RESEARCH REPORT
SWINE HEALTH

Title: Characterization of emerging porcine parvovirus types in the U.S. pig population – **NPB #12-027**

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Industry Summary

A new porcine parvovirus (PPV), provisionally designated as PPV5, was identified in U.S. pigs. PPV5 is most closely related to PPV4 with overall genomic identities of 64.1-67.3%. The amino acid identities between PPV5 and PPV4 were 84.6%-85.1% for ORF1 and 54.0%-54.3% for ORF2. Epidemiologic investigations of PPV4 and PPV5 in U.S. pigs of different ages indicated a slightly higher prevalence for PPV5 (6.6%; 32/483) compared to PPV4 (4.1%; 20/483), with detection of concurrent PPV4 and PPV5 in 15.6% (7/45) of lungs of infected pigs. Virus isolation using established conditions similar to those for classical parvovirus were unsuccessful. Experimental inoculation of pigs revealed a low viremia length and magnitude for PPV4 followed by normal seroconversion. Infection of pigs with PPV5 positive material did not result in a detectable infection.

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Keywords: Porcine parvovirus; cloning; comparison; pig model

Scientific Abstract:

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Introduction

Parvoviruses are ubiquitous and are associated with a broad spectrum of clinical diseases in animals. Five different groups of parvoviruses that infect pigs have been identified to date, including classic porcine parvovirus (PPV) type 1 (PPV1), PPV2, PPV3 (also known as porcine PARV4, hokovirus, or partetravirus), PPV4, and porcine bocaviruses (PBoV) (Xiao et al., 2013c). The knowledge on prevalence and diversity of PPV in the U.S. is very limited as sow vaccination for PPV is routinely conducted in most breeding herds and testing for PPV DNA is often restricted to aborted fetuses from litters with increased numbers of mummies.

PPV4 was initially identified in 2010 in U.S. pigs diagnosed with porcine circovirus (PCV) associated disease, and subsequently was also identified in China, Hungary, and Africa (Cheung et al., 2010, Huang et al., 2010, Blomström et al. 2012). PPV4 is unique in that its genome nucleotide sequence is most closely related to bovine parvovirus 2 (BPV2), but its genome organization, characterized by presence of an additional ORF3, resembles that of Bocavirus (Cheung et al., 2010). Further characterization revealed that PPV4 had a circular or a head-to-tail concatemeric template in its DNA(Cheung et al., 2010), which is different from the traditional head-to-head or tail-to-tail intermediates.

During a previous PPV prevalence investigation we identified a novel PPV isolate different from the recently described PPV4 strain (76% on the nucleotide level based on 704 bp region located in the replicase gene of Genbank accession:GQ387500) and tentatively called "PPV5" (Xiao et al., 2013a). Further epidemiologic investigations of PPV4 and PPV5 in U.S. pigs of different ages indicated a slightly higher prevalence for PPV5 (6.6%; 32/483) compared to PPV4 (4.1%; 20/483), with detection of concurrent PPV4 and PPV5 in 15.6% (7/45) of lungs of infected pigs (Xiao et al., 2013b). The prevalence of PPV4 in China was much lower (2.09%) and PPV4 was often not associated with disease (Huang et al., 2010).

Mixed infections (more than one PPV subtype in the same sample) with PPV4/PPV5, PPV1/PPV4, PPV1/PPV5 were identified in the investigated pig tissues (data not shown). The rather high combined prevalence (>20%) of PPV in the U.S. could indicate that, besides being associated with reproductive failure, these viruses alone or in combination with other pathogens may also contribute to performance deficiencies in pigs.

Objectives

The objective of this study was to further characterize PPV5 *in* vitro and to investigate the pathogencity of PPV5 in growing pigs and breeding animals.

Materials and Methods

A. Isolation of PPV4 and PPV5

Cases obtained from routine submissions to the Iowa State University Veterinary Diagnostic Laboratory were tested for all known PPVs (PPV1, PPV2, PPV3, PPV4, PPV5) and PCV2. Cases positive for only a single PPV type and negative for PCV2 were selected for virus isolation. Virus isolation attempts were conducted on primary porcine kidney cells.

B. Full sequencing of the PPV5 genome

Sequencing of the entire PPV5 genome was conducted and the sequence was deposited in GenBank. In brief, the known genomic sequences of PPV4 were downloaded from the GenBank and analyzed with the Lasergene package (DNASTAR Inc.). A pair of primers located in the conserved region of the replicase gene, was designed to amplify a 704 bp fragment of PPV4. The PCR products were separated on a 1% agarose gel by gel electrophoresis, the target bands were excised and purified with the QIAquick® gel extraction kit (Qiagen), and cloned into the pCR®II-TOPO® vector (Invitrogen). The recombinant plasmids were transformed into TOP10 *Escherichia coli* bacteria (Invitrogen) and propagated following the procedures of the cloning kit manual. The identified recombined plasmids were extracted using the QIAprep Spin MiniPrep kit (Qiagen) according to the manufacturers' instructions, quantified using a spectrophotometer (Nanophotometer, IMPLEN), and then sequenced.

The majority of the genome sequence of the newly identified PPV5 was obtained by sequence-independent single primer amplification (SISPA). Five of the obtained products were used as templates in a

subsequent PCR reaction, in which a method similar to primer walking was adopted. New sequencing primers were designed based on the first PPV5 genome obtained.

C. Comparison of PPV4 and PPV5 at the genomic level

The sequences were assembled and analyzed with the software DNASTAR (Lasergene®) and DNAMAN Version 7 (Lynnon Corporation). Sequences were aligned by ClustalW and phylogenetic analyses were carried out with MEGA 5.0 (Tamura et al., 2011). The evolutionary trees were constructed by the Maximum Likelihood method based on the nucleotide sequences of non-structural gene (ORF1).

D. Experimental infection of pigs with PPV5

Four 5 week old pigs were randomly divided into 2 rooms with 2 pigs in each room. One pig in each room was infected with PPV4 or PPV5 by using a combination of intramuscular (1 ml) and intralymphoid (0.5 ml) routes. The other pig served as a contact control pig. The inocula selected for this study (Table 1) were PPV4 or PPV5 positive serum samples from the ISU VDL that were negative for the other known PPVs (PPV1, PPV2, PPV4) and also negative for PCV2.

Table 1: Selected inocula for infection of pigs.

ISULIMS Accession #	Sample	PCV2 ORF1	PPV1	PPV2	PPV3	PPV4	PPV5
2013013971	11	0	0	0	0	22.8	0
2013012457	6	0	0	0	0	28.9	0
2013022953	24	0	0	0	0	0	22.3
2013022953	6	0	0	0	0	0	26.5
2013013975	1	0	0	0	0	0	26.8

Blood and fecal samples were collected on days 0, 3, 7, 10, 15, 20 and 23. On day 15, pigs were re-challenged using the same inocula and necropsy was conducted at day 23.

D. Protein expression, purification and enzyme-linked immunosorbent assay (ELISA) development

Based on the predicted antigenic sites as determined by analysis with DNASTAR, one pair of primers was designed to amplify the partial capsid gene of each PPV1, PPV4 and PPV5. The following primers were utilized:

PPV1F 5-CGGGATCCCTAATGGTCGCACTAGACAC-3
PPV1R 5- GCGTCGACATGCATGTTAGATTTCCCT-3
PPV4F 5-CGGGATCCGAATATCTAAAAAACAT -3
PPV4R 5-TAGTCGACAATGGATAGTGGTGGTG-3
PPV5F 5-CCGGATCCGAAAACATTGATAACAT-3
PPV5R 5-TAGTCGACAATAGATAAAGGGGGCG-3

The primer contained restriction sites for BamH I within the forward primers and for Sal I within the reverse primers, which correspond to proteins of 267 amino acids for PPV1, 106 amino acids for PPV4, and 96 amino acids for PPV5. The gene fragments were amplified, expressed and identified according to methods described previously (Giménez-Lirola et al., 2012) except for using the expression vector pHUE (a gift from Dr. R. Rowland, Kansas State University). The proteins were then purified with PrepEase® His-tagged Protein Purification Midi Kit-High Specificity (USB Corp. Germany) according to the manufactures' instructions and evaluated in an ELISA platform (Giménez-Lirola et al., 2012) for cross-reactivity between PPV1, PPV4 and PPV5 antibodies. Briefly, microtiter plates were coated with each of the three recombinant polypeptides, diluted in phosphate-buffered saline (PBS) at a concentration of 1 µg/ml each and incubated overnight at room temperature. Plates were blocked with a fetal bovine serum solution, washed, and then incubated with sera diluted 1:100 in PBS containing fetal bovine serum for 60 min at 37°C. After a wash step, a 1:10.000 dilution of peroxidase-conjugated goat anti-swine immunoglobulin G (IgG) (Jackson) was added and incubated at 37°C for

60 min. Finally, the peroxidase reaction was visualized by using a tetramethylbenzidine-hydrogen peroxide solution as a substrate (KPL).

Results

A. Isolation of PPV4 and PPV5

Isolation was attempted on primary porcine kidney cells but as indicated in Table 2 was not successful.

Table 2: Isolation of PPV4 and PPV5 during serial passage on primary porcine kidney cells. Numbers listed under "passage" indicate PPV ct values.

Isolate	Lab ID	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5
PPV4	377	23.59	Not done	Not done	Negative	Negative
PPV4	379	22.60	Not done	Not done	36	Negative
PPV5	361	34.53	Not done	Not done	35.4	Negative
PPV5	367	27.91	Not done	Not done	Negative	Negative
PPV5	370	28.19	Not done	Not done	Negative	Negative

B. Experimental infection of CDCD pigs with PPV4 and PPV5

None of the pigs developed clinical signs of disease and appeared healthy until termination of the study. PPV4 viremia was detected in the PPV4 challenged pig on days 15 and 20 but not in the contact control. All pigs remained negative for PPV5 DNA. The PPV4 DNA positive pig seroconverted by day 20 to PPV4 but all other pigs remained seronegative for the duration of the study. There was no evidence of gross or microscopic lesions in the tissue collected from the pigs at necropsy.

C. PPV5 characterization

The genome sizes of PPV5 obtained in the present study varied from 5,516 bp to 5,805 bp, with typical PPV genome organization of two predicted major ORFs, ORF1 and ORF2, and 5′ and 3′ UTRs. However, the ends of the genomes were arbitrarily assigned, as the left and right ends of the linear viral genome were not determinable from the head-to-tail or circular configuration obtained by inverse PCR, as described for PPV4 (Cheung et al., 2010) (Fig 1).

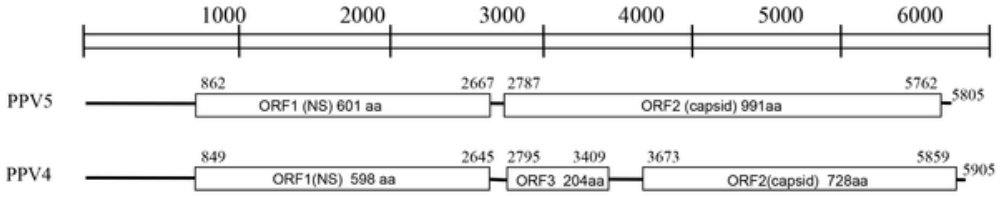

Fig. 1. Schematic representation of the genomes of PPV5 compared with PPV4.The position and size of putative ORFs are indicated. The GenBank accession numbers of the reference sequences are JX896321 (PPV5), GQ387499 (PPV4). aa: amino acid. NS: nonstructural gene.

D. Phylogenetic analysis of PPV4 and PPV5

PPV5 strains have the closest relationship with PPV4 strains, but form a separate subclade distinct from PPV4 in the trees (Fig. 2), supported by bootstrap tests.

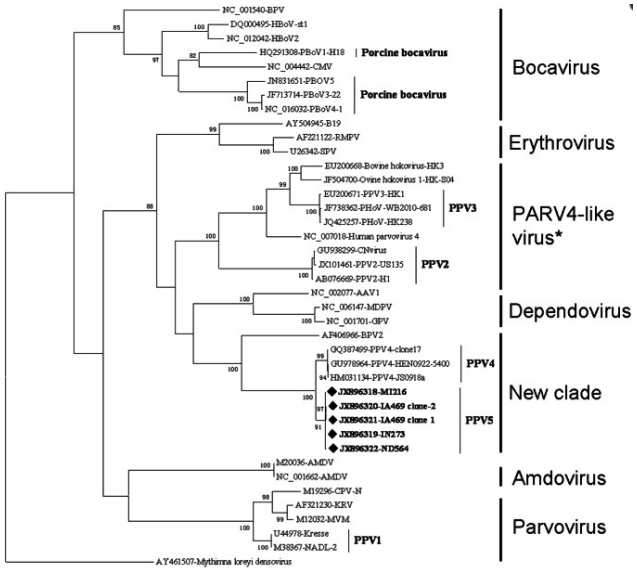

Fig. 2. The phylogenetic trees were constructed by using the Maximum Likelihood method based on the Poisson correction model, with the amino acid sequences of the nonstructural protein (ORF1, NS1).

D. Evaluation of cross-reactivity of the partial capid protein of PPV1, PPV4 and PPV5

Among the experimentally derived samples, all six negative control samples were negative by the PPV1 ELISA and the PPV4 ELISA while 1/6 samples were positive by the PPV5 ELISA. The three samples from pigs with passively derived antibodies were negative by the PPV1 ELISA and the PPV5 ELISA but one sample was positive for PPV4. Finally, among the positive controls, 9/9 were positive for PPV1 and 0/9 were positive for PPV4 and PPV5. Among the 22 field samples, 17/22 were positive for PPV1, 4/22 were positive for PPV4 (all four samples were also positive for PPV1) and 3/22 samples were positive for PPV5 (all three samples were also positive for PPV1 and PPV4). Of note, 3/22 field samples were positive for all three PPVs. These preliminary results indicate that the PPV1 ELISA appears to correlate well with the hemagglutination inhibition assay and that cross reaction with PPV4 and PPV5 is unlikely. However, testing of additional samples including known positive samples from pigs infected with either PPV4 or PPV5 is required for further evaluation.

Discussion

Recent evidence indicates that the age of the *Parvoviridae* family may exceed 40 to 50 million years (Shackelton et al., 2006). Moreover, the genomes of parvoviruses exhibit similar high mutation rates as RNA viruses (Shackelton et al., 2005 and 2006; Streck et al., 2011). These high mutation rates together with the long history may be the reason for the high diversity and the vast genetic divergence of the family *Parvoviridae.* Based on the present data, PPV5 could be inferred as an intermediate during the evolution of an ancestral PPV which evolved into PPV4, while the co-circulation of PPV4 and PPV5 in pigs might be evidence that the evolution of new PPVs is ongoing.

Our epidemiologic investigation based on U.S. pig samples revealed that based on DNA detection, PPV5 had a slightly higher positive rate than PPV4, specifically, 6.6% versus 4.1% in lung samples and 2.6% versus 1.9% in fecal samples (Xiao et al., 2013b). The overall prevalence of PPV4 DNA in our study was similar to the results reported previously in Hungary and China (Huang et al., 2010), which may indicate that PPV4 has similar prevalence rates in different geographical locations. Furthermore, the low detection in pre-suckling, suckling and nursery pigs suggests vertical transmission may not be a main route for both PPV4 and PPV5.

To the author's knowledge, serological assays have not been developed for any of the emerging parvoviruses in pigs to date. Based on a small evaluation in this study using serum samples from pigs experimentally infected with PPV1, it appears that antibodies against PPV1 do not react with PPV4 and PPV5 capsid proteins perhaps supporting that development of differential serological tools would be feasible and could be utilized in the future. Further investigations are urgently needed to determine the pathogenic

capabilities of both PPV4 and PPV5 under controlled conditions, and more extensive epidemiologic studies including analysis of samples from other geographic regions and countries are necessary to determine the distribution and transmission of PPV4 and PPV5.

PPV4 and PPV5 isolation attempts in cell culture failed. Experimental infection of pigs with PPV4 indicated a low viremia level followed by seroconversion while experimental infection of pigs with PPV5 was not successful. Further studies to better clarify the roles of PPV4 and PPV5 in pigs are underway.

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