 and bovine parainfluenza virus type 3 (bPIV3).

As new viruses emerging that causes severe diseases in pigs and have the potential to cross species barrier and infect other hosts including humans, it is important that these new viruses to be studied further. The objectives of my thesis is to perform molecular characterization and pathogenicity studies to understand the significance of these two strains of sPMV for swine health and to determine their taxonomic status and to examine the pathogenicity of the two
viruses in conventionally reared pigs. This will establish a foundation for a reverse genetics system to study pathogenesis and develop them into vaccine vectors.
Chapter 1

Literature Review

1.1 Introduction

In the last few decades, many novel paramyxoviruses have emerged causing catastrophic illnesses in different aquatic and terrestrial species of animals and some of them also made the species jump to humans. Members of Paramyxoviridae have been isolated from many host species and this family includes viruses which are found associated with central nervous and respiratory system diseases (Lamb and Parks, 2007). There are many important disease-causing viruses in this family, including animal pathogens that are economically important in poultry and livestock industry, such as Newcastle disease virus (NDV), Rinderpest virus (RPV) and Peste-des-Petits ruminants virus (PPRV), and human pathogens such as measles virus (MeV), mumps virus (MuV), respiratory syncytial virus (RSV) and parainfluenza viruses (PIV) (Lamb and Parks, 2007). Members in Paramyxoviridae family are not only important in economic and public health aspects, some of them such as MeV and NDV are also extensively studied to be developed as a vaccine vector or for use as anticancer agents (Cassel and Garrett, 1965; Elankumaran et al., 2006; Russell and Peng, 2009; Vigil et al., 2008).

Members of the family Paramyxoviridae are non-segmented, negative-strand RNA viruses. The paramyxovirus contains a lipid bilayer envelope that is derived from plasma membrane of the host cell during budding. Viral glycoprotein spikes are inserted into the envelope and can be visualized by electron microscopy. Inside the viral membrane is the helical filamentous nucleocapsid core formed by large polymerase, nucleocapsid, and phosphoproteins
bound to the genomic RNA. They have a close relationship with two other negative strand RNA viruses: Rhabdoviruses (for their unique nonsegmented genomes organization and its expression) and Orthomyxoviruses (for the biological properties of the envelope glycoproteins). The genomic RNA of Paramyxoviruses functions as a (i) template for synthesis of mRNA as well as (ii) the template for the antigenome strand for making further copies of the genomic RNA. This virion structural pattern and replication strategy are common in all negative-stranded RNA viruses (Lamb and Parks, 2007).

1.2 Classification of Paramyxoviruses

Paramyxoviruses are enveloped RNA viruses possessing non-segmented negative-strand genomes in the order Mononegavirales (Lamb et al., 2005b). Based on virion morphology, genome organization, and structure and sequence relatedness, family Paramyxoviridae can be divided into two subfamilies, Paramyxovirinae and Pneumovirinae. Currently, there are five genera within the subfamily Paramyxovirinae: Rubulavirus, Avulavirus, Respirovirus, Morbillivirus, and Henipavirus, and two genera within the subfamily Pneumovirinae: Pneumovirus and Metapneumovirus (Lamb and Parks, 2007) (Table 1). Respiroviruses and rubulaviruses demonstrate both hemagglutinating (HA) and neuraminidase (NA) activity. Morbilliviruses and henipaviruses exhibit only HA but lack NA activity. Pneumoviruses do not cause detectable HA in mammalian and avian erythrocytes and their narrower nucleocapsids also morphologically distinguish them from Paramyxovirinae. In addition, the differential coding potential of the P genes, and the presence of an extra gene (SH) are also considered for the classification (Lamb and Parks, 2007).
Some recently identified paramyxoviruses: Menangle virus (MenV) (Philbey et al., 1998), Tupaia paramyxovirus (Tidona et al., 1999), Tioman virus (TioV) (Chua et al., 2001), Mossman virus (Miller et al., 2003), J-virus (J-V) (Jack et al., 2005; Jun et al., 1977), Beilong virus (BeV) (Li et al., 2006b), Mapuera virus (MPRV) (Karabatsos, 1985), Salem virus (Glaser et al., 2002) and Fer-de-lance virus (Clark et al., 1979) are also officially classified into Paramyxoviridae by International Committee on the Taxonomy of Viruses (ICTV); however, the subfamily and genera are still need to be assigned (Lamb et al., 2005a; Lamb and Parks, 2007). Two more prospective members are, T. truncates parainfluenza virus type 1 (TtPIV-1) isolated from bottlenose dolphins (Nollens et al., 2008), and Atlantic salmon paramyxovirus (ASPV) (Nylund et al., 2008).

1.3 Emerging Paramyxoviruses

A large and diverse host species are infected by paramyxoviruses, including avian, porcine, canine, bovine, equine, ovine, reptiles, aquatic species and humans (Franke et al., 2001; Horwood et al., 2008; Lamb and Parks, 2007; Nollens et al., 2008; Nylund et al., 2007; Nylund et al., 2008). Many bat-associated paramyxoviruses have emerged and some of them caused diseases in animals and humans (Wang et al., 2001). There are also extensive records of paramyxoviruses derived from sick pigs. Mapuera virus (MPRV) was isolated in 1979 from an apparently healthy tropical fruit bat (Sturnira lilium) in Brazil (Karabatsos, 1985). Genomic studies showed that MPRV was closely related to La Piedad Michoacán paramyxovirus (LPMV) (Wang et al., 2007), also known as porcine rubulavirus (PoRV), the only well studied neurotropic paramyxovirus isolated from pigs prior to the 1990s (Moreno-Lopez et al., 1986).
The host range and disease-causing potential of MPRV is still unknown. There was also concurrent infection of porcine reproductive and respiratory syndrome virus (PRRSV) and a paramyxovirus in Germany in the 1990s that had been subsequently named “SER” virus (Heinen et al., 1998; Tong et al., 2002).

In 1972, a disease outbreak characterized by respiratory distress, lethargy, and death occurred on a snake farm in Zurich, Switzerland (Clark et al., 1979). A virus subsequently was isolated from lung tissue of one of the dead snakes. This virus initially was classified as a paramyxo-like virus and designated Fer-de-Lance Virus (Clark et al., 1979). Since the original disease outbreak and identification of a viral etiology in 1972, ophidian paramyxoviruses (OPMV) have emerged as important pathogens of viperid snakes. The first report of OPMV infection in the United States occurred in 1980 in a private collection of snakes in Florida (Jacobson et al., 1980). Similar viruses also have been isolated from non-viperid snakes including a black mamba, corn snakes, beauty snakes, and Moellendorff’s rat snakes (Jacobson et al., 1980). Clinical signs of OPMV include a sudden gaping of the mouth, followed by violent convulsions, regurgitation, and expulsion of a brownish fluid from the glottis. Death usually follows within hours of the first convolution. A variety of lesions have been described in snakes dying with OPMV infections, including proliferative pneumonia, encephalitis, and pancreatic hyperplasia (Jacobson et al., 1981).

In late 1994 in Australia, Hendra virus (HeV) caused an outbreak of severe respiratory disease resulting in the death of 13 horses and their trainer (Murray et al., 1995), followed by sporadic HeV outbreaks in horses and humans (Field et al., 2007a). Subsequent serosurveillance of wild-caught wildlife showed evidence that flying foxes (genus Pteropus, order Chiroptera)
were the natural hosts of HeV (Field et al., 2007b). A closely related virus, Nipah virus (NiV) from Malaysia caused severe febrile encephalitis and death in pigs and humans (Chua et al., 2000), which spread to Bangladesh and India (Chadha et al., 2006; Hsu et al., 2004). HeV and NiV were assigned as a genus, *Henipavirus*, in *Paramyxovirinae* (Eaton et al., 2006).

In 1997, another paramyxovirus, named Menangle virus (MenV) has been isolated in Australia from still-born pigs with deformities (Philbey et al., 1998), and associated human illness (Chant et al., 1998). Neutralizing antibodies against this virus could be detected in several fruit bat species (Philbey et al., 1998). In 2000, Tioman virus (TioV) was isolated from urine collected beneath a fruit bat colony on Tioman Island, Malaysia (Chua et al., 2001). Molecular characterization revealed that MenV and TioV are closely related novel members of the genus *Rubulavirus* (Bowden and Boyle, 2005; Chua et al., 2001; Chua et al., 2002). J-virus (JV), isolated from wild mice in Australia, and Beilong virus (BeV), originally isolated from human mesangial cells in China and subsequently detected in rat mesangial cells, may represent a new group of paramyxoviruses (Basler et al., 2005; Jack et al., 2005; Jun et al., 1977; Li et al., 2006b; Schomacker et al., 2004).

Recently, *T. truncates* parainfluenza virus type 1 (TtPIV-1) isolated from bottlenose dolphins with severe respiratory illness (Nollens et al., 2008), and Atlantic salmon paramyxovirus (ASPV) suffering from proliferative gill inflammation (Kvellestad et al., 2003; Nylund et al., 2008) are also suggested to be classified in *Paramyxovirinae*. However, most of these recently identified paramyxoviruses need to be further classified by the ICTV. For many of the new and emerging viruses, the host range, pathogenicity and geographic distribution are unknown.
1.4 Clinical Signs and Lesions

The clinical signs associated with paramyxovirus infections are essentially similar across species. Many members of the family *Paramyxoviridae* were recognized as important respiratory tract pathogens in many species of animals and humans, while some can also lead to neurological symptoms. **Parainfluenza virus 1 to 3** are major causes of croup, known as severe acute laryngotracheobronchitis, and hPIV3 can also cause pneumonia and bronchiolitis, in infants and children (Chanock, 1956; Chanock et al., 1958). hPIV4 is less severe and can cause mild upper respiratory tract illness in young adults (Ruth A. Karron, 2007). hPIV1, 2, or 3 can produce sensitive and permissive infections in hamsters experimentally. In addition, guinea pigs, cotton rats, and ferrets are also semipermissive, however mice are poorly permissive (Ruth A. Karron, 2007). Chimpanzees and monkeys can also be infected with hPIV1-3 but only hPIV3 causes symptomatic illness in chimpanzees and African green monkeys. Infection usually remains subclinical and pulmonary pathology is minimal or undetectable in most experimental animals due to the limited virus replication (Lamb and Parks, 2007).

**Canine parainfluenza virus 2** (known as PIV5) is one of several etiologic agents of kennel-cough (Appel and Percy, 1970). Infected dogs exhibit clinical signs ranging from dry cough to pneumonia with fever, malaise, coughing with copious amounts of nasal discharge (Appel and Percy, 1970; Rosenberg et al., 1971). A canine PIV was isolated from the cerebrospinal fluid of a dog that experienced incoordination and posterior paresis (Evermann et al., 1980), and this isolate induced hydrocephalus with central nervous system (CNS) depression and severe inspiratory-expiratory dyspnea in dogs after experimental inoculation (Baumgartner et al., 1982a; Baumgartner et al., 1982b).
Canine distemper is caused by the **canine distemper virus** (CDV) which is a contagious, incurable, often fatal, multisystemic viral disease that affects the respiratory, gastrointestinal and central nervous systems, and was the leading cause of death in unvaccinated dog of all ages worldwide (Appel, 1969). CDV occurs among domestic dogs and many other carnivores, including raccoons, skunks, foxes (Appel, 1969), and large cats such as lions, leopards, cheetahs, and tigers (Myers et al., 1997a), but does not cause disease in domestic cats (Bart et al., 2000). The development of a vaccine in the early 1960s led to a dramatic reduction in the number of infected domestic dogs, and canine distemper occurs now only as sporadic outbreaks (Kapil et al., 2008; Norris et al., 2006). Young puppies between 3 and 6 months old are most susceptible to infection and disease, and are more likely to die than infected adults. Nonimmunized older dogs are also highly susceptible to infection and disease (Appel, 1970).

**bPIV3** was first isolated from nasal mucus of calves showing clinical signs of shipping fever including rapid fever, respiratory symptoms, nasal discharge, cough, lacrimation, conjunctivitis and inappetence (Andrewes et al., 1959; Reisinger et al., 1959). bPIV3 can also be isolated from clinically normal cattle (Rosenberg et al., 1971). It is generally accepted that co-infection of bPIV3 along with other viruses and Mannheimia/Pasturella species could result in a clinical disease known as shipping fever (Battrell, 1995; Woods, 1968). A variety of factors such as environmental temperature, transportation, hygiene, stocking density, co-mingling, host immune status can contribute to increased susceptibility to secondary bacterial infection and severity of clinical disease (Kahn and Line, 2005).

**Mumps** was a worldwide common childhood disease, characterized by painful salivary gland swelling (95% of symptomatic cases), and orchitis in infected teenage and adult males
before introduction of the mumps vaccine (Philip et al., 1959). One third of MuV infections are subclinical (Center for Disease Control (CDC) Mumps- United States, 1984-1986). Multiple organs can be symptomatically involved during infection including testes, CNS, epididymis, prostate, ovary, liver, pancreas, spleen, thyroid, kidneys, labyrinth, eyes, thymus, heart, mammary glands, lungs, bone marrow, and joints (Carbone and Rubin, 2007). Virulent strains of MuV commonly target CNS and result in seizures or remain asymptomatic (Azimi et al., 1969). Pancreatitis (Feldstein et al., 1974), arthritis (Gordon and Lauter, 1984), abortion (Philip et al., 1959), renal dysfunction (Utz et al., 1964), deafness (Kirk, 1987), obstructive hydrocephalus (Bistrian et al., 1972), and transient electrocardiogram abnormalities (Arita et al., 1981) may also occur.

**NDV** is one of the most important avian pathogens known. The clinical signs of NDV varies depending on the tissue tropism, strain virulence and susceptibility of the avian order (Cross, 1991). NDV strains display a spectrum of virulence ranging from mild, inapparent infections to severe disease with high mortality (Brugh and Beard, 1984; Cross, 1991). Viscerotropic velogenic NDV causes 100% morbidity and mortality and considered to be the most devastating disease in poultry industry. Infected birds rapidly become anorectic and listless with cyanotic and edematous combs and wattles, followed by severe respiratory distress and conjunctivitis (Cross, 1991). Neurotropic velogenic NDV is another acute, generally lethal infection of chickens of all ages affecting respiratory and neurologic tissues. It can cause 100% morbidity but mortality is generally far less with extremes of 50 percent in adult birds and 90 percent in young chickens (Cross, 1991). Mesogenic NDV is less virulent and result in respiratory signs, CNS signs and reduction in egg production (Cross, 1991). Lentogenic NDV is
milder than mesogenic NDV infection and CNS signs are rarely present (Brugh and Beard, 1984).

The records of paramyxoviruses isolated from pigs with encephalomyelitis are extensive from many parts of the world including Japan (Sasahara et al., 1954), Canada (Greig et al., 1971), and Israel (Lipkind et al., 1986). LPMV, responsible for blue eye disease, is the most thoroughly studied neurotropic paramyxovirus isolated from pigs. LPMV was first isolated in central Mexico in the early 1980s (Moreno-Lopez et al., 1986), and it has become endemic in Mexico (Linne et al., 1992). Clinical signs include reproductive failure, interstitial pneumonia, encephalitis and corneal opacity in infected pigs (Stephano, 1990). Nursing piglets are most susceptible. Morbidity is between 20% and 50% and mortality is between 87% and 90% (Linne et al., 1992).

**Hendra virus** was discovered in 1994 in Australia and caused the death of 13 horses and a trainer (Murray et al., 1995). In horses, infection caused pulmonary oedema and congestion, while, in humans, hemorrhage and edema of the lungs, and encephalitis were seen. A total of nine outbreaks of Hendra virus have occurred since 1994, all involving infection of horses. Four of these outbreaks have spread to humans as a result of direct contact with infected horses. **Nipah virus** was identified in 1999 when it caused an outbreak of neurological and respiratory disease on pig farms in peninsular Malaysia, resulting in 105 human deaths and the culling of one million pigs (Chua et al., 1999). Symptoms of infection from the Malaysian outbreak were primarily encephalitic in humans and respiratory in pigs. Later outbreaks have caused respiratory illness in humans, increasing the likelihood of human-to-human transmission and indicating the existence of more dangerous strains of the virus (Chadha et al., 2006; Hsu et al., 2004).
Several paramyxoviruses have been isolated from rodents since 1960s. **Mossman virus** (MoV) was first isolated from pooled organs of trapped native rats (Miller et al., 2003) and **J-virus** (J-V) was isolated from trapped moribund *Mus musculus* (Jun et al., 1977) both in 1970s in Queensland, Australia. **Beilong virus** (BeV) was first isolated from the human mesangial cell line. However, the presence of BeV in a rat mesangial cell line used in the same laboratory prior to the isolation from the human mesangial cells suggests it to be of rodent origin (Li et al., 2006b). **Tupaia paramyxovirus** (TPMV) was isolated from the kidneys of an apparently healthy tree shrew captured in Thailand (Tidona et al., 1999). Phylogenetic analysis places these four viruses between or within the genera *Morbillivirus*, *Respirovirus* and *Henipavirus*, but the taxonomic status is still unclear and they have not been officially classified into any of the existing genera (Li et al., 2006b; Tidona et al., 1999).

**Atlantic salmon paramyxovirus** (ASPV) was first isolated in 1995 from Atlantic salmon (*Salmo salar* L.) experiencing respiratory disease, proliferative gill inflammation (PGI) and the outbreaks increased in past years (Nylund et al., 2008). PGI shows signs of inflammation and proliferation and cell death of gill tissue in Atlantic salmon. Affected fish had reduced growth rate and even mortality in severe cases (Nylund et al., 2008). ASPV was one of the agents isolated from some PGI cases although the primary causative agent has not yet been identified (Kvellestad et al., 2003; Kvellestad et al., 2005).

**T. truncates parainfluenza virus type I** (TtPIV-1) was isolated from 19-year old male Atlantic bottlenose dolphin suffering from respiratory illness including raspy, foul-odored breath and cream-colored exudates from the blowhole (Nollens et al., 2008). Foamy mononuclear cells were observed in blood smear and neutrophils and monocytes were detected in blowhole swabs.
Phylogenetic analysis classified TtPIV-1 in the cluster of *Paramyxovirinae*, genus *Respirovirus*, and significant relationship with bPIV3 (Nollens et al., 2008).

1.5 Molecular Biology of Paramyxovirus

1.5.1 Virion Structure and Genome Organization

The family *Paramyxoviridae* has a typical negative-stranded RNA virus structural pattern (Figure 1). The enveloped virus particle contains a left-handed, helically symmetrical ribonucleoprotein core, called nucleocapsid (NC), which consists of non-segmented single-negative-stranded viral RNA genome surrounded by nucleocapsid proteins and associated with phosphoproteins and large polymerase protein (Lamb and Parks, 2007). The NC of *Paramyxovirinae*, 18 nm in diameter, 1µm in length, and a pitch of 5.5 nm in diameter, is enclosed by an outer lipoprotein envelope (Lamb and Parks, 2007). The helical NC of paramyxoviruses presents “herring bone” morphology when viewed under an electron microscope. *Pneumovirinae* subfamily can be differentiated from *Paramyxovirinae* subfamily morphologically because they have narrower nucleocapsids (Lamb and Parks, 2007). The virions of *Paramyxoviridae* are pleiomorphic in shape being spherical or filamentous (Figure 1). In general, spherical virions are 150-350 nm in diameter (Lamb and Parks, 2007).

The genome length of family *Paramyxoviridae* ranges from 15,000 to 19,000 nucleotides (nt), encoding six to ten tandemly linked genes in the order 3'-N-P/C/V-M-F-HN-L-5'. Interestingly, the genome length of most members of *Paramyxoviridae* is divisible by six, known
as “rule of six” (Calain and Roux, 1993; Kolakofsky et al., 1998; Kolakofsky et al., 2005), i.e.
their genomes must be of polyhexameric length \( (6n+0) \) to replicate efficiently. Each nucleoprotein subunit associates with six nucleotides and that makes the RNA chain length as an even multiple of six when completely encapsidated. There is a \( \sim 50 \) nt 3’ extracistronic leader region and a 50 to 171 nt 5’ extracistronic trailer region (or \([-\]) \) leader) at the two ends of the genome. The leader and trailer are considered to be \( cis \)-acting signals and are essential for transcription and replication. Between the gene boundaries, there are intergenic regions (IGS), which are strictly conserved trinucleotides in \textit{Respirovirus, Henipavirus} and \textit{Morbillivirus} but quite variable in length for the \textit{Rubulavirus} and \textit{Pneumovirinae}, ranging from 1 to 56 nt. The newly discovered paramyxoviruses, such as TioV, have an even longer IGS, which may be up to 70 nt in length.

The glycoprotein complexes are stalk-like spikes on the surface of virions. Fusion protein (F) and an attachment protein, called hemagglutinin-neuraminidase (HN) or hemagglutinin (H) or glycoprotein (G) depending on the virus genera (Lamb and Parks, 2007), are the major components of the spikes. The F and HN/H/G proteins may be in the form of trimers (F) and tetramers (attachment protein), respectively. The envelope glycoproteins are responsible to mediate virus attachment and penetration during infection. The internal helical nucleocapsid core contains the RNA genome and nucleocapsid (N), phosho- (P) and large (L) polymerase proteins. Another structural protein, matrix (M) protein, is located between the envelope and the core, and is important in virion architecture and in infection process. Accessory proteins are mostly generated from overlapping open reading frames within P gene transcriptional units by a process called RNA editing or by alternative translation initiation. These accessory proteins are important in viral morphogenesis, RNA synthesis, and pathogenesis (Lamb and Parks, 2007).
1.5.1.1 Nucleocapsid Protein

The nucleocapsid protein (N) is one of the most abundant proteins in paramyxoviruses and is present as the first transcribed gene, adjacent to the leader sequence at the 3’ end of viral genomic RNA for all paramyxoviruses, except the pneumoviruses, in which two more nonstructural genes, NS1 and NS2 will be transcribed first. The N protein is composed of 489 to 553 amino acids (aa) with the predicted molecular weight of 53-58 kDa. The N-terminal region of the N protein is involved with encapsidation of viral RNA, coats full-length of viral [-] sense genomic and [+] sense antigenomic RNAs to form the helical nucleocapsid. This helical structure serves several functions including protection from nuclease digestion, alignment of distal RNA segments to create a functional 3’ end promoter and providing interaction with P and L proteins during transcription and replication, and association with M protein during virus assembly (Lamb and Parks, 2007).

1.5.1.2 The P gene and its encoded proteins

*Paramyxovirinae* P gene encoding multiple viral proteins can be considered as a remarkable example of exploiting the coding capacity of viruses. Sendai virus (SeV) P gene can direct the expression of at least seven polypeptides, including P, V, W, C’, C, Y1 and Y2. Other paramyxoviruses may express fewer proteins from the P gene, however this capability is always seen. Generally, two mechanisms involve production of multiple mRNA species from the P gene. One is known as alternative initiation, by which the family of C proteins is produced from an alternative initiation translation codon. The second mechanism involves pseudotemplated
insertion of multiple G residues at a specific position, known as mRNA editing site, to produce mRNAs of P, V proteins and virus-specific proteins variously called W/I/D proteins, depending on the virus genus. P, V, W/I/D are a set of proteins that are amino co-terminal and the mRNAs are only different by inserted G nucleotides that shift the translation reading frame after the insertion site (Lamb and Parks, 2007).

Viral accessory proteins and their interactions with the host

Type I interferon (IFN) is one of the most important antiviral response which has two general phases: a primary transcriptional phase of induction of IFN synthesis, and a secondary transcriptional phase through type I IFN signaling pathway (Biron and Sen, 2007). Paramyxovirus accessory proteins can counteract the host cell IFN pathways at both levels (Biron and Sen, 2007). The paramyxovirus V protein cysteine-rich domain can limit dsRNA-induced activation of the IFN-beta promoter by direct interaction with melanoma differentiation-associated gene 5 (MDA5) (Komatsu et al., 2004). The expression of C proteins in SeV and W proteins in NiV can also reduce IFN-beta promoter activation in response to dsRNA (Komatsu et al., 2004). IFN signaling is initiated by binding of secreted IFN and its cognate receptor on the cell surface leading to phosphorylation of latent transcription factors, STAT1 and STAT2, which are signal transducers and activators of transcription (Karron and Collins, 2007; Lamb and Parks, 2007). The V protein of some paramyxoviruses such as PIV5, MuV, SV41, hPIV2, NDV can target one of the STATs for degradation to block the IFN signaling (Biron and Sen, 2007) while some like henipaviruses and measles virus can form a V and STAT protein binding complex and prevent its translocation to the nucleus (Palosaari et al., 2003; Rodriguez et al., 2003; Shaw et al.,
The C protein of respiro- and morbilliviruses are involved in blocking IFN signaling by a different mechanism (Garcin et al., 2001; Garcin et al., 2002; Gotoh et al., 2003; Takeuchi et al., 2001). SeV and hPIV3 can alter STAT phosphorylation patterns (Gotoh et al., 2003), interact with STAT1 (Garcin et al., 2001; Takeuchi et al., 2001), and induce ubiquitination and degradation of STAT1 in some mouse cells (Garcin et al., 2002).

Phosphoprotein

The paramyxovirus P protein is generally 400 to 600 aa long. It is heavily phosphorylated at serine and threonine residues predominantly within the N-terminal region (Lamb and Parks, 2007). The C-terminal module and N-terminal domain of P protein play important roles as polymerase cofactor and in nascent chain assembly, respectively. The C-terminal polymerase cofactor module is conserved in the predicted secondary structure for all Paramyxovirinae members. It contains domains for P-P multimerization, for L protein interactions and for N-RNA template binding (Horikami et al., 1992). The N-terminal region is believed to act as a chaperone to facilitate interactions with unassembled N in order to prevent N aggregation and to prevent uncontrolled encapsidation of non-viral RNA (Curran et al., 1995).

V protein

The V protein is a 25 to 30 kDa polypeptide that is amino co-terminal with P protein but with a variable C-terminal domain. The C-terminal V-specific domain is highly conserved among paramyxoviruses with invariantly spaced histidine and cysteine residues forming a
domain (cys-rich domain) that binds two zinc molecules per V protein (Fukuhara et al., 2002; Li et al., 2006a; Liston and Briedis, 1994; Paterson et al., 1995). The V protein plays important roles in virus replication (Baron and Barrett, 2000; Curran et al., 1991), functions as a negative regulator to inhibit RNA synthesis (Baron and Barrett, 2000; Delenda et al., 1997; Durbin et al., 1999), and inhibits host cell antiviral response by interacting with cellular proteins (Andrejeva et al., 2002; Lin et al., 1998).

**W/D/I protein**

The W and D proteins are expressed by inserting 2 G residues at the P gene mRNA editing site in respiro-, morbilli-, and henipaviruses. The W protein in SeV is found to interact with unassembled N0, suggesting an inhibitory role in viral RNA synthesis (Horikami et al., 1996). In PIV3, insertion of 2 G residues at the editing site produces a protein called D protein (Galinski et al., 1992). In rubulaviruses, insertion of one or four G residues during RNA editing produces I protein (Paterson and Lamb, 1990; Thomas et al., 1988). The role of W/D/I in viral growth cycle has not been understood (Lamb and Parks, 2007).

**C protein**

The C proteins are generated using alternative translation initiation codons in *Respiro-, Henipa-, Morbillivirus* (Lamb and Parks, 2007). In SeV, C’, C, Y1 and Y2 comprise a nested set with a shared C-terminal. The C proteins are small basic polypeptides that may involve in the
viral growth cycle, control of viral RNA synthesis (Lamb and Parks, 2007), counteracting host cell antiviral pathways (Komatsu et al., 2004) and facilitating the release of virus from infected cells (Garcin et al., 1997; Kato et al., 2001).

1.5.1.3 Matrix Protein

The M protein is the most abundant protein in the virion, comprising of 341 to 375 residues with a molecular weight of 38.5-41.5 kDa. The net charge at neutral pH is +14 to 17 making it a basic protein. Although there is no transmembrane domain that has sufficient length to span a lipid bilayer, M protein is somewhat hydrophobic and peripherally associated with membranes (Lamb and Parks, 2007). The M protein is considered to be the central organizer of viral morphogenesis interacting with the cytoplasmic tails of integral membrane protein, F and HN, the lipid bilayer and the nucleocapsids (Blumberg et al., 1984; Cathomen et al., 1998; Sanderson et al., 1993; Schmitt et al., 1999; Stricker et al., 1994).

1.5.1.4 L Protein

The L protein, located at the most promoter distal part in the genome, is the least abundant protein in virus particles. A paramyxovirus particle contains only ~50 copies of L (Lamb et al., 1976). Overexpression of L protein may inhibit virus growth (Banerjee and Barik, 1992). It is an essential subunit of paramyxovirus RNA-dependent RNA polymerase (RdRP). The L gene consists of ~2200 aa with a molecular weight of 220-250 kDa. The L protein possesses all the
enzymatic activities needed for polymerization, 5’ end capping, methylation and 3’ end polyadenylation of mRNA (Grdzelishvili et al., 2005; Ogino et al., 2005). The L protein associates with P protein to form the active viral polymerase and the polymerase complex can recognize the helical N-RNA template (Hamaguchi et al., 1983; Poch et al., 1990).

1.5.1.5 Viral Glycoproteins

**Fusion Protein**

The F glycoprotein is a type I integral membrane protein mediating viral penetration by fusion between the virion envelope and the host cell plasma membrane at neutral pH. The F protein is composed of 540 to 580 aa residues. It is synthesized as an inactive precursor, F0, which must be proteolytically cleaved to produce the active fusion protein, which consists of disulfide-linked F1 and F2 polypeptides to be biologically functional (Scheid and Choppin, 1974). This cleavage event is required for progeny virions to become infectious (Garten et al., 1980; Nagai et al., 1976). The cleavage of F0 is a candidate to be a key determinant for infectivity and pathogenicity for certain viruses. The Paramyxoviridae has a cleavage signal sequence located at the N-terminal of F with multibasic or single basic residue at the cleavage site. The cleavage of F containing multibasic residues at the cleavage site occurs intracellularly by furin, a subtilisin-like endoprotease (Klenk and Garten, 1994), while F with a single basic residue at the cleavage site must be expressed at the cell surface and incorporated into released virions and then can be cleavage activated by the addition of exogenous protease (Scheid and Choppin, 1974). For NDV, the nature of the cleavage site correlates with the virulence of the virus strains.
The ones with multibasic residues at the cleavage site show virulence and readily disseminate through the host whereas strains with single basic residue at the cleavage site are avirulent and tend to be restricted to the respiratory and alimentary tracts where the necessary secreted protease is present (Nagai and Klenk, 1977). The C-terminal of F has a hydrophobic transmembrane (TM) domain that anchors the protein into the membrane leaving a short cytoplasmic tail (~20-40 residues). A 4-3 (heptad) pattern of hydrophobic repeats, designated heptad repeats HRA and HRB can be found between the fusion peptide and the TM domain, with approximately 250 aa apart from each other (Lamb and Parks, 2007). Biophysical data and crystallographic studies have shown that HRA and HRB can form a helical hairpin or six-helix bundle (6HB) structure (core trimer) in F protein homotrimers and this 6HB formation is tightly linked to the merger of lipid bilayers and is believed to couple the free energy released on protein refolding to membrane fusion (Melikyan et al., 2000; Russell et al., 2003).

**Attachment Protein**

Attachment protein is another glycoprotein in *Paramyxoviridae* involved in cell attachment process, which is also an integral membrane protein (Lamb and Parks, 2007). The attachment protein of *Respirovirus*, *Avulavirus* and *Rubulavirus* binds to cellular sialic acid-containing receptors and are capable of agglutinating erythrocytes. Besides, these attachment proteins also have neuraminidase activity that cleaves sialic acid from the progeny virus particles to prevent viral self-aggregation (Scheid and Choppin, 1974) and enable release of virions from the cell membrane. Thus, the protein has been designated hemagglutinin-neuraminidase (HN). HN is a type II membrane protein composed 565 to 582 residues that span the membrane once. It
contains an N-terminal cytoplasmic tail, a single N-terminal transmembrane domain (TM domain), a membrane-proximal stalk domain that supports a C-terminal globular head domain. The globular head domain contains both receptor binding and enzymatic activity (Hiebert et al., 1985a). HN presents on the surface of virions and infects cells as a tetramer consisting of pairs of homodimers (Thompson et al., 1988).

In *Morbillivirus*, attachment protein, H, can cause agglutination of primate erythrocytes but lacks neuraminidase activity. This restricted host range, representative in measles virus for primate cells, makes the sialic acid unlikely to be the primary receptor. It is now believed that CD150, also known as signaling lymphocyte activation molecule (SLAM), is the principal receptor for unadapted isolates of lymphotropic measles virus (Oldstone et al., 2002; Yanagi et al., 2002). The attachment protein, G, in *Henipavirus* has neither hemagglutinating nor neuraminidase activity. Ephrin B2 is considered as the cellular receptor for both HeV and NiV. The widespread occurrence of ephrin B2 in vertebrates, particularly in arterial endothelial cells and in neurons, provides an explanation for the wide host range of henipaviruses and their ability to cause systemic infections (Eaton et al., 2006).

The *Pneumovirus*, RSV, does not cause detectable HA. The cellular receptor for RSV is not completely understood yet, but it is believed that it involves interactions with heparan sulfate, a glucosaminoglycan that is part of the extracellular matrix. The structure of G protein in *Pneumovirus* is quite different from the attachment protein of *Paramyxovirinae*. Both membrane bound and proteolytically cleaved soluble forms can be found in infected cells. Extensive carbohydrate modification is one distinguishing feature of the G protein of RSV, which makes the protein migration on SDS-PAGE with a dramatic increase in molecular weight than that of
the predicted polypeptide. These studies also show that G protein is not essential for virus assembly or growth in tissue culture or animals, although, it does confer a growth advantage (Lamb and Parks, 2007). Interestingly, the observation that G protein deletion mutant of RSV and HN deleted virus-like particle (VLP) of SeV can infect cells via the asialoglycoprotein receptor suggests that some paramyxovirus F proteins may also show binding activity (Leyrer et al., 1998).

For most paramyxoviruses, the receptor binding protein (HN, N, or G) is required to mediate the fusion reaction (Lamb et al., 2006; Morrison, 2003). The precise role of HN/N/G in stimulating the F conformational change remains to be understood. However, HN may execute its function by stabilizing the F prefusion stalk in a receptor-dependent manner (Lamb and Parks, 2007). Several models were studied to rationalize the involvement of HN in fusion promotion (Lamb, 1993; Lamb and Parks, 2007). A model in NDV proposed a second sialic acid binding site in addition to the active site (Crennell et al., 2000). The location of this new site at the HN dimer interface, together with observation that changes in the HN structure that appear to be generated by catalysis alters the association of the HN dimer or tetramer and may also propagate a change in the stalk region that triggers the fusion protein, suggests a model for how sialic acid binding by HN might trigger conformational changes in F (Zaitsev et al., 2004).

**Other Envelope Proteins**

The rubulaviruses PIV5 and mumps virus contain a small gene located between F and HN genes, designated “small hydrophobic” (SH) gene (Hiebert et al., 1985b; Hiebert et al.,
Mutants lacking SH of PIV5 is attenuated in vivo, and can induce apoptosis in L929 and MDCK cells through a tumor necrosis factor alpha-mediated extrinsic apoptotic pathway (He et al., 2001; Lin et al., 2003). Mumps SH may have a similar role as in PIV5 (Wilson et al., 2006). Avian paramyxovirus type 6 (APMV6), a member in Avulavirus also contains the SH gene in its genome. However, NDV and other members in the genus Avulavirus do not encode SH. NDV was used to be classified in Rubulavirus. The presence of the SH gene in APMV6 to some extent justifies the close relationship between this two genera and also suggests that APMV-6 might play an intermediate role between the evolution of Avulavirus and members of the genus Rubulavirus (Chang et al., 2001). Members of the Pneumovirinae also encode a SH protein, however, it does not necessarily mean a commonality in function with Rubulavirus SH and the role of SH in the RSV life cycle is not completely understood (Lamb and Parks, 2007). M2 gene, encoding two partially overlapping ORFs, M2-1 and M2-2, can be found in members of the Pneumovirinae and two more nonstructural proteins, NS1 and NS2, can be exclusively found in RSVs. M2-1 and M2-2 play an important role in transcription and RNA replication (Bermingham and Collins, 1999; Fears and Collins, 1999) and NS1, NS2 show importance in suppression of type I interferon induction and RNA synthesis (Atreya et al., 1998; Bossert et al., 2003; Spann et al., 2004; Teng and Collins, 1999; Whitehead et al., 1999).

J-V possesses two unique proteins that are not observed in other paramyxoviruses. Putative transmembrane protein (TM) is 258 aa long protein located between SH and G protein genes in J-V genome (Jack et al., 2005). J-V “G” gene has an extraordinarily long putative 3’ UTR in which a second open reading frame (ORF), termed ORF-X, has been identified commencing immediately after the stop codon for the putative G protein (Jack et al., 2005). ORF-X encodes a putative protein of 704 aa within which the first methionine is the 30th aa
residue (Jack et al., 2005). No significant amino acid sequence homology was identified between TM or X and other known proteins in sequence data banks by BLASTp search and this sequencing based prediction need to be experimentally verified (Jack et al., 2005).

1.5.2 Strategy of replication

Paramyxoviruses encode and package their own RNA dependent RNA polymerase (RdRP). The synthesis process is activated only after the virus being uncoated in the infected cell and the replication occurs in the cytoplasm. In cell cultures, a single cycle of growth takes ~14-30 hours for most paramyxoviruses, but with virulent NDV strains, this can be as short as 10 hours. Viral intracellular replication begins with viral RdRP packaged in the virion transcribing the N encapsidated genome RNA into 5’ capped and 3’ polyadenylated mRNA. This virion RNA being encapsidated with the N and associated with P and L proteins forms the transcriptase complex which is the minimum transcriptional unit of paramyxovirus (Lamb and Parks, 2007). The transcription signals are located at gene-start (GS) and gene-end (GE), which border individual genes. Starting from the 3’ end of the genome, viral RdRP begins the transcription process in a sequential manner to produce capped and polyadenylated viral mRNAs by terminating and reinitiating at each gene junction, also known as a start-stop-restart mechanism. A controlled attenuation of transcription occurs in each intergenic region, where a fraction of elongating polymerases are released from the genomic templates, producing mRNA levels that progressively decrease from N to L (Lamb and Parks, 2007), resulting in a transcriptional gradient, commonly referred to as “polarity of transcription”. After primary transcription and translation, as unassembled N protein accumulates, genomic RNA synthesis will be activated.
The RdRP will then ignore all the gene junctions to produce an exact complementary antigenome chain in a fully assembled nucleocapsid.

1.6 Parainfluenza viruses

1.6.1 History of parainfluenzaviruses

Human parainfluenza virus contains 4 serotypes, hPIV1 to hPIV4. They were first recovered from infants and children with lower or upper respiratory tract diseases, referred as “croup associated” (CA) viruses at that time (Chanock, 1956; Chanock et al., 1958; Johnson et al., 1960). The hPIV1, 2, and 3 viruses are second to human respiratory syncytial virus (hRSV) as a common cause of lower respiratory tract disease in young children, which are major causes of croup (hPIV1 to 3) and pneumonia bronchiolitis (hPIV3). However, hPIV4 causes less severe disease at less frequency. Bovine parainfluenza virus type 3 is one of the major pathogens in bovine shipping fever complex and it is a close relative of hPIV3.

Sendai virus (SeV) was first isolated from mice inoculated with an autopsy specimen from an infant with respiratory disease and it appeared to be a murine relative of hPIV1 rather a natural human pathogen. Simian virus 5 (SV5) and Simian virus 41 (SV41) were all originally isolated from primary monkey kidney tissue culture (Hull et al., 1956; Nishio et al., 1990). Both could be detected in a proportion of human population indicating capability for human infection. SV5 can also cause croup in dogs and is a canine relative of hPIV2. However, SV41 shows more close relationship to hPIV2 than SV5 in antigenic and structural properties (Tsurudome et al., 1990).
Newcastle Disease was recognized as an emerging poultry disease in Indonesia and England in 1926 (Doyle, 1927) and is now known as serotype 1 of nine distinct serotypes (Alexander, 1982; Alexander et al., 1983) of avian PIV (now redesignated avian paramyxoviruses (APMV)). Besides, mumps virus was also historically considered as a member of PIV group due to the morphologic and antigenic relativities. These viruses were classified in a single genus historically and then identified into distinguished genera after the addition of related viruses and molecular confirmation. Nowadays, it is widely acknowledged that within PIV group there are two divisions of mammalian PIV genera: Respirovirus and Rubulavirus, and one avian viruses genus Avulavirus (Ruth A. Karron, 2007).

1.6.2 Bovine parainfluenzavirus type 3

1.6.2.1 bPIV3 as a pathogen

bPIV3 is a respiratory pathogen associated with bronchopneumonia in cattle and it is one of the important pathogens of the bovine respiratory disease syndrome called “shipping fever”, which causes severe economic losses to the cattle industry in North America and other parts of the world. Clinical signs of shipping fever include rapid fever, respiratory symptoms, nasal discharge, cough, lacrimation, conjunctivitis and inappetence (Andrewes et al., 1959; Reisinger et al., 1959). bPIV3 can be isolated from clinically normal cattle, (Reisinger et al., 1959). Gross lesions in cattle experimentally infected with bPIV3 include consolidation of the anteroventral portions of the lungs (Bryson et al., 1979; Marshall and Frank, 1975), congestion of respiratory mucosa, and enlargement of the bronchial and retropharyngeal lymph nodes (Marshall and
Frank, 1975). Mononuclear and polymorphonuclear cells and multinucleated giant cell can be observed in affected lung lobes by histopathologic study (Tsai and Thomson, 1975). It is generally accepted that co-infection of bPIV3 along with other viruses and Mannheimia/Pasturella species in addition to a variety of other factors as environmental temperature, transportation, hygiene, stocking density, co-mingling and host immune status could cause shipping fever and can contribute to increased susceptibility to secondary bacterial infection and severity of clinical disease (Battrell, 1995; Woods, 1968).

1.6.2.2 Cross-species infection of bPIV3

PIV3 infections have been serologically demonstrated in a wide variety of mammals including cattle, human, sheep (Lyon et al., 1997), goats (Yener et al., 2005), bison (Zarnke and Erickson, 1990), guinea pigs (Ohsawa et al., 1998), black and white rhinoceros (Fischer-Tenhagen et al., 2000), moose (Thorsen and Henderson, 1971), bighorn sheep (Parks et al., 1972) and camels (Eisa et al., 1979). A novel parainfluenza virus isolated from bottlenose dolphin (Nollens et al., 2008) was phylogenetically closely related to PIV3.

Besides, cross-species infection has also been reported in numerous instances, including hPIV3-GPv isolated from lung homogenates of sentinel guinea pigs placed together with seropositive asymptomatic individuals (Ohsawa et al., 1998), and bPIV3 in a child experiencing pneumonia (Ben-Ishai et al., 1980). bPIV3 in lambs can cause rapid, shallow respiration, dyspnea and sporadic coughing (Stevenson and Hore, 1970). No respiratory signs were observed in calves experimentally infected with ovine PIV3 (Stevenson and Hore, 1970). Macroscopic
lesions of pneumonia were present in bPIV3 inoculated lambs and ovine PIV3 inoculated calves (Stevenson and Hore, 1970).

1.6.2.3 Virion Structure and Genome organization

PIV3 has spherical to pleiomorphic virions of approximately 150-200 nm in diameter and morphologically indistinguishable from paramyxoviruses (Karron and Collins, 2007). The genetic organization of bPIV3 is very similar to hPIV3, composed of 6 genes in the order of 3’-N-P-M-F-HN-L-5’, and the overall length and nt and amino acid sequences are also broadly conserved among PIV3 viruses. There is a high degree of identity between bPIV3 and hPIV3, ranging from 58.6% for P protein to 89.7% for M protein (Bailly et al., 2000). They also share extensive sequence identity in their cis-acting noncoding regulatory elements, 3’ leader (86.3%), 5’ trailer (93.2%) and trinucleotide intergenic sequence (100%). All bPIV3 and hPIV3 have a semi-conserved gene-start sequence and semi-conserved U-rich gene-end sequence. Interestingly, majority of variable amino acid residues found in bPIV3 and hPIV3 showed polymorphisms, either showing host specific or variable within the species at the given position (Bailly et al., 2000; Coelingh and Winter, 1990; Swierkosz et al., 1995). These residues might be products of distinct selective pressures during PIV3 evolution in their respective host. The host-specific sequences are likely responsible for the host range differences and growth efficiency in different hosts. Using reverse genetics to import these sequences from bPIV3 into hPIV3 was shown to be very useful in the development of vaccines (Bailly et al., 2000).
The complete nucleotide sequence for four isolates of bPIV3 has previously been determined (Bailly et al., 2000; Horwood et al., 2008; Sakai et al., 1987; Suzu et al., 1987). With the increasing availability of the sequence information, two distinct bPIV3 genotypes were proposed, designated as bPIV3-a and bPIV3-b (Horwood et al., 2008). However, the genotype b is an isolated variant lineage that has only been identified in Australia and could hypothetically be a lineage from a strain that recently crossed from another host species into cattle (Horwood et al., 2008). The newly identified genotypes have implications for the development of bPIV3 molecular detection methods and may also impact on bPIV3 vaccine formulations.

1.6.2.4 Antigenic relatedness with other parainfluenzaviruses

bPIV3 and hPIV3 shared approximately 25% antigenic relatedness. The Kansas/1562/84 (Ka) and Shipping Fever (SF) strains of bPIV3 replicated 100 to 1000 times less efficiently than hPIV3 in the upper and lower respiratory tract of rhesus monkeys and chimpanzees compared with hPIV3 (van Wyke Coelingh et al., 1988). Thus, bPIV3 was evaluated as a live vaccine against hPIV3. The bPIV3-Ka strain was highly attenuated in humans and was used in clinical trials as a candidate vaccine against hPIV3 (Karron et al., 1996). Although intranasally administered bPIV3 vaccine gave seroconversion rate to bPIV3 (57–65%), it did not give a robust response to the heterotypic human strain. However, bPIV3 vaccine being used as an attenuated backbone for insertion of human PIV3 HN and F proteins and F protein of RSV is promising and the effectiveness of this vaccine against both PIV3 and RSV challenge has been demonstrated in African green monkeys (Sato and Wright, 2008).
Monoclonal antibodies (Mabs) were used to identify the antigenic properties of hPIV3 and bPIV3 (Coelingh et al., 1986; van Wyke Coelingh et al., 1985). HI-Mabs, which are Mabs that have HI and neutralizing activities, can define 11 operationally unique HN epitopes which are organized into two topologically nonoverlapping antigenic sites (A and B) and a third bridging site (C) (van Wyke Coelingh et al., 1985). Using HN-Mabs, which has no known biological activity, three more sites (D to F) were identified (Coelingh et al., 1986). Two epitopes in site A and all epitopes in sites D and F are highly conserved among human clinical isolates examined as well as bovine strains (Coelingh et al., 1986). Nucleotide substitutions in the HN genes of antigenic variants selected with NI-Mabs were identified representing epitopes in site A, which are shared by human and bovine PIV3. The deduced amino acid substitutions in the variants were located in separate hydrophilic stretches of HN residues which are conserved in the primary structures of the HN proteins of both human and bovine PIV3 strains (Coelingh et al., 1986).

1.6.2.5 Diagnostic methods for bPIV3

A diagnosis of bPIV3 requires laboratory confirmation. Virus may be isolated when animals that are in the incubation or acute phases of infection are sampled. In live animals, virus can be isolated from nasopharyngeal swabs or broncho-alveolar washings, and in dead animals, from the lungs and trachea and their associated lymph nodes. The virus can be grown in a variety of cell cultures, such as bovine and ovine fetal lung, kidney and testis, and as well as in cell lines, such as MDBK and RK-13. Cytopathic effects are characterized by syncytia, intracytoplasmic and intranuclear inclusion bodies and cell destruction. Antigen detection methods such as
immunofluorescence, immunoperoxidase staining or enzyme immunoassay can be used (Haines et al., 1992). RT-PCR-based diagnostic tests enable accurate and timely detection of bPIV3 from nasopharyngeal swabs. Seroconversion can be detected 7-10 days following the onset of clinical signs by HI or virus neutralization or plaque reduction tests.

1.7 Swine Paramyxoviruses

1.7.1 History

The first record of the outbreak of one of two swine paramyxoviruses, ISU92, was described by Janke et al. (1992). A group of 400 pigs in a continuous-flow finishing barn showed signs of respiratory and CNS disease. Sick pigs showed mild cough with temperature from 103 to 104.5 °F. Injectable dexamethasone and tylosin were administered and total of seventeen pigs died within four days after the first appearance of clinical signs. Continued treatment with a combination of penicillin, dexamethasone, spectinomycin and atropine were used and no more deaths occurred. Wet, heavy, congested lungs with small areas of consolidation on the ventral tips of the lung lobes were observed at necropsy.

The disease continued to spread throughout the operation and infected the second finishing all-in/all-out unit. The entire 400 pigs in the finishing barn were infected within a few days. Severe dyspnea, coughing and CNS disturbance were observed. Several sick pigs showed persistent squealing, head pressing, whole body tremors, and hind-limb ataxia (Janke et al., 2001). In the breeding, farrowing, nursery and grower buildings, located ¼ mile north of the finishing barns, nursery age pigs showed dyspnea and less cough than finishing pigs; clinical
signs in nursing piglets in farrowing barn were more severe; the grower buildings were only mild affected; few sows and gilts became dyspneic, two or three abortions and two premature farrowing occurred, however, the cause was not investigated. Injectable antimicrobials were administered to all affected pigs immediately after first onset of clinical signs and there were no more death losses due to the outbreak (Janke et al., 2001).

1.7.2 Laboratory Examination

Two live pigs from the second finishing barn were sent to the Veterinary Diagnostic Laboratory of Iowa State University. CNS disorder was the major signs observed similar to those seen during the first outbreak. Pigs exhibited depression, whole body tremors, continuous squealing, and rear-end ataxia. No respiratory symptoms were observed. 20% of lung volume showed bilateral consolidation on ventral portions of cranial and middle lung lobes. No gross lesions were noticeable in brain or other internal organs. In histopathologic examination, moderate to severe bronchointerstitial pneumonia with thickening of alveolar septa containing mixed inflammatory cells and swollen pneumocytes and endothelial cells were observed. Mild diffuse gliosis and widespread, mild lymphocytic perivasculitis were seen in brain from both pigs.

No pseudorabies (PRV), Swine influenza virus (SIV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine respiratory coronavirus (PRCV) were detected by either virus isolation or fluorescent antibody test. No significant bacteria were recovered from the collected tissue samples. Paramyxo-like virus particles were isolated from fetal porcine
kidney (FPK) cell culture, which was inoculated with a pooled tissue homogenates. Cytopathic effect (CPE) was observed in infected cell cultures from second passage onwards (Janke et al., 2001).

1.7.3 Pathogenicity studies

Specific pathogen free (SPF) pigs were inoculated with the lung homogenate (Battrell, 1995). No clinical illness, gross or microscopic lesions were observed. No antibody against ISU92 was detected in the serum of before inoculation or at necropsy. Experimental inoculation of three-day-old gnotobiotic piglets with cell-culture propagated ISU-92 showed temporary and slight increase of rectal temperature in some infected pigs. Irregular tan-gray discoloration was observed in the lung lobes of infected pigs. Lungs with thickened alveolar septa and proliferation of pneumocytes and brain with glial nodules were observed on microscopic examination (Janke et al., 2001).

1.7.4 Prevalence studies

To identify the extent of prevalence of swine paramyxoviruses in USA, a virus neutralization test was developed (Battrell, 1995). Antibodies against ISU92 strain were detected in the serum of SPF pigs experimentally infected with ISU92 by 7 days post inoculation (DPI), and remained at detectable levels for the duration of the trial, 24 weeks PI. A seroprevalence study was performed with 876 serum samples collected from 36 swine farms in Iowa between
1988 and 1989. Only 6 samples, representing 5 swine herds, were shown to have anti-ISU92 antibody titers and the seroprevalence of sPMV was believed to be very low (Battrell, 1995).

1.7.5 Texas81 history

Texas81 virus was isolated from the brain of pigs that exhibited respiratory and neurological disease in Texas State, in 1981 (cited in (Janke et al., 2001). Further information on the history of this virus is not available.

1.8 Paramyxoviruses as vaccine vector

The first reverse genetic system of nonsegmented negative-strand RNA viruses (NNSV) was established in 1994, recovering infectious rabies virus from cloned cDNA (Schnell et al., 1994) (Figure 2). This milestone event made it possible to explore the great potential of NNSVs to be developed as vaccine vectors (Bukreyev et al., 2006). NNSV as vaccine vectors have many advantages to become promising viral vector candidates. First, a large number of well-understood NNSVs belong to this group, such as NDV, and Vesicular stomatitis virus (VSV). Some of them have the host range restrictively exclusive in human or much attenuated in primates making them widely acceptable for immunization in human population (Bukreyev et al., 2006). Many NNSV can induce cell-mediated protective immune responses, systemic IgG antibody as well as effective local IgA immune response via intranasal infection. Therefore, the vaccines could be intranasally administrated which will be safer and easier during an epidemic.
and especially superb in respiratory tract infection, such as SARS and influenza (Bukreyev et al., 2006). Due to the nature of NNSV replication strategy and genome organization, the possibility of vaccine genome integrating into host genome and recombination between vaccine and circulating viruses is extremely low (Bukreyev et al., 2006).

Three strategies were generally used (Figure 3): (i) Wild-type NNSV expressing foreign genes. Insert a foreign gene into a wild-type NNSV, which is either a naturally attenuated due to the host range restriction or a genetically engineered mutant. (ii) Antigenic chimeric viruses. A construct of the chimeric virus use foreign gene/genes in a pathogenic virus to replace the major protective surface antigen (s) of the vector. (iii) Antigenic chimeric virus expressing additional foreign gene/genes. It is a combination of the first two strategies that are commonly employed to produce bivalent vaccines (Bukreyev et al., 2006).

Antigenic chimeric virus B/HPIV3 is an example of application of the second strategy. It uses bPIV3-Ka, attenuated in human due to the host range restriction (Bailly et al., 2000; Skiadopoulos et al., 2003a; van Wyke Coelingh et al., 1988), as vector backbone with the replacement of F and HN glycoprotein from hPIV3, and has been evaluated clinically by the National Institutes of Allergy and Infectious Diseases as vaccine against hPIV3 (Haller et al., 2000; Pennathur et al., 2003). Besides, bPIV3 as backbone for replaced F and HN of hPIV3 and inserted F of RSV was also constructed. The effectiveness of this vaccine against both PIV3 and RSV challenge has been demonstrated in African green monkeys and is being evaluated in clinical trials (Sato and Wright, 2008).
1.9 Objectives

As new viruses emerge that cause severe disease in pigs and have the potential to cross species barrier and infect other hosts including humans, it is important that these new viruses need to be studied further. As a NNSV, the sPMV could be developed as a vaccine vector for many swine pathogens and even for other species. Understanding the significance of these two strains of sPMV for swine health and their molecular characterization are the essential steps to reach these goals. Therefore, the objective of this study is to undertake the biological and molecular characterizations of these two novel paramyxoviruses, and to study their pathogenicity in conventionally reared pigs, thus to establish a foundation for development of a reverse genetics system. Our longterm goal is to develop them into vaccine vectors using the reverse genetics system.

Objectives:

1. To undertake biological and molecular characterization of two strains of novel swine paramyxoviruses.

2. To examine the pathogenicity of the two viruses in conventionally reared pigs.
1.10 References


Garcin, D., J. B. Marq, et al. (2002). "All four Sendai Virus C proteins bind Stat1, but only the larger forms also induce its mono-ubiquitination and degradation." Virology 295(2): 256-65.


Family Paramyxoviridae.


Skiadopoulos, M. H., L. Vogel, et al. (2003). "The genome length of human parainfluenza virus type 2 follows the rule of six, and recombinant viruses recovered from non-


Stricker, R., G. Mottet, et al. (1994). "The Sendai virus matrix protein appears to be recruited in the cytoplasm by the viral nucleocapsid to function in viral assembly and budding." J Gen Virol 75 (Pt 5): 1031-42.


Table 1. Examples of Members of the *Paramyxoviridae* family

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<th>Family</th>
<th>Subfamily</th>
<th>Genus</th>
<th>Examples</th>
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<td><em>Rubulavirus</em></td>
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<td>Parainfluenza virus 5 (PIV5/SV5)</td>
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<td>Human parainfluenza virus type 2, type 4a and 4b (hPIV2/4a/4b)</td>
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<td>Porcine rubulavirus (La-Piedad-Michoacán virus, LPMV)</td>
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<td><em>Avulavirus</em></td>
<td>Newcastle disease virus (avian paramyxovirus 1, NDV)</td>
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<td>Sendai virus (mouse parainfluenza virus type 1, SeV)</td>
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<td>Human parainfluenza virus type 1 and type 3 (hPIV1/3)</td>
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<td><em>T. truncates</em> parainfluenza virus type1 (TtPIV-1) (?)</td>
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1.12 Figures

(A)

Figure 1. Virion structure of a Paramyxovirus.

(B) Electron micrograph of paramyxovirus virion.

Figure 1. Virion structure of a Paramyxovirus.
(A) Schematic diagram of a paramyxovirus (not drawn to scale) (from Field’s virology, 5th edition)
(B) Electron micrograph of paramyxovirus virion.
Figure 2. Reverse genetics system of NNSV (from (Bukreyev et al., 2006)).

A plasmid encoding a full length antigenomic RNA of a parainfluenza virus is shown at the top. The extragenic 3’ leader and 5’ trailer regions, individual genes, GS and GE are shown, respectively. The antigenomic cDNA is flanked at the left by a T7 promoter (T7 pr.), and a self-cleaving ribozyme and T7 terminator (T7 tr.) at the right. Three other T7 expression plasmids encode the N, P, and L support proteins needed to reconstitute a biologically active nucleocapsid. The T7 RNA polymerase is supplied from a cotransfected eukaryotic expression plasmid. The plasmids are transfected into a cell monolayer in which the plasmid-expressed viral RNA and protein components assemble into a functional nucleocapsid and launch a productive infection. Recovered virus is amplified by passage and can be plaque purified.
Figure 3. Strategies for designing a NNSV vector (from Bukreyev et al., 2006)).

(A) Wild-type NNSV expressing foreign genes.

(B) Antigenic chimeric viruses.

(C) Antigenic chimeric virus expressing additional foreign genes.
Chapter 2

Molecular Characterization of Glycoprotein Genes and Phylogenetic Analysis of Two Swine Paramyxoviruses Isolated From United States

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Abstract

Two swine Paramyxoviruses (sPMV) (81-19252 (Texas81), and 92-7783 (ISU92) were isolated from encephalitic pigs in the United States in 1981 and 1992. Antigenic, morphologic and biological characteristics of these two viruses were essentially similar to members of the family Paramyxoviridae. Antigenic analysis by indirect fluorescent antibody, immunoblot and one-way cross-neutralization tests placed these viruses along with bovine parainfluenza 3 (bPIV3) viruses. Purified virions were 50-300 nm in size and morphologically indistinguishable from other paramyxoviruses. These two viruses hemagglutinated red blood cells and had neuraminidase activity. The gene junctions of fusion (F) and hemagglutinin (HN) glycoprotein genes of these viruses contained highly conserved transcription start and stop signal sequences and trinucleotide intergenic regions similar to other Paramyxoviridae. The F gene of ISU92 was longer than Texas-81 due to insertion of a 24 nucleotide “U” rich 3’ UTR. Structure based sequence alignment of glycoproteins of these two sPMVs indicated that they are essentially similar in structure and function to parainfluenzaviruses. The Texas81 strain was closely related to bPIV3 Shipping Fever (SF) strain at nucleotide and amino acid level, while the ISU92 strain was more closely related to bPIV3-910N strain. The envelope glycoproteins of ISU92 had only ~92% and ~96% identity at nucleotide and amino acid levels with bPIV3-SF strain, respectively. The high sequence identities to bPIV3 indicated cross-species infection in pigs. Phylogenetic analyses based on both F protein and HN protein suggested the classification of these viruses into the subfamily Paramyxovirinae, genus Respirovirus and genotype A of bPIV3.

Keywords: Paramyxovirus, Bovine Parainfluenzavirus 3, Glycoproteins, Cross-species infection, Pigs.
2.1 Introduction

Paramyxoviruses are established pathogens of the central nervous and respiratory systems in many host species. In the last few decades, many novel paramyxoviruses have emerged causing catastrophic illnesses in different aquatic and terrestrial species of animals and some of them also made the species jump to humans. Members of the family *Paramyxoviridae* are enveloped viruses possessing a non-segmented negative-strand genome and are divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. Currently, there are five genera within the subfamily *Paramyxovirinae*: *Rubulavirus*, *Avulavirus*, *Respirovirus*, *Morbillivirus*, and *Henipavirus* (Lamb RA, 2005). La Piedad Michoacán paramyxovirus (LPMV) is the only well studied neurotropic paramyxovirus isolated from pigs prior to the 1990s. LPMV was first isolated in central Mexico in the early 1980s (Moreno-Lopez et al., 1986), and it has become endemic in Mexico (Linne et al., 1992). This virus induced interstitial pneumonia and encephalitis in pigs. There were extensive records of paramyxoviruses derived from the brain or nasal swabs of sick pigs in Japan, in 1950s (Philbey et al., 1998), Canada, in 1971 (Ellis et al., 1998), in Israel in 1986 (Janke et al., 2001) as well as the United States, in 1960s and 1980s in Texas (Janke et al., 2001). There was also concurrent infection of porcine reproductive and respiratory syndrome virus and a paramyxovirus in Germany in the 1990s (Heinen et al., 1998) that has been subsequently named “SER” virus (Tong et al., 2002).

Since 1994, four bat-associated paramyxoviruses have emerged, three of which caused disease in animals and humans (Wang et al., 2001). In late 1994 in Australia, Hendra virus (HeV) caused an outbreak of severe respiratory disease resulting in the death of 13 horses and their trainer (Murray et al., 1995), followed by sporadic HeV outbreaks in horses and humans.
(Field et al., 2007a). A closely related virus, Nipah virus (NiV) from Malaysia caused severe febrile encephalitis and death in pigs and humans (Chua et al., 2000), which spread to Bangladesh and India (Chadha et al., 2006; Hsu et al., 2004). In 1997, Another paramyxovirus, named Menangle virus (MenV) has been isolated in Australia from still-born pigs with deformities (Philbey et al., 1998), and associated human illness (Chant et al., 1998). In 2000, Tioman virus (TioV) was isolated from urine collected beneath a fruit bat colony on Tioman Island, Malaysia (Chua et al., 2001). Molecular characterization revealed that MenV and TioV are closely related novel members of the genus Rubulavirus (Bowden and Boyle, 2005; Chua et al., 2001; Chua et al., 2002). Mapuera virus (MPRV) was isolated in 1979 from the salivary glands of an apparently healthy fruit bat (Sturnira lilium), captured in the tropical rainforest of Brazil (Karabatsos, 1985). J-virus (JV), isolated from wild mice in Australia, and Beilong virus (BeV), originally isolated from human mesangial cells in China and subsequently detected in rat mesangial cells, represent a new group of paramyxoviruses (Basler et al., 2005; Jack et al., 2005; Jun et al., 1977; Li et al., 2006b; Schomacker et al., 2004). Recently, novel Paramyxoviruses were also isolated from Atlantic bottlenose dolphins and Atlantic salmons and characterized (Nollens et al., 2008; Nylund et al., 2007).

Two swine paramyxoviruses (sPMV) (81-19252 (Texas81), and 92-7783 (ISU92)) were isolated from the brain of pigs that experienced respiratory and central nervous system disease in the 1980s and 1990s from South and North Central United States, respectively. ISU92 virus was isolated from a swine operation in the 1990s in north central United States. The outbreak started in a continuous-flow finishing barn of 400 pigs with signs of respiratory disease. Affected pigs showed high fever and mild cough and seventeen pigs died within 4 days. Ten days later, the epizootic spread to a second finishing barn and affected all pigs of different ages. Encephalitic
signs observed from several pigs with persistent squealing, head pressing, whole body tremors, and hind-limb ataxia (Janke et al., 2001). Texas-81 virus was isolated from the brain of pigs that exhibited respiratory and neurological disease in Texas State, in 1981. Further information on the history of this virus is not available. Antigenic analysis by indirect fluorescent antibody assay (IFA) at the National Veterinary Services Laboratory (NVSL), Ames, Iowa indicated that these two viruses are closely related to human parainfluenza virus (hPIV) type 1 and 3 and bovine parainfluenza virus type 3 (bPIV3).

As new viruses emerge that cause severe diseases in pigs and have the potential to cross species barrier and infect other hosts including humans, it is important that these new viruses need to be studied further. Although paramyxoviruses have been isolated from swine in United States and other parts of the world, their importance as swine pathogens or zoonotic agents remains to be determined. To understand the significance of these two strains of sPMV for swine health and to determine their taxonomic status, we have initiated molecular characterization studies. Here, we describe the biological and molecular characterization of the fusion (F) and hemagglutinin (HN) glycoprotein genes and their phylogenetic identity with other paramyxoviruses.

2.2 Materials and Methods

2.2.1 Viruses and Cells

Texas-81 and ISU-92 were received from the NVSL, Animal and Plant Health Inspection Service (APHIS), United States Department of Agriculture (USDA), Ames, Iowa. Porcine
Kidney (PK15) cells, tested free of porcine circovirus (PCV) were obtained from Dr. X. J. Meng, Virginia Polytechnic Institute and State University, and used to propagate these swine paramyxoviruses. Vero cells (ATCC #C1008) were used for fusion and plaque assays.

2.2.2 Virus purification

Iodixanol gradients (OptiPrep, Sigma) were prepared in phosphate buffered saline (PBS) (14% to 26%). Cell lysates from virus stock (plaque purified) were layered onto the top of the gradient and centrifuged for 1.5 h at 250,000 × g in a SW41 Ti rotor. Virus fraction was collected from the gradient and was either examined by transmission electron microscopy or analyzed for protein content by immunoblot.

2.2.3 Transmission Electron Microscopy

Purified virus was adsorbed to glow discharged carbon films on 400 mesh grids. The grids were then washed in PBS and fixed with 2% paraformaldehyde (10 min) and extensively washed with distilled water before negatively stained with 1% phosphotungstic acid. All specimens were observed in Philips EM 420 transmission electron microscope operating at 100 KV.
2.2.4 Plaque Assay

Confluent Vero cells in 6-well plates were infected with virus stocks prepared from the third limiting dilution passage in PK15 cells. Viruses were diluted in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), incubated at 37 °C, in a 5% CO₂ incubator for 1 h, washed with PBS, and then overlaid with 0.8% methyl cellulose in DMEM with 2% FBS. The cells were fixed with methanol-acetone (1:1) at 6 days post-infection (dpi) and stained with 1% crystal violet. The mean number of plaque forming units (pfu) ml⁻¹ for each virus was determined.

2.2.5 Hemagglutination (HA) Assay

Swine red blood cells (sRBCs) were washed 3 times with PBS and resuspended in PBS as a 1% suspension. Texas81 and ISU92 virus stocks prepared in Vero cells were titrated in doubling dilutions against sRBC in V-bottom microtiter plates (Nunc).

2.2.6 Fusion Index Assay

The fusogenicity of Texas81 and ISU92 viruses was examined as described by Kohn (Kohn and Fuchs, 1969). Viruses were inoculated into confluent Vero cells in 6-well plates at a MOI of 0.1. Cells were maintained in DMEM with 2% FBS at 37 °C in a 5% CO₂ environment. After 72 h, the medium was removed, and cells were washed once with 0.02% EDTA and then incubated with 1 ml of 0.02% EDTA for 2 min at room temperature. The cells were then washed with PBS and fixed with methanol and stained with hematoxylin-eosin (Hema 3, Sigma). Fusion
was quantitated by expressing the fusion index as the ratio of the total number of nuclei to the number of cells in which these nuclei were observed (i.e., the mean number of nuclei per cell). The formation of syncytia was visualized by staining virus-infected cells at 48 h with Hema 3 without EDTA treatment.

Fusogenicity of Texas81 and ISU92 F genes were also tested in a plasmid-based system. The open reading frames of the F and HN genes of Texas81 and ISU92 viruses were cloned into pCAGGS plasmid and transfected into Vero cells (1µg of each plasmid) using Lipofectamine 2000 (Invitrogen). At 72 h post-transfection, fusion index was calculated as described above.

2.2.7 Neuraminidase (NA) activity

The NA activity of the Texas81 and ISU92 strains was determined by a fluorescence-based NA assay according to the procedures of Potier et al. (Ferraris et al., 2005; Potier et al., 1979). Newcastle Disease Virus (NDV) Beaudette C strain was used as a standard control virus. Briefly, serial dilutions of virus strains in dilution buffer (32.5mM MES (2-(N-morpholino)ethanesulfonic acid, sodium salt, Sigma–Aldrich) pH 5.8, 4mM CaCl₂) were prepared in a 96-well black flat bottom plates. Then, the same volume of substrate buffer (2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid, sodium salt (MUN, Sigma–Aldrich) prepared in 32.5mM MES pH 5.8, 4mM CaCl₂ buffer) was added to each well. The plate was gently shaken on a mechanical vibrator and then incubated for 1 h at 37 °C. The reaction was terminated by adding 150µl of 50mM glycine buffer pH 10.4. The results were read in a
VIKTOR multilabel reader (TECAN Safire²) with an excitation wavelength of 360nm and an emission wavelength of 450nm.

### 2.2.8 Indirect Fluorescent Antibody (IFA) Test

Virus stocks were inoculated into confluent Vero cells in 8-well chambers at a MOI of 1.0, 0.1, and 0.01. Cells were maintained in DMEM with 2% FBS at 37 °C in a 5% CO₂ environment for 48 h and observed for cytopathic effects (CPE). The cells were then washed with PBS and fixed with methanol-acetone followed by 3 washes with PBST (0.1% Tween 20 in PBS). The cell monolayers were blocked overnight at 4 °C with PBST containing 5% skim milk powder (PBSM). After 3 brief washes with PBST, the cells were incubated with anti-bovine PIV3 polyclonal antibody (NVSL) diluted 1:100 in PBSM and incubated overnight at 4 °C. Then the cells were washed 3 times with PBST and incubated with FITC-anti bovine IgG (KPL) (1:32 dilution in PBSM) at 37 °C in a moist environment for 1 h. After 3 washes with PBST, the slides were layered with glycerol-PBS (1:1) and observed under a Nikon Eclipse TS-100 epifluorescence microscope.

### 2.2.9 Immunoblot

OptiPrep purified virions were subjected to immunoblotting using standard procedures. Briefly, the proteins were separated in a 4-20% sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P (Millipore) membranes. The viral
proteins were probed with anti-bovine PIV3 polyclonal bovine antibody (NVSL) (1:100 dilution), followed by HRP labeled anti-bovine IgG (KPL) (1:500 dilution) secondary antibodies. The blots were visualized with chemiluminiscent ECL Western blotting system (GE Healthcare).

2.2.10 One-way Cross-neutralization Test

Monospecific convalescent sera were obtained from pigs inoculated intranasally with 5x10^7 TCID50 of Texas81 or ISU92 virus stock. One way-cross neutralization test was performed in 96-well plates against 500 TCID50 of respective viruses and heat-inactivated, two-fold diluted sera. The cells were fixed on day 5 and stained with Hema 3 to determine the virus-neutralization titer.

2.2.11 Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) and Sequencing

RNA extraction and reverse transcription (RT) reactions were performed using standard procedures. Briefly, virus-infected PK15 cells were scraped into the medium and subjected to three cycles of freezing and thawing. After initial clarification at 3000 x g for 15 min, polyethylene glycol (PEG) 8000 (Sigma) was added to the cell lysate to a concentration of 10 % and the lysate was incubated for 4 h at 4 °C. The virus was pelleted at 12, 000 x g for 60 min at 4 °C, and the viral genomic RNA was extracted from the virus pellet using RNeasy Mini Kit (QIAgen). Oligonucleotide primers were designed based on consensus bPIV3 F and HN gene nucleotide sequences (primer sequences available upon request). The complementary DNA
(cDNA) copies of the genomic RNA of the two virus strains were synthesized using the specific oligonucleotide primers and Superscript III reverse transcriptase (Invitrogen). Subsequently, Platinum Taq DNA Polymerase (Invitrogen) was used to amplify the F or HN gene. The PCR products were purified using Qiaquick PCR purification kit (Qiagen). The PCR products were cloned using the TA cloning system (Invitrogen). Both purified PCR products and at least 10 TA clones were sequenced in both directions in an automated sequencer (Core Laboratory Facility at Virginia Bioinformatics Institute, Virginia Tech). Based on the sequencing results, primer-walking strategy was employed and new sets of oligonucleotide primers were designed for sequencing the middle part of the genes.

**2.2.12 Determination of antigenic relatedness**

The antigenic relatedness of sPMV strains were expressed by R value, calculated using the following formula created by Archetti and Horsfall (1950)(Archetti and Horsfall, 1950).

\[ R = \sqrt{r_1 \times r_2} \]

where \( r_1 \) is the ratio of the heterologous titer obtained with virus 2 to the homologous titer obtained with virus 1; \( r_2 \) is the ratio of the heterologous titer obtained with virus 1 to the homologous titer obtained with virus 2; \( R \) is a geometric mean of ratio \( r_1 \) and \( r_2 \), which is used to express the antigenic relatedness between two viruses when both antigens and antisera were used in indirect FA test.
R values were interpreted by the method of Brooksby (Brooksby, 1967) for similarity comparison of two viruses. The criteria are as follows: 1) R value of 1 or close to 1 indicates antigenic identity between two tested viruses; 2) R value >70% means little or no difference between two viruses tested; 3) R value between 33% and 70% indicates a minor subtype difference; 4) R value between 11% and 32% represents a major subtype difference; 5) R value between 0% and 10% represents a serotype difference.

2.2.13 Data Analysis

Nucleotide sequence editing, alignments and prediction of amino acid sequence and analyses were conducted using the software package DNASTAR (Lasergene). Phylogenetic relationships of these two viruses within Paramyxoviridae family were constructed by Phylogenetic Analysis using Parsimony (PAUP 4.01) software with 1000 bootstrap replicates. The protein functional analyses of deduced amino acid sequences were done using online resources available at ExPASy Proteomics tools (http://us.expasy.org/tools/).

2.2.14 Accession numbers for sequence analyses

The sequences of ISU-92 and Texas-81 were deposited in GenBank under accession # EU439428 and EU439429, respectively. The accession numbers for other viral sequences used for phylogenetic analysis are: Atlantic salmon paramyxovirus (ASPV), EF646380; Avian metapneumovirus (aMPV), NC_007652; Avian paramyxovirus 6 (aPMV6), NC_003043;
Beilong virus (BeV), NC_007803; Bovine parainfluenza virus 3-910N (bPIV3-910N), D84095; Bovine parainfluenza virus 3 strain Kansas/15626/84 (bPIV3-Ka), AF178654; Bovine parainfluenza virus Q5592 (bPIV3-Q5592), EU277658; Bovine parainfluenza virus 3 strain Shipping Fever (bPIV3-SF), AF178655; Bovine parainfluenza virus 3 strain SK-217 (bPIV3-SK217), U31671; bovine respiratory syncytial virus (bRSV), NC_001989; Canine distemper virus (CDV), NC_001921; Dolphin morbillivirus (DMV), NC_005283; Fer-de-lance virus (FDLV), NC_005084; Hendra virus (HeV), AF017149; Human metapneumovirus (hMPV), NC_004148; Human parainfluenza virus 1 strain Washington/1964 (hPIV1-Wa), NC_003461; Human parainfluenza virus 2 (hPIV2), NC_003443; Human parainfluenza virus 3 (hPIV3), AB012132; Human parainfluenza virus 3-GPv (hPIV3-GPv), NC_001796; Human parainfluenza virus 3-JS (hPIV3-JS), Z11575; Human respiratory syncytial virus (hRSV), NC_001781; J-virus (J-V), NC_007454; Measles virus (MeV), NC_001498; Menangle virus (MenV), NC_007620; Mossman virus (MoV), NC_005339; Mumps virus (MuV), NC_002200; Newcastle disease virus (NDV), NC_002617; Nipah virus (NiV), NC_002728; Peste-des-petits-ruminants virus (PPRV), NC_006383; Pneumonia virus of mice, AY573818; Porcine Rubulavirus (PoRV/LPMV), NC_009640; Rinderpest virus (RPV) (strain Kabete O), NC_006296; Sendai virus (SeV), NC_001552; Simian parainfluenza virus 5 (SV5), NC_006430; Tioman virus (TioV), NC_004074; Tupaia paramyxovirus (TPMV), NC_002199.
2.3 Results and Discussion

2.3.1 Morphological and Biological Properties

Transmission electron microscopy revealed spherical to pleiomorphic virions approximately 50-300 nm in diameter morphologically indistinguishable from paramyxoviruses (Robert A. Lamb, 2007). Intact virions were enveloped and densely packed with surface projections representing viral glycoprotein spikes. Nucleocapsids were visible and exhibited a typical “herringbone” pattern (Figure 1). Both Texas81 and ISU92 viruses were able to agglutinate sRBCs. The HA titers of Texas-81 and ISU-92 were $2^4$ and $2^5$, respectively. The agglutinated sRBCs eluted after 1 h suggesting neuraminidase function. Neuraminidase assay also confirmed this. bPIV3 has been shown to have hemagglutinating and neuraminidase functions (Breker-Klassen et al., 1996). The fusion index of Texas81 was 63 nuclei per cell and ISU92 was 60 nuclei per cell (72 hpi, MOI, 0.1), indicating that these viruses are highly fusogenic (Figure 2). The fusion index with Texas81 and ISU92 F plasmids with homologous HN plasmids was 22.7 and 13.5 respectively. The viruses were able to form plaques in Vero cells at 6 dpi and grew to $2.62 \times 10^7$ pfu ml$^{-1}$ (Texas81) and $5.75 \times 10^7$ pfu ml$^{-1}$ (ISU92). Both viruses produced similar sized plaques in Vero cells (Figure 2).

2.3.2 Antigenic analysis

Antigenic analysis at the National Veterinary Services Laboratory (NVSL), Ames, Iowa indicated that they were closely related to human parainfluenza virus (hPIV) type 1 and 3 and bPIV3 (Table 1). Cross-reactivity in several epitopes in F and HN proteins with hPIV3 and
bPIV3 has been reported earlier (Coelingh et al., 1986). Bovine anti-bPIV3 serum was able to detect the cells infected by these viruses (Figure 2) by IFA. Besides, using bovine anti-bPIV3 serum, all the viral proteins were detectable in OptiPrep purified virion preparations (Figure 2). The protein designations described were based on studies with virion proteins of PIV3 (Storey et al., 1984; Wechsler et al., 1985). Homologous sera could neutralize virus infectivity of similar titers and heterologous serum (bPIV3-SF) neutralized virus infectivity of Texas81 at 1:32 and ISU92 at 1:8, suggesting antigenic variation. The R values of Texas81 and ISU92 strains with bPIV3-SF strain were 100% and 35%, respectively by IFA. These observations suggest that Texas81 and ISU92 were antigenically closely related to bPIV3. However, antigenic analysis by IFA also suggested that ISU92 is a minor subtype of bPIV3, according to the criteria of Brooksby (1967) (Brooksby, 1967).

2.3.3 Transcriptional start and stop sequences

The nucleotide sequence of F and HN genes of both Texas81 and ISU92 viruses were determined from RT-PCR products amplified from viral genomic RNA. Individual genes are transcribed by a start-stop mechanism controlled by conserved sequences at the gene borders in members of the family Paramyxoviridae. The F and HN genes of Texas81 and ISU92 shared the universal pattern of paramyxovirus genome structure, flanked at 3’ end with a conserved gene-start (GS) sequence and at 5’ end with a gene-end (GE) sequence with an intergenic sequence (IGS) in between. The GS and GE sequences of the F and HN genes were essentially similar to bPIV3. Only the variable fifth and sixth positions of the GS sequence showed host-specificity (Table 2). All of them terminated with U-rich GE sequences. The trinucleotide IGS (3’-GAA) of
these two viruses were also identical to members of the genera Respirovirus, Morbillivirus, and Henipavirus (Bailly et al., 2000). A long U-rich sequence was identified in the 3’ UTR of F gene of ISU92. This is a characteristic feature commonly found in bPIV3 and hPIV3. This has been shown to result in read-through transcripts of M gene in bPIV3 and hPIV3 (Sakai et al., 1987; Suzu et al., 1987).

2.3.4 F gene

The F gene of Texas81 strain was 1869 nucleotides (nt) in length with a single ORF of 1620 nt beginning at position 211 (Table 3), capable of encoding a 540 amino acid protein. The F gene of ISU92 strain was 1893 nt in length with a 1620 nt ORF but with a longer 3’ untranslated region (235 nt). The long 3’ UTR in ISU92 strain had a 24 nt “U” rich insertion compared to the Texas81 strain. The F protein of ISU92 had a predicted molecular weight of 60,039 Da and an estimated isoelectric point (pI) of 6.797. For Texas-81 strain, the uncleaved F0 protein had a predicted molecular weight of 60,189 Da and an estimated pI of 6.539. The size determination by immunoblotting confirmed this (Figure 2). The Texas81 strain has 100% identity with bPIV3-SF strain both in nucleotide and deduced amino acid sequences. ISU92 strain had 98.7% identity in nucleotide sequence and 99.1% identity in amino acid sequences with bPIV3-SK217 strain, while it had 90.9% and 95% identity with bPIV3-SF strain in nucleotide and amino acid sequences, respectively (Table 4) for F gene.

The F protein mediates fusion of virus and cell membrane in paramyxoviruses. Fusion activation is dependent on the cleavage of F0 protein into disulfide-linked subunits F2-s-s-F1. The
furin, a subtilisin-like endoprotease, is believed to be one of the proteases that cleaves most F proteins intracellularly (Klenk and Garten, 1994; Ortmann et al., 1994). In Texas81 and ISU92 viruses, the F cleavage motif L/SRTKR was located between amino acid residues 105 to 109, and cleavage occurs between residues 109(R) and 110(F). This cleavage site conformed to the pattern of consensus motif for cleavage by furin (Hosaka et al., 1991), R-X-K/R-R, which is conserved in the majority of Paramyxovirinae (Robert A. Lamb, 2007). The predicted F cleavage site was immediately followed by a 25-aa hydrophobic fusion peptide, highly conserved in all paramyxovirus F proteins (Horvath et al., 1992; Robert A. Lamb, 2007). The cleaved F₁ protein was approximately 51kDa in size. The F protein of both strains, like the F protein of other paramyxoviruses, was predicted to be a type I membrane protein. The transmembrane region was near the carboxyl terminal (amino acid residues 497-517), which was thought to serve as an anchor in the viral envelope, leaving a 23 amino acid cytoplasmic tail (Figure 3).

The F proteins of swine paramyxoviruses are essentially similar in structure and function to other paramyxoviruses, and especially to BPIV3. Peptides corresponding to the heptad repeat regions A and B (HRA and HRB) from SV5 (Baker et al., 1999), hRSV (Zhao et al., 2000) and hPIV3 (Yin et al., 2005), were conserved in the F protein of sPMV. F proteins assembled into stable six helical bundles (6HBs) (the F₁ core), and their structure in hPIV3 had been determined. Structure based sequence alignment of the sPMV F protein sequences with these 6HB fragments revealed only minor differences. The conserved blocks in F₁ and F₂ (CBF₁, CBF₂) were also preserved in sPMV as in other paramyxoviruses which strengthens the hypothesis that these conserved blocks had conserved functions, either in the membrane fusion or in the folding and processing of the F protein (Gardner and Dutch, 2007). Cysteines, which were important for disulfide bond formation and secondary structure, were also identical to bPIV3 and hPIV3.
Among the five potential conserved N-linked glycosylation sites (N101, N238, N359, N446, and N508) in the F protein (Suzu et al., 1987), all but one had the N-X-T motif. The one at position 446 had the N-X-S motif. It should be noted that only one potential glycosylation site (at N101) was located before the F protein cleavage site, and one site was located in the transmembrane domain (N508). The other three were located prior to the transmembrane domain in the F1 protein and were, therefore, likely to be exposed on the surface, as in other members of the genus Respirovirus (Robert A. Lamb, 2007).

2.3.5 HN gene

The HN gene was 1888 nt in length with a single ORF beginning at position 74 that could encode a 572 amino acid protein (Table 3). Texas81 strain shared 99.8% identity with bPIV3-SF strain both in nucleotide and predicted amino acid sequences. ISU92 strain had 98.4% nucleotide sequence identity and 99.1% amino acid sequence identity with bPIV3-SK217 strain. Identity of ISU92 with bPIV3-SF strain is 91.7% at nucleotide level and 95.8% at amino acid level (Table 4). Comparing with hPIV3, the two swine viruses had 80.1% ~ 82.7% identities in the HN gene nucleotide sequences and 76.4% ~ 77.6% identity at deduced amino acid sequences.

The deduced amino acid sequence of the HN protein was 572 residues in length. The HN protein of ISU92 had a predicted molecular weight of 64,652 Da and an estimated pI of 7.343. The HN protein of Texas81 strain had a predicted molecular weight of 64,624 Da and an estimated pI of 7.720. The active site residues predicted from NDV or hPIV3 HN crystal structure were also conserved in the swine viruses. The major transmembrane region of HN
protein was predicted to be from amino acid residues 36 to 54 of the protein as in other type II membrane glycoproteins. The transmembrane domain at the N-terminal end of the HN protein contained several conserved substitutions among the examined viruses (Figure 4).

The disulfide bonds in the HN protein C190–C214 (A), C256–C269(C), C355–C469 (D), C463–C473(E), C535–C544(F), C159–C571(W) and C350–C363(X) were conserved as in NDV HN or hPIV 3 HN crystal structures (Crennell et al., 2000; Lawrence et al., 2004). Predicted N-glycosylation sites were conserved in HN proteins as in other members of Paramyxovirinae. In swine viruses, potential N-linked glycan sites observed at all predicted sites (N8, N308, N351, N448 and N523) were similar to hPIV3. The counterpart to N351 in NDV HN is N341, which is glycosylated in that molecule. NDV HN contains a N-linked glycosylation site at N481, but in hPIV3 HN the sequence at this site is N-P-T and in swine viruses, it is N-P-S, the asparagine moiety of which is thus not expected to be glycosylated. Texas81 strain had an additional (N15) N-linked glycosylation acceptor site in the HN protein, which appears to be host specific.

Whether all of the potential N-linked glycan sites are glycosylated awaits future studies. Both Texas81 and ISU92 viruses contained the conserved NRKCS neuraminidase active site motif (Jorgensen et al., 1987). Up to date, all analyzed members of Respirovirus and Rubulavirus had this sequence (Langedijk et al., 1997).

The sequence analysis showed that Texas81 and ISU92 had higher levels of identity with bPIV3 than hPIV1 or hPIV3. The 100% identity at nucleotide level between the F protein of Texas81 and the SF strain of bPIV3 indicated that cross species transmission might occur among different hosts. However, further studies will be needed to confirm this. On the other hand, ISU92, which was closely related to the SK217 and 910N strains of bPIV3, possessed host-
specific amino acid residues in the F and HN proteins differing from both bPIV3 strains. There were three host specific amino acid residues (Y100, A437, and V501) in F protein and 3 in the HN protein (K70, S88 and S387) of ISU92 strain, compared to bPIV3 and hPIV3 (Figures 3 and 4). bPIV3 strains SK217 and SF/Ka were shown to differ in virulence (Breker-Klassen et al., 1996; Shibuta et al., 1981). This virulence difference had been associated with a change in amino acid 193 in HN protein which had a dramatic effect on syncytium inducing activity, neuraminidase activity and hemagglutinating activity (Breker-Klassen et al., 1996). ISU92 had I at this position, unlike SK217 or 910N, but identical with Texas81 and bPIV3-SF/Ka. Further, ISU92 strain was more fusogenic than the Texas81 strain. The importance of these residues in host specificity awaits future reverse genetic studies.

2.3.6 Phylogenetic analysis

Phylogenetic analyses of the full length F and HN genes of these viruses with other members of the *Paramyxoviridae* were performed by parsimony analysis. Phylogenetic analysis of Texas81 and ISU92 based on the deduced amino acid sequences of the F and HN proteins placed them on the same clade along with bPIV3 (Figure 5). Recently, it has been reported that there are two genotypes of bPIV3: genotype A and genotype B (Horwood et al., 2008). However, two subgenotypes of bPIV3 were discernible in genotype A; one represented by 910N-like viruses and the other represented by Ka, and SF viruses. The Texas81 and SF/Ka strains formed the first genetic group while ISU92 and 910N strains formed the second genetic group. Phylogenetic reconstruction with HN gene also confirmed these groupings. Antigenic analysis by IFA has also confirmed that there are subtypic differences between bPIV3 and sPMV strains as
well. The above facts support the classification of these two swine viruses in the genus *Respirovirus, Paramyxovirinae* subfamily.

The phylogenetic analyses also indicated that these two strains are very likely the host-adapted variant strains transmitted from cattle to pigs. The host-specific sequence elements might contribute to the growth of the virus in heterogeneous hosts (Bailly et al., 2000). Cross-species infection of bPIV3 in a human (Ben-Ishai et al., 1980), bPIV3 in sheep and ovine PIV-3 in cattle (Stevenson and Hore, 1970) has been reported. This happens to be the first report of cross-species infection of bPIV3 in pigs. Several studies using reverse genetic system to recover recombinant PIV3 have been successfully constructed and employed including hPIV3 and bPIV3 (Skiadopoulos et al., 1998; Skiadopoulos et al., 1999). Similar approaches can be undertaken to determine the importance of the individual or combined effects of host-specific sequence changes in the pathogenesis and host-adaptation of bPIV3. Further, this would also help in developing an attenuated phenotype as a vaccine candidate for these viruses.

### 2.4 Acknowledgments

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2.5 References


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Table 1. Antigenic relationship of ISU92 and Texas81 with other paramyxoviruses

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</table>

*Indirect fluorescent antibody test was performed at the National Veterinary Services Laboratory, Ames, IA against a panel of paramyxoviruses.
Table 2. Comparison of the gene-start and gene-end sequences of sPMV, bPIV3 and hPIV3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Strain</th>
<th>Gene-start</th>
<th>Gene-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>Texas81</td>
<td>UCCUA\text{GUUC}^a</td>
<td>UU\text{CAUGUUUUU}U</td>
</tr>
<tr>
<td></td>
<td>ISU92</td>
<td>UCCUA\text{GUUC}</td>
<td>UU\text{CAUGUUUUU}U</td>
</tr>
<tr>
<td></td>
<td>bPIV3-SF/Ka</td>
<td>UCCUA\text{GUUC}</td>
<td>UU\text{CAUGUUUUU}U</td>
</tr>
<tr>
<td></td>
<td>bPIV3-910N</td>
<td>UCCUA\text{GUUC}</td>
<td>UU\text{CAUGUUUUU}U</td>
</tr>
<tr>
<td></td>
<td>hPIV3</td>
<td>UCCUGU\text{UUUC}</td>
<td>CUA\text{AAUAAUUUUU}</td>
</tr>
<tr>
<td>HN</td>
<td>Texas81</td>
<td>UCCUU\text{GUUC}</td>
<td>UU\text{AUAAUUUUU}U^b</td>
</tr>
<tr>
<td></td>
<td>ISU92</td>
<td>UCCUU\text{GUUC}</td>
<td>UU\text{AAUGUUUUU}U</td>
</tr>
<tr>
<td></td>
<td>BPIV3-Ka/SF</td>
<td>UCCUU\text{GUUC}</td>
<td>UU\text{AAUAAUUUUU}U</td>
</tr>
<tr>
<td></td>
<td>BPIV3-910N</td>
<td>UCCUU\text{GUUC}</td>
<td>UU\text{AAUGUUUUU}U</td>
</tr>
<tr>
<td></td>
<td>hPIV3</td>
<td>UCCU\text{CAUUUC}</td>
<td>UU\text{UAUAAUUUUU}</td>
</tr>
<tr>
<td>Consensus:</td>
<td></td>
<td>UCCU\text{NNNUUU}C</td>
<td>NUNNUNU\text{UUUUU}U</td>
</tr>
</tbody>
</table>

a. Host-specific nucleotide residues between hPIV3 and bPIV3 are underlined;
b. Positions that display variability within the host group are in bold-face type.
Table 3. Comparison of nucleotide sequences between sPMV, bovine and human PIV3 viruses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Virus</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>3'UTR(nt)</th>
<th>ORF(nt)</th>
<th>5' UTR(nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Texas81</td>
<td>1869</td>
<td>540</td>
<td>211</td>
<td>1620</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>ISU92</td>
<td>1893</td>
<td>540</td>
<td>235</td>
<td>1620</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>bPIV3-SF/Ka</td>
<td>1869</td>
<td>540</td>
<td>211</td>
<td>1620</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>bPIV3-910N</td>
<td>1893</td>
<td>540</td>
<td>235</td>
<td>1620</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>hPIV3</td>
<td>1851</td>
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<td>193</td>
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<td>572</td>
<td>73</td>
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<tr>
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<td>ISU92</td>
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<td>572</td>
<td>73</td>
<td>1716</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>bPIV3</td>
<td>1888</td>
<td>572</td>
<td>73</td>
<td>1716</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>hPIV3</td>
<td>1888</td>
<td>572</td>
<td>73</td>
<td>1716</td>
<td>99</td>
</tr>
</tbody>
</table>
Table 4. Nucleotide and Amino acid identities between sPMV, bPIV3 and hPIV3

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>F</th>
<th></th>
<th>HN</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nucleotide</td>
<td>Amino acid</td>
<td>Nucleotide</td>
</tr>
<tr>
<td></td>
<td>bPIV3-910N</td>
<td>91.4%</td>
<td>95.2%</td>
<td>91.8%</td>
<td>96.2%</td>
</tr>
<tr>
<td></td>
<td>bPIV3-SK217</td>
<td>92.2%</td>
<td>95.9%</td>
<td>92.1%</td>
<td>96.2%</td>
</tr>
<tr>
<td>Texas81</td>
<td>bPIV3-SF</td>
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<td>100.0%</td>
<td>99.8%</td>
<td>99.8%</td>
</tr>
<tr>
<td></td>
<td>bPIV3-Ka</td>
<td>97.8%</td>
<td>98.3%</td>
<td>98.3%</td>
<td>98.3%</td>
</tr>
<tr>
<td></td>
<td>hPIV3</td>
<td>82.2%</td>
<td>82.7%</td>
<td>80.1%</td>
<td>76.4%</td>
</tr>
<tr>
<td></td>
<td>bPIV3-910N</td>
<td>98.0%</td>
<td>98.9%</td>
<td>98.3%</td>
<td>98.8%</td>
</tr>
<tr>
<td></td>
<td>bPIV3-SK217</td>
<td>98.7%</td>
<td>99.1%</td>
<td>98.4%</td>
<td>99.1%</td>
</tr>
<tr>
<td>ISU92</td>
<td>bPIV3-SF</td>
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<td>95.0%</td>
<td>91.7%</td>
<td>95.8%</td>
</tr>
<tr>
<td></td>
<td>bPIV3-Ka</td>
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<td>95.6%</td>
<td>91.4%</td>
<td>94.9%</td>
</tr>
<tr>
<td></td>
<td>hPIV3</td>
<td>82.0%</td>
<td>82.5%</td>
<td>82.7%</td>
<td>77.6%</td>
</tr>
</tbody>
</table>
2.7 Figures

![Images of ultrastructure of swine paramyxoviruses](image)

**Figure 1. Ultrastructure of swine paramyxoviruses.** Purified virions were negatively stained with 1% phosphotungstic acid and viewed under a transmission electron microscope. (A, C) ISU92 virus, (B, D) Texas81 virus. Intact virion with fine surface projections (arrow a) representing the viral glycoprotein spikes. Highly pleomorphic viral particles with extruded nucleocapsids were indicated by arrow b. Nucleocapsids exhibit a typical “herringbone” pattern.
Figure 2. **(A-C) Syncytium formation in Vero cells by Texas81 and ISU92.** The fusogenicity of Texas81 and ISU92 viruses was examined in Vero cells. A. ISU92; B. Texas81; C. Mock-infected Vero cells. **(D-F) Plaque Assay.** Confluent Vero cells in 6-well plates were infected with serially diluted virus stocks and then overlaid with 0.8% methyl cellulose in DMEM with 2% FBS. Plaque sizes of Texas81 and ISU92 viruses were similar D. ISU92; E. Texas81; F. mock infected. **(G-J) Antigenic analysis of swine paramyxoviruses.** (G-I) Indirect fluorescence antibody assay was performed by infecting Vero cells with swine paramyxoviruses at a MOI of 1.0, 0.1, and 0.01. Virus-specific antigens to the swine viruses were probed with anti-BPIV3 polyclonal bovine antibody (NVSL) (1:100 dilution) followed by anti-bovine FITC-conjugated antibody. (G) ISU-92, (H) Texas-81, (I) mock infected Vero cells. (J) Optiprep purified virions of Texas81 (Lane 2) and ISU92 (Lane 3) were used for immunoblotting. The viral proteins were probed with anti-bPIV3 polyclonal bovine antibody (NVSL), followed by HRP labeled anti-bovine IgG (KPL) secondary antibodies.
Figure 3. Schematic of the predicted domain structure of the swine paramyxovirus $F_0$ protein. Identification of conserved block in F1 (CBF$_1$), conserved block in F2 (CBF$_2$) and fusion peptide (FP) (Gardner and Dutch, 2007; Yin et al., 2005) regions were obtained from structure based sequence alignment of the F protein of sPMV with other paramyxoviruses. Domains are indicated as DI to DIII. TM indicates transmembrane domain. HRA, HRB, and HRC indicate heptad repeat regions. Conserved amino acid residues are shaded.
**Figure 4.** Schematic of the HN protein globular head region domain structure of swine paramyxovirus. (A) The transmembrane domain, sialic acid site (NRKSCS motif) and the predicted alpha helix regions (Garnier-Robson) of swine paramyxovirus were given as colored boxes (Crennell et al., 2000). Disulfide bonds followed those used in a structure-based sequence alignment and the crystal structure of NDV HN protein (Crennell et al., 2000). (B) The corresponding position of the active site residues in the deduced amino acid sequence of the sPMV and other paramyxoviruses.
Figure 5. Phylogenetic analysis. Phylogenetic analysis was performed using Parsimony (PAUP 4.01) software with 1000 bootstrap replicates. (A) Phylogenetic analysis based on F gene deduced amino acid sequences compared to other Paramyxoviridae family members. (B, C) Phylogenetic analysis of PIV3 genotypes based on F and HN gene sequences, respectively.
Chapter 3

Complete Genome Sequence and Pathogenicity of Two Swine Parainfluenzaviruses Isolated From Pigs in the United States

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²Dept. of Veterinary Diagnostic & Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA.

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Abstract

Two novel paramyxoviruses 81-19252 (Texas81) and 92-7783 (ISU92) were isolated from pigs that experienced respiratory and central nervous system disease in the 1980s and 1990s from South and North Central United States. The complete genome of Texas81 virus was 15456 nucleotides (nt) and ISU92 was 15480 nt in length consisting of six non-overlapping genes coding for the nucleo- (N), phospho- (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large polymerase (L) protein in the order 3'-N-P/C/V-M-F-HN-L-5'. The features related to virus replication and found to be conserved in most members of *Paramyxoviridae* were also found in swine viruses. These include: conserved and complementary 3’ leader and 5’ trailer regions, trinucleotide intergenic sequences, highly conserved gene start and gene stop signal sequences. The length of each gene of these two viruses were similar except for the F gene, in which ISU92 had an additional 24 nt “U” rich 3’ untranslated region (UTR). The P gene of these viruses were predicted to express P protein from the primary transcript and edit a portion of its mRNA to encode V and D proteins, and the C protein was expected to be expressed from an alternate translation initiation codon from the P gene as in respiroviruses. Sequence specific features related to virus replication and host specific amino acid signatures in viral proteins indicated that these viruses probably originated from bovine parainfluenzavirus 3. Pairwise comparison of deduced amino acid sequences of swine viral proteins with members of *Paramyxoviridae* and phylogenetic analysis based on the individual gene as well as predicted amino acid sequences suggested that these swine parainfluenzaviruses (sPIV3) are novel members of the genus *Respirovirus* of the *Paramyxovirinae* subfamily and can be grouped into two subgenotypes of genotype A of bovine parainfluenza virus 3. The mild clinical signs and undetectable gross and microscopic lesions observed in sPIV3-infected pigs indicate the
inapparent nature of these viruses in conventionally reared pigs. Seroprevalence studies in serum samples collected from pig farms in Minnesota and Iowa in 2007-2008 by indirect ELISA revealed that sPIV3 are not circulating in these farms. The mild pathogenicity of sPIV3 can facilitate its development as a vaccine vector.
3.1 Introduction

Outbreaks of many novel paramyxoviruses causing catastrophic illnesses have been reported all over the world in the last few decades. A large and diverse host species are involved, including avian, porcine, canine, bovine, equine, ovine, human, reptiles and aquatic species (Franke et al., 2001; Horwood et al., 2008; Nollens et al., 2008; Nylund et al., 2007; Nylund et al., 2008; Robert A. Lamb, 2007). Cases of cross-species transmission and pathogen jumping to humans were also reported (Chua et al., 1999; Field et al., 2007a), demonstrating the value of characterizing new animal pathogens, even if their pathogenic potential was currently unknown. Prior to 1990s, only La Piedad Michoacán paramyxovirus (LPMV) has been well studied as a neurotropic paramyxovirus isolated from pigs. Many paramyxoviruses as porcine pathogen have been reported since 1950s all over the world, including Japan (Philbey et al., 1998), Canada (Ellis et al., 1998), Israel (Janke et al., 2001) as well as the United States in Texas (Janke et al., 2001). There was also concurrent infection of porcine reproductive and respiratory syndrome virus (PRRSV) and a paramyxovirus in Germany in the 1990s (Heinen et al., 1998) that has been subsequently named “SER” virus (Tong et al., 2002). Four bat-associated paramyxoviruses were reported to cause animals and human diseases in 1994 (Wang et al., 2001). Hendra virus (HeV) and Nipah virus (NiV), which were identified to cause an outbreak of severe respiratory disease and death in horses and their trainer, and severe febrile encephalitis and death in pigs and farmers, respectively, have been classified as Henipavirus as a genus in subfamily Paramyxovirinae (Chadha et al., 2006; Chua et al., 2000; Field et al., 2007a; Hsu et al., 2004; Murray et al., 1995).

Some recently isolated viruses, such as Menangle virus (MenV) (Philbey et al., 1998),
Tupaia paramyxovirus (TPMV) (Tidona et al., 1999), Tioman virus (TioV) (Chua et al., 2001), Mossman virus (MoV) (Miller et al., 2003) J-virus (J-V) (Jack et al., 2005; Jun et al., 1977), Beilong virus (BeV) (Li et al., 2006b), Mapuera virus (MPRV) (Karabatsos, 1985), *T. truncates* parainfluenza virus type 1 (TtPIV-1) isolated from bottlenose dolphins (Nollens et al., 2008), and Atlantic salmon paramyxovirus (ASPV) (Nylund et al., 2008) remain unclassified below subfamily level. Most of them have been well characterized genetically, and complete genome sequences are available. All members of the subfamily *Paramyxovirinae*, have six invariant genes in the order 3′– N – P – M – F – A – L – 5′, indicating the nucleocapsid, phospho-, matrix, fusion, attachment and large polymerase proteins, respectively (Lamb and Parks, 2007).

Recently, we have reported the antigenic and molecular characterization of glycoprotein genes of two novel swine parainfluenza type 3 viruses (sPIV3) isolated from the pigs that experienced respiratory and central nervous system (CNS) disease (Qiao et al., 2009). These two sPIV3 strains were antigenically and genetically closely related to bovine parainfluenzavirus 3 (bPIV3) and were closely related to the genus *Respirovirus* (Qiao et al., 2009). However, the pathogenicity of these sPIV3 in conventionally reared pigs and the complete genome sequences of these isolates are presently unknown.

In cattle, bPIV3 infection results in asymptomatic infection to severe respiratory disease, but no neurological disease has been reported due to bPIV3 in cattle (Dinter and Morein, 1990). Limited sequence polymorphism has been reported among bPIV3 strains (Coelingh and Winter, 1990; Swierkosz et al., 1995). Recently, after analysis of Australian isolates of bPIV3, two distinct genotypes of bPIV3: genotypes A and B, have been proposed (Horwood et al., 2008). In this study, we have performed a complete genome sequence analysis of these novel sPIV3 and
determined their pathogenicity in conventionally reared pigs. Our analysis indicated that there are two distinct genetic groupings discernible within genotype A, represented by bPIV3 shipping fever strain (bPIV3-SF)-like and bPIV3 910N strain (bPIV3-910N)-like viruses with one swine virus strain in each of these groups. Several species-specific amino acid residues were identified that may dictate host range. But, both swine viruses induced a very mild respiratory illness without any neurological signs in young piglets, suggesting that co-infection with other infectious agents or the presence of other environmental factors may be required to precipitate clinical disease.

3.2 Materials and Methods

3.2.1 Viruses and Cells

Texas 81-19252 (Texas81), and ISU-92-Minnesota isolate 92-7783 (ISU92) viruses were obtained from the National Veterinary Services Laboratory (NVSL), Animal and Plant Health Inspection Service (APHIS), United States Department of Agriculture (USDA), Ames, Iowa. Porcine Kidney (PK15) cells, tested free of porcine circovirus (PCV) were obtained from Dr. X. J. Meng, Virginia Polytechnic Institute and State University, and used to propagate these viruses for the first three passages. Vero cells (ATCC #CCL-81) were used for all other tests as described earlier (Qiao, Janke et al, 2009).
3.2.2 Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) and Sequencing

RNA extraction and reverse transcription (RT) reactions were performed using standard procedures. Briefly, virus-infected PK15 cells were scraped into the medium and subjected to three cycles of freezing and thawing. After initial clarification at 3000 x g for 15 min, polyethylene glycol (PEG) 8000 (Sigma) was added to the cell lysate to a concentration of 10 % and the lysate was incubated for 4 h at 4 °C. The virus was pelleted at 12, 000 x g for 60 min at 4 °C, and the viral genomic RNA was extracted from the virus pellet using RNeasy Mini Kit (QIAgen). Oligonucleotide primers were designed based on consensus bPIV3 and hPIV3 nucleotide sequences (Table 1). Based on the sequencing results, new sets of oligonucleotide primers were designed and primer-walking strategy was employed for sequencing the gap areas. The leader and trailer sequences were determined according to the RACE method of Li et al (Li et al., 2005). Briefly, a single set of adaptor and identical set of reagents were used. The two RACE methods differ only in the order of adaptor ligation and cDNA synthesis. In the 5’ RACE, adaptor ligation was carried out after cDNA synthesis whereas in the 3’ RACE the order was reversed (Figure 1).

The complementary DNA (cDNA) copies of the genomic RNA of the two virus strains were synthesized using the specific oligonucleotide primers and Superscript III reverse transcriptase (Invitrogen). Subsequently, Platinum Taq DNA Polymerase (Invitrogen) was used to amplify each fragment with the following cycling parameters: 94°C for 4 min, followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and a final 7 minutes extension at 72°C. The PCR products were purified using Qiaquick PCR purification kit (Qiagen). To ensure the consensus sequence, every nucleotide in the genome was sequenced.
once from purified PCR products and at least from 3 independent TA clones in both directions in an automated sequencer (Core Laboratory Facility at Virginia Bioinformatics Institute, Virginia Tech).

3.2.3 Data Analysis

Nucleotide sequence editing, alignment, prediction of amino acid sequences and analyses were conducted using the software package DNASTAR (Lasergene). Phylogenetic reconstructions of these two viruses were performed using Parsimony (PAUP 4.01) (Swofford, 2002) software with 1000 bootstrap replicates.

3.2.4 Accession Numbers for Sequence Analysis

The sequences of ISU92 and Texas81 were deposited in GenBank under accession # EU439428 and EU439429, respectively. The accession numbers for other viral sequences used for phylogenetic analysis are: Atlantic salmon paramyxovirus (ASPV), EF646380; Avian metapneumovirus (aMPV), NC_007652; Avian paramyxovirus 2 (aPMV2), EU338414; Avian paramyxovirus 6 (aPMV6), NC_003043; Beilong virus (BeV), NC_007803; Bovine parainfluenza virus 3 strain 910N (bPIV3-910N), D84095; Bovine parainfluenza virus 3 strain Kansas/15626/84 (bPIV3-Ka), AF178654; Bovine parainfluenza virus strain Q5592 (bPIV3-Q5592), EU277658; Bovine parainfluenza virus 3 strain Shipping Fever (bPIV3-SF), AF178655; Bovine respiratory syncytial virus (bRSV), NC_001989; Canine distemper virus (CDV),
NC_001921; Dolphin morbillivirus (DMV), NC_005283; Fer-de-lance virus (FDLV), NC_005084; Hendra virus (HeV), AF017149; Human metapneumovirus (hMPV), NC_004148; Human parainfluenza virus 1 strain Washington/1964 (hPIV1-Wa), NC_003461; Human parainfluenza virus 2 (hPIV2), NC_003443; Human parainfluenza virus 3 (hPIV3), AB012132; Human parainfluenza virus 3-GrpV (hPIV3-GrpV), NC_001796; Human parainfluenza virus 3-JS (hPIV3-JS), Z11575; Human respiratory syncytial virus (hRSV), NC_001781; J-virus (J-V), NC_007454; Measles virus (MeV), NC_001498; Menangle virus (MenV), NC_007620; Mossman virus (MoV), NC_005339; Mumps virus (MuV), NC_002200; Newcastle disease virus (NDV), NC_002617; Nipah virus (NiV), NC_002728; Peste-des-petits-ruminants virus (PPRV), NC_006383; Pneumonia virus of mice, AY573818; Porcine Rubulavirus (PoRV/LPMV), NC_009640; Rinderpest virus (RPV) (strain Kabete O), NC_006296; Sendai virus (SeV), NC_001552; Simian parainfluenza virus 5 (SV5), NC_006430; Tioman virus (TioV), NC_004074; Tupaia paramyxovirus (TPMV), NC_002199.

The consensus nucleotide sequence of hPIV3 Wash/47885/57 (hPIV3-Wa) was assembled from individual gene sequences from GenBank (Bailly et al., 2000) including, M11849 (N gene), M14552 (N gene), X04612 (N gene), M14890 (P gene), X04721 (P gene), D00130 (M gene), M16458 (M gene), M16569 (M gene), Y00119 (M gene), M14892 (F gene), S82195 (F gene), M21649 (F, HN, L gene and 5’ genomic termini), Z26523 (HN gene), M17641 (HN gene), M20402 (HN and partial L gene), X03967 (3’ genomic termini).
3. 2.5 Pathogenicity Studies in Pigs

A total of eighteen 6-weeks old Yorkshire crossbred pigs (less than 25 lbs body weight) were obtained from the Swine facility at Virginia Tech and randomly divided into 3 groups of 6 piglets. They were determined to be free of antibodies to PRRSV, Swine influenza virus, and PCV-2. The pigs were allowed to acclimatize for a week before the start of the experiment. The rectal temperatures were recorded two times a day (morning and evening). Clinical signs were observed three times a day (every 8 h) after inoculation until day 10. Treatment groups were inoculated intranasally with 2 ml (5x10^7 TCID50/ml) of virus stock with 1 ml in each nostril. The control group was inoculated intranasally with 2 ml phosphate buffered saline (PBS) with 1 ml in each nostril. From each of these groups, three pigs were euthanized on day 6 and 10, respectively. The clinical signs were scored on a 0 to 3 scale for physical appearance (normal, dull, lameness, recumbency), activity (alert, anorectic, mild response, no response), respiratory signs (none, sneezing, heavy nasal discharge, dyspnea) and other signs/body weight loss (none, <10% weight loss, 10-15% weight loss and >15% weight loss).

Blood was collected before inoculation and at necropsy. Serum was tested against the respective virus strains by virus neutralization (VN) test as described (Battrell, 1995). Trachea, lung, heart, brain, liver, spleen, and other internal organs were collected at necropsy, fixed in 10% neutral buffered formalin and used for histopathologic examination. Paraffin embedded tissue sections were employed for immunohistochemistry using bovine anti-bPIV3-SF polyclonal antiserum (NVSL, Ames, IA). Nasal and fecal swabs, blood, and 20% suspensions of lung and brain were subjected to virus isolation in Vero cells. The serum samples were examined by VN test in 96-well plates using homologous viruses.
3.2.6 Development of indirect Enzyme-linked immunosorbent assay (ELISA) for serology

The ISU92 virus stock prepared in Vero (1x10^8 pfu/ml) cells was used as an antigen to coat the polystyrene ELISA plates (Nunclon). The optimum dilution of antigen and serum were determined by checkerboard titration. The indirect ELISA is performed as follows: Briefly, a pre-determined concentration of antigen in coating buffer (KPL, Kirkegaard & Perry Laboratories, Inc.) was used to coat each well. The plates were then covered with parafilm and incubated overnight at 4°C. The plates were washed once with wash buffer (KPL) in an ELISA plate washer (TECAN, HydorFLEX). After blocking the unbound sites with a commercial blocking buffer (KPL) for 1 h, 100ul of 1 in 50 dilution of serum was added and the plates were incubated at 37°C for 1 h. Duplicate wells were set up for each sample and blank wells were set up with only diluent buffer. The plates were then washed three times with a brief soak and shake between each wash. The bound antigen-antibody complexes were probed with anti-species IgG horseradish peroxidase (HRP) secondary antibody diluted 1:500 and the plate was incubated for a further 1 h at 37°C. After washing the plates 3 times, 100 ul of TMB microwell substrate (KPL) was used to develop color for 10 min and then 100 ul BlueSTOP™ Solution (KPL) was added to each well to stop the reaction. The absorbances were read at 650 nm immediately (TECAN, Safire^2). A cut-off value of two times the mean of negative serum absorbance + 1 standard deviation was chosen based on the results of checkerboard titrations with known positive and negative sera samples. One hundred randomly selected pig serum samples from different age groups obtained from 5 swine farms in Minnesota and Iowa in 2007-2008 were screened for the presence of sPIV3 antibodies. The sera samples from pigs experimentally inoculated with ISU92 or Texas81 were also subjected to ELISA. The mean values from duplicate wells were used for analysis.
3.3 Results

3.3.1 Genome Features

The complete genome of Texas81 is 15456 nucleotides (nt) in length and 15480 nt for ISU92. The genome length is divisible by six and consistent with the “rule of six” as described for most other members of Paramyxoviridae (Calain and Roux, 1993; Kolakofsky et al., 1998; Kolakofsky et al., 2005). The genome contains six discrete, non-overlapping transcription units. The coding capacity of the genome is 93.3% in Texas81 and 93.2% in ISU92. Both viruses have conserved gene start (GS), gene end (GE) and strictly conserved trinucleotide intergenic sequence (IGS), for all the 6 genes compared to bPIV3 (Table 2). ISU92 shared the identical GS and GE with bPIV3-910N strain, while Texas81 shared the identical GS and GE with bPIV3-SF/Ka strain, except the GS of L gene, which is identical with bPIV3-910N strain.

The genomic termini of members of Paramyxoviridae are observed to have complementarity between the 3’ and 5’ termini (Li et al., 2005). These conserved terminal sequences, especially the first 12–13 nt, are believed to contain the genome and anti-genome promoters essential for replication and transcription (Robert A. Lamb, 2007). The swine viruses have a 55 nt 3’ leader before the transcription start site for the N gene, the length of which is conserved among almost all of the members of subfamily Paramyxovirinae. The 5’ trailer of swine viruses is 44 nt long following the L gene transcription stop site and the length is variable among Paramyxovirinae subfamily but conserved among parainfluenza type 3 (PIV3) viruses. Overall, the swine viruses possess a 96.4% (53 nt out of 55 nt) identity in the leader region with bPIV3, while a 92.7% (51 nt out of 55 nt) identity with hPIV3. In trailer region, there is a 97.7% (43 nt out of 44 nt) identity between swine viruses and bPIV3, and 90.9% (40 nt out of 44 nt)
identity with hPIV3. The exact complementarity at the first 14 nt of 3’ genomic leader and overall 65.9% (ISU92)/63.6% (Texas81) complementarity (29(ISU92)/28 (Texas81) nt out of 44 nt) between the 3’ leader and 5’ trailer termini suggest conserved elements in the 3’ promoter regions of the genome and antigenome (Figure 2).

3.3.2 Genome organization

The genome organization of swine viruses can best be described as 3’-N-P/V/D-M-F-HN-L-5’ and can potentially encode 9 proteins. By inserting a broad distribution of Gs at the mRNA editing site, V and D proteins were predicted to express from the P gene of both ISU92 and Texas81. The features of six genes and corresponding proteins of two viruses were shown in Table 3. The identity of Texas81 with ISU92 genome is 94.1% at nucleotide level. The identity between swine viruses and bPIV3 is 98.2% at the highest (between Texas81 and bPIV3-SF) level, while the identity between swine viruses and hPIV3 is 80.1% at the highest (between Texas81 and hPIV3-JS) level. Analysis of the start sites of each gene and the P gene editing site of Texas81 and ISU92, and the hexamer phasing positions of “2,1,1,1,2,2”, which is shown to be genus-specific within the Paramyxovirinae (Harcourt et al., 2001; Kolakofsky et al., 1998) are identical with hPIV3 and bPIV3 (Table 4).
3.3.2.1 The Nucleoprotein (N) Gene

The N gene of both Texas81 and ISU92 is 1646 nt long and encodes a N protein of 515 amino acid (aa) long, with a predicted molecular weight (MW) of 57.3 k daltons (kDa) and pI of 5.0 (Texas81) and 5.2 (ISU92), respectively. The N protein tightly binds to the entire length of genomic and antigenomic RNA to form the nucleocapsid and it is also associated with the polymerase complex during transcription and replication. A highly conserved motif located near the middle of all members of *Paramyxovirinae* N protein, which is thought to be essential in N-N self assembly and N-RNA interaction process, is F-X4-Y-X3-O-S-O-A-M (where X is any residue and Ø is an aromatic amino acid) (Myers et al., 1997b; Robert A. Lamb, 2007). This motif is also seen within the central domain of the N protein of swine viruses, presented as FAPGNYPALWSYAM\textsuperscript{336}. In SeV and other paramyxoviruses, the first residue of this motif F324 (annotated in SeV) is needed for correct self-assembly and another residue Y260 (annotated in SeV) (Myers et al., 1997b) is critical for N-viral RNA binding. These two residues are conserved in swine viruses as F323 and Y259, respectively. The last 24% sequence of carboxy terminal of N (aa 394 to 515) has a very low identity with other members of *Paramyxoviridae*. This region with consistently low similarity is where most of the phosphorylation and antigenic sites of the protein (Robert A. Lamb, 2007; Ruth A. Karron, 2007) are located. The N protein of ISU92 and Texas81 has 97.1% amino acid sequence identity with each other. When aligned with selected members from *Paramyxoviridae*, the identities decreased in order: subfamily *Paramyxovirinae*, *Respirovirus* (60.6%-100%); *Morbillivirus* (18.9%-23.4%); *Henipavirus* (19.8%-20.2%); *Avulavirus* (17.7%-20.6%); *Rubulavirus* (17.4%-19.2%); subfamily *Pneumovirinae*, *Metapneumovirus* (11.5%-12.2%); *Pneumovirus* (11.1%-11.8%); unclassified viruses, ASPV (23.4%-24.8%), BeV (23.3%-23.8%), J-V (20.2%-21.5%) (Table 5).
3.3.2.2 The Phosphoprotein (P) Gene and P/C/V Editing

The P gene of the two strains of sPIV3 is 1995 nt long with a major ORF of 1788 nt encoding the large P protein of 596 aa long with a calculated size of 66.3 kDa (ISU92) and 69.2 kDa (Texas81) and pI of 5.4 and 5.6, respectively (Table 3). The sequence identity between two sPMV P genes is 87.6% and when compared with other Paramyxoviruses P proteins, it is poorly conserved (Table 5).

The N terminal of P protein of paramyxoviruses are heavily phosphorylated at serine and threonine residues (Robert A. Lamb, 2007). There are 44 serine and 16 threonine residues in Texas81 P protein and 50 serine and 17 threonine residues in ISU92 P protein identified as potential phosphorylation sites using NetPhos 2.0 server (Blom et al., 1999) (http://www.cbs.dtu.dk/services/NetPhos/).

The P gene contains an mRNA editing site, 5’AAAAAAGGG3’(mRNA sense) in both Texas81 and ISU92 (794-802 nt), which is identical to those of other respiroviruses (Figure 3). By sequencing at least 40 clones of cDNA from the mRNA of two strains, we found that Texas81 had 1-4 G insertions while ISU92 had a broader distribution of G insertions, (1-9 Gs) at the editing sites. The insertion of G residues during mRNA synthesis can shift the translational reading frame and thus potentially generate V (+1/4/7 Gs) protein, predicted to be 412 aa long of 47.8 kDa in ISU92 and 46.7 kDa in Texas81, with identity of 83.3% between two strains, and D (+2/5/8 Gs) protein, 367 aa long of 41.3 kDa in ISU92 and 41.5 kDa in Texas81 with identity of 84.0% between two strains. The predicted V and D proteins will be amino co-terminal with P (+0/3/6/9 Gs) protein (first 241 aa). In sPIV3, the C protein is predicted to be initiated 10 nt downstream of the P protein start codon by alternate translation initiation AUG codon and
predicted to be 201 aa long with MW of 23.6 kDa in ISU92 and 23.7 kDa in Texas81 with identity of 91.1% between two strains.

At the C-terminal of V protein, sPIV3 contains all seven conserved invariantly placed cysteine residues, highly conserved motifs H-R-R-E and W-C-N-P, known among paramyxovirus V proteins (Figure 4). This cysteine-rich C-terminal is shown to be involved in coordinating two zinc molecules per V protein and may play an important role in viral pathogenesis and blocking of host interferon defense mechanisms (He et al., 2002; Patterson et al., 2000; Poole et al., 2002; Robert A. Lamb, 2007).

3.3.2.3 The Matrix protein (M) Gene

The M gene is 1149 nt long with a single ORF of 1053 nt. The encoded protein is 351 aa long with a predicted MW of 39.3 kDa and a pI of 9.5 (Texas81) and 9.6 (ISU92). The M protein is the most abundant virion structural protein located in the inner surface of envelope. It interacts with the cytoplasmic tails of the integral membrane proteins, lipid bilayer and the nucleocapsids, and plays an important role in virion assembly, budding, release as well as transport of viral components (Robert A. Lamb, 2007; Ruth A. Karron, 2007). M protein is considered to be the most conserved parainfluenza viral protein (Spriggs et al., 1987). The nuclear localization signal (NLS) (\textsuperscript{245}KMGRMYSEYCKQIEK\textsuperscript{261}) of M protein in swine viruses is highly conserved in PIV3 (Coleman and Peeples, 1993; Peeples et al., 1992). The ISU92 virus M protein has 96.6% amino acid sequence identity with Texas81. The amino acid sequence identity with members of the other genera of \textit{Paramyxovirinae} decreased in the following order: Respiroviruses (63.1%-

118
100%); Morbilliviruses (33.5%-37.2%); Henipaviruses (31.9%-32.7); Avulaviruses (17.9%-22.9%); Rubulaviruses (18.1%-19.9%); with unclassified viruses, ASPV (39.2%-40.1%), J-V (35.7%-36%), BeV (35.7%-36%). There was only less than 10% identity when sPIV3 were compared with the members of *Pneumovirinae*.

### 3.3.2.4 The Fusion Protein (F) Gene and Hemagglutination-Neuraminidase Protein (HN) Gene

We have recently reported the molecular characterization of the envelope glycoprotein genes of these two swine viruses (Qiao et al., 2009). The molecular features of these two genes are shown in Table 3. There were several amino acid changes in the antigenic sites of the F protein differing from hPIV3 but conserved as in bPIV3. They include: E101N, V/T367I (only in ISU92), S418Q, T492A, T513V.

### 3.3.2.5 The Large Polymerase Protein (L) Gene

The L gene of sPIV3 is 6795 nt long, with a major 6699 nt long ORF encoding a 2233 aa protein with a pI of 6.3 for Texas81 or 6.2 for ISU92, and MW of 25.6 kDa. The L protein of parainfluenzaviruses is the major RNA polymerase (RNAP) component and it is responsible for nucleotide polymerization, mRNA capping, methylation and polyadenylation (Robert A. Lamb, 2007). It is proposed that there are six highly conserved domains (DI-DVI) in nonsegmented negative-strand virus (NNSV) and each domain may be individually responsible for each of the L protein multiple functions (Poch et al., 1990; Sidhu et al., 1993; Svenda et al., 1997). Pairwise
alignment of sPIV3 along with the other paramyxoviruses revealed all the six domains, highly conserved A to D subdomains within DIII and a highly variable hinge region between DII and DIII (Figure 5). A highly conserved stretch (positions 543 to 562) within DII, proposed to be a template recognition site, was present in sPIV3 L protein and matched the pattern of basic and hydrophobic amino acid pair repeats every four residues as described (Poch et al. 1990). The highly conserved $^{772} \text{GDNQ}^{775}$ motif, which is believed to be the active site for nucleotide polymerization (Chattopadhyay and Shaila, 2004; Malur et al., 2002), was present in subdomain C in DIII. The L protein of swine viruses also contained a putative ATP binding site with motif $^{1786} \text{K-X21-G-E-G-A-G}^{1810}$ (Poch et al., 1990) in DVI. The L protein of Texas81 has 99.2% amino acid sequence identity with ISU92, 59.7%-98.7% with Respiroviruses, 38.8%-39.7% with Henipaviruses, 37.2%-42.5% with Morbilliviruses, 28.3%-29.8% with Rubulaviruses, 26.2%-27.2% with Avulaviruses, 48.3%-48.5% with ASPV, 38.6%-38.8% with J-V, 37.9%-38% with BeV, 16.7%-17.0% with Metapneumoviruses, and 15.1%-15.5% with Pneumoviruses.

### 3.3.3 Phylogenetic analysis

Phylogenetic trees were generated based on N, P, M, F, HN, L nucleotide sequence, deduced amino acid sequences, and complete genome sequence of sPIV3 with other representative members of all the five genera of family Paramyxoviridae and found to be similar. The sPIV3 strains were phylogenetically closely related to genus Respirovirus within Paramyxovirinae subfamily, and Texas81 and ISU92 belonged to two subgenotypes of genotype A and the Australian bPIV3 strain Q5592 formed the genotype B (Figure 6). The phylogenetic reconstructions of representative trees are shown (Figure 6).
3.3.4 Pathogenicity

Conventionally reared pigs infected intranasally with either of the two viruses resulted in mild respiratory signs only. None of the infected pigs developed neurological signs or elevated body temperatures throughout the experimental period (Figure 7). Two pigs from each of the infected groups developed mild respiratory signs at 2 days post inoculation (DPI) (mean clinical score 1). One pig from Texas81 group had diarrhea on day 2 and another on day 3 (mean clinical score 1). The gross lesions at necropsy on 6 and 10 DPI from virus-infected pigs were unremarkable, such as segmented, thickened small intestine and greenish milky intestinal contents. Histologically, no microscopic lesions were observed. Virus isolation attempts were unsuccessful on swab and tissue samples collected at day 6 and 10 DPI except from one nasal swab sample from Texas81 virus infected pig on day 6. None of the lung tissues examined by immunohistochemistry using polyclonal anti-bPIV3-SF serum revealed the presence of virus-specific antigen. Pre-inoculation serum samples were negative for sPIV3-specific antibodies by VN test. The homologous neutralization titers of sera samples at 10 DPI ranged from 1:8 to 1:16, but none of the infected pigs had VN antibodies at 6 DPI.

3.3.5 Serum antibody responses to sPIV3 in pigs detected by indirect ELISA

None of the 100 serum samples obtained from pigs aged 19 days to 70 days from the 5 swine farms designated A, B, C, D, E in Minnesota and Iowa in 2007-2008 were positive by ELISA suggesting the absence of prevalence of sPIV3 in these farms. The sera samples from experimentally inoculated pigs were positive at 6 and 10 dpi and the pre-inoculation samples and
sera from mock-infected pigs were negative. These results confirmed the neutralization test results of sera from experimentally infected pigs. However, the fact that 6 dpi samples also showed positive result in ELISA but not in neutralization test suggests that ELISA is capable of detecting non-neutralizing antibodies. A large number samples need to be screened by both tests to determine the relative merits of ELISA over virus neutralization test for prevalence studies. But, the indirect ELISA can be used as a simple screening test for the presence of sPIV3 antibodies.

3.4 Discussion

The recent insurgence of novel paramyxoviruses of zoonotic importance necessitates the close monitoring of viral pathogens with respiratory and neurological tropism in animals. Although, several paramyxoviruses have been isolated from swine in USA and other parts of the world, many except a few have shown the potential to be extremely virulent and infect across species (Field et al., 2007b). The epidemiologic significance of many of the swine paramyxovirus isolates has never been examined except for the initial case reports (Janke et al., 2001; Philbey et al., 1998).

In this study, we have performed detailed analysis of two swine viruses isolated in the United States between 1980 and early 1990s. Our preliminary study of the morphologic, antigenic characteristics and sequence analysis of the envelope glycoproteins indicated that these two viruses belonged in the genus Respirovirus, subfamily Paramyxovirinae. We have further characterized these isolates for their complete genomic sequence and features, and their
pathogenicity in conventionally reared pigs. Our comprehensive studies revealed that these sPIV3 are host-adapted variants of bPIV3, possibly transferred from cattle to pigs but failed to establish an active enzootic state.

The mild clinical signs and undetectable gross and microscopic lesions observed in sPIV3-infected pigs indicate the inapparent nature of these viruses in pigs. It has been reported that bPIV3 can be isolated from clinically normal cattle (Rosenberg et al., 1971). It is generally accepted that co-infection of bPIV3 along with other viruses and Mannheimia/Pasturella species could precipitate the classical shipping fever syndrome (Battrell, 1995; Woods, 1968). A variety of factors such as environmental temperature, transportation, hygiene, stocking density, co-mingling, and host immune status can contribute to increased susceptibility to secondary bacterial infections and severity of clinical disease (Battrell, 1995; Woods, 1968). The outbreak of disease in 1980s and 1990s in pigs with these sPIV3, therefore, might have resulted from coinfection with other pathogens. Initial pathogenicity studies in gnotobiotic piglets with ISU92 strain also resulted in mild respiratory disease and microscopic pathology (Janke et al., 2001), suggesting this possibility. Further, our limited serological prevalence studies in sera samples collected between 2007 and 2008 also indicated that sPIV3 are not prevalent in the field. An earlier study examining the seroprevalence of pigs from Iowa indicated that there was a very low seroprevalence. From 876 serum samples collected from 36 swine farms in 1988 to 1989, only 6 samples, representing 5 swine herds, were detected to have anti-ISU92 antibody titers (Battrell, 1995).

The complete genome of sPIV3 is 15456 nt in Texas81 and 15480 nt in ISU92, which is typical of genome sizes (15500 nt) in paramyxoviruses. However, the recently determined
sequences of aPMV6 (16236 nt) (Chang et al., 2001), aPMV3 (16272 nt) (Kumar et al., 2008), NiV (18246 nt) (Harcourt et al., 2001; Wang et al., 2001), HeV (18234 nt) (Wang et al., 2001), J-V (18954 nt) (Jack et al., 2005), BeV (19212 nt) (Li et al., 2006b), MoV(16650 nt) (Miller et al., 2003), TPMV (17904 nt) (Tidona et al., 1999) and ASPV (16965 nt) (Nylund et al., 2008) show a greater genome length ranging from ~16300 nt to ~19200 nt. The lengths of the genome of sPIV3 conform to the “rule of six” which is consistent in members of subfamily Paramyxovirinae (Calain and Roux, 1993) and this is thought to be associated with nucleocapsid organization in which each N protein monomer bind to six nucleotides of the viral genome. Recombinant PIV with a genome length that was not an even multiple of six has been shown to mutate to conform to the rule (Skiadopoulos et al., 2002; Skiadopoulos et al., 2003b).

The genome organization of these two strains of sPIV3 is essentially consistent with members of the subfamily Paramyxovirinae. The transcriptional start signal is conserved as in the consensus sequence of genus Respirovirus. The trinucleotide IGS between the gene boundaries is an exact resemblance to the ones of Respirovirus, Morbillivirus and Henipavirus, but differ from the variable-length intergenic regions found in the Rubulavirus (1-47nt), Avulavirus (1-63nt) and Pneumovirus (1-56nt) (Robert A. Lamb, 2007). The length of the 3’ leader is consistent in most known paramyxoviruses (55nt), except hPIV2 (70nt) (Robert A. Lamb, 2007). The length of 5’ trailer in Paramyxoviridae is more variable, ranging from the shortest 21 nt in hPIV2 (Ruth A. Karron, 2007) to 707 nt in aPMV3 (Kumar et al., 2008) as the longest trailer among members of family Paramyxoviridae. The 5’ trailer of sPIV3 is 44 nt within the 25-58 nt range seen in Respirovirus, Morbillivirus, Rubulavirus and Henipavirus genera. The overall complementarity between 3’ and 5’ termini was as high as 65.9%
(ISU92)/63.6% (Texas81) in sPIV3, and this high complementarity was also seen in most members of the *Paramyxovirinae*.

The total coding percentage of the sPIV3 is 93% which is similar to what is found in other *Paramyxovirinae* with an average of 92% coding capacity (Wang et al., 2000). The genome contains six genes: N, P, M, F, HN, L, as found in most members of *Paramyxoviridae*. In some of the members, instead of HN, the H or G proteins serve as attachment proteins and some *Rubulaviruses, Avulaviruses* (APMV6) and all *Pneumoviruses* also encode a third integral membrane protein, SH, in the genome. The genome of swine viruses can potentially encode 9 proteins including the six invariant structural proteins and three accessory proteins, V, D, C, which are all derived from P/C/V gene by a mechanism known as mRNA editing (V and D) or alternative translation initiation (C).

The mRNA editing in P/C/V gene is a common feature in *Respiroviruses, Morbilliviruses, Henipaviruses and Avulaviruses*, and is considered as a remarkable example of exploiting the coding capacity of viruses (Robert A. Lamb, 2007). The V protein plays important roles in virus replication (Baron and Barrett, 2000; Curran et al., 1991), function as a negative regulator to inhibit RNA synthesis (Baron and Barrett, 2000; Delenda et al., 1997; Durbin et al., 1999), and inhibit host cell antiviral response by interacting with cellular proteins (Andrejeva et al., 2002; Lin et al., 1998). The C-terminal V-specific domain is highly conserved among paramyxoviruses with invariantly spaced histidine and cysteine residues forming a domain (cys-rich domain) that binds two zinc molecules per V protein (Fukuhara et al., 2002; Li et al., 2006a; Liston and Briedis, 1994; Paterson et al., 1995). In sPIV3, the C-terminal of V protein contains all of the seven conserved cysteine residues and motifs H-R-R-E and W-C-N-P. The presence of
these domains suggests a similar function for the V protein of swine viruses as in other paramyxoviruses. The role of D in viral growth cycle has not been understood (Lamb and Parks, 2007). The C protein ORF is commonly observed in *Respiroviruses and Morbilliviruses* (Robert A. Lamb, 2007). C proteins are small basic polypeptides that may involve in the viral growth cycle, control of viral RNA synthesis (Lamb and Parks, 2007), counteracting host cell antiviral pathways (Komatsu et al., 2004) and facilitating the release of virus from infected cells (Garcin et al., 1997; Kato et al., 2001). Both the editing site and the predicted size of accessory proteins of sPIV3 highly resemble that of hPIV3 and bPIV3 and may have similar functional roles.

The amino acid identities with the corresponding proteins of other *Paramyxoviruses* clearly place the sPIV3 as novel viruses in the genus *Respirovirus* with a high level of identity to bPIV3. The overall conservation of homologous proteins in paramyxoviruses is generally consistent with the description in the most recent ICTV report, V>C>L>M>F>N, and HN>V>P (Lamb, 2005). Limited sequence polymorphism has been reported among bPIV3 strains (Bailly et al., 2000; Coelingh and Winter, 1990; Swierkosz et al., 1995). The host specific amino acids identified in bPIV3 (Bailly et al. 2000) were invariable between bPIV3 and sPIV3 but, further amino acid changes noticed in envelope glycoproteins (Qiao, Janke et al, 2009) and P/N473H in P gene and amino acid residues A326T and I924M in the L gene, suggest that the sPIV3 is adapting to the swine host. The bPIV3-910N strain was a plaque-type variant and reported to have intermediate fusion capabilities but avirulent in mice (Shibuta et al., 1981). The bPIV3-SF strain was originally obtained from a calf showing signs of shipping fever (Breker-Klassen et al., 1996). The antigenic site amino acid residues in F protein (Van Wyke Coelingh and Winter, 1990) were conserved in both strains of sPIV3 as in bPIV3. The fusogenicity of both Texas81
and ISU92 were comparable (Qiao, Janke et al, 2009). Detailed epitope mapping studies with F and HN monoclonal antibodies are needed to elucidate host-specific antigenic site variations.

The genomic organization, amino acid identities of homologous proteins, phylogenetic analysis based on the genome sequences and antigenic analysis all support the classification of these two novel strains of sPIV3 into Respirovirus genus in Paramyxovirinae subfamily and Paramyxoviridae family.

This mild pathogenicity of sPIV3 can facilitate its development as a vaccine vector. The bovine/hPIV3 chimeric virus (Bailly et al., 2000; van Wyke Coelingh et al., 1988), as a vector backbone with the replacement of F and HN glycoprotein from hPIV3 has been evaluated as a vaccine against hPIV3 (Haller et al., 2000; Pennathur et al., 2003). Besides, bPIV3 as backbone with replaced F and HN of hPIV3 and inserted F of RSV were also constructed. The effectiveness of this vaccine against both PIV3 and RSV challenge has been demonstrated in African green monkeys and is being evaluated in clinical trials (Sato and Wright, 2008). The promising outcome of the vectored vaccines using paramyxoviruses and the biological and molecular characteristics of swine viruses described here suggest a great potential for sPIV3 to be developed as a vaccine vector. To fulfill this goal, the prevalence of sPIV3 in human population needs to be ascertained and potential risk of increased pathogenicity by co-infection need to be further studied. The screening ELISA method developed by us with sPIV3 antigen can be used to detect seroprevalence in human and animal populations.
3.5 Acknowledgments

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3.6 References


Skiadopoulos, M.H., Tao, T., Surman, S.R., Collins, P.L. and Murphy, B.R. (1999) Generation of a parainfluenza virus type 1 vaccine candidate by replacing the HN and F
glycoproteins of the live-attenuated PIV3 cp45 vaccine virus with their PIV1 counterparts. Vaccine 18(5-6), 503-10.


(1990) Focus on: swine blue eye disease. Stephano, H.A.


Stricker, R., Mottet, G. and Roux, L. (1994) The Sendai virus matrix protein appears to be recruited in the cytoplasm by the viral nucleocapsid to function in viral assembly and budding. J Gen Virol 75 (Pt 5), 1031-42.


Thorsen, J. and Henderson, J.P. (1971) Survey for antibody to infectious bovine rhinotracheitis (IBR), bovine virus diarrhea (BVD) and parainfluenza 3 (PI3) in moose sera. J Wildl Dis 7(2), 93-5.


### Table 1. Primer list

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### Primers for RACE

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a. All primer sequences were given from 5' to 3'. The numbers in superscript indicate the expected position of the primer in bPIV3-SF/Ka genome. DT88 and DT89 were the primers used in Li et al., 2005 for RACE.
Table 2. Comparison of the gene-start and gene end signals of sPIV3, bPIV3 and hPIV3.

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|      | ISU92           | UCCUAAUUC           | UUCAUUCUUUUU     |
|      | bPIV3-Ka/SF     | UCCUAAUUC           | UUCAUUCUUUUU     |
|      | bPIV3-910N      | UCCUAAUUC           | UUCAUUCUUUUU     |
|      | bPIV3-Q5592     | UCCUAAUUC           | CUAUUCUUUUU⁵     |
|      | hPIV3           | UCCUAUUUUC          | UUCAUUCUUUUU     |
|      | Consensus:      | UCCUNNUUNNC         | NUNNUNNUUUUU     |

| M    | Texas81         | UCCUACUUUC          | UUUU----------AGUUUUU |
|      | ISU92           | UCCUACUUUC          | UUUU----------AGUUUUU |
|      | bPIV3-Ka/SF     | UCCUACUUUC          | UUUU----------AGUUUUU |
|      | bPIV3-910N      | UCCUACUUUC          | UUUU----------AGUUUUU |
|      | bPIV3-Q5592     | UCCUAAUUUC          | UUUU----------AGUUUUU |
|      | hPIV3           | UCCUAAUUUC          | UUAUUCUUUUU     |
|      | Consensus:      | UCCUNNUUNNC         | NUNNUNNUUUUU     |

| F    | Texas81         | UCCUAGUUUC          | UUCAUUGUUUUU     |
|      | ISU92           | UCCUAGUUUC          | UUCAUUGUUUUU     |
|      | bPIV3-Ka/SF     | UCCUAGUUUC          | UUCAUUGUUUUU     |
|      | bPIV3-910N      | UCCUAGUUUC          | UUCAUUGUUUUU     |
|      | bPIV3-Q5592     | UCCUAGUUUU          | UUAUUCUUUUU     |
|      | hPIV3           | UCCUAGUUUU          | CUAUAAUUUUU     |
|      | Consensus:      | UCCUNNUUNNC         | NUNNUNNUUUUU     |

| HN   | Texas81         | UCCUGGUUUC          | UUAUUAUUUUUU     |
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|      | bPIV3-910N      | UCCUGGUUUC          | UUAUUAUUUUUU     |
|      | bPIV3-Q5592     | UCCUGGUUUC          | UUAUUAUUUUU     |
|      | hPIV3           | UCCUGGUUUC          | UUAUUAUUUUU     |
|      | Consensus:      | UCCUNNUUNNC         | NUNNUNNUUUUU     |

| L    | Texas81         | UCCUCUUUUC          | UUCAUUCUUUUU     |
|      | ISU92           | UCCUCUUUUC          | UUCAUUCUUUUU     |
|      | bPIV3-Ka/SF     | UCCUCUUUUC          | UUCAUUCUUUUU     |
|      | bPIV3-910N      | UCCUCUUUUC          | UUCAUUCUUUUU     |
|      | bPIV3-Q5592     | UCCUCUUUUC          | UUAUUCUUUUU     |
|      | hPIV3           | UCCUCUUUUC          | UUAUUCUUUUU     |
|      | Consensus:      | UCCUNNUUNNC         | NUNNUNNUUUUU     |

a. Host-specific nucleotide residues between hPIV3 and bPIV3 are in red;
b. Positions that display variability within the host group are in blue;
c. Positions that unique in bPIV3-Q5592 are in bold.
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\(^a\) bPIV3 indicates bPIV3 shipping fever stain if not specified.
Table 4. Subunit hexamer phasing positions for gene start sites and P editing sites of a selection of *Paramyxovirinae*

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Table 5. Amino acid identities of swine parainfluenzaviruses with analogous proteins in other paramyxoviruses.

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<tr>
<td></td>
<td>FDLV</td>
<td>21.4</td>
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^a^ A, attachment protein. The attachment protein for morbilliviruses is H; for henipaviruses, metapneumoviruses, pneumoviruses, BeV, J-V is G; for remaining is HN.

^b^ T indicates Texas81, I indicates ISU92, the identities in the row sPIV3 indicates the identities between the two swine strains.
3.8 Figures

**Figure 1. Strategy for rapid Amplification of cDNA Ends (RACE)** (adapted from Li et al. 2005). For 5'RACE, cDNA synthesis was carried out first using a virus-specific primer-1 (VSP51), followed by RNaseH treatment, then ligation of adaptor (DT88). In 3' RACE, adaptor ligation was carried out first directly with the viral RNA, followed by cDNA synthesis using the DT89 primer complementary to adaptor DT88. The remaining steps of the primary and hemi-nested PCRs are the same for both RACE methods with DT89 and VSPs using standard procedures.
(A) 3' leader

bPIV3-910N 3' - UGGUUUGUUCUCUUCUCUGAAGCGACCCUUAUAUUAUGCUGUAAUUUAUUGAA
bPIV3-Ka 3' - UGGUUUGUUCUCUCUCUGAAGCGACCCUUAUAUUAUGCUGUAAUUUAUUGAA
bPIV3-SF 3' - UGGUUUGUUCUCUCUCUGAAGCGACCCUUAUAUUAUGCUGUAAUUUAUUGAA
bPIV3-Q5592 3' - UGGUUUGUUCUCUCUCUGAAGCGACCCUUAUAUUAUGCUGUAAUUUAUUGAA
hPIV3-Wash 3' - UGGUUUGUUCUCUCUCUGAAGCGACCCUUAUAUUAUGCUGUAAUUUAUUGAA
hPIV3-JS 3' - UGGUUUGUUCUCUCUCUGAAGCGACCCUUAUAUUAUGCUGUAAUUUAUUGAA
ISU92 3' - UGGUUUGUUCUCUCUCUGAAGCGACCCUUAUAUUAUGCUGUAAUUUAUUGAA
Texas81 3' - UGGUUUGUUCUCUCUCUGAAGCGACCCUUAUAUUAUGCUGUAAUUUAUUGAA

(B) 5' trailer

bPIV3-910N GUAUUAUAUAUAUAUGCUGUAAUGGCAAACCCAAACCA-5'
bPIV3-Ka GUAUUAUAUAUAUAUGCUGUAAUGGCAAACCCAAACCA-5'
bPIV3-SF GUAUUAUAUAUAUAUGCUGUAAUGGCAAACCCAAACCA-5'
bPIV3-Q5592 GUAUUAUAUAUAUAUGCUGUAAUGGCAAACCCAAACCA-5'
hPIV3-Wash GUAUUAUAUAUAUAUGCUGUAAUGGCAAACCCAAACCA-5'
hPIV3-JS GUAUUAUAUAUAUAUGCUGUAAUGGCAAACCCAAACCA-5'
ISU92 GUAUUAUAUAUAUAUGCUGUAAUGGCAAACCCAAACCA-5'
Texas81 GUAUUAUAUAUAUAUGCUGUAAUGGCAAACCCAAACCA-5'

(C) Genomic termini

ISU92
3' - UGGUUUGUUCUCUCUCUGAAGCGACCCUUAUAUUAUGCUGUAAUUUAUUGAA
5' - ACCAAACAGAGAAAAACUCUGUUGGUAUAUAUAUAUAUG
Texas81
3' - UGGUUUGUUCUCUCUCUGAAGCGACCCUUAUAUUAUGCUGUAAUUUAUUGAA
5' - ACCAAACAGAGAAAAACUCUGUUGGUAUAUAUAUAUG

Figure 2. Nucleotide sequence of the 3' leader (A), 5' trailer (B) regions and genomic termini (C) of PIV3 genomic RNA. Sequences are 3' to 5' in negative sense. (A,B) Nucleotide residues in blue are host-specific (bPIV3 or hPIV3). Host-specific positions are those where bPIV3 strains share an assignment and hPIV3 strains share a different assignment. Positions that display variability within a host species are in red. (C) Shading indicates complementary base pairs.
**Figure 3. Schematic of P protein structure, P gene expression strategy and RNA editing site sequence of swine viruses compared with other paramyxoviruses.** The scale bar at the top indicates the amino acid of P protein, and the boxes below indicate possible reading frames of protein P, V, D and C. Known functional domains are indicated for chaperoning unassembled N proteins during the nascent chain assembly of genome replication (N), self-assembly as a tetramer (4’mer), L protein-binding site (L), and N-RNA-binding site (N:RNA) based on the study of sendai virus (Robert A. Lamb, 2007). In the editing site sequences, AₙGₙ elements are spacing between Aₙ and Gₙ to facilitate visual comparison (Hausmann et al., 1999; Robert A. Lamb, 2007).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Editing Site Sequence</th>
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<tbody>
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The table shows the RNA editing site sequences for various viruses compared to other paramyxoviruses. The sequences include AₙGₙ elements to facilitate visual comparison.
Figure 4. Amino acid sequence alignment of the conserved cysteine-rich C-terminal region of selected paramyxovirus V proteins. Positions of the conserved seven conserved cysteine residues that are involved in Zn binding are underlined.
Figure 5. Schematic overview of important features and conserved domains found in the predicted L-protein amino acid sequences. Positions of the conserved RNA-dependent RNA polymerase major domains DI-DVI, and subdomains A–D within DIII (Poch et al., 1990) are indicated. In addition, the template recognition site in DII, conserved GDNQ sequence in subdomain C, and a putative ATP-binding site motif (K–X21–G–E–G–A–G) in major domain VI (Harcourt et al., 2001) are shown.
Figure 6. Phylogenetic analysis. Phylogenetic analysis was performed using Parsimony (PAUP 4.01) analysis with 1000 bootstrap replicates, based on M gene (A) nucleotide sequences compared with other members of Paramyxoviridae. (B, C) Phylogenetic analysis of PIV3 genotypes based on N and M gene nucleotide sequences, respectively. The numbers over the branches indicate the percentage of 1000 bootstrap replicates that support each phylogenetic branch. Strain information and GenBank accession numbers are presented in Materials and Methods.
Figure 7. Rectal temperature in experimentally inoculated pigs. The rectal temperatures of Texas81 and ISU92 or mock-inoculated pigs were recorded two times per day during the experiment and the mean ± standard deviation of temperatures for each group at each time point were plotted.
General Conclusion

In conclusion, the complete genome of Texas81 virus was 15456 nucleotides (nt) and ISU92 was 15480 nt in length consisting of six non-overlapping genes coding for the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large polymerase (L) protein in the order 3'-N-P/C/V-M-F-HN-L-5'. The features related to virus replication and found to be conserved in most members of *Paramyxoviridae* were also found in swine viruses. These include: conserved and complementary 3’ leader and 5’ trailer regions, trinucleotide intergenic sequences, highly conserved gene start and gene stop signal sequences. The length of each gene of these two viruses were similar except for the F gene, in which ISU92 had an additional 24 nt “U” rich 3’ untranslated region (UTR). The P gene of these viruses were predicted to express P protein from the primary transcript and edit a portion of its mRNA to encode V and D proteins and the C protein was expected to be expressed from alternate translation initiation from the P gene as in Respiroviruses. Sequence specific features related to virus replication and host specific amino acid signatures in N, F, HN, L proteins indicated that these viruses probably originated from bovine parainfluenzavirus 3. Phylogenetic analysis based on the individual genes as well as predicted amino acid sequences suggested that these swine parainfluenzaviruses are novel members of the genus *Respirovirus* of the *Paramyxovirinae* subfamily, genotype A of bovine parainfluenza virus type 3 and distributed into two subgenotypes of genotype A. The mild clinical signs and undetectable gross and microscopic lesions observed in sPIV3-infected pigs indicate the inapparent nature of these viruses in pigs. The mild pathogenicity and limited replication of sPIV3 in the lungs of pigs can facilitate its development as a mucosal vaccine vector.