Increasing porcine PARV4 prevalence with pig age in the U.S. pig population

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1. Introduction

Paroviruses are small, non-enveloped, single-stranded DNA viruses, with a genome size of approximately 4,000 to 6,000 nucleotides (nt) that contain terminal palindromic sequences (Tijssen et al., 2011). Several of the paroviruses are ubiquitous and may be associated with many clinical manifestations in animals (Mengeling et al., 2000; Tijssen et al., 2011). The family Paroviridae is composed of two main subfamilies, the subfamily Parovirinae, infecting vertebrates, and the subfamily Densovirinae infecting arthropods. The Parovirinae subfamily is further divided into five genera: Parovirus, Erythrovirus, Dependovirus, Amudovirus and Bocavirus (Tijssen et al., 2011).

In 2008, a novel parovirus was discovered in samples obtained from domestic pigs and cattle in Hong Kong (Lau et al., 2008) and later was also identified in domestic pigs in the United Kingdom (Szelei et al., 2010) and the wild boar populations in Germany (Adlhoch et al., 2010) and Romania (Cadar et al., 2011). Originally, this virus was known as Hokovirus (Lau et al., 2008), later it was proposed to be renamed as partetravirus (Tse et al., 2011), while some researchers use the name PPV3 (Cheung et al., 2010; Cságoła et al., 2012). According to the Ninth Report of the International Committee on Taxonomy of Viruses (chapter 39), none of these names is accepted and therefore the virus is called porcine PARV4 in this study. Viruses resembling PARV4 contain a single-stranded DNA genome of approximately 5 kb and the genome has two open
reading frames (ORFs) coding for non-structural and capsid proteins. They differ from other parvoviruses by their relatively large predicted viral protein 1 (VP1) region and the presence of a unique small putative protein (Lau et al., 2008). Molecular analysis of bovine PARV4 and porcine PARV4 confirmed that these viruses are related to human PARV4, with nucleotide identities of 61.5 to 62.3% (Lau et al., 2008). Human PARV4 was initially identified in 2005 in the plasma of a hepatitis B positive drug user with an acute viral infection (Jones et al., 2005) and was subsequently detected in plasma from AIDS patients and hepatitis C virus infected people. Human PARV4 was further determined to have at least three genotypes: genotype 1, genotype 2 (previously termed PARV5) and genotype 3 (Fryer et al., 2007; Longhi et al., 2007; Panning et al., 2010; Simmonds et al., 2008). Interestingly, human PARV4 can be frequently detected in human coagulation factor concentrates prepared from older plasma samples and is suspected to be a potential cause of encephalitis in children (Benjamin et al., 2011; Schneider et al., 2008). Recently, ovine PARV4 and an additional genotype of bovine PARV4 were identified (Tse et al., 2011). PARV4 and related viruses are currently regarded as unclassified genus in the Parvovirinae subfamily (Tijsen et al., 2011).

The currently estimated prevalence rates of porcine PARV4 in domestic pigs and wild boar populations are rather high. In domestic pigs in Hong Kong, the overall prevalence of porcine PARV4 was found to be 44.4% (148/333) based on testing of lymph nodes, livers, serum samples, nasopharyngeal swabs and feces (Lau et al., 2008). Similarly, the prevalence of porcine PARV4 in Germany, based on testing of liver and serum samples obtained from wild boars, was 32.7% (Adlhoch et al., 2010). Recently, porcine PARV4 prevalence rates ranging from 22.8% to 50.5% were identified in samples of lymph nodes, lungs, liver, kidneys, spleen and tonsils collected from wild boars in Romania in 2006 through 2007 or in 2010 through 2011, respectively (Cadar et al., 2011).

In the present study, we investigated the prevalence of porcine PARV4 in lung tissues obtained from U.S. pigs of different ages and with different disease manifestations sampled during May and June 2011. Additionally, we further characterized the genomes of three U.S. porcine PARV4 strains by genomic sequencing. The results of this study contribute to a better understanding of the ecology of porcine PARV4 in pigs.

### 2. Materials and methods

#### 2.1. Sample origin

Four-hundred-eighty-three lung tissues were obtained from pigs within five different age groups located in the U.S (Table 1). The tissues were randomly selected from routine submissions to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) between May and June 2011. The pigs included in this study originated on 178 farms located in 16 U.S. states including Colorado, Iowa, Illinois, Indiana, Kansas, Michigan, Minnesota, Missouri, North Carolina, North Dakota, Nebraska, Ohio, Oklahoma, Pennsylvania, Texas, and Wisconsin. The majority of the submissions (53.8%; 260/483) came from Iowa due the geographic location of the ISU-VDL. Fifty-seven of the 483 samples were part of an anonymous influenza A surveillance program and the farm ID was not available. The cases used in this study were further classified into different disease manifestations based on the submission history. For the purpose of this study, the influenza A surveillance samples were classified as respiratory disease. There were a total of 29 cases of abortion or reproductive failure, 37 cases of enteric disease, 374 cases of respiratory disease, and 43 cases of systemic or central nervous disease.

#### 2.2. Sample processing

Approximately 1 gram of lung tissue was minced by scissors and diluted 1:10 in Dulbecco’s Modified Eagle Medium (DMEM), homogenized by using a Stomacher® 80 (Seward Laboratory Systems Inc., Bohemia, NY) and centrifuged at 1500 g for 10 min to obtain the supernatant. All samples were stored at −80°C.

#### 2.3. Viral DNA extraction

DNA was extracted from 50 μl of tissue homogenates using the 5 × MagMAX™ 96 Viral Isolation Kit (Ambion) according to the manufactures’ instructions on an automated extraction platform (KingFisher Flex; Thermo Fisher Scientific Inc.). DNA was eluted in 50 μl of elution buffer provided in the kit. All DNA extraction procedures included a positive and three negative controls (containing only water) in each run.

*Table 1* Summary of the history (pig age, number of farms and states) and prevalence of porcine PARV4 DNA in lung tissue samples obtained from 483 pigs located in the U.S.

<table>
<thead>
<tr>
<th>Age group</th>
<th>No. of farms/U.S. states</th>
<th>No. of samples</th>
<th>Porcine PARV4 prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuses (&lt;1 days)</td>
<td>12/7</td>
<td>28</td>
<td>0/28 (0%)</td>
</tr>
<tr>
<td>Suckling pigs (1–20 days)</td>
<td>7/3</td>
<td>15</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td>Nursery pigs (21–55 days)</td>
<td>55/14</td>
<td>178</td>
<td>10/178 (5.6%)</td>
</tr>
<tr>
<td>Grow-finish pigs (8–25 weeks)</td>
<td>88/14</td>
<td>235</td>
<td>44/235 (18.7%)</td>
</tr>
<tr>
<td>Mature pigs (&gt;25 weeks)</td>
<td>16/7</td>
<td>27</td>
<td>6/27 (22.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>178/16</td>
<td>483</td>
<td>60/483 (12.4%)</td>
</tr>
</tbody>
</table>

* Different superscripts (abc) indicate significant (P < 0.05) differences in prevalence rates between groups.

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2.4. Development of the quantitative real-time PCR assay for porcine PARV4

After multiple alignments of known genomic sequences of porcine PARV4, bovine PARV4 and ovine PARV4 obtained from the GenBank, a pair of detection primers (porcine PARV4-DF/porcine PARV4-DR) and a probe specific for porcine PARV4 were designed (Table 2), which covers a fragment of 223 nucleotides located in a conserved region within the VP1 gene. Selected positive PCR products were separated by electrophoresis on a 1% agarose gel, the target bands were excised and purified using the QIAquick\textsuperscript{TM} Gel Extraction Kit (QIAGEN Inc.), and the purified PCR products were cloned into the pGEM-T easy vector (Promega). The recombinant plasmids were transformed into TOP10 Escherichia coli bacteria (Invitrogen) and propagated following the instructions of the cloning kit manual. The plasmids were extracted using the QIAprep Spin Mini-preps Kit (QIAGEN Inc.) according to the manufacturers' instructions, quantified using a spectrophotometer (Nanophotometer, IMPLEN), and sequenced. The plasmids confirmed to contain porcine PARV4 were used as standards for the real-time PCR assay.

All real-time PCR reactions were carried out in 96-well plates. Standards were run in triplicate. Each reaction consisted of a total volume of 25 \( \mu \)l, containing 12.5 \( \mu \)l of the TaqMan Universal PCR Master Mix (Applied Biosystems), 2.5 \( \mu \)l of the sample or standard DNA, 1 \( \mu \)l of 10 \( \mu \)M of each of the two primers, 0.5 \( \mu \)l of the 10 \( \mu \)M probe and 7.5 \( \mu \)l distilled water. Amplification and quantification reactions were performed using the ABI 7500 Fast Real Time PCR System (Applied Biosystems) under universal conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A sample was considered negative if no threshold cycle (\( C_T \)) was detected in 40 amplification cycles.

The sensitivity of the quantitative real-time PCR was determined by testing ten-fold serial dilutions of the DNA standards (5 to 5 \times 10^{-6} copies). The specificity of the probe was confirmed by BLAST analysis and by testing samples positive for other DNA viruses and bacteria, including porcine circovirus type 2 (PCV2), torque teno sus virus (TTSuV), Mycoplasma hyopneumoniae and classical porcine parvovirus (PPV).

2.5. Genome sequencing of porcine PARV4

Based on multiple alignments of porcine PARV4, two pairs of primers were designed to amplify the near full-length genome sequences (4,982 bp), covering the entire protein coding regions (Table 2). The PCR products were purified and cloned into the pGEM-T easy vector, and the recombinated plasmids were propagated and verified as described in Section 2.4. Sequencing of the plasmids was performed by primer walking. The genetic distance between sequences and phylogenetic analysis were carried out with MEGA 5.0 as described (Tamura et al., 2011). The phylogenetic tree was constructed by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993).

2.6. Nucleotide sequence GenBank accession numbers

The nucleotide sequences of the nearly full-length genomes of the porcine PARV4 obtained in the present study have the following GenBank accession numbers: JQ425257 (porcine PARV4-238), JQ425258 (porcine PARV4-187), and JQ425259 (porcine PARV4-133).

2.7. Statistical analysis

For data analysis, SAS\textsuperscript{®} software version 9.2.0 SAS Institute, Cary, NC, USA) was used. Differences in porcine PARV4 prevalence rates among age groups or disease manifestations were investigated using the Fisher’s Exact test. First an overall assessment of on association was done using all groups and if significant (\( P < 0.05 \)), this was followed by pairwise comparisons.

3. Results

3.1. Validation of the real-time PCR assay

Real-time PCR on ten-fold serial dilutions of the DNA standards revealed a detection limit of 50 porcine PARV4 genome equivalents occurring around a \( C_T \) of 37 in all three replicates. Determined average slope, 92 and intercept value of the standard curve were -3.71, 0.999 and 43.4, respectively. No amplification was observed with DNA templates of any of the other non porcine PARV4 pathogens. This indicates that the present real-time PCR assay allows specific, efficient, and sensitive detection of porcine PARV4 species down to 50 genome equivalents or less in a 2.5 \( \mu \)l sample preparation input volume.

3.2. Porcine PARV4 is widely distributed in the United States

Prevalence data of porcine PARV4 obtained with the real-time PCR assays on lung tissues is summarized in...
Table 1. Among field samples, the C_T values of porcine PARV4 DNA positive samples varied from 18.3 to 39.8, corresponding to $5.8 \times 10^6$ to 9 viral copies per PCR reaction (approximately $2.32 \times 10^{10}$ to $3.6 \times 10^9$ viral copies per gram). All fetuses and suckling pigs examined were negative for porcine PARV4 DNA and the prevalence rate in the other age groups was 5.6% for nursery pigs, 18.7% for grow-finish pigs, and 22.2% for adult pigs. Porcine PARV4 was detected in 36 of the 178 sites located in 10 of the 16 states (Colorado, Iowa, Illinois, Indiana, Minnesota, North Carolina, North Dakota, Nebraska, Pennsylvania, and Texas) from which samples were received. Lack of detection in some states (Kansas, Michigan, Missouri, Ohio, Oklahoma, and Wisconsin) was likely due to lower numbers of samples and sites tested from these areas. An overall significant difference in porcine PARV4 prevalence rates among the different age groups was identified ($P < 0.001$) and the porcine PARV4 prevalence in adult pigs was significantly higher compared to nursery pigs (Table 1). This was not unexpected as increase in pathogen prevalence with age is commonly observed (Nieto et al., 2011; Xiao et al., 2012) unless there is particular tropism for fetuses. Across age groups, porcine PARV4 was detected in 12.4% of the lung tissues received.

Based on disease manifestations, porcine PARV4 DNA was not detected in abortion/reproductive failure cases ($n = 29$) and was present in 2.7% (1/37) of the pigs suffering from enteric disease, 11.6% (5/43) with systemic/central nervous system disease manifestation, and 14.4% (54/374) with respiratory disease. There was an overall significant ($P = 0.02$) difference in porcine PARV4 prevalence rates among the different disease manifestations. Specifically, the prevalence rates in respiratory disease were significantly ($P < 0.05$) higher compared to those associated with enteric disease or abortion. Moreover, within pigs suffering from respiratory disease, the proportion of porcine PARV4 positive pigs was significantly ($P < 0.05$) higher in adult pigs (21.7%; 5/23) and grow-finish pigs (18.3%; 40/218) compared to nursery pigs (6.9%; 9/130). Interestingly, the highest porcine PARV4 DNA loads (around $2.32 \times 10^{10}$ copies per gram) were identified in two pigs with respiratory disease and in one pig with systemic disease. Each of these pigs was coinfected with other pathogens which varied between pigs.

3.3. Molecular characteristics and phylogenetic analysis of porcine PARV4

The near full-length genomes from three of the 60 porcine PARV4 positive samples which originated in two different U.S. states, Iowa (porcine PARV4-133 and porcine PARV4-238) and North Carolina (porcine PARV4-187) were sequenced. The three obtained porcine PARV4 sequences were 4,982 nucleotides long and had sequence homology of 98.5–98.8% with each other. When compared to available porcine PARV4 sequences from other regions, the U.S. porcine PARV4 strains also showed high homology (97.6–99.1%). Additionally, overall nucleotide identities of 98.1–98.4% were demonstrated with porcine PARV4 sequences (GenBank accession numbers FJ982249 and FJ982250) collected in the U.K. in 1994.

The two large non-overlapping ORFs, ORF1 encoding a putative non-structural polyprotein NS1 (636 aa, amino acids) and ORF2 encoding overlapping putative VP1 (925 aa) and VP2 (555 aa) capsid proteins, were also identified in the three U.S. strains, with identities of 99.2–99.7% and 99.4–99.7% between each other, and 97.3–99.2% and 98.7–99.7% to the putative NS1, VP1 and VP2 proteins of other available porcine PARV4 sequences, respectively.

Phylogenetic analysis was conducted based on the multiple sequence alignment of the near full-length genome sequences of different PARV4, which included the three U.S. porcine PARV4s characterized in the present study and 30 additional PARV4 sequences obtained from pigs, humans, cattle, and sheep available in the GenBank database. The phylogenetic tree (Fig. 1) showed four distinct clades with high bootstrap values (100%) within the proposed genus PARV4-like: human PARV4, ovine PARV4, bovine PARV4 and porcine PARV4. Although the differences were overall minimal, United States isolates porcine PARV4 -187 and porcine PARV4-238 showed the highest relationship with U.K. strains Cl2001A, Cl2001B and Cl2001C (GenBank accession numbers FJ982246, FJ982247 and FJ982248), while strain porcine PARV4-133 showed a close relationship with the Romanian strain EU WB2010-681 (GenBank accession number JF738362) which was identified in 2010 (Fig. 1).

4. Discussion

In previous reports, porcine PARV4 prevalence rates of 44.4% in domestic pigs in Hong Kong (Lau et al., 2008), 58.6% in suckling pigs in the mainland of China (Li et al., 2012), 32.7% in German wild boars (Adlhoch et al., 2010), and 22.8% (during 2006/2007) and 50.5% (during 2010/2011) in Romanian wild boars (Cadar et al., 2011) were reported. In the present study, porcine PARV4 was identified and characterized in domestic pigs located in the U.S. The overall prevalence rate of porcine PARV4 in lung tissues was 12.4% (60/483). Increasing porcine PARV4 prevalence rates were observed with increasing pig age, which is supported by previous studies (Adlhoch et al., 2010; Cadar et al., 2011; Lau et al., 2008). However, in this study the overall prevalence of porcine PARV4 was lower compared to previously reported prevalence rates. This may be due to the overall younger age of the animals examined in the current study as essentially all of the pigs in this study were less than one year of age. Differences in samples types utilized for the testing may also account for the different results among studies. While lung tissues were utilized in the current study, serum samples, lymph nodes, liver and nasopharyngeal swabs were used previously (Adlhoch et al., 2010; Cadar et al., 2011; Lau et al., 2008). Interestingly, a recent investigation of liver tissues from Chinese pigs less than 20 days of age indicated a high porcine PARV4 prevalence rate (58.6%) in this age group (Li et al., 2012).

This study was designed as a pilot study with the main goal to determine if porcine PARV4 is present in North America. In order to accomplish this goal, lung tissues from pigs with various disease manifestations were tested by PCR for presence of porcine PARV4 DNA. Lung tissues were
chosen as this type of tissue is most commonly submitted with a variety of disease conditions and access to sufficient numbers of tissues was guaranteed for this study. While many pathogens tend to be distributed multisystemically when associated with severe disease, lung tissues are not necessarily always a good choice to investigate non-respiratory pathogens associated with enteric disorders or others. Healthy control pigs were not included in this study so it is not possible to conclusively associate porcine PARV4 with any specific symptoms or lesions in pigs. In this study, porcine PARV4 was identified in 14.4% of respiratory cases often concurrently infected with other respiratory pathogens including *Mycoplasma hyopneumoniae*, PRRSV and swine influenza virus (data not shown), which may indicate a possible role of porcine PARV4 in the swine respiratory disease complex which continues to be a major problem for pig producers. However, in stressful conditions such as those that can occur during infection with respiratory pathogens (Avitsur et al., 2011; Godbout and Glaser, 2006), the conditions for viral replication in lungs may be also optimized for non-respiratory viruses and caution is needed when interpreting this finding.

It has recently been suggested that the closely related human PARV4 is a potential cause of encephalitis in children (Benjamin et al., 2011) and in the current study we detected porcine PARV4 in 11.6% of the pig cases with a history of systemic/central nervous system disease. It is important that we continue to advance the body of

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knowledge on porcine PARV4 by doing experimental inclusions and thoroughly examining all organ systems in singularly-inoculated pigs. Transmission of human PARV4 has been associated with parenteral administration and the incidence of human PARV4 infection in injection drug users is high (Sharp et al., 2009; Simmonds et al., 2007). Blood transfusion in pigs is a rare and negligible event although feeding of porcine plasma as part of the diet for suckling and recently weaned pigs is common due benefits on growth performance and health (Peace et al., 2011).

In this study, porcine PARV4 was not detected in fetuses and suckling pigs and the prevalence rate in nursery pigs (5.6%) was low. In grow-finish pigs and mature pigs the infection rate of porcine PARV4 increased compared to the younger age groups. This could possibly be due to recent contact with porcine PARV4 shedders, consumption of porcine PARV4 contaminated plasma products through feed, or porcine PARV4 persistence in the environment and infection of pigs as they get older and lose passive immunity. Serological tests to profile herds for porcine PARV4 infection status are not available for pigs to date and assessment of the pig environment for presence of porcine PARV4 has not been done to the author’s knowledge. For this reason, the exact source of porcine PARV4 infection and route of transmission in pig herds needs further investigation.

Based on genome sequence comparisons, U.S. porcine PARV4 sequences have the same genomic organization and coding potential as the porcine PARV4 sequences reported previously (Adlhoch et al., 2010; Cadar et al., 2011; Lau et al., 2008; Tse et al., 2011). The high sequence identities and the close relationship of the U.S. strains with those from the U.K. and Romania, and the high identity with the strains collected in 1994 suggest that U.S. porcine PARV4 strains recently diverged from European strains. The high relatedness of the virus across geographically different regions suggests a highly efficient transmission route, most likely by live pig export/import or exchange of contaminated commercial biological products like vaccines. However, the origin of the U.S. porcine PARV4 and routes of porcine PARV4 transmission need further investigation including checking for the presence of porcine PARV4 in commercially available biological products. Availability of serological assays to determine individual immune responses to this virus and herd profiles would be very helpful in this regard.

5. Conclusions

To the authors’ knowledge, this is the first investigation confirming the presence of porcine PARV4 in the U.S. In the present investigation, the overall porcine PARV4 prevalence rate was 12.4% in lung tissues. The epidemiological data obtained through this study needs to be evaluated with caution and direct comparison with data obtained in other geographic regions may be hindered by differences in sample types and ages of the pigs sampled. The clinical and pathological significance of porcine PARV4 are both still poorly understood and serological investigation and biological assessment of PARV4 by experimentally infecting pigs are needed.

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