Widespread detection and characterization of porcine parainfluenza virus 1 in pigs in the USA

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Porcine parainfluenza virus 1 (PPIV1) was first identified in 2013 in slaughterhouse pigs in Hong Kong, China. Here, two near-complete genomes were assembled from swine exhibiting acute respiratory disease that were 90.0–95.3 % identical to Chinese PPIV1. Analysis of the HN gene from ten additional PPIV1-positive samples found 85.0–95.5 % identity, suggesting genetic diversity between strains. Molecular analysis identified 17 out of 279 (6.1 %) positive samples from pigs with respiratory disease. Eleven nursery pigs from a naturally infected herd were asymptomatic; however, nasal swabs from six pigs and the lungs of a single pig were quantitative reverse transcriptase (qRT)-PCR positive. Histopathology identified PPIV1 RNA in the nasal respiratory epithelium and trachea. Two serological assays demonstrated seroconversion of infected pigs and further analysis of 59 swine serum samples found 52.5 % and 66.1 % seropositivity, respectively. Taken together, the results confirm the widespread presence of PPIV1 in the US swine herd.

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 family Paramyxoviridae (Lamb & Parks, 2007). The subfamily Paramyxovirinae consists of seven genera: Respirovirus, Rubulavirus, Avulavirus, Morbillivirus, Aquaparamyxovirus, Ferlaviruses and Henipavirus. The genus Respirovirus consists of five recognized species: bovine parainfluenza virus 3 (BPIV3), human parainfluenza virus 1 (HPIV1), human parainfluenza virus 3, Sendai Virus (SeV), Simian virus 10 (Lamb & Parks, 2007) and the proposed porcine parainfluenza virus 1 (PPIV1). While swine are the primary reservoir of porcine rubulavirus, cross-species transmission of paramyxoviruses from their hosts to swine have been reported, including Nipah virus, 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 Nipah virus and Hendra virus, have high fatality rates in humans (Chadha et al., 2006; Murray et al., 1995).

Swine have been shown to serve as intermediate hosts for Nipah virus (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.

While PPIV1 has been identified in the USA by veterinary diagnostic laboratories in recent years, little is known about its epidemiology and role in clinical disease. There is only a single published report on the detection of the genetic signature of PPIV1 in pigs from the USA using microarray technology (Jaing et al., 2015).

Here, a commercial swine operation in Oklahoma experiencing recurrent disease in pigs of 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 Iowa State University Veterinary Diagnostic Laboratory where they tested negative for influenza A virus (IAV) and positive for PPIV1.
by reverse transcriptase PCR (RT-PCR). Samples were transferred to Kansas State University Veterinary Diagnostic Laboratory (KSVDL) and metagenomic sequencing was performed as previously described (Hause et al., 2015a; Neill et al., 2014) 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 4236 contigs which were analysed by BLASTN. Viruses identified were porcine astrovirus 4 (PA4), 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 a mean coverage of 353x.

A commercial swine operation in Illinois with similar clinical symptoms to the farm in Oklahoma 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 mapping to Sus scrofa, yielded 134 contigs following de novo assembly. BLASTN analysis identified multiple contigs with highly significant E values to PA4, porcine kobuvirus, porcine haemagglutinating encephalomyelitis virus and PPIV1. Templated assembly of PPIV1 using JX857411 afforded only approximately 45% genome coverage. Of the viruses found in these symptomatic pigs, porcine circovirus 2 and haemagglutinating encephalomyelitis virus have been shown to contribute to respiratory disease.

Asymptomatic pigs from a commercial breeding herd in Nebraska that were quantitative RT-PCR (qRT-PCR) positive for PPIV1 were transferred to the University of Nebraska-Lincoln to monitor PPIV1 pathogenesis. A pool of nasal swabs was 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 PA4, porcine astrovirus 5, porcine bocavirus 3, porcine bocavirus 5 and a small circular DNA virus most similar to one identified in fur seal faeces. PA4 was identified in all three PPIV1 positive samples analysed by metagenomic sequencing. PA4 was previously shown to be commonly detected in swine diarrhoea samples in the USA (Xiao et al., 2013). PA4 was also readily identified in both diarrhoeic and normal swine faeces in China (Zhang et al., 2014). An aetiological role for PA4 in clinical disease has not been established.

To investigate the prevalence of PPIV1, 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 Oklahoma resulted in cycle threshold (Ct) 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 Ct 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 Ct 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 Ct 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 homogenates, oral fluid or nasal swab samples of unknown infection status were screened by qRT-PCR for PPIV1. Seventeen samples tested positive (6.1%). Of the qRT-PCR positive samples, 12 were nasal swabs, four were oral fluids and one was lung homogenate. Virus isolation was attempted on swine testicle, Vero, porcine alveolar macrophage and primary porcine kidney cells. No cytopathic effects were evident. Viral titres were monitored by the N gene qRT-PCR. Following two passages, all samples were negative by qRT-PCR.

The near full genome sequences determined from the nasal swabs from pigs originating in Oklahoma (strain 1438-1) and Nebraska (3103-1) were aligned using CLUSTAL W and phylogenetic analysis was performed with MEGA 6.06 software using the maximum-likelihood method with topology verified by 1000 bootstrap replicates (Tamura et al., 2013). The two nearly full genome sequences used in the phylogenetic analysis were submitted to GenBank under accession numbers KT749882 (1438-1) and KT749883 (3103-1). The genomes had 97.7% pairwise identity to each other and 90.0–95.3% nucleotide identity to three PPIV1 sequences from China. Phylogenetic analysis of the genome sequences found that the two US sequences formed a well-supported clade that was most similar to Chinese PPIV1 strains S119N and S206N (Fig. 1a). The PPIV1 clade was most closely related to a clade consisting of HPIV1 and SeV.

An approximate 1760 bp fragment of the haemagglutinin-neuraminidase (HN) gene was sequenced from an additional 10 USA PPIV1-positive samples and nucleotide identities ranged from 93.7–99.9% for samples from the USA and 85.0–95.5% identity to Chinese PPIV1. The sequences 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). 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 (Fig. 1b). 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 Oklahoma (1438-1, 1438-4E1, 1438-2E6, 1438-4E6, 1438-3C11) and Nebraska (3103-1, 3103-D0A4, 3103-D0A7), little diversity between samples was observed.
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).

Eleven randomly selected weaned pigs (18–19 days old) from a farm naturally infected with PPIV1 were transferred to the University of Nebraska-Lincoln animal research unit. Throughout the 2 week observation period, no clinical symptoms of disease such as coughing, sneezing, nasal discharge or lethargy were observed. 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 the 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 porcine endemic diarrhoea virus (PEDV) (Jung et al., 2015). Nasal swabs collected on day 0 (22–23 days of age) were positive for PPIV1 for six of the 11 pigs (55 %) (Table 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}=53.2). Histopathology was examined on lung, trachea and nasal turbinates for eight out of the 11 animals (A4–11). Animals A4, A7, A8 and A9 animals had marked atelectasis in the lung tissues but this pathology is not likely 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 genetic material in turbinate respiratory...
epithelial cells (Fig. 2a) and to a lesser extent in the trachea (Fig. 2b) in pig A8 (Advanced Cell Diagnostics). In addition, haematoxylin and eosin (H&E) staining of nasal turbinate slides from this animal found neutrophils present in the respiratory epithelium and lymphocytes and plasma cells in the submucosa surrounding the glands (Fig. 2c). Similar findings were seen, to a lesser extent, in the trachea (Fig. 2d). 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. Consequently, the inflammation seen in the histopathology results cannot solely be attributed to PPIV1. 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 aetiological agents, we were unable to show clinical significance or specific pathological lesions that could be attributed to PPIV1 infection.

Serological analysis was performed using an immunoprecipitation coupled to PCR detection assay (ICPD) modelled after the luciferase immunoprecipitation system using a PPIV1-positive nasal swab pool as antigen (Bur belo et al., 2009, 2011). 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 PBS 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 Illinois with recurrent unexplained acute respiratory disease in pigs which tested positive for PPIV1. Of the three pigs bled on day 14 of the study, two (pigs A1 and A3) 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 fusion (F) protein peptide. The ELISA was performed similarly to assays previously described (Hause et al., 2015b; Lin et al., 2005). 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 ($3 \times SD$ above the mean). All but four of the animals on day 0 of the study were positive for PPIV-1 F antibodies. While animals A1 and A3 tested negative at the beginning of the study for PPIV-1 F antibodies by ELISA, over the 2 week observation period, these animals shed PPIV1 and subsequently seroconverted. These results were further verified using ICPD, with these same animals testing negative on day 0 and 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 days 0 and 14 on the ELISA however was negative at both time points by ICPD. Increased sensitivity associated with the ELISA and the presence of waning maternal antibodies could explain the discrepant results for pig A2. This hypothesis is also consistent with the finding of seven of 11 pigs positive on the ELISA at day 0 and subsequent PPIV1 infection.

To investigate the prevalence of PPIV1 antibodies in commercial swine, 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 and 39 samples (66.1 %) by the PPIV1 F ELISA. Agreement between the two assays was assessed by Cohen’s kappa coefficient which suggested moderate agreement ($\kappa=0.57$). In conjunction with the 6.1 % PPIV1 prevalence determined by qRT-PCR, these data suggest that PPIV1 is prevalent in the US swine herd.

Several swine viruses identified in China have recently emerged in the USA, including PEDV and mutant porcine circovirus 2b, illustrating the ease with which swine pathogens can move intercontinentally (Huang et al., 2013; Xiao et al., 2012). No clinical symptoms were observed during the course of our pathogenesis study, raising the possibility that PPIV1 infection is asymptomatic without additional cofactors.

Other paramyxoviruses have also caused disease in pigs in the USA. In the 1980s and 1990s, two 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.

**Table 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 $C_t < 37$.

<table>
<thead>
<tr>
<th>Pig</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 11</th>
<th>Day 14</th>
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<tr>
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<td>28</td>
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*ND, Not detected.
Further genetic analysis of additional genomes, both contemporary and archived, is needed to resolve the evolutionary history of PPIV1. Likewise, additional studies are needed to determine the ecology, host susceptibility and pathogenesis of PPIV1.

Acknowledgements

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References


