The application of metagenomic sequencing to detect and characterize emerging porcine viruses

by

Rachel Palinski

B.S., The University of Georgia, 2012

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
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Abstract

Emerging viral diseases threaten the health of the US swineherd and have the potential to impact the industry. Parvoviruses are capable of infecting birds, livestock and humans, however, in swine, parvoviruses cause reproductive failure and contribute to a devastating set of diseases termed porcine circovirus associated disease (PCVAD). Here, a divergent porcine parvovirus, porcine parvovirus 7 (PPV7), distantly related to known parvovirus sequences, was identified in market pigs in the US. The PPV7 non-structural protein displayed 42.4% similarity to *Eidolon helvum* parvovirus 2 and 37.9% similarity to turkey parvovirus. Conserved parvovirus replicase motifs including three rolling circle replication (RCR), two NTP-binding motifs and a helicase-binding domain, were present in PPV7. Analysis by qPCR of 182 porcine samples found 16 (8.6%) positive, suggesting moderate nucleic acid prevalence in US swine.

Paramyxoviruses are capable of infecting various species including cattle, pigs and humans, causing respiratory disease and importantly, can overcome species barriers causing disease. In 2013, a novel paramyxovirus sequence was described in Hong Kong, China in slaughterhouse pigs, and subsequently named porcine parainfluenza virus 1 (PPIV1). The second study identifies two complete PPIV1 genomes in US pigs originating in Oklahoma and Nebraska that display 90.0-95.3% identity to the Chinese strains. Molecular analysis by qPCR resulted in 6.1% prevalence in 279 porcine respiratory samples. Further serological analysis revealed 66.1% of 59 porcine sera samples were positive by PPIV1 F ELISA. Eleven 3-week old nursery pigs from a PPIV1 naturally infected herd were monitored for signs of infection. No clinical signs were seen in the animals, however, six pigs and the lungs of one animal tested qPCR positive by the conclusion of the study. Taken together, PPIV1 is moderately prevalent in US swine-herds.

Previously known to infect avian species, canines and swine, recent reports have identified circoviruses in bats, mink, and human feces. In pigs, porcine circovirus 2 (PCV2) is essential to PCVAD, a group of diseases including reproductive failure, respiratory disease complex (PRDC), porcine dermatitis and nephropathy syndrome (PDNS) and postweaning multisystemic wasting syndrome (PMWS). Additionally, PCV2 nucleic acid has been detected in mammalian species other than swine such as cattle and mink. The final study focuses on the identification and characterization of a divergent circovirus, porcine circovirus 3, identified in aborted mummies taken from sows displaying clinical and histological signs of PDNS. Putative capsid and replicase open reading frames display 37% and 55% identity to PCV2, respectively. A retrospective study of 48 PDNS cases, PCV2 negative by immunohistochemistry (IHC), identified 45 positive and 60% of a subset, positive for PCV3 by IHC. Molecular and serological prevalence studies revealed 12.5% nucleic acid and 55% antibody prevalence in US swine samples. Collectively, these studies identify emerging porcine viruses with the potential to cause disease using metagenomic sequencing. The results of these studies will help to mitigate the risk attributed to emerging swine viruses causing disease outbreaks.

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Dedication

To my family who always believes in me and always supported my decision to pursue a doctoral degree. You equipped me with the determination to keep going and the skill to see a bright side of things. To Tom and Chris Day for giving me peace of mind by caring for three needy animals. To the two friends who ensured my survival throughout this process. I would not have made it this far without your support. To my advisor, Bob Rowland and my committee members, Ben Hause, Bill Wilson, Mike McIntosh and Beth Davis. You provided me with both a path and encouragement to become a better scientist.

Chapter 1 - Literature Review

Introduction

Numerous viruses can infect swine and cause disease. Recently, emerging viral diseases have been identified in the US causing considerable economic concern. Porcine circovirus 2 (PCV2) was first identified in pigs with postweaning multisystemic wasting syndrome (PMWS) in the 1990's (Harding, 1996). Subsequently, outbreaks of severe disease were reported across the globe. The disease was first described in the US in 1998 (Allan *et al.*, 1998; Kiupel *et al.*, 1998; Sato *et al.*, 2000; Segalés *et al.*, 1997). In 2003, a genotype shift occurred moving from predominantly PCV2a to PCV2b, causing, on many farms, a second outbreak of a more severe systemic disease (Cheung *et al.*, 2007; Cortey *et al.*, 2011; Segalés *et al.*, 2013; Timmusk *et al.*, 2008). Although preventative measures temporarily controlled the disease, in 2012, an investigation of PCV2 vaccine failures led to the discovery of a mutant PCV2 (Xiao *et al.*, 2012).

One of the most rapid and devastating outbreaks of disease in the US swine industry can be attributed to porcine endemic diarrhea virus (PEDV). The first description of PEDV occurred in the 1970s in England and Belgium, however, the virus was not identified in the US until 2013 (Pensaert & de Bouck, 1978; Stevenson *et al.*, 2013; Wood, 1977). Within a single year following PEDV discovery in the US, it is estimated that 10% of the domestic pig population had died of PEDV related disease. This equates to approximately 7 million piglets (Jung *et al.*, 2015).

In addition to wasting disease and diarrhea, emerging respiratory diseases in US swine such as Influenza A virus (IAV) H1N1 are of great economic and public health concerns. Influenza viruses can infect a wide variety of species including humans and pigs, and most concerning, can transmit between mammalian species. In 2009, a pandemic outbreak of IAV H1N1 occurred by transmission from swine to humans and vice versa causing respiratory disease

and death in both species (Kong *et al.*, 2015). While these emerging viral diseases pose an economic threat to the health of the US swineherd, the threat to human health, as seen in the IAV H1N1 outbreaks strengthens the need for a greater focus on emerging pathogens in swine.

Emerging swine viral diseases have the potential to threaten the economy by impacting the health of the swineherds, including, as discussed here, parvoviruses, paramyxoviruses and circoviruses. Parvoviruses are some of the smallest and most diverse organisms infecting birds, livestock and humans. Parvovirus infections act as both primary pathogens or contribute to severe diseases by co-infecting with other pathogens contributing to a set of economically important swine diseases termed porcine circovirus associated disease (PCVAD) (Opriessnig *et al.*, 2014). One factor that contributes to the significance of paramyxoviruses as an emerging swine viral disease is their propensity to cross species barriers causing disease. This has been shown to occur between pigs and cattle with bovine parainfluenza virus 3 (Coelingh *et al.*, 1986; Janke *et al.*, 2001; Qiao *et al.*, 2009, 2010). Furthermore, BPIV is capable of infecting humans (Ben-Ishai *et al.*, 1980; Schmidt *et al.*, 2000). Although it is currently unclear whether circoviruses infect humans, one member, PCV2, causes severe economic devastation to the swine industry as an essential component in PCVAD. Taken together, it is important to rapidly identify viruses with the potential to cause disease in swine to minimize the likelihood of outbreaks or epidemics in the swineherds, and to protect human health.

Research Objectives

The studies in this dissertation are a part of diagnostic investigations using metagenomic sequencing conducted in the Kansas State Veterinary Diagnostic Laboratory (KSVDL). The investigations involve sequencing of various swine samples such as nasal swabs, rectal swabs, aborted fetus tissue homogenates, mummy tissue homogenates, serum, or tissues from pigs that died of unknown causes. Novel or divergent viral sequences identified were verified by PCR followed by Sanger sequencing. If a full genome sequence was not obtained, additional methods such as rolling circle amplification (RCA) for circular viral genomes or rapid amplification of cDNA ends (RACE) were utilized to acquire the most complete genomic sequence possible. These methods allow for the identification of numerous novel or divergent viral species in swine samples. The studies in this dissertation are characterization experiments performed following the discovery of unrecognized viral sequences using the methods outlined above.

In the first study, healthy porcine rectal and nasal swabs were submitted to KSVDL to characterize the virome of the pigs on a commercial swine farm. Data analysis identified unique sequences in a healthy pig rectal swab most closely related to parvoviruses recently identified in turkey feces and bat urine. Interestingly, this viral sequence was no more than 40.4% identical to any known porcine parvoviruses. The objectives for this project were to:

- Obtain the genomic sequence of this virus, and identify conserved parvovirus domains to verify the identity of this divergent species
- Analyze the phylogeny and similarity of the novel parvovirus to known parvoviruses
- Evaluate the nucleic acid prevalence in various swine samples from different geographic locations

In the second study, US swine tissue samples were submitted to KSVDL from pigs displaying mild respiratory disease, testing negative for common respiratory pathogens, were subjected to metagenomic sequencing. Data analysis identified complete viral genomes for porcine parainfluenza virus 1 (PPIV1), previously identified only in China. The objectives for this project were to:

- Analyze the similarity of the full PPIV1 genomes to those from China and other viruses in the *Paramyxovirinae* family.
- Characterize PPIV1 disease and pathology in pigs from a naturally infected herd
- Evaluate the molecular and serological prevalence of PPIV1 in US swine

The third study describes the discovery of a divergent circovirus genome obtained from commercial swine farm samples taken from pigs displaying PDNS-like clinical signs. The pigs tested negative for viral agents normally associated with PDNS and subsequent metagenomic sequencing revealed a circovirus sequence, designated porcine circovirus 3 (PCV3), most similar to a partial circovirus genome taken from ground pork. The objectives of this project were to:

- Determine the phylogenetic relationship and similarity of this novel circovirus to known *Circoviridae* species
- Determine the association of PCV3 with PDNS using cases of PDNS previously testing negative by immunohistochemistry for the normally associated viral pathogens
- Investigate the molecular and serological prevalence of PCV3 in swine samples from different geographical origins

Porcine Viral Diseases

The focus of this dissertation is the identification and characterization of novel viruses potentially associated with porcine diseases with the use of metagenomic sequencing. The following literature review covers background information necessary to understand the sequencing methods and viral families investigated in these studies. Specifically, this review covers the *Parvoviridae, Paramyxoviridae,* and *Circoviridae* families and provides information on metagenomic sequencing as it related to novel virus discovery.

Metagenomic Sequencing for viral discovery

The use of sequencing to determine the collection of all biological organisms in environmental samples is known as metagenomic sequencing. The first published use of metagenomic sequencing analyzed the viral communities in sea water samples from two locations on the San Diego coast (Breitbart *et al.*, 2002). Over 65% of the sequences found in the study were not significantly similar to any known sequences suggesting little was known of marine viral populations. A year later, in 2003, the technique was applied to human fecal samples, again finding most of the sequences were previously unidentified (Breitbart *et al.*, 2003). Since then, different techniques coupled to metagenomic sequencing have led to the discovery of novel parvoviruses, astroviruses, circoviruses, picornaviruses, and aneloviruses (Amimo *et al.*, 2016; He *et al.*, 2013; Wang *et al.*, 2016b).

Techniques used for sequence-independent viral identification

Multiple techniques have been used to non-specifically amplify sequences in samples to discover unique viral sequences including sequence independent single-primer amplification (SISPA), linker amplified shotgun library (LASL), arbitrary primer PCR (AP-PCR), random

PCR amplification, and rolling circle amplification (RCA) (Bexfield & Kellam, 2011; Delwart, 2007). All of these methods are relatively fast and simple and, with the exception of RCA, do not bias towards a viral family. This allows for the discovery of both known viruses and highly divergent viral sequences. A challenge of these techniques involves viral nucleic acid enrichment, as it can be difficult to separate viral from host genetic materials. Despite this, viral enrichment techniques prior to nucleic acid amplification have allowed for data enhancement. The improved data aided in novel viral discovery using the metagenomic sequencing approach.

Single-primer amplification methods

Advances in deep sequencing techniques such as SISPA and LASL have aided in viral discovery (Bexfield & Kellam, 2011; Delwart, 2007). Samples are treated with nucleases to degrade unprotected nucleic acids resulting in the incorporation of mainly viral genetic material into the SISPA workflow (Figure 1.1) (Hause *et al.*, 2015a). Samples must then undergo cDNA synthesis with random primers by reverse transcription creating single stranded cDNA. Following second strand synthesis, the SISPA primer is added for an additional round of amplification yielding products that can be barcoded, through introduction of a short coded stretch of nucleotides and incorporated into the standard library preparation procedure. A general outline of a metagenomic sequence workflow with SISPA is represented in figure 1.2. Related to SISPA, LASL first shears the dsDNA of purified viruses at random sites then repairs the ends using T4 DNA polymerase and T4 polynucleotide kinase. A linker is ligated to the end of these sequences and PCR amplification with a complementary primer yields products that can be sub-cloned into a plasmid for sequencing. Recently, SISPA techniques have been popularized due to its ability to amplify viral sequences in low quantities within a sample and has been used to

characterize novel astroviruses, hepatitis viruses and posaviruses (Hause *et al.*, 2015b; Matsui *et al.*, 1993; Reyes *et al.*, 1990).

Rolling circle amplification (RCA)

Circular DNA genomes can be amplified with RCA using PhiX29 polymerase. After complete amplification of the circular genome, PhiX29 is displaced at the 5' end but continues to amplify the genome. Random primers can then anneal to the displaced strand, creating double stranded DNA and cut with restriction enzymes yielding single fragments of the exact length of the circle. Despite these findings, the amplification of circular DNA genomes only is limiting. Additionally, RCA has inefficient amplification that may not detect viral sequences in low quantities. Polyomaviruses, circoviruses and geminiviruses all possess circular genomes have been classified using RCA (Haible et al., 2006; Johne et al., 2006a, b).

Metagenomic sequencing for the discovery of viruses

Metagenomic sequencing has aided in the discovery of novel parvoviruses, paramyxoviruses and circoviruses in the recent past. In 2014, metagenomic sequencing of slow loris tissue samples led to the discovery of a parvovirus most similar to *Dependoparvoviruses* and *Tetraparvoviruses* but divergent enough to represent a separate parvovirus genus (Canuti *et al.*, 2014). Caribou parvovirus was discovered in the nasal swabs of Alaskan caribou that same year, phylogenetically grouping most similar to BPV3 (Schürch *et al.*, 2014). More pertinent to the US livestock industry, porcine parvovirus 6 was identified in the serum samples of pigs from US farms and aborted fetuses in China using metagenomic sequencing techniques (Schirtzinger *et al.*, 2015). In 2012, Sunshine virus, a divergent paramyxovirus was discovered in the tissues of pythons that died of neurorespiratory disease originating in Australia (Hyndman *et al.*, 2012). That same year a novel circovirus was identified in the tissue of European catfish that died of

unknown causes (Lőrincz *et al.*, 2012). Most recently, in 2015, divergent circoviruses sequences in the respiratory fluids of Brazilian free-tailed bats was identified using a similar approach with metagenomic sequencing (Lima *et al.*, 2015). Metagenomic sequencing can be used to identify potential novel viral pathogens to prevent widespread outbreaks. In an industry as large as the swine-production industry, preventative measures are essential to maintain the health, well being and economic benefits provided by these livestock.

Parvoviruses

The Parvovirdae family

As important pathogens in the US swine industry, viruses classified as members of the family *Parvoviridae* are small, nonenveloped viruses with single stranded DNA (ssDNA) genomes of approximately 5kb. The *Parvoviridae* family is divided into two subfamilies, *Parvovirinae* and *Densovirinae,* which infect vertebrates and invertebrates, respectively. Eight genera make up the *Parvovirinae* subfamily; *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus,* and *Tetraparvovirus* (Cotmore *et al.*, 2014; King *et al.*, 2012a). Recent studies have identified two new putative genera within *Parvovirinae*, *Marinoparvovirus* and *Chapparvovirus* (Palinski *et al.*, 2016; Phan *et al.*, 2014). Currently, *Marinoparvovirus* is made up of a single species identified in feces taken from a California sea lion pup suffering from malnutrition and pneumonia was designated sesavirus. The putative *Chapparvovirus* genus comprises viruses detected in turkey feces, *Eidolon helvum* feces and swine samples (Baker *et al.*, 2013; Palinski *et al.*, 2016; Reuter *et al.*, 2014).

Genome structure and organization

The linear, single-stranded DNA genome of viruses classified in the *Parvoviridae* family has a number of unique characteristics. *Parvovirinae* species have genomes possessing two major open reading frames (ORF) flanked by 3' and 5' inverted terminal repeats. The left-handed REP ORF encodes for the nonstructural genes while the right handed CP ORF encodes for the structural capsid proteins (Figure 1.3a). With the exception of certain *Densoviruses*, which contain ORFs encoded by both DNA strands, all other species classified in *Parvoviridae* encode both ORFs on the same DNA strand (Berns & Parrish, 2013). The ssDNA genome (*Parvovirinae*) is packaged within an icosahedral virion made up of 60 copies of the capsid protein spanning only 18-26nm in diameter (Figure 1.3b). Parvovirus capsid proteins contain regions functioning as nuclear localization signals for the virion upon host cell infection. Additional calcium-dependent phospholipase A2 (PLA2) motifs, exposed during cell entry, allow for more efficient viral release (Berns & Parrish, 2013; Zadori et al., n.d.). Despite the PLA2 motif function, a few new unclassified members of *Parvovirinae* do not contain this region (Palinski et al., 2016; Reuter et al., 2014). It is not known how a missing PLA2 motif affects the viral infectivity. Highly conserved sequence regions on the structural REP ORF are also characteristic of parvovirus species, including three replication initiation motifs (I, II, and III), two NTP-binding motifs (A and B), and a helicase motif (H) (Ilyina & Koonin, 1992; Momoeda et al., 1994; Xiao et al., 2013). Host neutralizing antibodies target both linear and conformational epitopes on the parvovirus capsid proteins and antigenic variability prevents these antibodies from being cross protective among parvovirus species (Berns & Parrish, 2013).

Host receptors contributing to viral infection

The capsid protein mediates the host cell tropism, which is variable among parvoviruses and is determined by binding to cellular receptors. Canine parvovirus (CPV) and feline parvoviruses (FPV) have been shown to use transferrin receptors (TfR) for cell infection although species receptor variations prevent cross-infection (Goodman *et al.*, 2010; Qing *et al.*, 1999). These receptors are present in dividing cells. In addition, CPV, FPV and minute virus of mice (MVM) can bind sialic acids on some erythrocytes (Löfling *et al.*, 2013; López-Bueno *et al.*, 2006). The tropism of adeno-associated dependoparvovirus-2 is dependent on efficient binding to heparan sulfate proteoglycans, sharing a common receptor to B19 (Summerford & Samulski, 1998). Other receptors for AAV-2 include human fibroblast growth factor receptor 1 and αVβ5 integrin (Qing *et al.*, 1999; Summerford *et al.*, 1999). After receptor binding, conformational changes provide the necessary means for viral entry into the dividing host cell.

Viral transmission and clinical presentation

Parvovirus species are known to infect dividing host cells in the S phase of cell division. This is common among parvoviruses despite the broad tissue tropism shown by certain species (Berns & Parrish, 2013). While dividing cells are a requirement for parvovirus replication the virus utilizes host replication machinery, parvoviruses have been identified in host lymphoid tissues, heart, respiratory tract, oropharynx, and intestine (Christensen *et al.*, 2013; Deng *et al.*, 2012; Mori *et al.*, 1991; Smith *et al.*, 1993; Streck *et al.*, 2013). As parvoviruses are capable of infecting many host tissues, viral shedding can occur through a variety of methods. Transmission may occur by contact with infected animals, urine, and feces (Compton *et al.*, 2012; Ueno *et al.*, 1997; Yang *et al.*, 1995). Moreover, the environmental stability of parvoviruses contributes to host infection despite time, inactivation treatments and sanitization procedures. Porcine

parvovirus has been shown to be infective after heat treatments as high as 60°C, possibly contributing to the widespread infection seen with these viral species (Blümel *et al.*, 2002).

After infection, parvovirus associated disease manifestation is variable based on factors included host species, viral species, host age and clinical status (Hueffer & Parrish, 2003). In humans, parvovirus B19 infection is characterized by erythema in children, fetal hydrops and arthralgia coupled with polyarthritis (Macé *et al.*, 2014; Thammasri *et al.*, 2013). Feline panleukopenia virus, mink enteritis virus and CPV all cause enteritis, myocarditis and ataxia (Nandi & Kumar, 2010; Stuetzer & Hartmann, 2014; Wang *et al.*, 2013). While goose parvovirus and duck parvovirus also cause myocarditis, an additional host symptom for these viral species includes hepatitis (Glávits *et al.*, 2005). Infection of pregnant sows with porcine parvoviruses has been shown to cause fetal death, abortion and infertility although clinical signs are dependent on the time of infection (Van-Leengoed *et al.*, 1983). Interestingly, Aleutian mink disease displays clinical symptoms inconsistent with other parvoviruses as Aleutian mink disease virus (AMDV) infection causes immune complex-mediated disease in which phagocytosed immune complexes comprising viral antigen bound by host antibodies are not degraded causing lesions in the host (Best & Bloom, 2005). Taken together, parvovirus species are capable of causing a broad range of clinical signs, in some cases, causing severe disease.

Porcine parvoviruses

Currently, six porcine parvoviruses have been classified within the family *Parvovirinae*, although for a majority of these viruses, an association with disease in swine has yet to be resolved. Porcine parvovirus 1 (PPV1), however, is a major etiologic agent in reproductive failure of swine farms and was first discovered in the 1960s in sows in Germany (Mayr *et al.*, 1968; Ren *et al.*, 2013). Decades later, in 2001, a second porcine parvovirus was identified,

porcine parvovirus 2 (PPV2) from swine sera in Myanmar (Hijikata *et al.*, 2001). In 2008, the exponential growth of porcine parvoviruses started with the discovery of porcine parvovirus 3 (PPV3) in various swine samples including lymph nodes, nasal swabs, fecal swabs and serum samples (Lau *et al.*, 2008). Since then, porcine parvovirus 4 (PPV4) was identified in the lung lavage of a deceased pig from the US, porcine parvovirus 5 (PPV5) in swine tissue samples from the US and porcine parvovirus 6 (PPV6) in aborted pig fetuses from China (Cheung *et al.*, 2010; Ni *et al.*, 2014; Xiao *et al.*, 2013). Little is known regarding the pathogenicity of PPV2-PPV6, however, the prevalence of these potential pathogens in the US swine herd merits further research.

Recent parvovirus discovery

Developing viral discovery techniques have allowed for the detection of a number of novel parvovirus species in bats, turkeys, chicken, pigs and sea lions. In 2013, a partial sequence of a novel parvovirus designated *Eidolum helvum* parvovirus 2 was identified in throat and urine swabs of bats and remains unclassified within a *Parvovirinae* genera due to the low sequence identity to known parvoviruses (Baker *et al.*, 2013). Similarly in 2013, metagenomics sequencing of turkey feces identified a virus now known as Turkey parvovirus TP1-2012/HUN, also divergent from known parvovirus sequences (Reuter *et al.*, 2014). The same study identified a divergent parvovirus in chickens. A fecal swab obtained in 2014 from a seal lion pup afflicted with pneumonia and suffering from malnutrition contained a parvovirus genome known as sesavirus that is the sole member of the putative *Marinoparvovirus* genera (Phan *et al.*, 2014). More recently, in 2015, PPV6 was identified in porcine reproductive and respiratory virus positive serum samples taken from pigs in North America (Schirtzinger *et al.*, 2015).

Considering the diversity of parvoviruses and severity of disease produced by infection, rapid identification of parvoviruses is crucial to maintain the health of US livestock species.

Paramyxoviruses

The Paramyxoviridae family

In addition to parvoviruses, paramyxoviruses can also be found in swine afflicted with disease. The family *Paramxyoviridae* is a diverse group of single stranded RNA (ssRNA) viruses known to infect animals including humans, cattle, pigs and poultry. Members of *Paramyxoviridae* are divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, of which *Paramyxovirinae* has seven genera: *Aquaparamyxovirus, Avulavirus, Ferlavirus, Henipavirus, Morbillivirus, Respirovirus* and *Rubulavirus.* The *Pneumovirinae* subfamily is divided into two genera, *Orthopneumovirus* and *Metapneumovirus*. *Paramyxovirinae* genera capable of infecting mammals include *Henipavirus, Morbillivirus, Respirovirus* and *Rubulavirus* (King *et al.*, 2012b; Lamb & Parks, 2013) . Infamous and representative viral species in these *Paramyxovirinae* genera are Hendra virus (HeV), Measles virus, Sendai virus and Mumps virus, respectively.

Genome structure and Organization

Viruses classified in the *Paramyxoviridae* family have genomes of approximately 15-19kb in length, commonly encoding six genes flanked by 3' and 5' extracistronic regions (up to ten genes can be encoded by pneumoviruses) (King *et al.*, 2012b). Approximate sizes of the various proteins encoded by the six genes are displayed in figure 1.4b. Intergenic regions are three nucleotides for *Respiroviruses* and *Morbilliviruses* while *Rubulaviruses* and *Pneumoviruses* are more variable (1-47nt and 1-56nt, respectively) (Lamb & Parks, 2013). A

diagram of the genome and approximate sizes of the proteins of a representative *Respirovirus* is shown in figure 1.4a. The pleomorphic virion is approximately 150 to 350nm protecting a ribonucleoprotein (RNP) between the matrix protein layer and lipid envelope (Figure 1.4c). Nucleocapsid (N) protein monomers protect the RNA from degradation by associating with a six nucleotide sequence in the paramyxovirus genome (King *et al.*, 2012b; Lamb & Parks, 2013). A combination of phosphoprotein (P) and large polymerase (L) attached to nucleoprotein (N) associating with genome RNA to form the RNP that is necessary for replication. Viral attachment to the host cell is carried out by hemagglutinin-neuraminidase (HN) proteins and the fusion (F) proteins that reside on the surface of the virions. In addition to the previously mentioned proteins, paramyxoviruses may encode up to seven polypeptides (Sendai virus) within the *P* gene using several species dependent mechanisms. The major proteins encoded by the *P* gene are P, V, and C although virus-specific proteins W, I and D have been identified (Lamb & Parks, 2013). Only HN and F proteins are shown to elicit neutralizing antibodies in humans and animals although antibodies against most other proteins are present in post-infection sera as well (Ambrose *et al.*, 1991; Porotto *et al.*, 2003; Ray *et al.*, 1988; Spriggs *et al.*, 1987). Immunization of cotton rats, guinea pigs and hamsters with purified HN and F proteins induces protective immunity *in vivo* (Ambrose *et al.*, 1991; Ray *et al.*, 1988).

Host Receptors necessary for viral infection

The HN and F paramyxovirus proteins function to attach the virus to multiple host cell receptors. Previous studies have identified molecules containing sialic acid serve as the cell surface receptors for human parainfluenza viruses among other paramyxovirus species (Alymova *et al.*, 2012). Gangliosides may function in attachment and receptor binding for Sendai virus (Markwell *et al.*, 1981). Sialic acid expression on cell surfaces is seen on many cell types in

vetebrates, possibly contributing to the broad host ranges of paramyxoviruses. Additionally, MeV H has been shown to bind to CD46, CD150/SLAM or Nectin-4 in a viral strain and tissue dependent manner (Noyce & Richardson, 2012; Seki *et al.*, 2016). These host receptors are important as they contribute to the broad host ranges and cellular tropisms of paramyxoviruses. The signaling lymphocyte activation molecule (CD150/SLAM) is present on lymphocytes, monocytes and dendritic cells where as Nectin-4 is associated with epithelial cells. Using both CD150/SLAM and Nectin-4 as cellular receptors, paramyxoviruses can infect a broad range of cells, perhaps causing disease. Nipah virus (NiV) and HeV G proteins may bind to Ephrin B2 and Ephrin B3 as well (Bose *et al.*, 2015; Xu *et al.*, 2012). Importantly, Ephrin B2 is expressed on the surfaces of endothelial cells and neurons while Ephrin B3 expression occurs on lymphoid cells, both contributing to the severity of disease associated with NiV/HeV infection.

Clinical signs associated with paramyxovirus infection

A range of clinical symptoms are associated with paramyxovirus infection although signs are species dependent and vary with the severity of infection. In general, paramyxoviruses are spread through direct contact with an infected animal as the viruses are not environmental stable (Lamb & Parks, 2013). In pigs, respiratory disease is common or encephalitis to a lesser extent (Rivera-Benitez *et al.*, 2013). Mild diarrhea combined with mild respiratory infection was displayed in pigs infected with swine parainfluenza virus-3 (SPIV3) (Qiao *et al.*, 2010). The outbreak of La Piedad-Michoacan-Mexico virus (LPMV), a variant of porcine rubulavirus was accompanied by more severe clinical signs including interstitial pneumonia and encephalitis although the disease was most severe in pigs less than two weeks old (Allan *et al.*, 1996). In humans, respiratory disease is also characteristic of parainfluenza virus infection (Henrickson, 2003). Menangle virus infection in workers in a piggery in Australia was characterized by

influenza-like illness and rashes (Chant *et al.*, 1998). The single individual that died in the original outbreak of HeV displayed interstitial pneumonia upon autopsy (Murray *et al.*, 1995; Selvey *et al.*, 1995). The infected horses in contact with the individual had similar interstitial pneumonia upon necropsy. Interestingly, years later in Brisbane, Australia, a HeV outbreak occurred in horses displaying predominately neurological disease, later reflected in humans infected in the same outbreak (Field *et al.*, 2010).

Cross species infection among paramyxoviruses

The diversity of host cellular receptors utilized by paramyxoviruses may contribute to their ability to cross species barriers to infect economically important livestock such as swine. Viral species classified with the *Avulavirus, Henipavirus, Respirovirus* and *Rubulavirus* genera are capable of infecting swine although swine only serve as the primary reservoirs for *Respirovirus* (porcine parainfluenza virus 1) and *Rubulavirus* (porcine rubulavirus) species (Allan *et al.*, 1996; Lau *et al.*, 2013). Paramyxoviruses classified within the genera listed have been implicated to cross species barriers to infect swine, including, *Avulavirus* (Newcastle Disease Virus), *Henipavirus* (NeV), *Respirovirus* (bovine parainfluenza virus 3), and *Rubulavirus* (Menangle virus, Tioman Virus) (Chua *et al.*, 1999; Philbey *et al.*, 1998; Qiao *et al.*, 2010; Yaiw *et al.*, 2008; Yuan *et al.*, 2012). The discovery of novel paramyxovirus species such as porcine parainfluenza 1 (PPIV1) in pigs in the United States is concerning as these viruses have the potential to overcome species barriers.

Transmission of paramyxoviruses to pigs

Paramyxoviruses are present in multiple species throughout the world, leading to the discovery of a number of novel species, particularly in pigs. In Mexico in 1980, LPMV, a novel *Rubulavirus*, was reported to cause respiratory disease in piglets (Moreno-López *et al.*, 1986).

Meanwhile, in the United States, SPIV3, a variant of BPIV3 was isolated from pigs displaying mild respiratory infection (Qiao *et al.*, 2010). In the eastern hemisphere, Menangle virus was identified in pigs and humans in Australian piggeries in 1997 on a farm displaying an outbreak of reproductive disease (Philbey *et al.*, 1998). More recently, an outbreak of a variant of parainflunza virus 5, designated porcine parainfluenza virus 5, occurred in 2011 in South Korean piglets displaying respiratory illness (Lee *et al.*, 2013). A year earlier, in 2010, Newcastle disease virus was isolated from a pig located in the Henan Province, China (Yuan *et al.*, 2012). Novel paramyxovirus viral species are identified regularly; most recently, NeV and HeV have been popularized due to their ability to infect humans and swine or horses, respectively. Some of the most notorious respiratory diseases to humans and animals are classified within the *Paramyxoviridae* family, as paramyxoviruses readily cross species boundaries causing disease around the globe, rapid identification and characterization is essential.

Novel paramyxovirus discovery

Identification of new paramyxovirus sequences has been occurring at a growing rate. In 2014, Belinga bat virus was discovered in the tissues of African sheath-tailed bats with hemorrhagic lesions (Maganga *et al.*, 2014). Furthermore, a henipa-like virus was classified in rats in 2014 and named Mojiang paramyxovirus, expanding the spectrum of species capable of infection with henipaviruses (Wu *et al.*, 2014). Last year, new paramyxovirus sequences were discovered in feline, anaconda and bat species further confirming the expansion in diversity of paramyxoviruses in recent times (Amman *et al.*, 2015; Hyndman *et al.*, 2012; Sieg *et al.*, 2015). Further, a biologist was infected with an unknown agent causing manculopapular rash and oropharynx ulcerations that was later attributed to a novel unclassified paramyxovirus, Sosuga virus (Albariño *et al.*, 2014). As the diversity of paramyxovirus species expands, research should

focus on characterizing and identifying these species rapidly, in particular, those associated with economically important animals such as livestock.

Circoviruses

The Circoviridae family

Containing one of the most significant viruses in the US swine industry, viruses classified in the family *Circoviridae* are the smallest known circular viruses that infect avian species, bats, dogs and pigs. The two genera, *Circovirus* and *Cyclovirus*, are differentiated based on genome sense as circoviruses are ambisense and cycloviruses are negative sense (Meng, 2013a). There are currently 22 viral species associated with *circovirus* including beak and feather disease virus (BFDV), canine circovirus, human feces associated circovirus, porcine circovirus 1 (PCV1), and porcine circovirus 2 (PCV2). Twenty-nine known viral species have been formally classified in the *Cyclovirus* genera, including a diverse array of mammalian viruses, bovine cyclovirus, chimpanzee feces associated cyclovirus, goat cyclovirus, human cyclovirus (types 1-3) and human feces associated cyclovirus (types 1-8) (King *et al.*, 2012c; Meng, 2013a). Recently, the International Committee for Viral Taxonomy has reorganized the *Circoviridae* hierarchy, replacing the *Gyrovirus* genera with *Cyclovirus* as the sole *Gyrovirus* species is chicken anemia virus, now classified within the family *Anelloviridae* (Meng, 2013a).

Genome structure and organization

The circular DNA genome of circoviruses is approximately 1.7-2kb in size, encoding two major ORFs, a replicase protein and a capsid protein, oriented on opposite strands of the genome (King *et al.*, 2012c; Meng, 2013a)(Figure 1.5a). The 5' intergenic region contains the origin of replication (*ori*) characterized by a stem loop structure that possesses a relatively conserved

nonanucleotide sequence in the loop region. The conserved *ori* is important to initiate rolling-circle replication utilized by circovirus species. During transcription, the *rep* gene is capable of producing multiple transcripts using alternate splicing and alternative promoters. For instance, the PCV2 *rep* gene creates nine RNA transcripts; Rep, Rep', Rep 3a, Rep 3b, Rep3c, NS515, NS627, and NS0. Of these PCV2 *rep* transcripts, only Rep and Rep' are essential for viral replication (Cheung, 2003, 2012). It is unknown if other PCV2 RNA transcripts produce polypeptides or what role they play in PCV2 replication. After translation, the circovirus capsid proteins can produce virus like particles (VLPs), lacking genome DNA, via self-assembly and are the target of host neutralizing antibodies (Bucarey *et al.*, 2009; Yin *et al.*, 2010).

The non-enveloped, icosahedral circovirus virion is between 15 and 20nm in diameter encompassing the circular DNA genome, of which the outermost protein, the capsid, aids in viral attachment. Entry into host cells is mediated by recognition of the circovirus capsid proteins by host receptors including heparan sulfate, chondroitin sulfate B and glycosaminoglycans (GAG) heparin (Misinzo *et al.*, 2006). Host cells targeted by PCV2 include dendritic cells; specifically blood DCs and plasmacytoid DCs, macrophages and epithelial cells to a lesser extent (Meng, 2013b). In pigs, PCV2 can be detected in many tissues throughout the body including the lymphoid system (tonsil, spleen, peyer's patches, and lymph nodes), the respiratory system, the nervous system (brain), the digestive system and reproductive system (ovaries) (Hansen *et al.*, 2013; Harms *et al.*, 2002; Madson & Opriessnig, 2011; Phaneuf *et al.*, 2007). Furthermore, the systemic nature of the virus provides broad clinical signs for the manifestation of disease.

Clinical signs associated with circovirus infection

Minimal research has been done regarding the pathogenicity of cycloviruses, however, a few circovirus species have been shown to cause devastating diseases in their hosts. Although

cycloviruses have been detected in foxes with meningoencephalitis and the cerebrospinal fluid of patients with neurological disorders, a clear link to disease was not found (Bexton *et al.*, 2015). Cycloviruses have also been detected in human stool samples from healthy individuals and US commercial meat products, again provided no link to disease (Macera *et al.*, 2016). Conversely, circoviruses have been linked to vasculitis and hemorrhage in dogs and are known to cause one of the most economically significant clinical diseases in pigs, designated porcine circovirus associated disease (PCVAD) (Li *et al.*, 2013; Segalés, 2012). Economic devastation is associated with the wasting animals and reproductive failure in livestock. Even more concerning is the ubiquitous nature of PCV2 is swineherds throughout the world. PCV2 has been detected in the Europe, China, Asia and North America (Beach & Meng, 2012; Dupont *et al.*, 2008; Wang *et al.*, 2009). As the diversity of circovirus species continues to expand, prompt viral characterization is crucial.

Cross species transmission among circoviruses

Although infection status is unknown, circoviruses have been detected in multiple species beyond the host organism. Recently, circoviruses most similar to avian circoviruses and porcine circoviruses were discovered in human stool samples from Nigeria, Pakistan, Tunisia and the United States (Li *et al.*, 2010). Porcine circoviruses were found in rats cohabitating with pigs on farms in Brazil, Hungary, South Korea and China (Lőrincz *et al.*, 2010; Pinheiro *et al.*, 2013; TRUONG *et al.*, 2013; Zhai *et al.*, 2016). A study investigating PCV2 presence in bovid serum found PCV2 DNA in buffalo serum samples in China (Zhai *et al.*, 2014). Interestingly, in 2016 PCV2 was identified in the tissues of mink that died of diarrhea (Wang *et al.*, 2016a). This study suggests that PCV2 may have overcome species barriers, causing disease. Coupled with the

recent discovery of a vast array of novel circovirus sequences, the identification of circoviruses in multiple mammalian species is concerning.

Recent circovirus identification

In past years, with the development of techniques for viral discovery, an assortment of novel members of *Circoviridae* have been identified. In 2016, a circovirus grouping most similarly to members of the *Circovirus* genera was discovered in the feces of kiwi in New Zealand (White *et al.*, 2016). Interestingly, the badger circovirus-like virus was the closest relative to the kiwi circovirus and cross-species viral transmission was suggested. Two divergent circovirus genomes and two cyclovirus genomes were identified in bat fecal samples taken in Brazil (Lima *et al.*, 2015). One of these genomes clustered most closely to dragonfly circovirus. As novel circovirus species are continually identified research should focus on characterization of these species as many *Circoviridae* members are linked to severe disease and are capable of overcoming species barriers.

This collection of studies addresses emerging swine viruses identified in United States pigs with the use of metagenomic sequencing. The first study identifies a novel parvovirus in the rectal swabs of market pigs with no defined disease symptoms. Although no *in vivo* work was performed with porcine parvovirus 7 (PPV7), porcine parvoviruses are associated with disease. The second study draws a link between PPIV1 and mild respiratory disease in pigs from the United States. Finally, a divergent circovirus, porcine circovirus 3 (PCV3), is associated with PDNS, a severe dermatitis and nephropathy disease in pigs with unknown causative agents. The results of these studies identify novel viruses in US swine and may explain the presence of disease despite vaccination for closely related viral pathogens.

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Figure 1.1 Schematic of the sequence independent single primer amplification (SISPA) technique.

The workflow starts with total nucleic acid. Sequence primers are shown to the right of the appropriate step. The random hexamer on the end of the first strand primers are displayed in blue. Transcribed sequences are represented in red. Reverse transcriptase (RT) ia labeled in the first step.

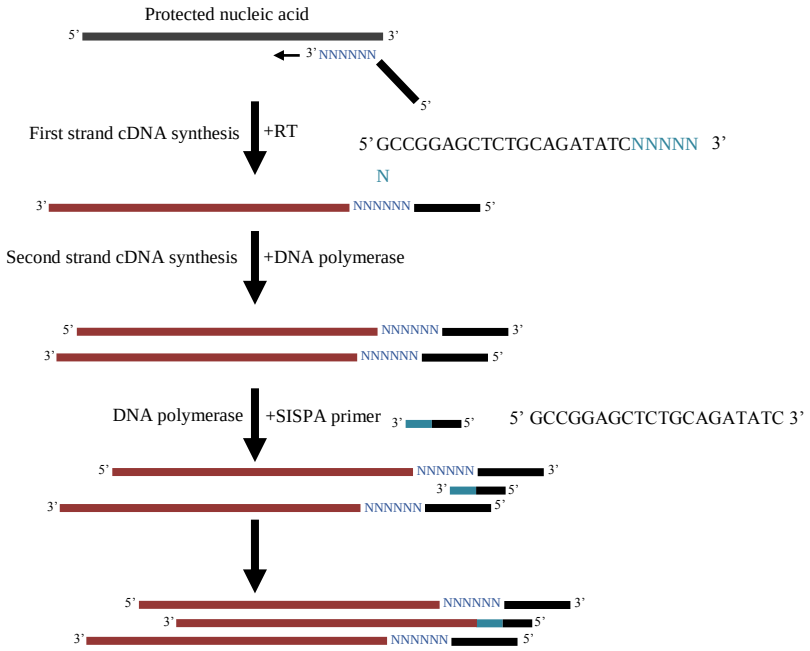


Figure 1.2 Metagenomic sequencing workflow with sequence independent single primer amplification (SISPA).

An example outline of a SISPA metagenomic sequence workflow starting with field samples. Red lines represent host nucleic acid and blue lines represent the library barcodes.

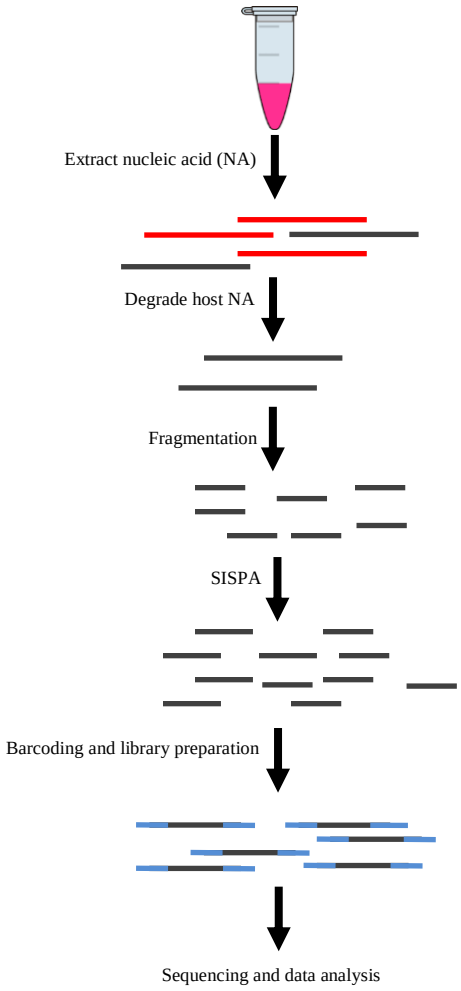


Figure 1.3 Diagram of the genome organization and the virion structure of *Parvovirdae*.

The parvovirus genome structure is composed of two open reading frames flanked by 3' and 5' inverted terminal repeats (A). The genes of members of *Parvoviridae* are encoded in a conserved order, 3' replicase and 5' capsid. The parvovirus capsid proteins form a virion housing the genomic DNA necessary for viral replication (B).

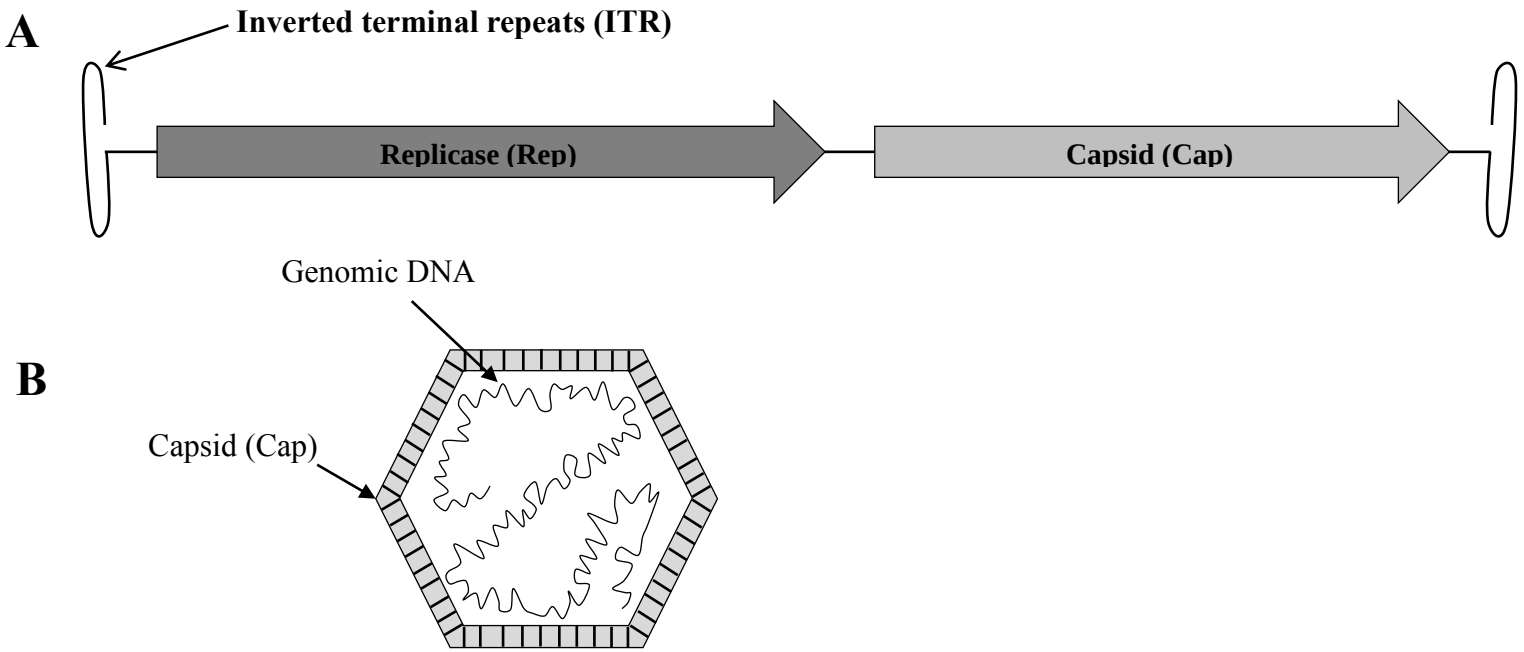
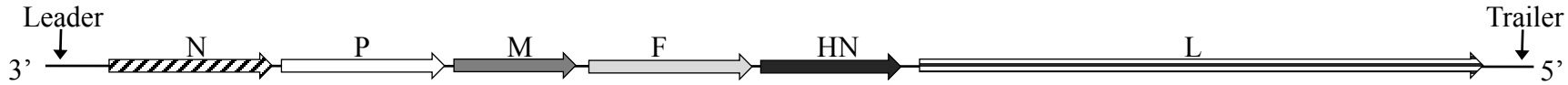


Figure 1.4 Genome structure and organization members of *Paramyxoviridae*.

The general gene organization of paramyxoviruses (A) containing a 3' leader and 5' trailer. Paramyxovirus genes are encoded in a conserved order; nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large polymerase (L). Approximate protein size encoded by each gene is given as amino acid (aa) values (B). The structural organization diagram (C) displays the viral ribonucleoprotein (RNP) which is formed from the nucleocapsid, phosphoprotein and large polymerase.

A



B

Protein	Approximate Size (aa)
N	489-553
P	400-600
M	341-375
F	540-580
HN	565-582
L	2200

C

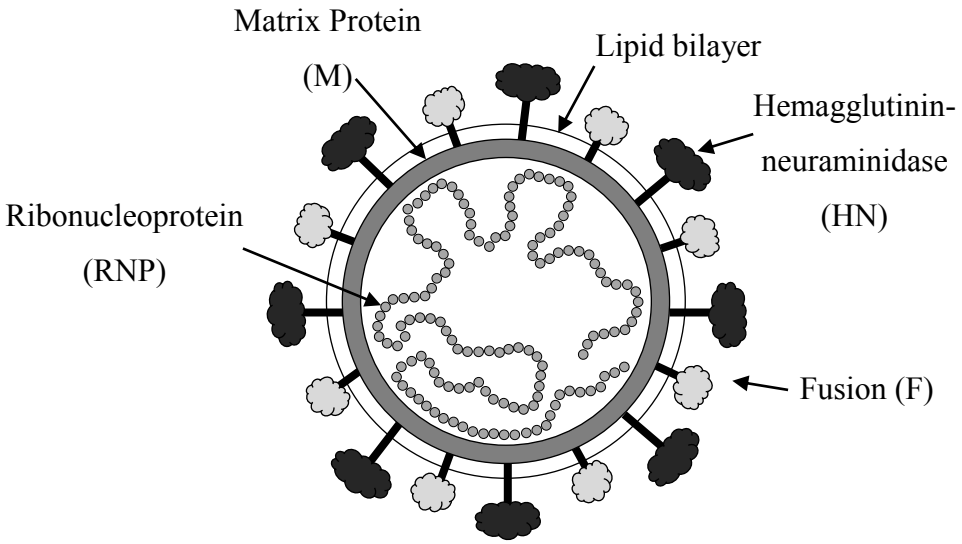
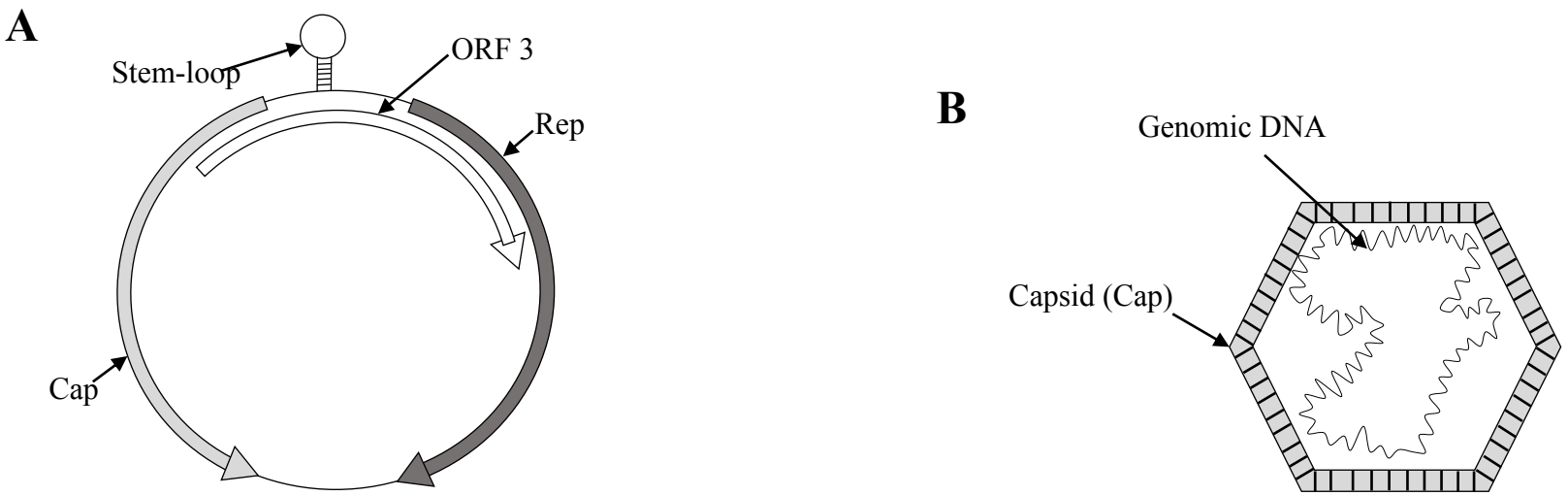

Matrix Protein
(M)
Lipid bilayer
Hemagglutinin-
neuraminidase
(HN)
Ribonucleoprotein
(RNP)
Fusion (F)

Figure 1.5 General genome and virion structure of *Circoviridae* viral species.

The circovirus structure is composed of two major open reading frames (ORF) in an ambisense orientation, the replicase (Rep) and capsid (Cap) (A) A third ORF has been identified in a number of species as well and lies between the Rep and the Cap ORFs (ORF 3). At the 5' end of the genome, within the ORF 3 coding region lies a characteristic stem-loop structure necessary for replication. The circovirus capsid proteins house the circovirus genomic DNA, together making up the virion (B).



Chapter 2 - Discovery of a novel *Parvovirinae* virus, porcine parvovirus 7, by metagenomic sequencing of porcine rectal swabs

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Abstract

Parvoviruses are a diverse group of viruses containing some of the smallest known species that are capable of infecting a wide range of animals. Metagenomic sequencing of pooled rectal swabs from adult pigs identified a 4,103-bp contig consisting of two major open reading frames (ORFs) encoding proteins 672 and 469 amino acids (aa) in length. BLASTP analysis of the 672-aa protein found 42.4% identity to fruit bat (*Eidolon helvum*) parvovirus 2 (EhPV2) and 37.9% to turkey parvovirus (TuPV) TP1-2012/HUN NS1 proteins. Additionally, conserved sequence motifs within the ORF1 of parvoviruses were identified in the novel viral sequence including three motifs used for rolling circle replication (RCR), two for NTP-binding and one for interaction with a helicase. The 469 aa protein had no significant similarity to known proteins. Taken together, the novel viral species was provisionally designated porcine parvovirus 7 (PPV7). Genetic and phylogenetic analyses suggest that PPV7, EhPV2, and TuPV represent a novel genus in the family *Parvoviridae*. Quantitative PCR (qPCR) screening of 182 porcine

diagnostic samples found a total of 16 positives (8.6%). Together, these data suggest that PPV7 is a highly divergent novel parvovirus prevalent within US swine.

Introduction

Parvoviruses are among the smallest viruses capable of infecting many animal species. These non-enveloped, icosahedral viruses are classified in the family *Parvoviridae* which is further divided into the subfamilies *Parvovirinae* and *Densovirinae* (Cotmore *et al.*, 2014; King *et al.*, 2012). Members of the *Parvovirinae* subfamily infect vertebrates while members of the *Densovirinae* subfamily infect invertebrates. Recent publications have proposed that *Parvovirinae* is comprised of nine genera: *Dependoparvovirus, Copiparvovirus, Bocaparvovirus, Amdoparvovirus, Aveparvovirus, Protoparvovirus, Tetraparvovirus, Erythroparvovirus,* and *Marinoparvovirus* (Cotmore *et al.*, 2014; Phan *et al.*, 2014). Additionally, there are a number of unassigned members in the *Parvovirinae* subfamily.

The parvovirus genome is composed of a linear single-stranded DNA molecule of between 4 and 6.3 kb in length typically containing two gene cassettes (King *et al.*, 2012). The left open reading frame (ORF) encodes the non-structural protein(s) (NS) which has replicase (REP) activity while the right-hand ORF encodes the structural or capsid protein (s) (CP). Terminal palindromic sequences form characteristic stem-loop and hairpin structures vital for DNA replication. The virion protein(s) range in size from 45 to 96 kDa and may contain a phospholipase A_2 (PLA_2) enzymatic core domain involved in intracellular trafficking or escape from endosomes (Cotmore *et al.*, 2014). Members of some genera as well as numerous unassigned vertebrate parvoviruses such as turkey parvovirus 260 lack the PLA_2 domain (Cotmore *et al.*, 2014; King *et al.*, 2012).

Eight species classified in the *Parvovirinae* family were identified in swine including ungulate protoparvovirus 1, ungulate tetraparvovirus 2, ungulate tetraparvovirus 3, ungulate copiparvovirus 2, ungulate bocaparvovirus 2, ungulate bocaparvovirus 3, ungulate bocaparvovirus 4, ungulate bocaparvovirus 5 (Cotmore *et al.*, 2014; Schirtzinger *et al.*, 2015). Within the US, the species prevalence of *Parvovirinae* infecting swine are variable based on tissue, animal age, farm, clinical status, and time of sample collection (Table 2.1). Furthermore, co-infections of two or more *Parvovirinae* members in a single swine farm are seen with some farms testing PCR positive to PPV1, PPV2, PPV3, PPV4 and PPV5 (Opriessnig *et al.*, 2014).

Although co-infections between parvoviruses are widespread, co-infections amongst porcine parvoviruses and porcine circovirus 2 (PCV2) are typical and can be economically significant. Porcine parvoviruses (PPV) alone are known to cause reproductive failure manifested by embryonic and fetal death in swine, impacting the swine industry worldwide (Hueffer & Parrish, 2003; Van-Leengoed *et al.*, 1983). A co-infection of a porcine parvovirus and PCV2 has been known to contribute to the appearance of a set of diseases and clinical signs, termed porcine circovirus associated disease (PCVAD), which economically impacts the swine industry. PCVAD comprises post-weaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), pneumonia, and reproductive failure (Harding, 2007; Harms *et al.*, 2002; Kim *et al.*, 2003; Madson & Opriessnig, 2011; Rosell *et al.*, 1999). Moreover, the potential economic impact and the identification of novel porcine parvoviruses recently warrant further investigation.

Application of new molecular techniques has accelerated viral discovery, with the identification of several new species of porcine parvoviruses including porcine parvovirus 4, 5 and 6 (PPV4-6) in recent years. These viruses were identified in a wide variety of tissues such as

lung lavages (PPV4), lung tissues (PPV5) and aborted fetuses (PPV6) (Cheung *et al.*, 2010; Ni *et al.*, 2014; Xiao *et al.*, 2013a). The NS of PPV4-6 are 28.1-49.8% identical to members of *Copiparvovirus* and have less than 30% identity to other genera, leading to their inclusion in the genus *Copiparvovirus* (Cheung *et al.*, 2010; Ni *et al.*, 2014; Schirtzinger *et al.*, 2015; Xiao *et al.*, 2013a). Metagenomic sequencing and particularly the development of sequence independent single primer amplification (SISPA) techniques has led to the rapid identification of novel viruses in clinical samples (Allander *et al.*, 2001). Both bovine parvovirus and bungowannah virus have been discovered using SISPA (Allander *et al.*, 2001; Kirkland *et al.*, 2007). Here, a sequence from a novel porcine parvovirus was obtained from the rectal swabs of adult pigs.

Materials and Methods

Ethics Statement

Porcine rectal swabs, lung lavages, nasal swabs and sera were obtained by veterinarians as part of routine diagnostic investigations and were submitted to Kansas State Veterinary Diagnostic Laboratory (KSVDL) or Iowa State University Veterinary Diagnostic Laboratory (ISUVDL).

Collection of Samples

A pool was assembled containing rectal swabs from five healthy adult pigs and subsequently used for metagenomic sequencing. A quantitative PCR (qPCR) assay was used to screen for the presence of porcine parvovirus 7 (PPV7) in 95 serum, 29 rectal swabs, 29 lung lavages and 29 nasal swabs taken from swine and submitted to ISUVDL for diagnostic purposes. The geographic origin and infection status of the qPCR samples is unknown.

Metagenomic Sequencing

Methods previously outlined were used for metagenomic sequencing on a pool of five porcine rectal swabs (Hause *et al.*, 2015; Neill *et al.*, 2014). The sample pool was enriched for viral nucleic acid with the addition of nucleases and an incubation of 37°C for 90 minutes. The remaining viral nucleic acids were extracted using the MinElute Viral Spin filter kit (Qiagen, Valencia, CA) as instructed by the manufacturer. Previously described primers were used to synthesize the first-strand cDNA from viral DNA with a Superscript III first-strand synthesis kit via the manufacturers instructions (Allander *et al.*, 2005). Sequenase 2.0 DNA polymerase was used to synthesize the second strand followed by cDNA purification using a selection size of >300 base pairs (bp) with Agencourt AMPure XP beads. Next, the double-stranded cDNA was amplified with TaKaRa DNA polymerase with primer sequences similar to first-strand synthesis but lacking the random hexamer. A similar size selection protocol of >300bp was used for a second round of purification, again with Agencourt AMPure XP beads. Amplicons were quantified using a Qubit fluorimeter and the standard protocol was used for library preparation with the Nextera XT library preparation kit. Libraries were pooled and sequenced on a Illumina Miseq instrument by paired 150bp reads.

Data Analysis

Barcodes added during library preparation were used to differentiate the reads based on sample and imported into CLC Genomics Workbench (CLC Bio version 7.0). Reads mapping to the host genome (*Sus scrofa*) were removed and contigs were assembled *de novo* from the unmapped reads then analyzed with BLASTN. ClustalW was used to align sequences that were subsequently used to construct phylogenetic trees with MEGA 6.06 software using the best fit

model LG+ F with gamma distribution and invariant sites and maximum-likelihood methodology verified by 1000 bootstrap replicates (Tamura *et al.*, 2013).

qPCR determination of PPV7 prevalence and overlapping PCR to obtain a near complete PPV7 genome

Viral nucleic acid was extracted according to the manufacturer with a MagMax-96 total nucleic acid isolation kit. The prevalence of PPV7 in various porcine samples was determined with a 5' nuclease assay targeting a 112bp region of the NS1 gene: probe, 5'-FAM- CAG GCA GTG GTA GTG AAG GAT CCC- Iowa Black-3'; Forward, 5'- AGC AGA GAC AAA CAC AGA CG-3'; Reverse, 5'- CCA GTT TGC ATT GTT CCC ATC-3'. The assay was performed with the Qiagen Quantitech PCR kit using the following conditions: 95°C, 15 minutes; 45 cycles of 94°C, 15 seconds and 60°C for 60 seconds. The primer sequences used for qPCR analysis are specific to PPV7 NS1 and contain at least ten nucleotide differences with even the closest *Parvovirinae* species. To verify and complete the PPV7 contig obtained by metagenomic sequencing, eight sets of primers creating approximately 800bp fragments overlapping by 100bp were utilized to span the putative complete genome sequence as seen in Table 1.2. The PCR was performed using TaKaRa DNA polymerase with the PPV7 primers as follows: 94°C, 2 minutes; followed by 40 cycles of 94°C, 20 seconds; 50-55°C, 30 seconds; 72°C, 1 minute; with a final extension of 72°C, 10 minutes. Sanger sequencing was performed on PCR products using forward and reverse primers used for PCR. Rapid amplification of cDNA ends (RACE) was attempted to complete both the 5' and 3' ends of the PPV7 genome, however, a viable product and sequence was never obtained.

GenBank Accession Numbers

The near complete sequence of PPV7 used in the phylogenetic analysis has been submitted to GenBank under accession KU563733. Previously published sequences used for phylogenetic analysis include: Adeno-associated virus-1 (AAV1) NC002077; Aleutian mink disease virus (AMDV) NC001662; Avian adeno-associated virus strain DA-1 (AAAV1) NC006263; Bombyx mori bidensovirus 2 (BmDNV) S78547; Bovine adeno-associated virus (BAAV) NC005889; Bovine hokovirus strain HK1 (BHoV) EU200669; Bovine parvovirus 1 (BPV1) NC001540; Bovine parvovirus 2 (BPV2) NC006259; Bovine parvovirus 3 (BPV3) AF406967; Bufavirus 3 (BuPV) AB982218; Canine parvovirus (CPV) NC001539; Chicken parvovirus ABU-P1 (ChPV) GU214704; Chipmunk parvovirus (ChpPV) U86868; Eidolon helvum parvovirus 2 (EhPV2) AGL97807; Feline parvovirus (FPV) EU659111; Galleria mellonella densovirus (GmDNV) NC004286; Goose parvovirus (GPV) NC001701; Human bocavirus (HBoV) NC007455; Human parvovirus 4 G1 (PARV4) EU2007018; Human parvovirus 4 G2 (PARV5) DQ873391; Human parvovirus B19 (B19) NC000883; Mouse parvovirus 1 (MPV1) NC001630; Mouse parvovirus 3 (MPV3) NC008185; Ovine hokovius strain HK-S04 (OHoV) JF504700; Parvovirus H1 (H1) NC001358; Porcine bocavirus strain 5/JS677 (PBoV) NC016647; Porcine hokovirus strain HK1 (PHoV) EU200671; Porcine parvovirus 1(PPV1) NC001718; Porcine parvovirus 2 (PPV2) GU938299; Porcine parvovirus 4 (PPV4) NC014665; Porcine parvovirus 5 isolate MI216 (PPV5) JX896318; Porcine parvovirus 6 isolate TJ (PPV6) NC023860; Protoparvovirus HK-2014 (protoparvovirus) KM254172; Rat parvovirus 1a (RPV1a) AF036710; Sesavirus (sesavirus) NC026251; Simian parvovirus (SPV) SPU26342; Turkey parvovirus TP1-2012/HUN (TuPV) KF925531; Turkey parvovirus 260 (TuPV) GU214706.

Results

Metagenomic Sequencing

A pool of rectal swabs from five healthy pigs, submitted to KSVDL, was subjected to metagenomic sequencing. A total of 1,312,482 paired reads were generated using a MiSeq instrument using 300bp chemistry, of which 232,493 reads mapped to the *Sus scrofa* reference genome and were subtracted from analysis. *De novo* assembly of the remaining reads using the CLC Genomics software package generated 2,162 contigs that were analyzed via BLASTN. A 4,103-bp contig comprised the highest number of reads (256,676 reads) and failed to show significant similarity to known organisms by BLASTN.

ORF analysis identified two adjacent ORFs encoding predicted 672 and 469 amino acids (aa). A third ORF was identified within the left-hand ORF and encoded a predicted 249-aa protein in an alternate reading frame. BLASTP query of the 672-aa left-end ORF found significant expectation (*E*) values of $<7e^{-72}$ for a fruit bat (*Eidolon helvum*) parvovirus 2 NS1 (AGL97807), a protoparvovirus HK-2014 NS1 (AIW53333), a turkey parvovirus TP-2012/HUN NS1 (AHF54687) and a partridge parvovirus PA147/ITA/2008 NS1 (ADZ49579). The conserved domain function in BLASTP also identified a partial parvovirus NS1 domain from amino acids 302-431 ($E=2.6e^{-20}$). BLASTP analysis of the 469-aa right hand ORF yielded no significant identity to known viral sequences or conserved protein domains. Likewise, BLASTP analysis of the 249-aa ORF failed to identify significant similarity to known proteins or domains. Other viruses identified in the sample include picorbirnavirus (KJ476131.1) ($E=1e^{-62}$), parechovirus-like virus (JX491648.1) ($E=4e10^{-101}$), small single stranded DNA virus (JF713717.1) ($E=0$), porcine bocavirus 3 (KF025389.1) ($E=0$), porcine circovirus 1

(DQ648032.1) (PCV1, *E*=0), porcine parvovirus 2 (KP765690.1) (PPV2, *E*=0), porcine torovirus (KM403390.1) (*E*=0) and posavirus 1 (KR019687.1) (*E*=0).

Genome Organization of Putative novel parvovirus

The nearly complete genome of the novel porcine parvovirus, designated porcine parvovirus 7 (PPV7) strain 42, included a partial 5' UTR (538 bp), a complete NS1 gene (2019 bp), a complete putative capsid gene (1410 bp), and a partial 3'UTR (119 bp) (Figure 2.1). The coding DNA sequence was confirmed by Sanger sequencing of amplicons obtained by primer walking using 800bp fragments overlapping by ~100bps. Similar to some unclassified parvoviruses, PPV7 lacks the PLA_2 motif in the capsid protein as determined by BLASTP. Nested PCR was conducted to obtain the 5' and 3' ends of PPV7 as previously descried but yielded no products and further attempts with RACE were unsuccessful (Cheung *et al.*, 2010).

Despite the low overall homology between PPV7 and other parvoviruses, conserved sequence motifs important for parvovirus function are present. Replication initiation motifs (I, II, III), NTP-binding motifs (A and B) and the helicase domains (H) seen in *Parvovirinae* are also found in PPV7 (Figure 2.2) (Ilyina & Koonin, 1992; Xiao *et al.*, 2013b). The replicase of ssDNA viruses that utilize rolling circle replication (RCR) are highly conserved and display three characteristic motifs (Ilyina & Koonin, 1992). The putative replicase encoding ORF1 in PPV7 possesses motifs needed for RCR including futltxxx (Motif I), xpHuHuuux, and uxxYuxkxxx where lowercase letters identify variability, x represents no consensus and u can be an I, L, V, M, F, Y, or W. In PPV7, RCR motif I corresponds to rep amino acids (aa) 76-84, motif II to aa 100-106, and motif III to aa 154-163. In addition to the RCR motifs, two NTP-binding (A and B) and one helicase domain (H) is necessary for parvovirus replication these motifs are tPuuuttnu, GxxxxGK(T/S), and (D/E)(D/E), respectively (Momoeda *et al.*, 1994). All three motifs are

present in the PPV7 putative replicase protein sequence between aa 319-326 (A), 361-366 (B) and 397-405 (H).

Phylogenetic Analysis

Phylogenetic analysis was performed on the NS1 proteins of 39 viruses within the *Parvoviridae* family (Figure 2.3). The analysis showed that PPV7 was most closely related to turkey parvovirus (TuPV, KF925531) and *Eidolon helvum* parvovirus 2 (EhPV2, AGL97807) and that these viruses represent a divergent lineage of *Parvoviridae*. A pairwise comparison of NS1 amino acid sequences revealed the highest identity (42.4%) to EhPV2 followed by HK-2014 (34.1%), and TuPV (37.9%) (Table 2.3). No more than 20.4% identity to PPV7 was determined for the remaining parvovirus NS1 sequences. In 2014, EhPV2 was identified in the urine and throat samples of fruit bats and was phylogenetically different from other known *Eidolon helvum* parvoviruses (Baker *et al.,* 2013). TuPV was recently recovered from a postmortem fecal sample of a 1-year old turkey with diarrhea (Reuter *et al.,* 2014). The aetiologic role of these viruses and their roles in clinical disease remain unknown (Baker *et al.*, 2013; Reuter *et al.*, 2014).

Quantitative PCR to determine PPV7 prevalence

To evaluate the prevalence of PPV7 in porcine samples submitted for diagnostic testing, 95 serum, 29 rectal swabs, 29 lung lavages or 29 nasal swabs from pigs with unknown infection status were subjected to PPV7 qPCR. The qPCR results determined two sera (2.1%), five rectal swabs (17.2%), five nasal swabs (17.2%), and four lung lavages (13.8%) to be positive for PPV7 DNA (Table 2.4). Of the positive samples, cycle threshold (C_t) values were as low as 8.8, suggesting high PPV7 titers for some samples. Overall, of the 182 samples, a total of sixteen samples were positive (8.6%).

Discussion

A novel porcine parvovirus, PPV7, was identified with metagenomic sequencing on porcine rectal swabs exhibiting the highest similarity to *Eidolon helvum* parvovirus 2 (AGL97807) and turkey parvovirus HK-2014 (AIF54687) both of which are unclassified members of *Parvovirinae.* BLASTP analysis of the remaining ORFs resulted in no similarity to known sequences. Based on the strong similarity of the left-hand ORF to parvovirus NS and genomic architecture, it is likely the right-hand ORF encodes a highly divergent CP. It is less clear if the remaining ORF (586-1335, 750 bp) encodes a parvoviral protein; however, for some parvoviruses, minor ORFs within REP have been identified (King *et al.*, 2012). Further analysis of the putative NS1 ORF of PPV7 identified conserved parvovirus sequence motifs possibly necessary for viral replication. Pairwise comparison of the nucleotide coding domains for *Parvovirinae* members showed PPV7 has <43% identity to current *Parvovirinae* species. Moreover, in a comparison of *Parvovirinae* protein sequences, PPV7 CP shares <17.5% to parvoviral species.

Interestingly, the proposed capsid gene of PPV7 is 1410bp while the gene for other members of *Parvoviridae* typically contain an approximately 2 kb capsid gene. A BLASTP search of the capsid protein resulted in no homology to known viral sequences; however the capsid proteins from EhPV2, TuPV and HK-2014 are not available in GenBank. As the genomic architecture of PPV7 is consistent with members of *Parvoviridae* and the NS1 protein sequences of PPV7, EhPV2, TuPV and HK-2014 are highly divergent, it is likely that the putative PPV7 capsid is novel.

A recent proposal outlined criteria for *Paroviridae* taxonomy (Cotmore *et al.*, 2014). Members of a genus should be monphyletic and have >30% pairwise identity in the NS1 protein

but <30% NS1 identity to members of other genera. Based on the genetic analysis here, TuPV and EhPV2 share >30% identity to PPV7 NS1 and are a monophyletic lineage. Likewise, these viruses have less than 30% identity to other *Parvoviridae* species. Taxonomical assignment of inclusion within a species requires >85% identity in NS1. Together, these results suggest that PPV7, TuPV and EhPV2 should be classified in a novel genus in the *Parvovirinae* family, proposed *Chapparvovirus* (*Chiroptera*, *Avian*, and *Porcine*), with PPV7 the prototype strain as it is the sole member with a complete coding DNA sequence determined.

As many of the recently identified porcine parvoviruses are found in co-infections with other viruses such as porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2, the association with clinical disease is often undetermined (Opriessnig *et al.*, 2013, 2014). Two other viruses found in the samples containing PPV7 are PPV2 and PCV1. Although not linked to clinical disease, PPV2 was identified in pigs suffering from "high fever disease" in China (Hijikata *et al.*, 2001; Wang *et al.*, 2010). Furthermore, PCV1 has been implicated as the causative agent of lung pathology in experimentally infected porcine fetuses (Saha *et al.*, 2011; Tischer *et al.*, 1974, 1982). Further research is needed to determine if there is any clinical significance associated with PPV7 infection.

Advances in sequencing techniques related to viral discovery have led to the identification of novel parvoviruses, including PPV4, PPV5 and PPV6 in recent years (Cheung *et al.*, 2010; Ni *et al.*, 2014; Xiao *et al.*, 2013a). The mutation rate of small ssDNA viruses has been shown to approach that of the mutation-prone RNA viruses which is a likely contributing factor to the expansion in parvovirus diversity. Although parvoviruses utilize the cellular machinery of the host for viral replication, incorrect methylation patterns may prevent them from taking advantage of host exonuclease repair mechanisms, possibly contributing to the high mutation

rate (Duffy & Holmes, 2008; Streck *et al.*, 2011). Mutation rates for the classical PPV are estimated to be $3\text{-}5x10^{-4}$ and 10^{-5} for CP and NS1 genes, respectively. The role of high mutation rates in parvovirus evolution is unknown, however, this is a possible explanation for PPV7 emergence. The moderate prevalence of PPV7 suggests the virus has a relatively broad tissue tropism as seen with many parvovirus species and commonly circulates in US pigs. Further research is needed to determine the *in vivo* affects of PPV7 infection.

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Table 2.1 Prevalence in the US of members of *Parvovirinae* known to infect pigs.

Prevalence of each specific virus (if known) is given as the percent positive animals in the referenced studies.

Species	Virus	Prevalence in US pigs	Reference
Ungulate protoparvovirus 1	Porcine parvovirus (PPV)	14.7%-26.9%	(Opriessnig 2014, identification)
Ungulate tetraparvovirus 2	Porcine parvovirus 3 (PPV3)	16.1%-19.2%	(Opriessnig 2014, identification)
	Porcine hokovirus (PHoV)	-	-
	Porcine PARV4	-	-
Ungulate tetraparvovirus 3	Porcine parvovirus 2 (PPV2)	1.6%-20.7%	(Opriessnig 2014, identification)
	Porcine partetravirus	-	-
	Porcine Cn virus	-	-
Ungulate copiparvovirus 2	Porcine parvovirus 4 (PPV4)	5.9%-7.5%	(Opriessnig 2014, identification)
	Porcine parvovirus 5 (PPV5)	5.4%-7.0%	(Opriessnig 2014, identification)
Ungulate bocaparvovirus 2	Porcine bocavirus 1 (PBoV1)	12.8%-56.4%	(Jiang 2014, high)
	Porcine bocavirus 2 (PBoV2)	3.6%-41.8%	(Jiang 2014, high)
	Porcine bocavirus 6 (PBoV6)	-	-
Ungulate bocaparvovirus 3	Porcine bocavirus 5 (PBoV5)	-	-
Ungulate bocaparvovirus 4	Porcine bocavirus 7 (PBoV7)	-	-
Ungulate bocaparvovirus 5	Porcine bocavirus 3 (PBoV3)	0.9%-88.9%	(Jiang 2014, high)
	Porcine bocavirus 4-1 (PBoV4-1)	1.8%-77.8%	(Jiang 2014, high)
	Porcine bocavirus 4-2 (PBoV4-2)	0.9%-88.9%	(Jiang 2014, high)
Unclassified	Porcine parvovirus 6 (PPV6)	13.2%	(Schirtzinger 2015)

Table 2.2 Overlapping primers to determine the near complete PPV7 genome from porcine rectal swabs.

Start and end locations are given as nucleotide locations within the PPV7 genome. Forward (F) and reverse (R) primer melting temperatures (T_m) are given in degrees celcius (°C).

Primer Set	Start Position	End position	Forward Primer (5'-> 3')	F T_m (°C)	Reverse Primer (5'-> 3')	R T_m (°C)
1	400	1256	GCA GCC GCT TCC TGG TGA G	61.5	CCA GGT CGG GTG CGT TTC	59.2
2	491	1352	TAA CCA GAC GAG ACA AGA G	50.9	TGT CTT GCA TGG ACC AGT TAT	54.9
3	990	1860	CAG GGA GCC TGA TGG AAT AC	55.0	CTG GAT CTG CGA ACG AAC	53.5
4	1935	2797	GCC AAG TGC CGG GAG GAC AAC	63.4	TGG TGC TGG TGC CCT CGA	62.9
5	2535	3300	TGT CCT ATC TAG AGC ATT G	48.1	GTT ATA CCA GCA GTG TTC	47.5
6	2601	3360	CAC GTT CAT GGC TTA CTG	50.9	GAT GTT GTG GCT GTA TCC	50.5
7	3073	3950	GCA CCC GAA ACA AAC TGG AC	57.0	GTC GTT GTG GTT GAC GAG TAT G	55.8
8	3244	4058	AAC GCC ATG AGC TTC CAC	55.8	GGG AGG CGG GTT AAT AAC AG	55.4

Figure 2.1 Organization of *Parvovirinae* viral genomes compared to porcine parvovirus 7 (PPV7).

A general outline of the key elements for parvovirus genome (a) and the open reading frames (ORF) encoded by the PPV7 genome (b). All ORF sizes are given in base pairs (bp). Untranslated regions (UTRs) are specified.

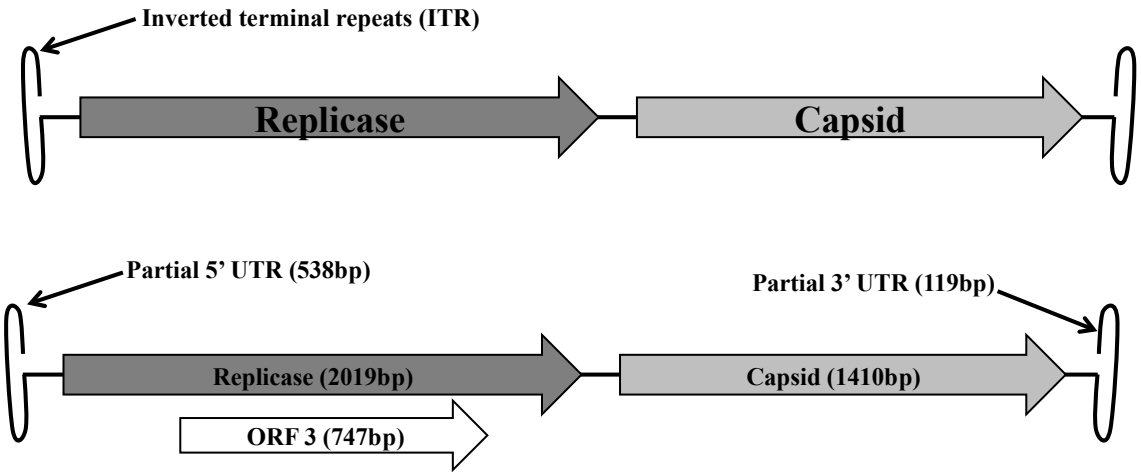

Figure 2.2 PPV7 Rep protein contains sequence motifs conserved among parvoviruses.

Rolling circle replication (RCR) motifs are labeled I, II and III, encompassing amino acids (aa) 76-83 (Motif I), 100-106 (Motif II), and 154-163 (Motif III). Amino acids necessary for the identification of the motifs are marked by black dots above the sequence while the putative active tyrosine residue (Y) is identified with a blue dot. The two motifs essential for NTP-binding are labeled A and B, encompassing residues 319-326 and 361-362, and residues used for identification indicated by stars above the sequence. The H identifies the helicase domain with the highly conserved proline (P) residue designated with a black diamond.

MEHPGSRRER KLERWRWIGN TVVQETAPLE SVALTPEQLE VAKAHLNMQQ WQGMVMMLSN GEGRPRTQPD

IRAIAFLLSQ LKTVRDWCFV AETNTDGILH YHCLVKTSQR SDALRDSVHR RWEQCKLAAM EDIEEPDPQI

EVLKSQKAHR PGSLMEYMMK GPLCFCAYSD TTMALGASIY LYNQGQRFAE KEKQKQKRKQ ILGPEVLQGA

HSLTRDLLGV IYTYNCQSAE DIFRNAPDLV VAHLHKPGFQ QIVKNCLGFV DATKDNWSMQ DNARRTPPDP

TAIHTCLAHQ GLDIDNFDAT MYAWITKKSD KRNTIVLWGP SNTGKTAFIR GLRQVVNCGE CCNGQIFCFE

GLCGKAIGIW EEPLISPECA EKAKQIFEGA DTQVPAKYKK PQDLPRTPII MTTNHAPWRF CTSEEGALRN

RMFIFIWDKD CTDGVFVRRS SGSCCQCRGC QGCGGGEVPA QQRGAGQVPG GQQSLQPVGA RLPGSGADVG

GGCSGPVSGG LESGDERLLC TSECGEDLSW SDDDLHRRCA SVSTLECAEL ARLGFSTGTA AGDEWGSGGD

HGSGYPNLRV YSAGGGDGVL VEPEQHRGGD GADPGGDGGG AIGAITGGPG GDREQHARGG DVVVLEQGSQ

HGHQMASEES GVGGEVDPVM VIPTKQHWCA YLSYLEHCFG DQ*

I I II A B H

Figure 2.3 A phylogenetic tree constructed from the NS1 protein sequence of 39 *Parvovirinae* genomes.

The tree was derived using maximum-likelihood analysis with 1000 bootstrap replicates. *Scale bars* indicate the number of nucleotide changes along the branches. *Vertical bars* represent the genera and Genbank accession numbers are in parentheses for the reference virus strains.

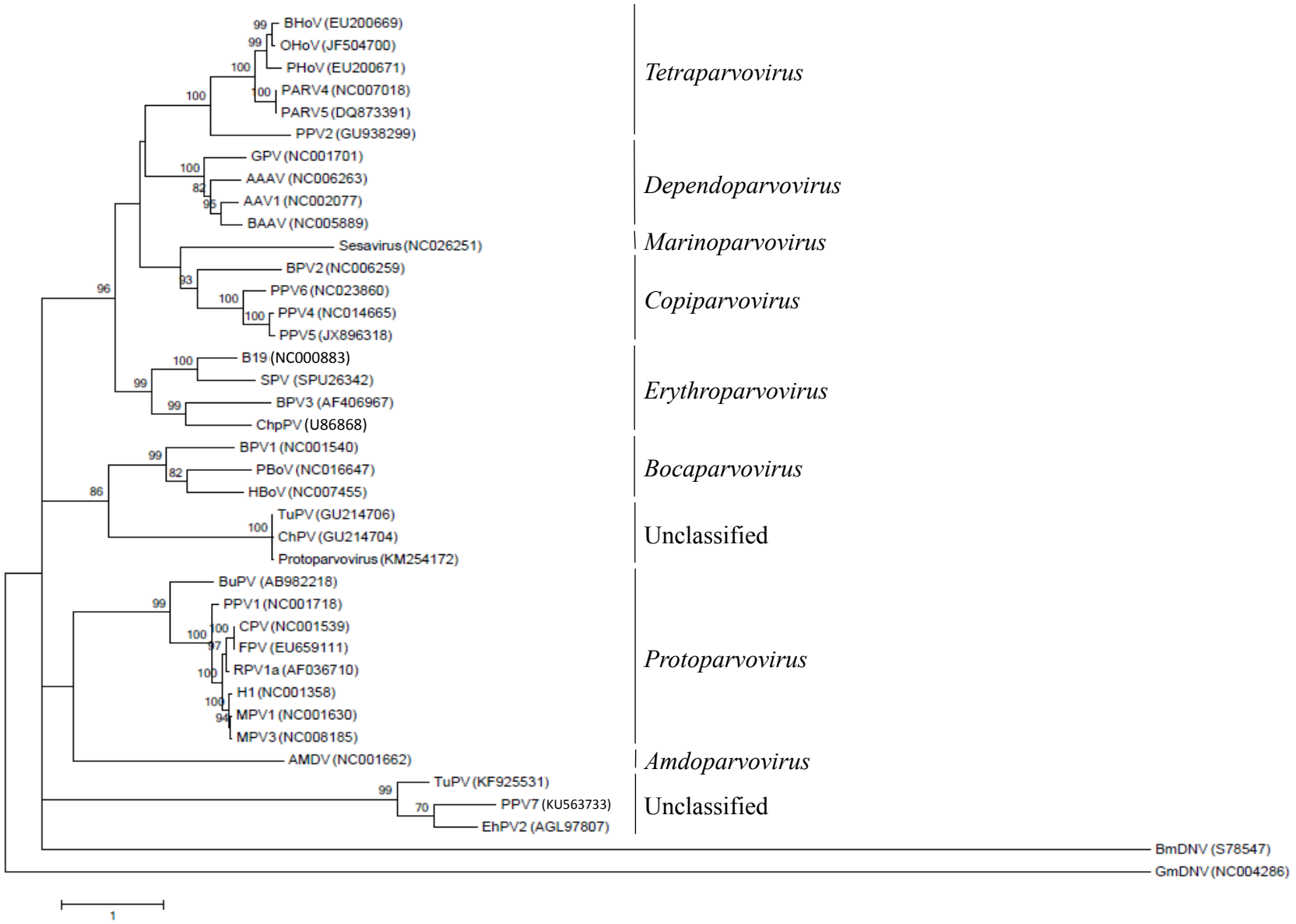
BHoV (EU200669)
OHoV (JF504700)
PHoV (EU200671)
PARV4 (NC007018)
PARV5 (DQ873391)
PPV2 (GU938299)
GPV (NC001701)
AAAV (NC006263)
AAV1 (NC002077)
BAAV (NC005889)
Sesavirus (NC026251)
BPV2 (NC006259)
PPV6 (NC023860)
PPV4 (NC014665)
PPV5 (JX896318)
B19 (NC000883)
SPV (SPU26342)
BPV3 (AF406967)
ChpPV (U86868)
BPV1 (NC001540)
PBoV (NC016647)
HBoV (NC007455)
TuPV (GU214706)
ChPV (GU214704)
Protoparvovirus (KM254172)
BuPV (AB982218)
PPV1 (NC001718)
CPV (NC001539)
FPV (EU659111)
RPV1a (AF036710)
H1 (NC001358)
MPV1 (NC001630)
MPV3 (NC008185)
AMDV (NC001662)
TuPV (KF925531)
PPV7 (KU563733)
EhPV2 (AGL97807)
BmDNV (S78547)
GmDNV (NC004286)
1
Tetraparvovirus
Dependoparvovirus
Marinoparvovirus
Copiparvovirus
Erythroparvovirus
Bocaparvovirus
Unclassified
Protoparvovirus
Amdoparvovirus
Unclassified

Table 2.3 Pairwise comparison of members of *Parvovirinae* to PPV7.

Replicase (Rep) and capsid (CP) nucleotide (nt) and amino acid (aa) percent identities are listed for various parvoviruses. The abbreviations are as follows: AAV1, adeno-associated virus 1; AMDV, aleutian mink disease virus; BPV1, bovine parvovirus 1; BPV3, bovine parvovirus 3; ChPV, chicken parvovirus; EhPV2, *Eidolon helvum* parvovirus 2; PPV1, porcine parvovirus 1; PPV2, porcine parvovirus 2; PPV3, porcine parvovirus 3; PPV4, porcine parvovirus 4; PPV5, porcine parvovirus 5; PPV6, porcine parvovirus 6; TuPV, Turkey parvovirus. Genbank accession numbers are in parenthesis. Partial sequences were not applicable (N/A) to the analysis.

	Species	**Rep (aa)**	**CP (aa)**	**Full Sequence (nt)**
AAV1 (NC002077)	*Dependoparvovirus*	18.3	15.5	40.2
AMDV (NC001662)	*Amdoparvovirus*	17.3	17.5	38.6
BPV1 (NC001540)	*Bocaparvovirus*	20.3	15.6	37.4
BPV3 (AF406967)	*Erythroparvovirus*	21.2	13.7	36.8
ChPV (GU214704)	Unclassified	20.3	15.5	37.6
EhPV2 (AGL97807)	Unclassified	42.4	N/A	N/A
PPV1 (NC001718)	*Protoparvovirus*	14.3	15.6	39.0
PPV2 (GU938299)	*Tetraparvovirus*	18.6	13.2	37.6
PPV3 (KU167029)	*Tetraparvovirus*	20.4	13.9	40.4
PPV4 (NC014665)	*Copiparvovirus*	17.1	16.4	35.0
PPV5 (JX896318)	*Copiparvovirus*	18.4	14.2	35.6
PPV6 (NC023860)	*Copiparvovirus*	18.5	11.9	33.2
Protoparvovirus (KM254172)	Unclassified	34.1	16.2	37.5
Sesavirus (NC026251)	*Marinoparvovirus*	18.6	14.3	39.5
TuPV (KF925531)	Unclassified	37.9	13.0	43.0

Table 2.4 Detection of PPV7 in diagnostic samples using qPCR targeting the NS1 gene.

All values are given as number of cycle thresholds (C_t) and samples were considered positive with a C_t<37.

Sample type	Cycle threshold (C_t)
Serum	35.3
	36.4
Rectal swab	8.8
	22.3
	24.5
	27.2
	32.7
Nasal swab	15.4
	17.6
	20.4
	23.1
	25.4
Lung lavages	22.2
	25.1
	25.5
	29.3

Chapter 3 - Widespread detection and characterization of porcine parainfluenza virus 1 in pigs in the United States

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Abstract

Porcine parainfluenza virus 1 (PPIV1) was first identified in 2013 in slaughterhouse pigs in Hong Kong, China. The pathogenesis or endemic potential of PPIV1 has not yet been assessed nor has the virus been formally identified outside of China. Here, metagenomic sequencing identified two complete PPIV1 genomes in samples from swine exhibiting acute respiratory disease out of facilities in Oklahoma and Nebraska. The genomes were 90.0-95.3% identical to the previously published Chinese strains. Phylogenetic analysis of a 1760bp segment of the HN gene from 10 additional PPIV1-positive samples found 85.0-95.5% identity, suggesting considerable genetic diversity between strains. Further molecular analysis by quantitative reverse transcription PCR (qRT-PCR) identified 17 positive samples out of 279 (6.1%) lung homogenate, oral fluid, or nasal swab samples from pigs with acute respiratory disease. Eleven

nursery pigs from a naturally infected herd were monitored for virus replication and pathogenesis. No clinical signs of illness were apparent however qRT-PCR detected PPIV1 in nasal swabs from six pigs and the lungs of one animal. *In situ* hybridization identified PPIV1 RNA in the nasal respiratory epithelium and trachea to a lesser extent. Serological analyses using immunocapture PCR and ELISA demonstrated seroconversion of infected pigs and further analysis of 60 swine serum samples representing at least eight states found 52.5% and 66.1% seropositivity, respectively. Taken together, the results confirm the widespread presence of PPIV1 in the United States swineherd.

Introduction

Paramyxoviruses are significant pathogens known to affect humans and a range of animals including livestock species such as cattle, pigs and poultry. These viruses are classified as members of the *Paramyxoviridae* family within the subfamily *Paramyxovirinae*. The subfamily *Paramyxovirinae* consists of seven genera: *Respirovirus*, *Rubulavirus*, *Avularvirus*, *Morbillivirus*, *Aquaparamyxovirus*, *Ferlavirus*, and *Henipavirus* (Lamb and Parks, 2007). The genus *Respirovirus* consists of five recognized species: bovine parainfluenza virus 3 (BPIV3), human parainfluenza virus 1 (HPIV1), human parainfluenza virus 3 (HPIV3), Sendai Virus (SeV), Simian virus 10 (SV10) (Lamb and Parks, 2007) and the proposed porcine parainfluenza virus 1 (PPIV1). Clinical symptoms are variable in terms of severity and presentation. While swine are the primary reservoir to porcine rubulavirus, cross species transmission of paramyxoviruses from their hosts to swine have been reported, including Nipah virus (NiV), Menangle virus, Newcastle disease virus and BPIV3 (Chua *et al.*, 1999; Ellis, 2010; Janke *et al.*, 2001; Philbey *et al.*, 1998; Stephan *et al.*, 1988). Importantly, many of these paramyxoviruses

are zoonotic, some of which, namely NiV and Hendra virus (HeV), have high fatality rates in humans (Chadha *et al.*, 2006; Murray *et al.*, 1995). Swine have been shown to serve as intermediate hosts for NiV (McCormack, 2005).

In 2013, three complete genome sequences of a novel porcine parainfluenza virus, designated PPIV1, were obtained from nasopharyngeal samples of slaughterhouse pigs in Hong Kong (Lau *et al.*, 2013). Phylogenetic analysis of the nucleocapsid (N) gene found PPIV1 to be most closely related to HPIV1 and SeV. While the nasal swabs were collected from deceased animals, nothing is known on the pathogenicity or ecology of PPIV1.

All viruses classified as *Paramyxovirinae* contain single stranded RNA genomes of approximately 15,000-16,000 nucleotides in length which encodes for six proteins in the conserved order 3'-N-P-M-F-HN-L-5' where N, P, M, F, HN, and L represent the nucleocapsid, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase proteins and the polymerase protein, respectively (Lamb and Parks, 2007). Both F and HN proteins are involved in receptor binding, possess neutralizing epitopes and are the most genetically diverse viral proteins.

While PPIV1 has been identified in the U.S. by veterinary diagnostic laboratories in recent years, little is known on its epidemiology and role in clinical disease. There is only a single published report on the detection of the genetic signature of PPIV1 in the USA using microarray technology (Jaing *et al.*, 2015). Here, two complete genomes of PPIV1 were assembled using next generation sequencing (NGS) performed on nasal swab samples submitted to the Kansas State Veterinary Diagnostic Lab (KSVDL) from nursery pigs with respiratory disease in two separate facilities. Additionally, the pathogenesis of the virus was assessed in

eleven naturally infected nursery pigs. Molecular epidemiological and serological analyses suggest the virus is common throughout the United States.

Materials and Methods

Ethics Statement

Porcine nasal swabs, sera and tissues were collected by veterinarians as part of routine diagnostic investigations and were submitted to KSVDL or Iowa State University Veterinary Diagnostic Laboratory (ISUVDL). Pathogenesis studies were carried out on pigs from a commercial swine operation that were naturally infected with PPIV1 and were transferred to the University of Nebraska-Lincoln (UNL). Experimental procedures were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee (#1016).

Collection of samples

Metagenomic sequencing was performed on a pool of two nasal swabs originating from a commercial swine operation in Oklahoma (OK). The samples were submitted to KSVDL from 10-21 day old pigs with acute respiratory disease that had previously tested positive by quantitative reverse transcription polymerase chain reaction (qRT-PCR) for PPIV1 at ISUVDL. Furthermore, these nasal swabs tested negative for influenza A virus (IAV) at ISUVDL. In addition, samples from four pigs with acute respiratory disease were collected from a commercial swine farm in Illinois (IL). A total of 279 porcine nasal swab, oral fluids or lung homogenate samples submitted to ISUVDL for diagnostic testing were screened for the presence of PPIV1 using a qRT-PCR targeting the N gene. The geographic origin of the 279 samples are unknown. The serum samples used for serology were submitted to ISUVDL for porcine reproductive and respiratory syndrome virus (PRRSV) qRT-PCR testing. The serum samples for

serology were obtained from eight states: Iowa (n=18), North Carolina (n=6), Nebraska (n=6), Missouri (n=1), Oklahoma (n=6), Minnesota (n=1), Indiana (n=1), Illinois (n=2), Mexico (n=5) and unknown (n=14). Sera (n=18) were also obtained from 3-week old pigs from a specific pathogen free research herd and used as negative controls.

Metagenomic Sequencing

Metagenomic sequencing was performed as previously described (Hause *et al.*, 2015b; Neill *et al.*, 2014). Nasal swab samples were pooled and treated with nucleases at 37°C for 90 minutes to enrich for viral genetic material. Viral nucleic acids were extracted using the MinElute Virus spin filter kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Synthesis of the first-strand cDNA from viral RNA was performed using the Superscript III first-strand synthesis kit as specified by the manufacturer with previously described primers (Allander *et al.*, 2005). Second strand synthesis was completed with Sequenace 2.0 DNA polymerase followed by cDNA purification using the Agencourt AMPure XP beads using the suggested protocol size selection >300 bp. The double stranded cDNA was amplified with TaKaRa DNA polymerase with primers identical those used for first strand synthesis but lacking the random hexamer. A second round of size selection and purification was performed using the Agentcourt AMPure XP beads again selecting for products >300bp. Amplicons were quantified using a Qubit fluorimeter and libraries were prepared by the standard Nextera XT library preparation kit protocol. Libraries were pooled and sequenced using paired 150 bp reads on an Illumina Miseq instrument.

Data Analysis

Raw sequence reads were parsed based on barcodes incorporated during library preparation and imported into CLC Genomics Workbench (CLC Bio version 7.0). Reads were

mapped to the host genome (*Sus scrofa*) and from the unmapped reads, contigs were assembled *de novo*. Contigs were analyzed by BLASTN. PPIV1 genomes were assembled using a reference-based assembler with the S119N strain of PPIV1 as a reference (GenBank JX857411). Sequences were aligned using ClustalW and phylogenetic trees were constructed using MEGA 6.06 software using the maximum-likelihood method with topology verified by 1000 bootstrap replicates (Tamura *et al.*, 2013).

RT-PCR for PPIV1 HN

Viral RNA was isolated using the MagMax-96 total nucleic acid isolation according to manufacturer's instructions. To determine the presence of PPIV1 in samples, a 5'-nuclease assay was designed targeting the N gene: probe, 5'-FAM- AGC AGA GGA GAT GGG AAA CAA CCA-Iowa Black-3'; Forward, 5'- CGG ATA CTT CAT CGT CAG TGT T-3'; Reverse, 5'-TGG AGA CAA CAA AGG GAG AAT AG-3'. Quantitative RT-PCR using the Qiagen Quantitect RT-PCR kit with the following conditions: 50°C, 30 minutes; 95°C, 15 minutes; and 45 cycles of 94°C, 15 seconds and 60°C for 60 seconds. The specificity of the assay was verified using samples positive for PPIV1 by metagenomic sequencing and common swine respiratory viruses, IAV and porcine reproductive and respiratory syndrome virus (PRRSV). To investigate PPIV1 genetic diversity, 1976 bp fragment of the HN gene was amplified using forward primer 1: 5'-TTA GGG TGC ACG ACA GTA AC-3' and reverse primer 1: 5'-GTC CAC AGG TCA CTT ATC-3'or forward primer 2: 5'-TTA GGG TGC ACG ACA GTA ACT C-3'and reverse primer 2: 5'-CCA CAG ATC ACC TGT CTC TAA G-3' which amplified a 1873 bp fragment. PCR products were sequenced by Sanger methodology using forward and reverse primers utilized for PCR. Sequencing of the HN gene fragment derived from HN primer set 2 required additional primers: 5'-CGG TGA GAA AGG ATG A-3' and 5'-CAA AGG GTC CTC TAG

AAG-3'. The RT-PCR was performed using the Superscript III One-Step RT-PCR kit with Platinum Taq with the HN primers and probes as follows: 45°C, 30 minutes; 94°C, 2 minutes; followed by 40 cycles of 94°C, 15 seconds; 55°C, 30 seconds; and 68°C for 30 seconds.

Virus Isolation

Virus isolation was attempted on swine testicle cells (ST), porcine alveolar macrophages (PAM), primary porcine kidney cells (PPK) and African green monkey kidney cells (Vero). Cell lines were maintained in minimal essential media (MEM) supplemented with L-glutamine and 5% fetal bovine sera. Porcine kidneys, sourced from an abattoir, were purchased from Innovative Research. Primary porcine kidney cells were cultured by treatment of finely minced kidneys with 0.25% trypsin at 37°C for 4 hours following transfer to cell culture flasks with MEM with L-glutamine and 100 units/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B. Cell culture fluids were removed from the 12-well plates (>80% confluency) and 25-100µL of sample (depending on available sample volume) was inoculated into 1mL of viral replacement media, which consisted of MEM and penicillin-streptomycin solution. Plates were incubated 5 days before being frozen, thawed, and passaged as above to fresh monolayers. Cells were observed daily for cytopathic effects (CPE) and PPIV1 growth was monitored by qRT-PCR.

Immunocapture PCR

Serological analysis was performed using an immunoprecipitation coupled to PCR detection assay (ICPD) modeled after the luciferase immunoprecipitation system (Figure 3.1) (Burbelo *et al.*, 2009, 2011). To measure antibody titers, 10µl serum was added to 40µl buffer A (50mM Tris, pH 7.5, 100mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100) in a 96-well microtiter polypropylene deep well plate. A pool of PPIV1 RT-PCR positive nasal swabs (C_t=29.3) served

as antigen; 50 µl was added to sample wells on the assay plate and incubated at 1800 rpm for 60 minutes at room temperature. Next, 500µl of Protein G magnetic Dynabeads® were resuspended in 1 ml of phosphate saline buffer with 0.05% Tween 20 (PBST) and 10µl was added to the bottom of each well of the assay plate. The plate was incubated with rotation for 20 minutes at room temperature. After incubation, magnetic beads were captured at the bottom of the wells by incubating the plate for 1 minute on a magnetic plate separator. The plate was washed with 100µl buffer A, incubated for 1 minute on a plate shaker at 1800 rpm and subsequently captured on the magnet for one minute. The wash liquid was discarded and the plate was washed two more times with buffer A and two times with PBST following the same procedure. After the final wash was discarded, bound antibodies were eluted by the addition of 20µl of 50mM Glycine pH 2.8 to each well. The plate was incubated at room temperature on a plate shaker for 2 minutes and captured on the magnetic plate separator. The supernatant was transferred to a clean 96-well plate. The pH was adjusted by adding 30µl of 1M Tris pH 7.5 and the resulting nucleic acid was purified using the Qiagen MagMax DNA/RNA isolation kit and subsequently tested for PPIV-1 by qRT-PCR.

Recombinant F protein ELISA

A fragment of the PPIV1 F gene encoding amino acids 44-245 was commercially synthesized and cloned into the pET28a (Novagen, Madison, WI) vector using the *BamHI* and *XhoI* restriction sites. The pET28a-F plasmid was transformed into *E.coli* BL21 (DE3) cells, grown in 2x yeast extract tryptone (YT) medium and induced by the addition of isopropyl β-d-1-thiogalactopyranoside (IPTG). Protein purification was performed using B-PER reagent extraction (Pierce, Rockford, IL) followed by Ni-NTA affinity purification using the N-terminal His tag from the pET28 vector. The Ni-NTA agarose (Qiagen, Valencia, CA) purification was completed following the manufacturer's instructions. Aliquots of purified protein were stored at -

80°C. Recombinant protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and stained with Coomassie brilliant blue G-250 (BioRad, Hercules, CA). Further verification was performed by Western blot detecting the His-tag at the N-terminal of the recombinant protein. Briefly, protein from the SDS-PAGE gel was transferred to a nitrocellulose membrane (Whatman Piscataway, NJ) by electroblotting and incubated at room temperature for two hours with anti-Histidine monoclonal antibody (Novagen, Madison, WI). The membrane was washed 3x with PBST and incubated for 2 hours at room temperature with IRDye 800CW-conjuagted goat anti-mouse antibody (LI-COR Biosciences, Lincoln, NE). The membrane was visualized using a digital imaging system (FLUOstar Omega, microplate reader, BMG Labtech).

The ELISA was performed similar to assays previously described with a few modifications (Hause *et al.*, 2015a; Lin *et al.*, 2005). Briefly, Corning EIA/RIA High-binding plates were coated with 100µl of 2µg/ml purified recombinant PPIV1 F protein diluted in 0.1M sodium carbonate buffer (pH 9.6) and incubated at room temperature for 20 hours. Plates were washed 3x in PBST and 100µl Starting Block (Thermo Fisher Scientific, Waltham, MA) was added to each well. After incubation for 1 hour at 37°C, plates were again washed in PBST. Serum samples were diluted 1:100 in PBST and 100µl/well was added to the plates. Plates were incubated for 1 hour at 37°C before being washed 3x with PBST. The goat anti-swine IgG secondary antibody labeled with horseradish peroxidase was diluted to 1:2,000 in PBST and 100µl was added to each well of the assay plate. Plates were incubated at 37°C for 1 hour and washed 3x with PBST. Plates were developed using a commercial peroxidase assay kit (ABTS ELISA HRP substrate, KPL, Gaitersburg, MD) for 10 minutes at room temperature. The reaction

was stopped by the addition of stop solution and measure at an absorbance of 405nm on a microplate reader.

In situ Hybridization (ISH)

A viral RNA probe targeting nucleotides 2-1206 of the N gene was synthesized for ISH analysis (Advanced Cell Diagnostics, Hayward, CA). The assay was performed on trachea and nasal turbinate tissue samples from the pathogenesis study. The assay was completed as specified by the manufacturer with a few modifications. Three pretreatment steps included Pretreatment 1 for 10 min at room temperature, Pretreatment 2 for 15 min at 100-105°C and pretreatment 3 for 30 min at 40°C. The RNA probe was added to cover sections on all slides and incubated at 40°C for 2 hours. The excess liquid was removed and slides were washed 2x in wash buffer at room temperature for 2 minutes. All slides went through six rounds of amplification with washes between each round alternating 30 minutes at 40°C and 15 minutes at 40°C. Then the chromagen, DAB, was applied for 10min at room temperature. Finally, slides were counterstained using Gill # 1 hematoxylin for 30 seconds and blued for 5 minutes in running tap water.

Pathogenicity studies in pigs

Eleven three-week old pigs from a farm that was qRT-PCR positive for PPIV1 were transported to the University of Nebraska-Lincoln facility for swine research and allowed to acclimate for four days prior to sampling. Pigs were observed daily for clinical signs of illness such as sneezing, coughing, and nasal discharge. Nasal swabs were collected on days 0, 2, 4, 6, 9, 11, and 14. Blood was collected on day 0 and day 14 for serological analysis (Figure 2.2). Two pigs were randomly sequentially sacrificed on days 2, 4, 6, and 9. Lungs, trachea and nasal turbinates were collected at necropsy and fixed with 10% buffered formalin before embedding in

paraffin. Slides were prepared from tissue blocks and stained with hematoxylin and eosin (H&E). Nasal swabs were assessed by qRT-PCR. Serum samples were analyzed for antibodies by ICPD.

Genbank Accession Numbers

The two nearly full genome sequences used in the phylogenetic analysis have been submitted to GenBank under acession numbers KT749882 (1438-1) and KT749883 (3103-1). In addition, the ten PPIV1 HN sequences used for phylogeentic analysis were submitted to GenBank as follows; KT749884 (1438-1), KT749885 (1438-4E1), KT749886 (1438-2E6), KT749887 (1438-4E6), KT749888 (1438-3C11), KT749889 (3103-1), KT749890 (3103-D0A4), KT749891 (3103-D0A7), KT749892 (5031-4) and KT749893 (5031-2). Previously published sequences used for phylogenetic analysis include: Swine parainfluenza virus 3 (SPIV3) EU439428; Bovine parainfluenza virus 3 (BPIV3) NC002161; Human parainfluenza virus 3 (HPIV3) NC001796; Sendai virus (SeV) NC 001552; Human parainfluenza virus 1 (HPIV1) NC003461; Porcine parainfluenza 1 (PPIV1) JX857409, JX857410, and JX 857411.

Results

Metagenomic sequencing

A commercial swine operation in OK experiencing recurrent disease in pigs approximately 10-21 days of age with clinical symptoms of a moderate cough, minor sneezing and a serous nasal discharge with unknown aetiology, submitted nasal swabs to ISUVDL where they tested negative for IAV and positive for PPIV1 by qRT-PCR. Samples were transferred to KSVDL and metagenomic sequencing was performed on a pool of two nasal swabs. The MiSeq run generated 8.8 million reads with 1.5 million mapping to the *Sus scrofa* reference genome. De

novo assembly of the remaining host subtracted reads resulted in 4,236 contigs which were analyzed by BLASTN. Viruses identified were porcine astrovirus 4 (Pas4), porcine circovirus 2, porcine kobuvirus, porcine stool associated circular virus and PPIV1, all with expectation (*E*) values of 0. Templated assembly using a PPIV1 reference sequence (JX857411) mapped 51,941 reads encompassing 99% of the PPIV1 genome with an average coverage of 353x.

A commercial swine operation in Illinois with similar clinical symptoms to the farm in OK submitted two approximately 10 day old pigs to KSVDL for diagnostic testing. Metagenomic sequencing of a nasal turbinate homogenate pool generated 2.9 million reads which, following subtraction of reads *Sus scrofa*, yielded 134 contigs following de novo assembly. BLASTN analysis identified multiple contigs with highly significant *E* values to Pas4, porcine kobuvirus, porcine hemagglutinating encephalomyelitis virus and PPIV1. Templated assembly of PPIV1 using JX857411 afforded only approximately 45% genome coverage.

Asymptomatic pigs from a commercial breeding herd in NE that were qRT-PCR positive for PPIV1 were transferred to UNL to monitor PPIV1 pathogenesis. A pool of nasal swabs were subjected to metagenomic sequencing. Of the 617,736 reads, 17,521 mapped to PPIV1 JX857411, resulting in 99% genome coverage. In addition to PPIV1, analysis of 58 host subtracted, *de novo* assembled contigs identified Pas4, porcine astrovirus 5, porcine bocavirus 3, porcine bocavirus 5 and a small circular DNA virus most similar to one identified in fur seal feces.

PPIV1 qRT-PCR

To investigate the differences seen in the metagenomic sequencing results, a Taqman qRT-PCR assay was designed to target the N region of the PPIV1 genome. Individual testing of

the five nasal swab samples from OK resulted in cycle threshold (C_t) values of 26.0, 28.1, 31.8, 28.9 and 26.0. A nasal swab pool for use in the serological assay had a C_t value of 29.3.

Nasal turbinate, lung and brain homogenates were tested individually from the two pigs from Illinois displaying acute respiratory disease with unknown aetiology. Only the nasal turbinate from one pig was positive with a C_t of 20.3. Additionally, nasal swabs were submitted from four pigs with similar clinical symptoms from the same site. All of the nasal swabs were positive for PPIV1 with C_t values of 26.5, 28.0, 32.6 and 34.9. The nasal swabs were negative for IAV by qRT-PCR.

To assess the prevalence of PPIV1 in porcine samples submitted for diagnostic testing, 279 lung homogenate, oral fluid or nasal swab samples of unknown infection status were screened by qRT-PCR for the PPIV1. Seventeen samples tested positive (6.1%). Of the qRT-PCR positive samples, twelve were nasal swabs, four were oral fluids and one was lung homogenate.

Virus Isolation

Virus isolation was attempted on ST, Vero, PAM, and PPK. No CPE was evident. Viral titers were monitored by the N gene qRT-PCR. Following two passages on cells all samples were qRT-PCR negative.

Phylogenetic Analysis

The near full genome sequences determined from the nasal swabs from pigs originating in OK (strain 1438-1) and NE (3103-1) had 97.7% pairwise identity to each other and 90.0-95.3% identity to three PPIV1 sequences from China at the nucleotide level. An approximate 1760 bp fragment of the HN gene was sequenced from 10 U.S. samples and nucleotide identities ranged from 93.7-99.9% for samples from the US and 85.0-95.5% identity to Chinese PPIV1.

Phylogenetic analysis of the genome sequences found that the two nearly complete US sequences formed a well-supported clade that was most similar to Chinese PPIV1 strains S119N and S206N (Figure 2.3a). S033N represented a divergent ancestral PPIV1. The PPIV1 clade was most closely related to a clade consisting of HPIV1 and SeV. Phylogenetic analysis was also performed on the HN gene of the parainfluenza virus samples collected in this study and other members of the genus *Respirovirus* (Figure 2.3b). Similar to the phylogeny of the genome sequences, all US HN genes formed a well-supported clade most closely related to Chinese strains S119N and S206N. Strains originating from the same state additionally formed well supported clades and for samples from OK (1438-1, 1438-4E1, 1438-2E6, 1438-4E6, 1438-3C11) and NE (3103-1, 3103-D0A4, 3103-D0A7), little diversity between samples was observed. As seen with the genome sequence phylogeny, strain S033N represented a divergent, ancestral member of PPIV1. The PPIV1 clade was well supported and was most closely related to a clade consisting of HPIV1 and SeV. Phylogenetic analysis of predicted HN amino acid sequences yielded identical topology (data not shown).

Pathogenesis of PPIV-1 in naturally infected pigs

Eleven randomly chosen weaned pigs (18-19 days old) from a farm naturally infected with PPIV1 were transferred to the UNL animal research unit. Throughout the two week observation period, no clinical symptoms of disease such as coughing, sneezing, nasal discharge or lethargy were observed. Nasal swabs collected on day 0 (22-23 days of age) were positive for PPIV1 for six of the eleven pigs (55%) (Table 2.1). Three additional pigs shed PPIV1 during the course of the study. The length of time for viral shedding was 2-10 days. qRT-PCR analysis of lung homogenates showed the presence of virus in the lungs of a single pig (A8, C_t=35.2). Histopathology was examined on lung, trachea and nasal turbinates for eight out of the eleven

animals (A4-11). Animals A4, A7, A8, and A9 animals had marked atelectasis in the lung tissues but this pathology is not associated with PPIV1. Animals A4, A7, A8, A9 and A11 displayed a subjective decrease in cilia, goblet cells or both in the trachea. *In situ* hybridization (ISH) using a probe designed to detect PPIV1 identified virus in turbinate respiratory epithelial cells (Figure 2.4a) and to a lesser extent in the trachea (Figure 2.4b) in pig A8. The sole pig positive for ISH (A8) displayed the highest amount of viral shedding at time of euthanasia (C_t=25). Mild lymphoplasmacytic rhinitis was observed for all pigs in the study and is likely a background lesion unrelated to the PPIV1 status.

Serological analysis suggests widespread infection of PPIV1 in swine herds

Sera collected at day 0 and day 14 of the pathogenesis study was subjected to ICPD serological analysis. All day 0 sera were negative for antibodies to PPIV1 as was the negative control where phosphate buffered saline replaced serum. Additional negative controls consisting of 18 serum samples from age-matched specific pathogen free pigs were also all negative. A positive control consisted of pooled sow sera from the farm in IL with recurrent unexplained acute respiratory disease in pigs that tested positive for PPIV1. Of the three pigs bled on day 14 of the study, two had a detectable antibody response as determined by ICPD with C_t values of 35.0 and 35.1. Pig A2 failed to seroconvert to PPIV1. PPIV1 antibodies in the same samples were also assayed with an indirect ELISA using a recombinant F protein peptide. Using 18 serum samples from age-matched specific pathogen free pigs, a value of >0.37 A_{405} was determined to be a positive result (3 standard deviations above the mean), all but four of the animals on day 0 of the study were positive for PPIV1 F antibodies. While animals A1 and A3 tested negative at the beginning of the study for PPIV1 F antibodies, over the two week observation period these animals seroconverted. These results were further verified using ICPD

assay with these same animals testing negative on day 0, tested positive by day 14 of the study. Pig A2 displayed different serological results as those seen for A1 and A3. Pig A2 tested positive for antibodies on day 0 and 14 on the ELISA however was negative at both time points by ICPD.

To investigate the prevalence of PPIV1 antibodies in swine production facilities, 59 serum samples collected from at least eight states were subjected to ICPD and ELISA serological analysis. PPIV1 antibodies were confirmed positive in 31 samples (52.5%) by ICPD. These results were similar to the PPIV1 F ELISA in which 39 samples (66.1%) tested positive for PPIV1 F antibodies. In conjunction with the 6.1% PPIV1 prevalence, these data suggest that PPIV1 is prevalent in swine production facilities throughout the United States.

Discussion

A number of novel paramyxoviruses causing outbreaks of lethal disease in livestock and humans have been described in recent decades. Some of these, such as NiV and HeV, can cross species barriers and cause disease (Chua *et al.*, 1999; Philbey *et al.*, 1998). For instance, HeV, thought to have originated in bats, caused an outbreak of severe respiratory disease in horses and humans in Australia, 1994 (Murray *et al.*, 1995; Selvey *et al.*, 1995). Furthermore, in 1998, NiV, also thought to have originated in bats, caused an outbreak of severe encephalitis and death in pigs and humans who had exposure to pigs in Malaysia (Chadha *et al.*, 2006; Chua *et al.*, 1999).

Paramyxoviruses have also caused disease in pigs in the United States. In the 1980s and 1990s two novel swine paramyxoviruses, Texas-81 (81-19252) and ISU-92 (92-7783), were isolated from an outbreak of respiratory and neurological disease in pigs (Janke *et al.*, 2001). These viruses were later determined to be BPIV3 (Coelingh *et al.*, 1986; Qiao *et al.*, 2009, 2010). BPIV3 has also been shown to infect humans (Ben-Ishai *et al.*, 1980; Schmidt *et al.*, 2000). Given the propensity of paramyxoviruses for interspecies transmission, further study is

warranted, in particular for paramyxoviruses with reservoirs in animals with which humans have frequent contact.

In 2013, a novel paramyxovirus, PPIV1, was identified in deceased pigs from a slaughterhouse in Hong Kong, China. No clinical or pathological data were available nor was there information on PPIV1 distribution outside China (Lau *et al.*, 2013). In the present study, we identified PPIV1 in 6% of samples from a collection of acute respiratory disease diagnostic submissions from geographically diverse swine facilities in the USA. Our serological results also support widespread circulation of PPIV1 in the U.S. swineherd, with over 50% of animals having detectable antibodies to PPIV1. It is unclear if PPIV1 is an emerging virus in the US or if it has circulated undetected for some time. Our phylogenetic analysis indicated that the Chinese PPIV1 strain S033N is an ancestor of contemporary US strains and that Chinese strains S206N and S119N had a close sister-clade relationship to US strains. Further genetic analysis of additional genomes, both contemporary and archived, is needed to resolve the evolutionary history of PPIV1. Several swine viruses identified in China have recently emerged in the US, including porcine epidemic diarrhea virus (PEDV) and mutant porcine circovirus 2b, illustrating the ease with which swine pathogens can move intercontinentally (Huang *et al.*, 2013; Xiao *et al.*, 2012)

While PPIV1 was detected in nursery-aged pigs with acute respiratory disease in the absence of common etiological agents such as IAV and PRRSV, metagenomic sequencing identified a number of other viruses present in nasal swabs. Pas4 was identified in all three PPIV1 positive samples analyzed by metagenomic sequencing. Pas4 was previously shown to be commonly detected in swine diarrhea samples in the US (Xiao *et al.*, 2013). Pas4 was also readily identified in both diarrheic and normal swine feces in China (Zhang *et al.*, 2014). An

etiologic role for Pas4 in clinical disease has not been established. The two samples from symptomatic pigs were positive for viruses shown to contribute to respiratory disease, porcine circovirus 2 and hemagglutinating encephalomyelitis virus. No clinical symptoms were observed during the course of our pathogenesis study, raising the possibility that PPIV1 infection is asymptomatic without additional cofactors. One difference between our study and PPIV1 positive pigs exhibiting clinical symptoms is the age of pigs. Clinically ill pigs positive for PPIV1 were 10-14 days of age while naturally infected pigs in our studies were approximately 22-26 days of age. Differences in clinical outcomes due to age have previously been seen in swine for viruses such as PEDV (Jung *et al.*, 2015). While our results demonstrate that PPIV1 replicates in respiratory epithelial cells of the upper respiratory tract and exhibits nasal shedding similar to established swine respiratory disease etiologic agents, we were unable to show clinical significance or specific pathological lesions that could have been due to PPIV1 infection.

In addition to PPIV1 identification and characterization, this study developed an innovative method of detecting antibodies using an immunocapture PCR coupled to antigen derived from nasal swabs. This is potentially useful for the large number of novel viruses being discovered which lack *in vitro* cultivation systems and has the advantage of using native antigen and circumvents potential conformational issues common for recombinant antigens. Further development of this serological method would provide a simple, cheap, and multiplexable method of detection of pathogen exposure in unprocessed animal samples.

Acknowledgments

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Figure 3.1 Immunoprecipitation coupled to PCR detection assay for the detection of viral antibodies in serum and nasal swabs.

An antigen pool is first incubated with the sample serum diluted 1:5 in buffer and incubated at room temperature for 1 hour to allow complexes to form. Following complex formation, protein G magnetic beads were added to the sample and incubated for 20 minutes at room temperature to capture the antibody-antigen complexes. Finally, antigen and antibodies are removed from the magnetic beads and subsequently analyzes for viral nucleic acid by quantitative PCR (qPCR).

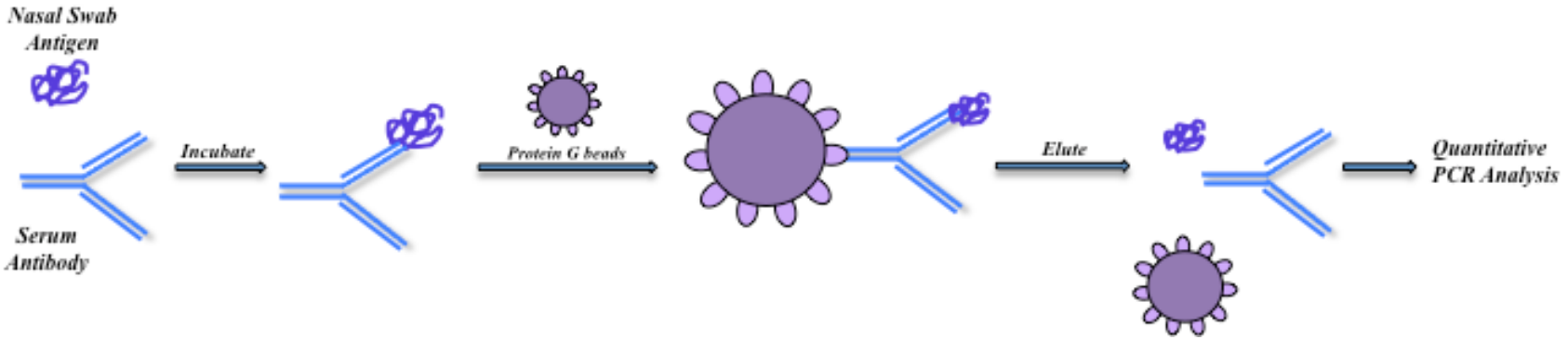


Figure 3.2 Outline of PPIV1 pathogenesis study using 11 three-week-old naturally infected pigs.

Pigs were allowed to acclimate for four days prior to the first collection day. Both serum and nasal swabs were collected on the sample days (0, 2, 4, 6, 9, 11, and 14). Two pigs were euthanized and lung, heart and nasal turbinates were collected during necropsies performed on days 2, 4, 6, and 9.

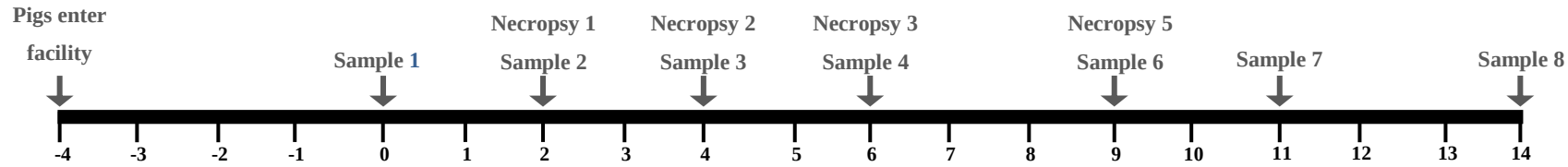


Table 3.1 Molecular detection (qRT-PCR) of PPIV1 in nasal swabs from pigs naturally infected with PPIV1.

All values are indicated as cycle threshold (C_t) values. A value was considered positive if C_t<37.

Pig	Day 0	Day 2	Day 4	Day 6	Day 9	Day 11	Day 14
A1	32	34	36	N.D.	N.D.	N.D.	N.D.
A2	N.D.*	N.D.	30	28	34	35	N.D.
A3	N.D.	N.D.	36	35	N.D.	32	32
A4	31	29	30	34	N.D.		
A5	N.D.	34	33	N.D.	N.D.		
A6	35	N.D.	36	N.D.			
A7	30	26	35	N.D.			
A8	35	29	25				
A9	N.D.	N.D.	N.D.				
A10	N.D.	N.D.					
A11	33	N.D.					

Note: *Not detected, N.D.

Figure 3.3 Phylogenetic analysis of the nucleotide sequences of PPIV1.

(a) nearly complete genome or (b) 1760bp fragment of the HN gene. The phylogenetic trees were constructed by maximum-likelihood analysis with 1,000 bootstrap replicates using MEGA 6.06 software. The bootstrap values are indicated by the numbers above the branches and the scale representing 0.1 nucleotide substitutions per site is in each panel. GenBank accession numbers are in parentheses and abbreviations are as follows: PPIV1, porcine parainfluenza virus 1; HPIV1,3, human parainfluenza 1, 3; SeV, Sendai virus; BPIV3, bovine parainfluenza 3; SPIV3, swine parainfluenza virus 3.

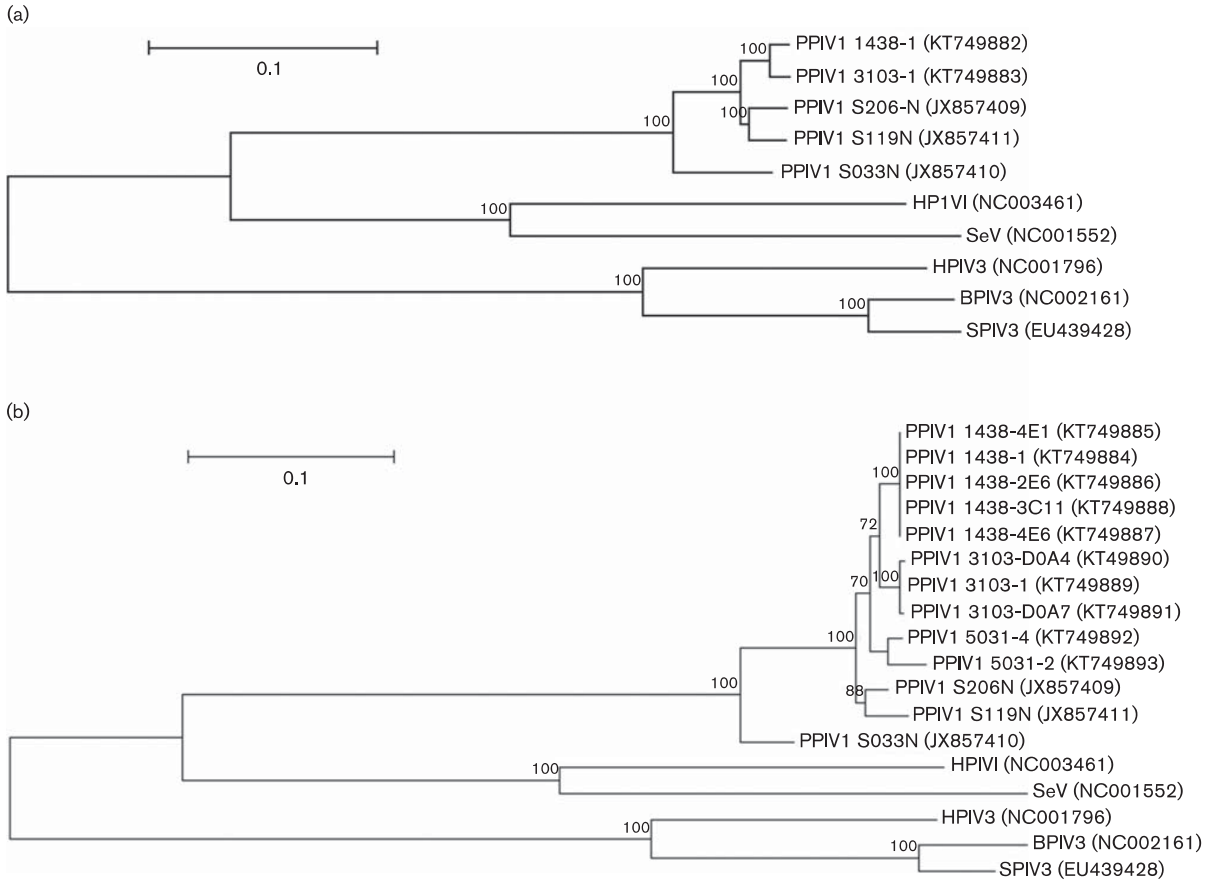
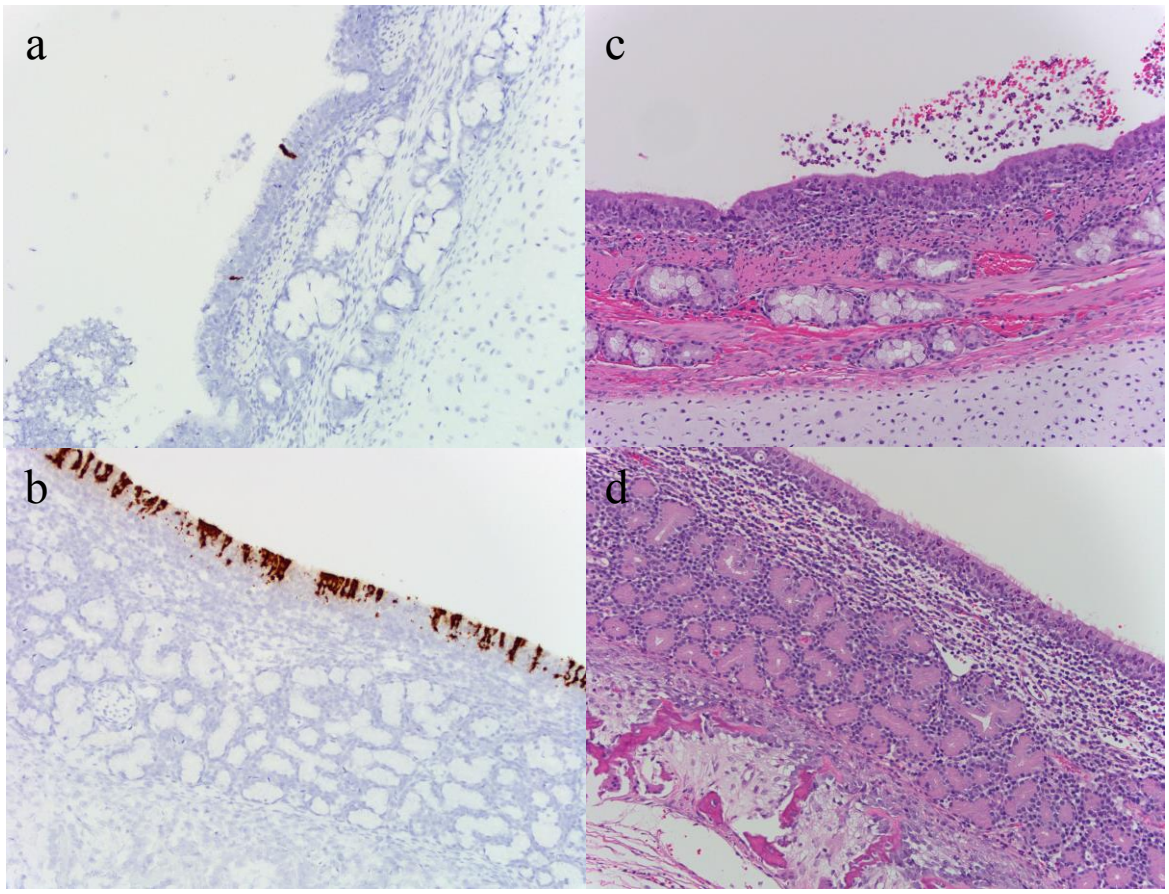


Figure 3.4 *In situ* hybridization analysis and H&E stain of a PPIV1 naturally infected 3-week old pig.

The presence of viral RNA can be seen in the respiratory epithelium of the nasal turbinates (a) of an animal from the pathogenesis study while lower levels are seen in the respiratory epithelium of the trachea (b) of the same animal (pig A8). The nasal turbinate (c) and trachea (d) of a naturally infected PPIV1 positive pig show neutrophils in the respiratory epithelium and lymphocytes and plasma cells in the submucosa surrounding the glands, although to a lesser extent in the trachea. Additionally, a small number of neutrophils are present in the tracheal lumen and there is a loss of cilia multifocally.



Chapter 4 - A novel porcine circovirus distantly related to known circoviruses is associated with porcine dermatitis and nephropathy syndrome and reproductive failure

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Abstract

Porcine circovirus associated disease (PCVAD) is clinically manifested by postweaning multisystemic wasting syndrome (PMWS), respiratory and enteric disease, reproductive failure, and porcine dermatitis and nephropathy syndrome (PDNS). Porcine circovirus type 2 (PCV2) is an essential component of PCVAD although an etiologic role in PDNS is not well established. Here, a novel circovirus, designated porcine circovirus 3 (PCV3), was identified in sows that died acutely with PDNS-like clinical signs. The capsid and replicase proteins of PCV3 share only 37% and 55% identity to PCV2 and bat circoviruses, respectively. Aborted fetuses from

sows with PDNS contained high levels of PCV3 ($7.57x10^7$ genomic copies/ml) and no other viruses were detected by PCR and metagenomic sequencing. Immunohistochemistry (IHC) on sow tissues identified PCV3 antigen in skin, kidney, lung and lymph nodes localized in typical PDNS lesions including necrotizing vasculitis, glomerulonephritis, granulomatous lymphadenitis and bronchinterstitial pneumonia. Further study of archived PDNS tissues, that were negative for PCV2 by IHC, identified 45 of 48 were PCV3 positive by qPCR with 60% of a subset also testing positive for PCV3 by IHC. Analysis by qPCR of 271 porcine respiratory disease diagnostic submissions identified 34 PCV3 positive cases (12.5%), and ELISA detection of anti-PCV3 capsid antibodies in sera found 46 positive samples of 83 tested (55%). These results suggest PCV3 commonly circulates within U.S. swine and may play an etiologic role in reproductive failure and PDNS. Due to the high economic impact of PCV2, this novel circovirus warrants further studies to elucidate its significance and role in PCVAD.

Importance

While first identified in sporadic cases of postweaning multisystemic wasting syndrome in Canada in the early 1990's, an epidemic of severe systemic disease due to porcine circovirus type 2 (PCV2) spread worldwide in the ensuing decade. Despite being effectively controlled by commercial vaccines, PCV2 remains one of the most economically significant viruses of swine. Here, a novel porcine circovirus (PCV3), which is distantly related to known circoviruses, was identified in sows with porcine dermatitis and nephropathy syndrome (PDNS) and reproductive failure. PCV2, which has previously been associated with these clinical presentations, was not identified. High levels of PCV3 nucleic acid was observed in aborted fetuses by quantitative PCR (qPCR) and PCV3 antigen was localized in histologic lesions typical of PDNS in sows by

immunohistochemistry (IHC). PCV3 was also identified in archival PDNS diagnostic cases which previously tested negative for PCV2 using IHC. The emergence of PCV3 warrants further investigation.

Introduction

Circoviruses (family *Circoviridae*, genus *Circovirus*) are circular, single stranded DNA viruses that are the smallest known autonomously replicating viruses (King *et al.*, 2012). Circoviruses have an ambisense genome organization encoding two major open reading frames (ORFs), *rep* and *cap*, on opposite strands of the double stranded DNA replicative intermediate. While the initial descriptions of circoviruses were mainly from avian species, numerous members of the family *Circoviridae* have been characterized in fish, insects and mammals (Garigliany *et al.*, 2015; Li *et al.*, 2010; Lőrincz *et al.*, 2012). Two species of circovirus are known to infect pigs. Porcine circovirus type 1 (PCV1) was first identified as a cell culture contaminate and has not been associated with clinical disease (Cheung, 2003a). In contrast, porcine circovirus type 2 (PCV2) is a ubiquitous, economically significant pathogen which has been associated with a diverse range of clinical diseases (Clark, 1996; Harding, 1996). Despite the marked difference in pathogenicity of the two porcine circoviruses, the nucleotide sequence identity between PCV1 and PCV2 *rep* and *cap* genes is ~83% and 67%, respectively (Cheung, 2003b).

PCV2 was first sporadically identified in pigs with postweaning multisystemic wasting syndrome (PMWS) in Canada in the mid 1990's (Harding, 1996). Epidemics of severe systemic disease, clinically presenting as wasting and failure to thrive, were subsequently observed in Europe and Asia followed by North America (Allan *et al.*, 1998a, b; Choi *et al.*, 2000; Ellis *et*

al., 1998; Harding, 1996; Kiupel *et al.*, 1998; Sato *et al.*, 2000; Segalés *et al.*, 1997). Histologic lesions typified by lymphoid depletion and lymphohistiocytic or granulomatous inflammation of multiple organs were described. PMWS diagnosis requires PCV2 antigen detection by immunohistochemistry (IHC) or *in situ* hybridization (ISH) (Sorden, 2000). PCV2 infection was subsequently associated with other clinical presentations, together termed porcine circovirus associated disease (PCVAD), which includes PMWS, pneumonia, porcine dermatitis and nephropathy syndrome (PDNS) and reproductive failure (Harding, 2007; Harms *et al.*, 2002; Kim *et al.*, 2003; Madson & Opriessnig, 2011; Rosell *et al.*, 1999).

The wide range of clinical disease under the PCVAD umbrella is in part due to co-infecting pathogens. By itself, PCV2 infections are typically subclinical (Segalés, 2012). Reproduction of PMWS in controlled conditions has included co-infection with pathogens such as porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV) or *Mycoplasma hyopneumoniae* (Allan *et al.*, 1999; Segalés *et al.*, 2004). While PDNS is considered a PCVAD due to the association between clinical disease and PCV2 detection, the etiologic role of PCV2 has yet to be established. Clinically, PDNS is distinctive, with apparent well demarcated red to dark macules and papules. The kidneys are often enlarged, pale and present cortical petechial. Histologically, PDNS is characterized by systemic necrotizing vasculitis along with glomerulitis and interstitial nephritis (Drolet *et al.*, 1999; Smith *et al.*, 1993; Thibault *et al.*, 1998). The pathogenesis of this systemic vasculitis as well as the fibrinous glomerulonephritis is thought to be immune-complex mediated. Importantly, PDNS has been reproduced experimentally without PCV2 using PRRSV and a tissue homogenate containing torque teno virus (TTV) (Krakowka *et al.*, 2008).

In 2015, sows on a farm located in North Carolina with chronic reproductive problems, including above average sow mortality and below average conception rates, experienced increased sow mortality with clinical signs consistent with PDNS. Concurrently, aborted mummified fetuses were collected from sows on the same farm with similar clinical signs. While histological lesions in the sows were consistent with PDNS, the tissues all tested negative for PCV2 by IHC and quantitative PCR (qPCR). Metagenomic sequencing revealed the presence of a novel, genetically divergent circovirus, designated porcine circovirus 3 (PCV3). In addition to this outbreak study, we performed a retrospective study in cases with lesions consistent with PDNS but negative for PCV2 by IHC and evaluated the prevalence of PCV3 nucleic acids in swine diagnostic submissions and anti-PCV3 antibodies in swine sera. The results obtained in these studies showed that PCV3 is widely distributed in the U.S. swine population.

Materials and Methods

Ethics Statement

Porcine tissue and sera were collected as part of a routine diagnostic investigation by licensed veterinarians and submitted to Kansas State Veterinary Diagnostic Laboratory (KSVDL) or Iowa State University Veterinary Diagnostic Laboratory (ISUVDL). Mouse immunizations were performed following a protocol approved by the Kansas State University Institutional Animal Care and Use Committee protocol.

Clinical outbreak of PDNS

Metagenomic sequencing was performed on a tissue homogenate consisting of three mummified fetuses originating from a commercial swine operation in North Carolina. The mummified fetuses were aborted from sows with skin lesions consistent with PDNS and were

sent to ISUVDL where they were negative for PCV2, PRRSV and PPV by quantitative polymerase chain reaction (qPCR). Tissue samples were also obtained from sows on the same farm that died with clinical signs of PDNS and were negative for PCV2, PRRSV and influenza A virus (IAV) by qPCR. The sows on the farm received multiple doses of a commercial PCV2 vaccine prior to first breeding but were not routinely vaccinated for PCV2 pre-farrowing.

PDNS retrospective study

A retrospective study was conducted on a total of 74 paraffin-embedded fixed tissues from 48 cases with histological lesions consistent with PDNS submitted to ISUVDL between 2010 and 2016. All samples selected for the retrospective study were negative for PCV2 by IHC.

PCV3 seroprevalence and nucleic acid detection in cases not associated with PDNS

A retrospective serological study was performed to establish seroprevalence and geographical distribution of PCV3. A total of 150 serum samples, representing at least eight states and Mexico (Iowa [n=19], Illinois [n=3], Indiana [n=1], Minnesota [n=1], Mexico [n=5], North Carolina [n=31], Nebraska [n=17], Oklahoma [n=31] and unknown [n=42]), were evaluated by a recombinant PCV3 ELISA. Sera (n=18) from 3-week old pigs from a specific pathogen free research herd that tested negative by PCV3 qPCR were used as negative controls. The prevalence of PCV3 in U.S. swine was investigated with 271 lung homogenate, oral fluid or nasal swab samples that were submitted to ISUVDL for respiratory disease diagnostic testing.

Metagenomic Sequencing

Metagenomic sequencing was performed as previously described (Hause *et al.*, 2015b; Neill *et al.*, 2014). First-strand cDNA synthesis of viral DNA was performed using Superscript III first-strand synthesis kit per the manufacturer's instructions with previously published primers (Allander *et al.*, 2005). Amplification of the double stranded cDNA was performed with

primers identical to those used for first strand synthesis but lacking the random hexamer and TaKaRa DNA polymerase.

Genetic Analysis

Raw sequence reads were separated based on barcodes added during library preparation and analyzed in CLC genomics Workbench (CLC Genomics version 7.0). Reads mapping to the host genome (*Sus scrofa*) were removed and unmapped reads were assembled *de novo* into contigs. Contigs were evaluated by BLASTN. Sequences were aligned using ClustalW and phylogeny was inferred using a Maximum likelihood method with the best fit general time reversible model with gamma distribution and invariant sites verified by 1000 bootstrap replicates.

Development of qPCR for specific detection of the PCV3 capsid gene

Viral DNA was isolated from clinical specimens using the MagMax-96 total nucleic acid isolation kit according to the manufacturer's protocol. Nucleic acid from formalin-fixed, paraffin-embedded tissues was extracted with the QIAamp DNA FFPE Tissue kit as instructed by the manufacturer (Qiagen, Valencia, CA). A 5'- nuclease assay was designed to target a 112bp region of the PCV3 *cap* gene nucleic acid in samples: probe, 5'-FAM-ACC CCA TGG-Zen- CTC AAC ACA TAT GAC C-Iowa Black-3'; Forward, 5'- AGT GCT CCC CAT TGA ACG-3'; Reverse, 5'-ACA CAG CCG TTA CTT CAC-3'. Quantitative PCR was performed with the Qiagen Quantitect PCR kit under the following conditions: 95°C, 15 minutes; and 45 cycles of 94°C, 15 seconds and 60°C for 60 seconds. The sensitivity and specificity of the assay was determined using a dilution series of a plasmid (pSF-CMV-cap) containing the entire PCV3 *cap* gene cloned into pSF-CMV-AMP (Oxford Genetics, UK) and PCV2 nucleic acid extracted

from cell culture. The cycle threshold (C_t) values determined from the plasmid dilution series were used to create a standard curve for the calculations to determine genomic copies/ml (gc/ml).

The complete genome for PCV3 was determined from a fetal tissue homogenate pool from the North Carolina PDNS-outbreak and from a sample in the prevalence study using Sanger sequencing of four overlapping amplicons generated with primers shown in table 1. The PCR was performed using TaKaRa Taq™ as follows: 94°C, 4 minutes; followed by 40 cycles of 94°C, 20 seconds; 50°C, 30 seconds; 72°C, 1 minute; and 72°C for 5 minutes. Sequencing to confirm select PCV3 positive samples was performed using a 330bp internal *cap* gene primer set: 5'-CCA CAG AAG GCG CTA TGT C-3' and 5'-CCG CAT AAG GGT CGT CTT G-3'. The *cap* gene PCR reactions were performed using TaKaRa Taq™ as outlined above. PCR products were Sanger sequenced for verification.

Virus Isolation

Virus isolation was attempted on swine testicles cells (ST) and porcine kidney cells (PK-15) maintained in minimal essential media (MEM) supplemented with L-glutamine and 5% fetal bovine sera. Cells were seeded onto 6-well plates (60-80% confluent) and 100µl of sample were inoculated into 1 ml viral replacement media, which consisted of MEM and penicillin-streptomycin solution. Cells were observed daily for cytopathic effects and PCV3 growth was monitored by qPCR and immunofluorescence.

Cloning, Expression and Purification of PCV3 capsid protein

To create the recombinant PCV3 *cap* construct, primers were designed to amplify a portion of the PCV3 gene encoding amino acids (aa) 35-214: F, 5'-AAA AAA GCT AGC GCT GGA ACA TAC TAC ACA-3'; R, 5'-AAA AAA GAA TTC TTA GAG AAC GGA CTTGTA ACG-3'. The 5' ends of the forward and reverse primers contained *NheI* and *EcoRI* restriction

sites (underlined), respectively. PCR products were cloned into the pET28a (Novagen, Madison, WI) vector to enable expression of the N-terminal truncated PCV3 *cap* as an N-terminal 6x histidine (His) fusion protein in *E.coli.* The pET28a-cap plasmid was transformed into *E.coli* BL21 (DE3) cells and expressed as previously described (Wu *et al.*, 2016). After expression, bacteria were harvested by centrifugation and lysed using the B-PER reagent (Pierce, Rockford, IL) following the Ni-NTA agarose (Qiagen, Valencia, CA) purification was completed as specified by the manufacturer. The purity and identity of the recombinant protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and Western blot detecting the His-tag at the N-terminal of the recombinant protein.

Production and *in vitro* characterization of an anti-PCV3 capsid monoclonal antibody

BALB/c mice were immunized with purified, truncated capsid protein (35-214aa). The mice were inoculated with 50μg of antigen mixed with Freund's incomplete adjuvant bi-weekly for a total of eight weeks. Subsequently, mouse splenocyte cells were fused with NS-1 myeloma cells. Monoclonal antibodies (MAbs) specific to PCV3 were identified by immunofluorescence antibody assays (IFA) using HEK293 cells expressing native PCV3 *cap*. HEK293 cells on a 6-well plate maintained in MEM with 5% fetal bovine serum (FBS) and antibiotics (ciprofloxacin, penicillin, streptomycin and gentamycin) were transfected with pSF-CMV-AMP or a plasmid derived from pSF-CMV-AMP which contained the complete PCV3 *cap* gene cloned into the *XhoI* restriction site (pSF-CMV-cap). On a 6 well plate, 10^6 cells were transfected with Lipofectamine™ 2000 (Invitrogen), per the manufacturer's instructions, and 10μg of DNA. After 48 hours, the plates were fixed with 80% acetone for 10 minutes at room temperature (RT) and allowed to dry. Transfected cells were incubated with undiluted anti-PCV3-cap MAbs at 37°C

for 1 hour, followed by three washes with phosphate buffered saline (PBS), and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc. West Grove, PA) diluted 1:100 in PBS at 37°C for one hour. Following a final wash, cells were visualized using an Eclipse TE2000-U inverted fluorescent microscope (Nikon). ST cells infected with PCV2 were used to evaluate the specificity of the PCV3 MAbs.

PCV3 detection and immunohistochemistry on PDNS cases

Tissues were fixed in 10% neutral buffered formalin at RT and tissue sections were embedded in paraffin until sectioning. The immunohistochemistry was performed via the standard protocol and slides were visualized with a LSM 700 confocal scanning microscope (Zeiss).

Development of a recombinant PCV3 capsid ELISA

The ELISA was performed similar to assays previously described using 2μg/ml of purified recombinant PCV3 capsid protein per well to coat the Corning EIA/RIA High-binding plates (Hause *et al.*, 2015a; Lin *et al.*, 2005; Palinski *et al.*, 2015)

Genbank Accession Numbers

The full genome sequences of PCV3 used in the phylogenetic analyses have been submitted to Genbank under the accession numbers KT869077 (PCV3 strain 29160) and KX458235 (PCV3 strain 2164). Previously published sequences used for phylogenetic analysis include: Barbel circovirus (BarCV) GU799606; Bat circovirus-1 (BtCV-1) JX863737; Bat circovirus-2 (BtCV-2) KC339249; Bat circovirus-3 (BtCV-3) JQ814849; Beak and Feather disease virus (BFDV) AF080560; Canary circovirus (CaCV) AJ301633; Canine circovirus (CanineCV) KC241983; Chimpanzee faeces associated circovirus (ChfaCV) GQ404851; Duck circovirus (DuCV) AY228555; European catfish circovirus (EcatfishCV) JQ011377; Finch

Circovirus (FiCV) DQ845075; Goose circovirus (GoCV) AJ304456; Gull circovirus (GuCV) DQ845074; Human faeces associated circovirus (HufaCV) GQ404856; Mink circovirus (MiCV) KJ020099; Columbid circovirus (PiCV) AF252610; Porcine circovirus 1 (PCV1) Y09921; Porcine circovirus 2(PCV2) AF027217; PorkNW2/USA/2009 HQ738638; Raven circovirus (RaCV) DQ146997; Starling circovirus (StCV) DQ172906; Swan circovirus (SwCV) EU056309; Zebra finch circovirus (ZfiCV) KP793918.

Results

Clinical and histological findings of an outbreak of PDNS-like disease in a commercial sow farm

During June 2015, a commercial swine operation in North Carolina experienced a 10.2% increase in sow mortality rate and 0.6% decrease in conception rate compared to historical farm averages due to an outbreak of PDNS. Clinically, affected sows were anorexic, and presented multifocal papules, macules and superficial dermatitis (Figure 4.1A). Tissue samples were submitted to the ISUVDL for diagnostic testing. Histologically, skin lesions were characterized by acute necrotic dermatitis and epidermatitis associated with lymphoplasmacytic perivascular cuffs. The kidneys displayed dilated cortical tubules, attenuation and regeneration of the tubular lining epithelium, and large clusters of lymphocytes and macrophages diffusely infiltrated the cortical interstitium and glomeruli. The farm experienced an increment of 1.19 aborted mummified fetuses per litter above the historical average abortion rate. The aborted litters contained mummified fetuses of varied gestational age, consistent with those previously described in PCV2 associated abortion (Figure 4.1B) (Pensaert *et al.*, 2004). While the gross and histological lesions observed in sows, as well as the presence of abortions, were consistent with PCVAD, all sow tissues, including kidney, lymph node, lung and skin, tested negative by IHC

and qPCR for PCV2, PRRSV and IAV. In addition, fetal tissues were negative for PCV2, PRRSV and PPV by qPCR.

Metagenomic Sequencing

A tissue homogenate pool, prepared from the three fetuses, was analyzed by viral metagenomic sequencing. The Miseq run generated 989,478 total reads with 926,380 mapping to the host genome, *Sus scrofa*. The remaining reads were assembled *de novo* resulting in 27 contigs. Approximately 54% of the reads mapped to a 1,246bp contig which was 98% similar to a partial circovirus genome identified in commercial ground pork, PorkNW2/USA/2009 (accession HQ738638), when analyzed by BLASTN. The remaining reads showed no similarity to any known eukaryotic virus.

Metagenomic sequencing was also performed on a pooled tissue homogenate from the sows with PDNS-like lesions. *De novo* assembly of sequences not mapping to the *Sus scrofa* reference (approximately 2.5 million sequences) yielded 735 contigs, which were analyzed by BLASTN. Two contigs had ~97% identity to torque teno virus 1 (TTV-1). Assembly using a PCV3 reference (KT869077) identified four reads mapping to the genome. The remaining reads showed no homology to known eukaryotic viruses.

Genetic Analysis

Rolling circle amplification followed by PCR and Sanger sequencing of the resulting amplicons allowed assembly of a 2,000 nucleotide (nt) circular genome from the fetal tissue homogenate. ORF analysis identified three ORFs encoding proteins greater than 200 amino acids (aa), with two ORFs showing homology to circovirus rep and cap proteins by BLASTP, orientated in opposite directions (Figure 4.2A). Within the 235 nt 5'-intergenic region between the *rep* and *cap* ORFs on the *rep* gene strand was a predicted stem-loop structure with a 9 nt

stem and loop nonomer identical to PCV1 (TAGTATTAC) (Li *et al.*, 2010). Following convention, the "A" residue at position 8 in the loop nonamer was defined as position "1" in the genome (Todd *et al.*, 2001).

The largest ORF encoded a predicted 297 aa protein which by BLASTP was 69.4% identical to a partial replicase protein of Circoviridae PorkNW2/USA/2009 (accession ADU77001, 221 aa) and 54% identical to a bat circovirus from China (accession AIF76248, 293 aa). The PorkNW2/USA/2009 genome was obtained from commercial pork meat products and encodes for a complete replicase and partial capsid gene most similar in organization and sequence to circoviruses (Li *et al.*, 2011). Conserved circovirus replicase and helicase domains were identified by BLASTP from aa 9-93 and 162-251, respectively, in the *rep* ORF. Further examination of the rep ORF protein sequence revealed conserved rolling circle replication (RCR) motifs and a P-loop motif similar to GoCV and PiCV (Ilyina & Koonin, 1992; Todd *et al.*, 2001). Of the three RCR motifs conserved among circoviruses, in PCV3 the FTLNN motif contained a single mutation, present as FTINN. This mutation is seen in other circoviruses such as GoCV (Todd *et al.*, 2001). The two other RCR motifs, HLQG and YCKK, are present in PCV3 as well. Moreover, three motifs conserved among circovirus replicase proteins but having unknown function were identified in PCV3 including, WWDGY (amino acids 196-200), DDFYGWVP (amino acids 209-216), and DRYP (amino acids 225-228). Interestingly, a canonical start codon was not identified. A GTC codon (encoding valine) is present at 5'-end of the ORF with the closest in-frame ATG present approximately 400 bp downstream. This alternative start codon was also seen in PorkNW2/USA/2009. Alternative initiation codons have been proposed for a number of avian circoviruses, including goose circovirus, pigeon circovirus

and beak and feather disease virus (Bassami *et al.*, 2001; Niagro *et al.*, 2014; Phenix *et al.*, 2001).

The putative *cap* ORF, in the opposite orientation of *rep*, encodes a 214 aa protein 87% identical to the partial capsid sequence (110 aa) of PorkNW2/USA/2009 and 36-37% identical to PCV2 and duck circoviruses (233 and 257 aa, respectively) by BLASTP (Fig.2A). Similar to other circovirus capsid proteins, the N-terminus contained numerous arginine residues and was highly basic. A conserved circovirus capsid domain was identified by BLASTP from aa 26-173. Additionally, the PCV3 cap protein had no predicted N-linked glycosylation sites but two predicted O-linked glycosylation sites at aa 146 and 150 (S and T, respectively). This contrasts to PCV2 which has two experimentally verified N-linked glycosylation sites (Lv *et al.*, 2014).

The third ORF, oriented on the same strand as the predicted *rep*, encodes a 231 aa protein that is 94% identical to an ORF identified in PorkNW2/USA/2009 and 39% identical to Murid herpesvirus M169, a protein of unknown function (Zhang *et al.*, 2014). Similar to *rep*, the initiation codon for ORF3 is unclear. The codon at the 5'-end is TCG (encoding serine). A methionine at ORF3 aa 55 is an alternate possible initiation site which would yield a 177 aa protein.

Owing to the genetic and structural similarity to the genus *Circovirus* and the <70% capsid aa identity to other species, we propose the novel species as porcine circovirus 3 (PCV3) (King *et al.*, 2012). The PCV3 genome sequences were submitted to Genbank under accession KT869077 and KX458235.

Phylogenetic Analysis

To investigate the evolutionary relationship of PCV3 to other members of *Circoviridae*, genome sequences from twenty-three members of the family and two PCV3 genomes were

analyzed. An analysis of the full circovirus genomes grouped both PCV3 sequences in a clade with PorkNW2/USA/2009 separate from all other members of the genus (Figure 4.2B). The phylogeny of the sequences indicated that PCV3 was most closely related to canine circovirus (KC241983), however, this lacked strong bootstrap support. The phylogeny also suggested that PCV3 and canineCV shares a common ancestor with a clade containing PCV1, PCV2 and BatCV-2 (KC339249). With the exception of human circovirus (HufaCV, GQ404856), mammalian and avian circoviruses belonged to separate, well supported clades.

Detection of PCV3 by PCR

To confirm the presence of PCV3 in porcine samples, a 5'-nuclease assay was designed to determine the presence of the PCV3 *cap* gene. The fetal tissue homogenate samples from the outbreak in North Carolina were strongly positive for PCV3 with C_t values between 16.7 and 21.3, corresponding to high levels of PCV3 of approximately to $1.88x10^8$ and $7.55x10^6$ gc/ml. Tissues from three of the sows with PDNS-like lesions were positive for PCV3, having between $2.13x10^4$ and $8.62x10^4$ gc/ml. In addition, 30 serum samples submitted to ISUVDL were analyzed for PCV3 by qPCR. The serum samples were positive for PCV3 with $5.63x10^2$ to $2.28x10^4$ gc/ml. The serum sample with the highest PCV3 titer was used amplified by PCR to generate overlapping amplicons to obtain a second complete PCV3 genome which was 99.0% identical to the original North Carolina PCV3. This second PCV3 genome was submitted to Genbank under accession KX458235.

Additionally, to investigate the prevalence of PCV3, a total of 271 samples submitted to ISUVDL for respiratory disease diagnostic testing, were analyzed by qPCR. Thirty-four (12.5%) of the samples were positive with titers of $3.00x10^2$-$1.52x10^7$ gc/ml.

Characterization of PCV3-cap MAb14

HEK293 cells transfected with the pSF-CMV-cap were incubated separately with four different MAb clonal cell supernatents and screened by IFA. Fluorescence localized to the nucleus (Figure 4.3A), as expected owing to the predicted highly basic nuclear localization signal (Liu *et al.*, 2001), was observed for clone 14 (MAb14). No fluorescence was observed for cells transfected with pSF-CMV-Amp (Figure 4.3B). In addition, ST cells infected with PCV2 had no detectable fluorescence.

Virus Isolation

Virus isolation was attempted on ST and PK-15 cells. Cells were inoculated with filtered fetal tissue homogenates and passaged three times. No cytopathic effects were evident and Ct values increased with each successive passage. No fluorescence was evident by IFA using MAb14.

Histological lesions associated with the presence of PCV3 antigen in PDNS cases

Tissue samples from North Carolina sows with PDNS-like lesions and archived PDNS cases were examined by H&E staining and IHC using PCV3 MAb14. Lungs showed variable degrees of bronchointerstitial pneumonia occasionally complicated by a secondary suppurative bronchopneumonia. The small and medium-size airways and small blood vessels were cuffed with peribronchiolar and perivascular aggregates of lymphocytes and plasma cells. Adjacent alveolar septa were infiltrated by lymphocytes and plasma cells. Within alveolar lumina there were abundant intraluminal edema intermixed with moderate numbers of foamy macrophages, rare multinucleated giant cells, and small clusters of neutrophils. Occasional lymphocytes and scatter macrophages showed moderate intracytoplasmic immunostaining against PCV3. In a section of skin, the dermis and subcutis presented necrotizing vasculitis with fibrinoid change

and transmural neutrophilic infiltration, hemorrhage, and fibrin exudation (Figure 4.4A). The inflammatory infiltrate often extended into the surrounding dermis and subcutis and often cuffed around vessels and dermal adnexa. Occasionally, the epidermis was hyperplastic with mild orthokeratotic hyperkeratosis. The dermal lymphocytic infiltration showed marked intracytoplasmic immunostaining against PCV3 (Figure 4.4B). Minimal background staining was evident when PCV3MAb14 was replaced with PBS (Figure 4.4C).

The lymph nodes showed diffuse granulomatous lymphadenitis, characterized by moderate cortical lymphoid depletion, occasionally replaced by histiocytes and numerous multinucleated giant cells (Figure 4.4D). The follicular and perfollicular lymphocytic population showed a diffuse, intense intracytoplasmic staining against PCV3 (Figure 4.4E) as compared to background staining where PCV3 MAb14 was replaced with PBS (Figure 4.4F) or lymph node tissue from a PCV3 qPCR negative pig (Figure 4.4G).

Kidneys presented diffuse membrane proliferative glomerulonephritis (Figure 4.4H), characterized by glomerulosclerosis and thickening of the Bowman's capsules, attenuation of the cortical tubules and variable interstitial fibrosis. The tubules were occasionally ectatic, lined by attenuated epithelium and occasionally presented marked proteinosis. Scattered throughout the sections were small to medium size clusters of lymphocytes and plasma cells in the interstitium. The tubular epithelium showed random areas of positive staining against PCV3 (Figure 4.4I). Minimal background fluorescence were observed for slides mock stained with PBS and goat anti-mouse FITC (Figure 4.4J) or kidney tissue from a PCV3 qPCR negative pig (Figure 4.4K).

Detection of PCV3 nucleic acid in tissues with PDNS lesions

To further investigate the etiologic role of PCV3 in PDNS, 48 cases with histological lesions consistent with PDNS that previously tested negative for PCV2 by IHC were evaluated.

Tissue scrolls from paraffin-embedded tissue blocks were assayed for PCV3 by qPCR. Forty five (93.8%) of the cases were positive for PCV3 with viral titers 1.60-3.47x10^4 gc/ml. To confirm these results, five of the samples with the highest viral titers were analyzed with a PCR targeting a 330bp fragment of the *cap* gene. The target amplicon was amplified in all samples evaluated and amplicon product sequences showed 100% identity with the PCV3 genome sequence. Tissues from five PCV3 PCR-positive cases were tested by PCV3-IHC. Three of the five were positive. All five cases were PCV2-qPCR positive.

PCV3 Seroprevalence

The prevalence of anti-PCV3 cap antibodies in swine sera were examined by ELISA using rPCV3-cap antigen. Eighteen sera samples from 3-week old pigs obtained from a specific pathogen free herd that tested PCV3 qPCR negative were used as negative controls and had an average absorbance of 0.49. The cutoff value differentiating positive and negative sera was determined as three standard deviations above the mean of the negative controls (0.87). Sera from ten sows from the farm in North Carolina collected three months after the PDNS outbreak were all positive with an average absorbance of 1.27. Additionally, 27 sera from gilts from a farm that supplies replacement animals to the sow farm were also tested, with seventeen animals (63%) having absorbances of 0.88-1.37. Anti-PCV3 cap antibodies were detected in 47 of 83 (56.6%) of samples submitted for unrelated diagnostic testing from multiple states. Of the positive samples, 13 originated from Iowa, one from Indiana, five from Mexico, four from North Carolina, five from Nebraska, one from Oklahoma and 18 were of unknown origin.

Discussion

First described in 1993 in Europe, PDNS has been reported in numerous countries worldwide (Meehan *et al.*, 2001; Smith *et al.*, 1993; White & Higgins, 1993). Although the prevalence of disease within a herd is typically low (<1%), mortality for afflicted pigs can be high. The incidence of PDNS may exceed that of PMWS in Europe and the United Kingdom (Gresham *et al.*, 2000). Although the etiology of PDNS is unknown, PCV2 nucleic acid is commonly detected in affected pigs using qPCR, while PCV2 antigen is inconsistently detected (Segalés *et al.*, 1998; Thibault *et al.*, 1998). This has led to speculation on the role of PCV2 in PDNS (Segalés & Domingo, 2002; Thomson *et al.*, 2001). Here, we identified a highly divergent new species of porcine circovirus, designated PCV3, from mummified fetuses aborted from sows with PDNS-like lesions and from sows which died acutely with clinical signs consistent with PDNS. PCV3 was the only virus identified by metagenomic sequencing of pooled fetal tissue, which was confirmed by qPCR. Cycle threshold values of 16.7-21.3 in fetal tissue pools indicate high viral titers of $7.55x10^6$-$1.88x10^8$ gc/ml of sample. There is a correlation between PCV2 titer in the fetuses and reproductive disease, with PCV2 levels of 10^7 PCV2 DNA copies/500ng fetal tissue or more associated with PCV2-associated reproductive failure including mummification (Brunborg *et al.*, 2007; Hansen *et al.*, 2010; Madson *et al.*, 2009; Segalés, 2012). Based on the amount of PCV3 nucleic acids and tissue distribution detected during this outbreak, we hypothesize a similar correlation for PCV3 and reproductive failure.

PCV3 was also detected by PCR and IHC in skin, lung, kidney, and lymph nodes of sows with PDNS-like lesions. The low amount of PCV3 in the sow serum samples (Ct=27.7-29.7) are likely responsible for the low number of PCV3 sequences identified by metagenomic

sequencing. Both PCR, IHC and metagenomic sequencing failed to identify PCV2 in the samples collected from North Carolina. These results suggest that PCV3 infection might contribute to the PDNS-like lesions and the presence of abortion and PCV3 in fetuses is the result of vertical transmission. In support of this potential etiologic role for PCV3 in PDNS lesions, screening of archived PCV2 IHC-negative PDNS cases found that PCV3 nucleic acid was highly prevalent (93.8%) and three out of the five cases examined for PCV3 by IHC were positive. We speculate the relatively low titers of PCV3 in the tissues may limit consistent detection.

Attempts to reproduce PDNS experimentally with PCV2 have been unsuccessful, however, PDNS has been reproduced experimentally in the absence of PCV2 using PRRSV and a tissue homogenate containing TTV (Krakowka *et al.*, 2008). Relatively little is known on the clinical significance of TTV infection. TTV is ubiquitous in pigs worldwide (Bigarré *et al.*, 2005; McKeown *et al.*, 2004; Taira *et al.*, 2009). While TTV is commonly detected in healthy pigs, several studies have suggested TTV infection moderates disease severity during co-infections. For example, inoculation of pigs with a tissue homogenate containing TTV followed by PCV2 resulted in PMWS while mono-infections did not (Krakowka *et al.*, 2008). The North Carolina sows with PDNS were infected with both PCV3 and TTV1. The impact of TTV1 co-infection is unknown. Interestingly, 93.8% (45 of 48) of the PDNS cases that we screened due to their PCV2 negative status by IHC were positive for PCV2 by PCR. It is unclear whether co-infections with genetically diverse small circular DNA viruses such as TTV1 (*Anelloviridae*), PCV2 and PCV3 influence the development of PDNS. The additive effects on disease severity of co-infections with PCV2 have been demonstrated for PMWS (Allan *et al.*, 1999, 2000; Kennedy *et al.*, 2000; Pogranichniy *et al.*, 2002). A co-infection of PPV and PCV2 has been shown to exacerbate disease (Allan *et al.*, 1999). PCV2 and PRRSV co-infections are a significant

component of the porcine respiratory disease complex (Allan *et al.*, 2000). Infection of a herd with these agents causes severe respiratory disease and economically devastating sow abortions and mortality.

The pathogenesis of PDNS, which includes characteristic necrotizing vasculitis, is thought to be a manifestation of an immune complex-mediated disorder involving PCV2 (Lee *et al.*, 2015; Wellenberg *et al.*, 2004). In a case control study, all pigs with clinical signs of PDNS were PCV2-PCR positive and had PCV2 antibody titers significantly higher than clinically normal pigs (60). Examination of the kidneys of PDNS positive pigs found increased fibrinoid deposits in the glomerula consisting of accumulated IgG1, IgG2, IgM, and complement factors C1q and C3, as compared to clinically normal pigs. Although PCV2 antigen was identified in lung tissue of these PDNS pigs by IHC, PCV2 antigen was not identified in the immune complexes. This result is similar to the inconsistent detection of PCV2 in renal tissues of PDNS pigs reported by others (Drolet *et al.*, 1999). Viral infections are known to contribute to immunological disorders, of which Aleutian Mink Disease (AMD), caused by Aleutian Mink Disease Virus (AMDV), has a similar pathogenesis to PDNS (Aasted, 1985; Wellenberg *et al.*, 2004). The pathogenesis of AMD has been associated with the overproduction of AMDV-specific IgG antibodies (Aasted, 1985). A role for PCV3 in a possible immune complex-mediated disorder resulting in the pathogenesis of PDNS needs further study.

PCV2 is one of the most economically significant swine viral pathogens worldwide (Gillespie *et al.*, 2009). The most common genotypes associated with PCVAD are PCV2a and PCV2b (de Boisséson *et al.*, 2004; Gagnon *et al.*, 2007; Olvera *et al.*, 2007). Before 2003, PCV2a was the principle genotype identified in the U.S. and Canada, although both PCV2a and PCV2b were found internationally (Beach & Meng, 2012; Gauger *et al.*, 2011; Patterson &

Opriessnig, 2010). Around 2003, a drastic shift occurred in the frequency of PCV2 genotypes globally from PCV2a to PCV2b coincident with severe systemic disease associated with PCV2b (Cheung *et al.*, 2007; Gauger *et al.*, 2011). The worldwide epidemic was successfully controlled with the development of commercial vaccines, which contain PCV2a antigen, that have been shown to be cross protective (Fort *et al.*, 2008; Opriessnig *et al.*, 2009). More recently, the novel genotype PCV2d, first detected in Switzerland in 1999, spread to China followed by the U.S (Grierson *et al.*, 2004; Wang *et al.*, 2009; Xiao *et al.*, 2012). Similar to seen with PCV2b, epidemiological studies suggest a genotype shift is in process with resulting reports of PCV2 vaccine failure and increased clinical disease (Grau-Roma *et al.*, 2012; Xiao *et al.*, 2015). PCV2d has been implicated in a more severe clinical signs and lesions (Guo *et al.*, 2012).

Circoviruses species are a genetically diverse and infect a broad range of hosts with documented cross-species transmission (Li *et al.*, 2010). Phylogenetic analysis suggests a closest evolutionary relationship between PCV3 and CanineCV. Interestingly, CanineCV was identified in the liver of a dog displaying necrotizing vasculitis and granulomatous lymphadenitis, both of which were observed in PCV3 infected sows as well as reported in PCV2 infections (Li *et al.*, 2013; Segalés, 2012). Similar to CanineCV, attempts to propagate PCV3 *in vitro* were unsuccessful. It is unclear whether PCV3 has been evolving in pigs undetected for some time or whether it originated via cross-species transmission or has arisen via recombination between unidentified parental circoviruses. PCV2 is capable of crossing species barriers causing fatal disease in species other than swine; a recent report identified PCV2 in six mink that died of diarrhea in China (Wang *et al.*, 2016).

The discovery of a novel porcine circovirus with a likely etiologic role in PDNS and reproductive failure is disconcerting. Retrospective studies suggest that PCV2 caused

sporadically systemic disease as earlier as 1985 before becoming an epidemic in the late 1990's. The possibility that PCV3 is on a similar trajectory deserves further research. Importantly, given the approximately 30% identity between the PCV2 and PCV3 capsid proteins, cross protection seems unlikely.

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Figure 4.1 Clinical signs of disease in sows at a North Carolina farm infected with PCV3.

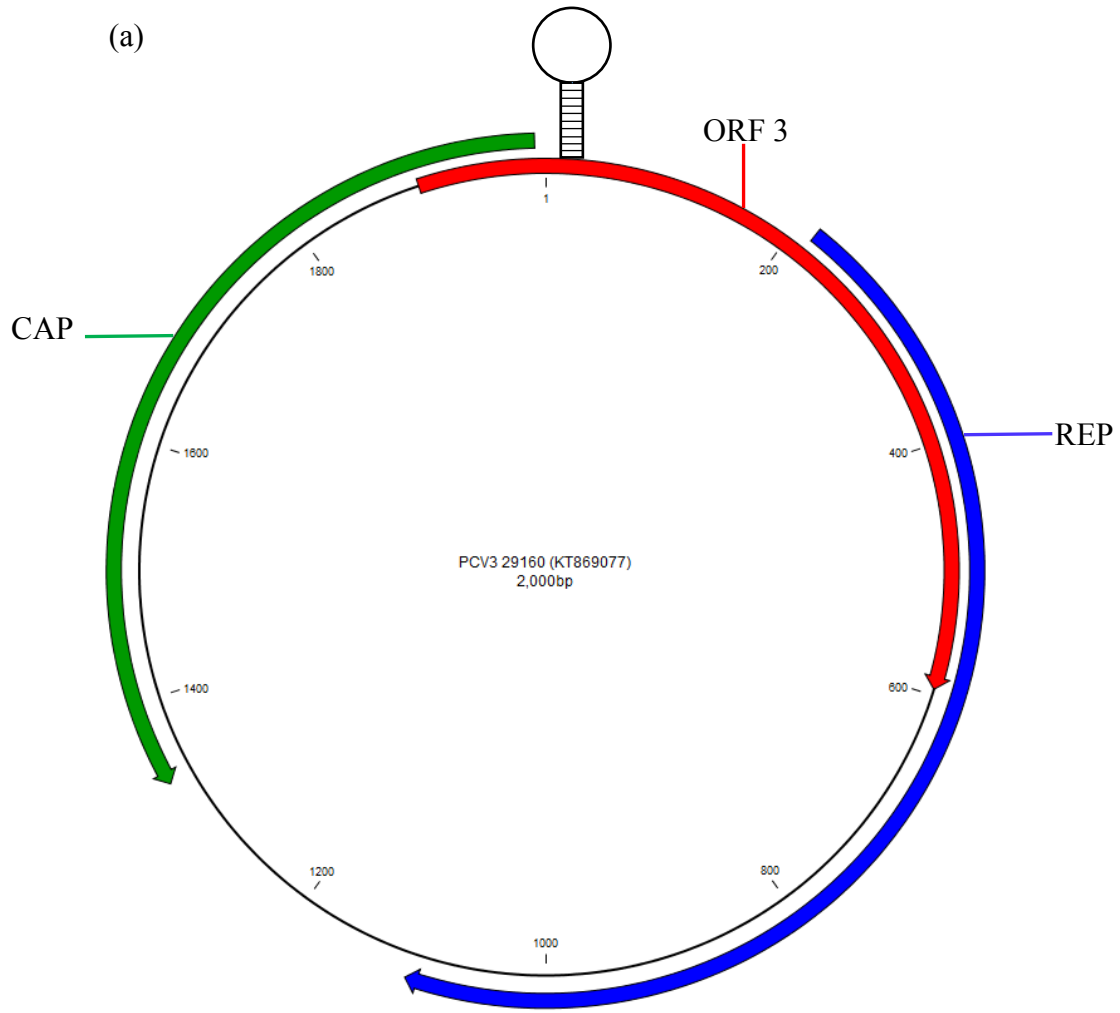
A) purple papules and macules B) mummified fetuses of varied crown-rump length aborted from sows with lesions similar to those seen with porcine dermatitis and nephropathy syndrome

Table 4.1 . Primer walking primers used to obtain the full length PCV3 genome.

Primer Set	Start Position	End position	Forward Primer (5'-> 3')	F T_m (°C)	Reverse Primer (5'-> 3')	R T_m (°C)
1	74	927	CAC CGT GTG AGT GGA TAT AC	52.5	CAA ACC CAC CCT TAA CAG	50.6
2	1609	433	GTC GTC TTG GAG CCA AGT G	56.0	CGA CCA AAT CCG GGT AAG C	56.1
3	965	8	TGT TGT ACC GGA GGA GTG	53.9	TGC CGG GTA ATA CTA GCC	53.9
4	594	1619	GAA GTT GCG GAG AAG ATG	50.7	TCC AAG ACG ACC CTT ATG	51.1

Figure 4.2 The genomic organization and phylogeny of PCV3.

A) The 2,000 nt ambisense genome contains three open reading frames (ORFs) greater than 200 amino acids encoding proteins capsid (cap), replicase (rep) and unknown function (ORF3). Phylogenetic analysis of predicted nucleotide sequences of the B) full genome. Phylogenetic trees were constructed by Maximum Likelihood analysis using the general time reversible with gamma distribution and invariant sites with tree topology evaluated using 1,000 bootstrap replicates. GenBank accession numbers are shown in parentheses.



(b)

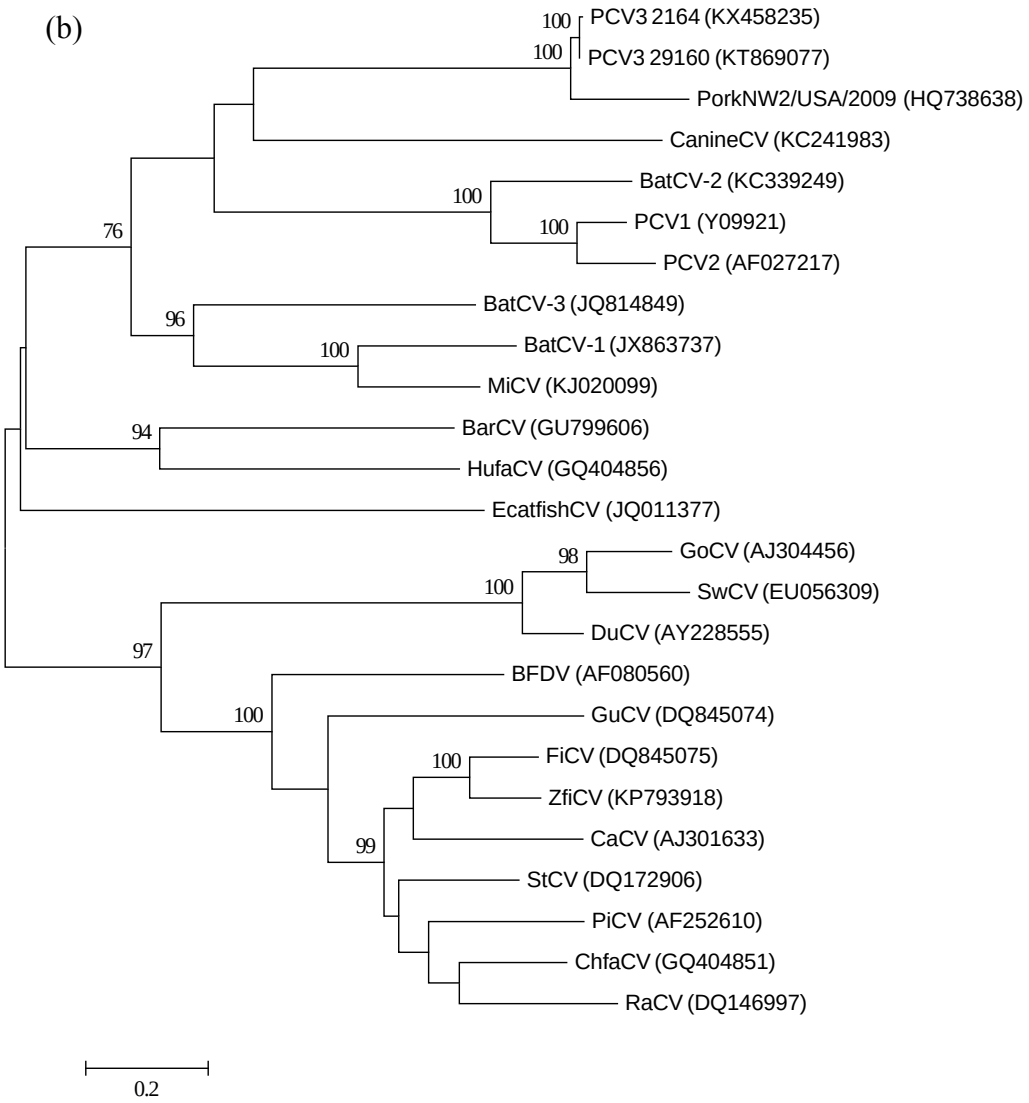

PCV3 2164 (KX458235)
PCV3 29160 (KT869077)
PorkNW2/USA/2009 (HQ738638)
CanineCV (KC241983)
BatCV-2 (KC339249)
PCV1 (Y09921)
PCV2 (AF027217)
BatCV-3 (JQ814849)
BatCV-1 (JX863737)
MiCV (KJ020099)
BarCV (GU799606)
HufaCV (GQ404856)
EcatfishCV (JQ011377)
GoCV (AJ304456)
SwCV (EU056309)
DuCV (AY228555)
BFDV (AF080560)
GuCV (DQ845074)
FiCV (DQ845075)
ZfiCV (KP793918)
CaCV (AJ301633)
StCV (DQ172906)
PiCV (AF252610)
ChfaCV (GQ404851)
RaCV (DQ146997)
100
100
100
100
76
96
100
94
98
100
97
100
100
99
0.2

Figure 4.3 Immunofluorescence of PCV3 capsid monoclonal antibodies.

Monoclonal antibodies against PCV3 were generated by immunization of mice with purified N-terminal truncated capsid protein (amino acids 35-214). Monoclonal antibodies were screened by indirect immunofluorescence assays using HEK293 cells transfected with A) a plasmid bearing a native PCV3 capsid expression cassette (pSF-CMV-cap) or B) empty plasmid (pSF-CMV-AMP).

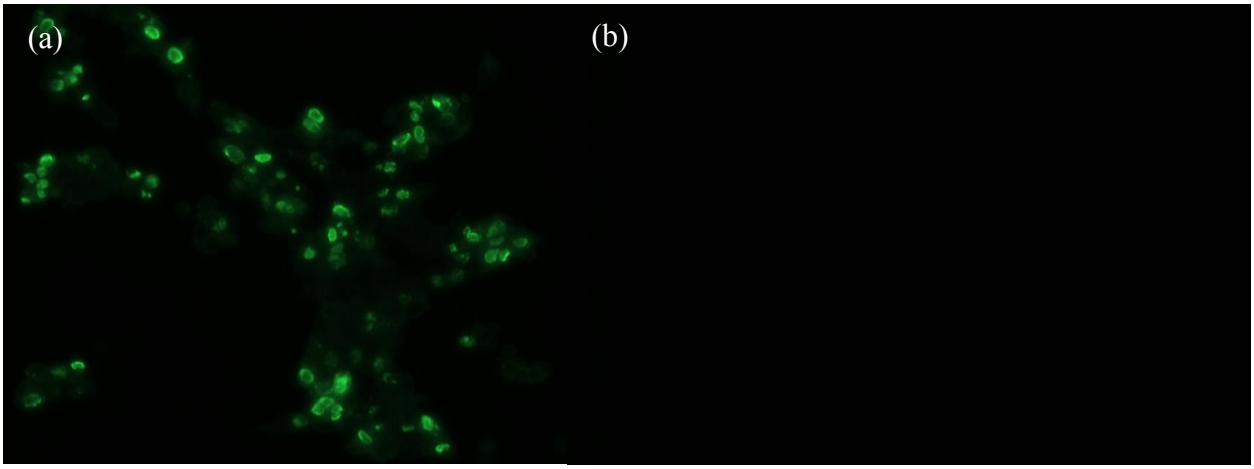


Figure 4.4 Immunohistochemistry on PDNS tissues using a PCV3 capsid specific monoclonal antibody

Paraffin-embedded sections of A-C) skin, D-G) lymph node, and H-K) kidney stained with hematoxylin and eosin (A, D, H) or PCV3 MAb14 (B, E, I) from porcine dermatitis and nephropathy cases. A) Section of skin are characterized by necrotizing vasculitis with fibrinoid changes (*) and scattered lymphoplasmacytic dermatitis (**). B) The dermal lymphocytic infiltration showed marked intracytoplasmic immunostaining against PCV3. D) There is a multifocal granulomatous lymphadenitis (*) with presence of multinucleated giant cells (arrow). E) The follicular and perifollicular lymphocytic population showed diffuse intracytoplasmic staining against PCV3. H) Kidneys are characterized by the presence of diffuse membranoproliferative glomerulonephritis (*) with minimal interstitial lymphopalsmacytic infiltration (**). I) The tubular epithelium showed random areas of positive staining against PCV3. Negative staining and background controls were performed in each tissue by elimination of primary PCV3 MAb 14, replaced by PBS followed by a goat anti-mouse secondary antibody (C, F, J). Lymph node and kidney tissues obtained from PCV3 q PCR negative animals were concurrently strained with PCV3 MAb14 and the goat anti-mouse secondary antibody to control for nonspecific antibody binding (G, K).

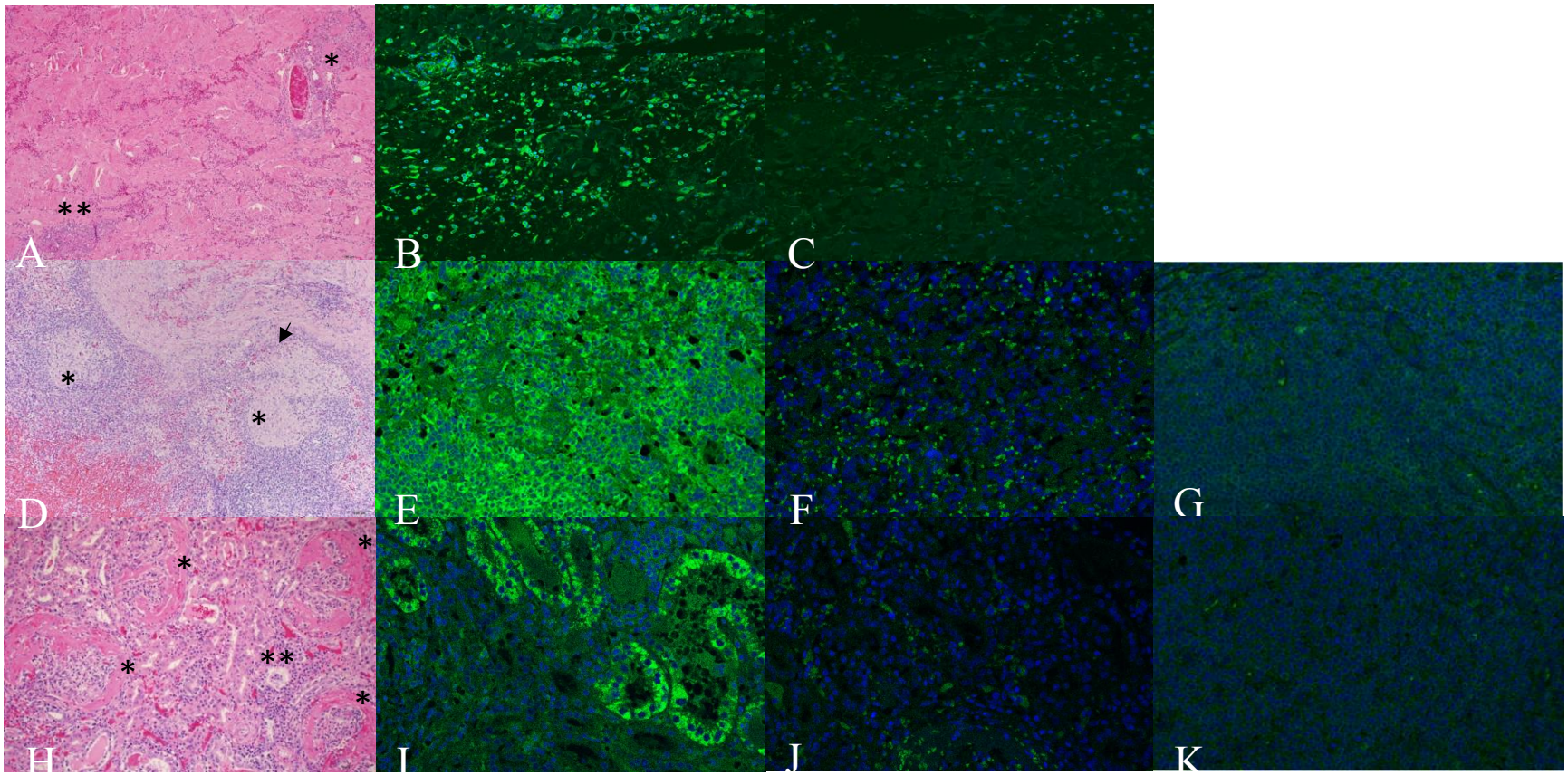
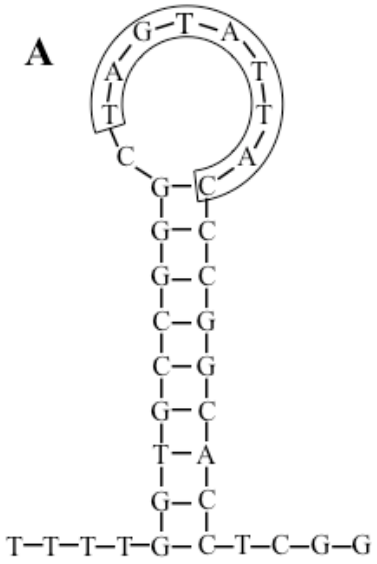

A
B
C
D
E
F
G
H
I
J
K

Figure 4.5 PCV3 Stemp-loop characteristics

(A) Stem-loop structure of the novel *Circoviridae* member, PCV3 and (B) nonamer sequences and stem lengths of for various circoviruses. Nucleotides in red are conserved in all circovirus sequences provided.

A



PCV3 stem-loop structure

B

Species	Nonamer Sequence	Stem Length (bp)
PCV1	TAGTATTAC	11
PCV2	AAGTATTAC	11
PCV3	TAGTATTAC	9
HufaCV	TAGTATTAC	9
ChfaCV	CAGTATTAC	14
BatCV-3	TAGTATTAC	9
PorkNW2/USA/2009	TAGTATTAC	9
CanineCV	TAGTATTAC	12
BarCV	TAGTATTAC	15
BFDV	TAGTATTAC	10
DuCV	TATTATTAC	10
GuCV	TAGTATTAC	10
RaCV	GAGTATTAC	19
StCV	CAGTATTAC	17

Chapter 5 - Discussion

The objective of the US swine industry is to provide a reliable and healthy food source to consumers. Towards this goal, prevention of disease outbreaks is crucial to secure the health of the animals. The number of viruses identified as potential pathogens is increasing as new technologies are developed for pathogen detection and discovery. The expanding diversity of mutant, novel or subtypes of viral species, may make it difficult to maintain the health of this staple food source. Therefore, it is essential to identify and characterize unique viral species as a first step in disease prevention.

The studies performed in this dissertation emphasize the detection of potential emerging swine viral diseases with metagenomic sequencing. Unknown viral species were discovered by investigating swine samples submitted to Kansas State Veterinary Diagnostic Laboratory (KSVDL) for diagnostic testing lacking the usual viral suspects. Furthermore, this dissertation explores the characteristics of three of these novel viral species.

In chapter 2, the study focuses on the identification of a divergent porcine parvovirus species from the rectal swabs of market pigs. Recently, porcine parvovirus diversity has expanded with the discovery of porcine parvovirus 4 (PPV4) in swine tissues, porcine parvovirus 5 (PPV5) in swine lung tissues and porcine parvovirus 6 (PPV6) in aborted fetal tissues (Cheung *et al.*, 2010; Ni *et al.*, 2014; Xiao *et al.*, 2013). Phylogenetic analysis of these viruses grouped PPV4-PPV6 together, sharing a clade with bovine parvovirus 2 (BPV2) later classified in the *Copiparvovirus* genera. In 2016, metagenomic sequencing on swine rectal swabs submitted to KSVDL for diagnostic testing revealed a partial sequence contig with the highest similarity to recently identified turkey parvovirus HK-2014 (AIF54687) and *Eidolon helvum* parvovirus 2 (AGL97807) (Baker *et al.*, 2013; Reuter *et al.*, 2014). Genome structural analysis revealed the

architecture and sequence motifs of that of members of *Parvoviridae* and the virus was designated porcine parvovirus 7 (PPV7).

The PPV7 genome encodes two major open reading frames (ORF), a 5' putative replicase of 2019bp and a 3' capsid of 1401bp. A third smaller ORF of 747bp is nested within the 5' putative replicase ORF. Within the 5' ORF, sequence motifs characteristic of parvovirus species were identified including replication initiation motifs (I, II, III), NTP-binding motifs (A, B) and a helicase domain (H) corroborating our previous results (Ilyina & Koonin, 1992; Xiao *et al.*, 2013). Pairwise comparison of the full genomes of porcine parvoviruses revealed PPV7 is <42.4% similar to any known porcine parvovirus displaying <20.4% similarity at the protein level.

Analysis of the PPV7 nucleic acid prevelance by qPCR of various porcine serum, rectal swab, nasal swab or lung lavage samples revealed an overall prevalence of 8.6%. The objective of this study was the molecular characterization of a divergent porcine parvovirus species. As porcine parvoviruses are known to contribute to disease complexes in swine, such as porcine circovirus associated disease (PCVAD), the discovery of a novel porcine parvovirus warrants concern.

Previous works have recognized porcine parvoviruses are present in animals, displaying symptoms of disease infected with the virus alone or as co-infections with known viral pathogens such as porcine circovirus 2 (PCV2) (Cutler *et al.*, 1983; Gillick, 1977; Opriessnig *et al.*, 2013, 2014). Porcine parvovirus 1 (PPV1) was initially discovered on a farm in Germany displaying reproductive failure in the 1960's, and is one of the only porcine parvoviruses shown to be the etiologic agent for reproductive disease (Mayr *et al.*, 1968; Ren *et al.*, 2013). PPV2 was present in pigs with "high fever disease" in China, however, both PCV2 and porcine reproductive and

respiratory syndrome virus (PRRSV) were present in the samples and no direct association to disease could be identified (Wang *et al.*, 2010). Earlier, in an acute outbreak of mortality in pigs from the US, PCV2 was identified with a novel species, designated PPV4 (Cheung *et al.*, 2010). In 2013, in a survey analyzing PPV4 in swine, porcine parvovirus 5 (PPV5) was identified in the tissues of pigs displaying respiratory disease, diarrhea, systemic disease or reproductive disorders. The association of PPV5 to disease is yet to be determined. Moreover, the samples of aborted fetal pigs in Hong Kong, China in 2014, were not co-infected with other pathogens, suggesting PPV6 was the cause of disease (Ni *et al.*, 2014). Unfortunately, the samples were not well characterized and swine samples from a study in the US identifying PPV6 also contained PRRSV (Schirtzinger *et al.*, 2015). The pathogenicity of PPV6 has not been addressed therefore, the role of PPV6 as the causative agent of disease in the Chinese pigs is unknown. Overall, the association of porcine parvoviruses as the etiologic agent of clinical disease is difficult to determine, although the role in disease complexes is well documented (Opriessnig *et al.*, 2014; Opriessnig & Langohr, 2013).

Future studies are necessary to address some of the questions raised in study. It would be interesting to determine the association between PPV7 and disease, if any. This pathogenesis study should first be addressed using an *in vivo* inoculation of piglets, therefore determining the viruses' pathogenicity alone. An interesting follow up study would be to determine the incidence of PPV7 in cases of PCVAD, as previous studies have identified an association between porcine parvoviruses and PCVAD (Opriessnig *et al.*, 2013). The information obtained in these studies could impact the treatment and prevention used on swine farms afflicted with this viral infection.

In chapter 3, a recently discovered porcine parainfluenza virus 1 (PPIV1), was detected in the nasal swabs of swine originating on a commercial farm in Oklahoma and were compared to

previously submitted sequences from China. In 2013, three complete PPIV1 sequences were determined from the nasopharyngeal samples of slaughterhouse pigs in Hong Kong (Lau *et al.*, 2013). Furthermore, a US commercial swine farm in Oklahoma was experiencing acute respiratory disease in 10-21 day old pigs with symptoms such as cough, sneezing and nasal discharge. Nasal samples were taken from two of the afflicted pigs which tested negative for Influenza A virus (IAV) but positive for PPIV1 by qRT-PCR. Metagenomic sequencing on these samples yielded a near complete PPIV1 genome. Nasal turbinates from pigs on a farm in Illinois displaying similar clinical symptoms to the pigs in Oklahoma were submitted to KSVDL for metagenomic sequencing. A second partial PPIV1 genome was obtained from these samples covering approximately 45% of the genome. Finally, eleven pigs from a PPIV1 naturally infected farm in Nebraska were monitored for clinical signs of disease. Nine of the eleven pigs tested qPCR positive by the conclusion of the study although no clinical signs of disease were observed. A third PPIV1 genome was obtained from the nasal swab samples of qPCR positive animals.

Phylogenetic analysis was performed on the full PPIV1 genome sequences obtained in this study. The Oklahoma and Nebraska complete genomes shared 97.7% pairwise identity and 90.0-95.3% identity to the PPIV1 sequences from China. Additionally, the US PPIV1 genomes grouped with the Chinese sequences S119N and S206N while the Chinese S033N was a more divergent sequence in the PPIV1 clade. The clade closest to PPIV1 contained human parainfluenza virus 1 (HPIV1) and Sendai virus (SeV).

No clinical signs were observed in the natural infection study for PPIV1. Histopathological examination revealed decreased cilia, goblet cells or both in the trachea and *in situ* hybridization identified virus in the turbinate respiratory epithelial cells and trachea to a

lesser extent. Despite these observations, a clear link between the lesions observed and PPIV1 was not seen. Molecular analysis found PPIV1 nucleic acid in 17 of 279 various swine samples (6.1%) and serological analysis identified up to 63.3% seroprevalence. These data suggest PPIV1 is moderately prevalent in US swine.

Paramyxovirus species are known for their ability to cause mild to severe respiratory disease in swine and other mammals. In swine, young animals display more severe symptoms than older animals, resulting in a decrease in weight gain and in some cases death (Lamb & Parks, 2013). One of the most concerning aspects of paramyxoviruses in swine is the propensity for cross-species transmission among paramyxoviruses. The first description of Menangle virus occurred in Australians working on a piggery with influenza-like symptoms and rash (Chant *et al.*, 1998). Concurrently, the virus was isolated from stillborn piglets on the same farm (Philbey *et al.*, 1998). Nipah virus, one of the most recently identified and notorious paramyxoviruses, is known to infect and cause fatal disease in humans and pigs (Centers for Disease Control and Prevention (CDC), 1999; Chua *et al.*, 2000). For these reasons, the identification of PPIV1 in US swine is concerning, not only to swine health but to human health as well.

Follow-up studies for PPIV1 should focus on characterizing the viruses' pathogenicity. The *in vivo* natural infection study, in chapter 3, characterized the subclinical disease on one farm and provided a glimpse into the potential of PPIV1 to cause disease. It is not known how this herd became infected with PPIV1. A study in which pigs are experimentally inoculated would be a beneficial follow-up experiment as many more factors can be controlled. Likewise, the age of the pigs in our studies (22-26 days) differed from the animals displaying clinical symptoms (10-14 days). As differences in clinical outcome have been shown for pathogens such as porcine endemic diarrhea virus (PEDV), an experimental infection might provide us with

different results (Jung *et al.*, 2015). Further research is necessary to characterize PPIV1 as an emerging swine pathogen, however, our research contributes to the knowledge needed to develop vaccines and other disease mitigation techniques.

The final study, outlined in chapter 4 is the first description of a divergent porcine circovirus designated porcine circovirus 3 (PCV3). In 2015, a farm in North Carolina displaying poor sow reproductive performance experienced an outbreak of disease with clinical signs consistent with porcine dermatitis and nephropathy syndrome (PDNS) and higher than average mortality rates. Aborted mummified fetuses were collected from sows on the same farm displaying signs of PDNS. The fetuses were negative for PCV2 by IHC and qPCR, representing the normal suspect for this set of clinical symtoms. Furthermore, the fetuses were also negative for PRRSV and PPV by qPCR. Tissue samples were obtained from sows that died of PDNS on the same farm that tested negative for PCV2, PRRSV and IAV. A mummy tissue homogenate was subjected to metagenomic sequence which resulted in the discovery of a 1,246bp contig most similar to a partial circovirus genome. Completion of the circovirus genome using rolling circle amplification (RCA) followed by Sanger sequencing yielded a 2kb complete genome.

Genome analysis of the 2kb PCV3 contig identified three ORFs in an organization consistent with circoviruses. The largest ORF encodes for a 297aa protein which is 69.4% identical to the partial replicase protein of a circovirus identified in commercial pork meat, PorkNW2/2009/USA (ADU77001) (Li *et al.*, 2011). Additionally a 54% similarity was determined between the PCV3 ORF and a bat circovirus from China (AIF76248). Conserved circovirus motifs characteristic of circovirus replicases were identified in this ORF such as rolling circle replication motifs and P-loop motifs. A stem loop region was found at the 5' intergenic region between the *rep* and *cap* ORFs of PCV3 with a loop nonamer identical to

porcine circovirus 1 (PCV1) (Li *et al.*, 2010). Interestingly, the *rep* ORF lacks a conventional ATG start codon, instead utilizing the alternative codon GTG, identical to PorkNW2/2009/USA and similar to avian circoviruses (Bassami *et al.*, 2001; Niagro *et al.*, 2014; Phenix *et al.*, 2001). The putative *cap* ORF encodes for a 214aa protein and lies on the opposite strand of the *rep* ORF. This ORF is 87% similar to the partial capsid of PorkNW2/2009/USA and 36-37% identical to PCV2 and duck circoviruses. The third ORF encodes for a 231aa protein, in the same orientation of the *rep*, that is 94% identical to a third PorkNW2/2009/USA ORF and 39% similar to a Murid herpesvirus M169 protein of unknown function. Phylogenetic analysis identified PCV3 and canine canine circovirus (KC241983) share a distant common ancestor most closely related to the clade containing PCV1, PCV2 and BatCV-2.

To determine if PCV3 is associated with PDNS, PCV3 nucleic acid and antigen presence were assessed in forty-eight PDNS cases. Of these cases, forty-five (93.8%) were positive for PCV3, however forty-seven of the forty-eight (97.9%) were positive for PCV2 including forty-four (97.8%) of the PCV3 positive tissues. Five of the PCV3/PCV2 qPCR positive case tissues were analyzed by IHC resulting in three of five positive samples. In the index case, IHC displayed PCV3 antigen in PDNS lesions including necrotizing vasculitis, glomerulonephritis, granulomatous lymphadenitis and bronchinterstitial pneumonia in either the skin, kidney, lung and lymph node tissues. To determine PCV3 nucleic acid prevalence in US swine without PDNS signs, 271 diagnostic submissions were evaluated by qPCR and thirty-four (12.5%) displayed positive results. Finally, the seroprevalence of PCV3 was assessed using a recombinant PCV3 capsid protein ELISA. Of the 83 serum samples assessed, originating in multiple states, 47 were positive (56.6%). Taken together, the PCV3 prevalence data suggests moderate nucleic acid and seroprevalence and a high occurrence in PDNS cases within the US swine herd.

Of utmost concern and interest is the discovery of PCV3 in the third study, putatively linked to clinical signs associated with PDNS. PDNS is classified as a PCVAD as qPCR detects PCV2 in many cases of PDNS (Segalés *et al.*, 1998; Thibault *et al.*, 1998). Porcine circoviruses are one of the most economically important pathogens afflicting the US swine industry today, causing a disease complex known as PCVAD (Gillespie *et al.*, 2009). Symptoms categorized in this broad group include reproductive failure, neurologic signs, respiratory disease and wasting (Harding, 2007; Harms *et al.*, 2002; Kim *et al.*, 2003; Madson & Opriessnig, 2011; Rosell *et al.*, 1999). The link to a viral cause for PDNS has eluded researchers and the search for a causative agent continues. However, PDNS has been experimentally reproduced in pigs with samples lacking PCV2 (Krakowka *et al.*, 2008; Segalés *et al.*, 1998; Thibault *et al.*, 1998). Our studies characterizing PCV3 may bridge the gap in knowledge attributed to PDNS. Our study found 45 of the 48 PDNS tissues were PCV3 positive by qPCR and three of five of these tissues tested displayed PCV3 antigen in the tissue lesions. These data support the need for further pathogenesis studies for this putative pathogen as our results showed a 12.5% nucleic acid prevalence and 55% seroprevelence in US swine samples. Despite a majority of the samples containing other viral pathogens, the original mummy sample in which PCV3 was identified contained no other viral agents, suggesting PCV3 contributed to the reproductive failure in this animal. Future *in vivo* pathogenesis studies should focus on describing the pathogenesis and clinical presentation of PCV3. If PCV3 were to take a similar trajectory as PCV2, the economic impact would be devastating.

The focus of future research on PCV3 should be to experimentally evaluate the viruses' pathogenicity. An *in vivo* study inoculating pigs and examining the occurrence of disease is the

most logical next step. Subsequent *in vivo* studies should address the high co-infection rate between PCV2 and PCV3 for these cases as well. A co-infection study would be a viable option.

Using these studies as examples, metagenomic sequencing has exponentially contributed to the knowledge and diversity of emerging swine viral diseases. Methods such as SISPA using viral enrichment have revolutionized viral sequence identification. Since 2002, the addition of viral enrichment such as ultracentrifugation and enzymatic digests prior to SISPA have increased method utility for viral discovery. Ultracentrifugation, as an enrichment tool, has been shown to concentrate viral nucleic acid using a filter size of 160-450nm (Allander *et al.*, 2005; Breitbart *et al.*, 2002). A downfall of this method is the likelihood of contaminating bacterial genetic material as the largest virus and the smallest bacterium are both approximately 400nm in size (Scola *et al.*, 2003). This is only a minor downfall depending on the filter size used as the largest viruses and smallest bacteria would be filtered out similarly. Enzymatic digestion can be a more beneficial option as the method is capable of selecting between DNA and RNA genetic material. The principle suggests the addition of DNAse I or RNAseH, depending on the targets, will remove naked and unprotected nucleic acids from the sample. In this case, vial capsids are protected from digestion as they are shielded from the enzymes. Enzymatic treatment has been used to remove contaminating nucleic acid from serum, feces and seawater (Allander *et al.*, 2001; Cann *et al.*, n.d.; Culley *et al.*, 2006). Unfortunatley, one concerning drawback of using metagenomic sequencing is that the sample must possess viral genetic material for detection to occur, therefore, a number of samples may have to be tested to identify a novel viral species. As serological viral identification is difficult if not impossible, metagenomic sequencing will be maintained as a viable option for viral discovery. Metagenomic sequencing for viral discovery is

becoming a mainstay in the scientific community as recent advancements have decreased price, time, efficiency and ease of preparation related to these techniques.

Although a number of factors contribute to the emergence of porcine diseases, one main factor is swine management practices. A change in herd size or weaning age can have moderate effects on the health of the animals. With group A rotaviruses, larger herds, younger weaning age and all-in-all-out facilities had an increased probability of animals testing positive for porcine rotavirus (Dewey *et al.*, 2003). Herd size also affected the occurrence of influenza A virus on swine farms in Vietnam. Farms with greater than 1,000 animals had a higher chance of testing influenza A virus positive. This observation was suggested to affect reassortment events as there would be an increased chance of reassortment in a larger group of hosts (Takemae *et al.*, 2016). Additionally, a higher turnover rate of animals within a facility allows potentially naïve animals to be exposed to pathogens such as influenza, further sustaining the disease and allowing for the introduction of new putative pathogens (Pitzer *et al.*, 2016). This change in operation produces a potential environment for a novel pathogen and can be provided from not only the introduction of new animals but the introduction of contaminated products. For instance, a number of studies have been done examining the introduction of porcine reproductive and respiratory syndrome virus (PRRSV) into the naïve animals of New Zealand, particularly by the importation of infected commercial pork meat (Cobb *et al.*, 2015). As a means to mitigate the negative impacts of herd management changes, the implementation of biosecurity measures positively correlates to increased herd size (Laanen *et al.*, 2013). Biosecurity measures have allowed New Zealand livestock to remain PRRSV free. Although the reason for the appearance of PPV7, PPIV1, and PCV3 is unknown, the number of emerging pathogens and diseases will continue to grow as swine management practices are dynamic.

The overarching goal of these studies is the identification and characterization of emerging swine viral diseases as a first step to prevent the spread of potentially pathogenic agents. In addition to inhibiting the spread of disease in swine, the possibility of viral transmission to humans is limited as well. The pervasive nature of many of these emerging viruses is concerning and relevant to human health due to the capacity for swine viruses to transmit to humans. By characterizing these potential pathogens, we have provided a means to develop surveillance assays to monitor swine herds for potential outbreaks of disease. Surveillance assays have been implemented in Europe and US monitoring swine influenza virus and influenza A H3N2, respectively (Cauchemez *et al.*, 2013; Henritzi *et al.*, 2016). A PCV2 surveillance program has been suggested in Malaysia (Jaganathan *et al.*, 2011). Additionally, the results of our studies contribute to the development of preventative vaccines in the case of clinical disease outbreaks. Furthermore, the identification of potential emerging pathogens for the development of preventative vaccines is necessary to secure the health of the animals, provide a reliable food source to consumers, and protect the health of the human population.

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