

SWINE HEALTH

Title: Epidemiological investigation of the role of retroviremia in endemic diseases of swine. **NPB #07-065**

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Industry Summary:

Endogenous retroviruses are present in all bird and mammalian species including the pig. In humans most are benign but some are thought to be associated with genetic, autoimmune, and other disease syndromes. In the pig three categories of endogenous retroviruses exist. These are designated as Porcine Endogenous Retrovirus A, B, & C. (PERV-A, B, & C). Exogenous retroviruses are infectious and have the ability to become a part of the host species genome by incorporating their genetic code into the genetic code of the host. The endogenous retroviruses are believed to be remnants once infectious or exogenous retroviruses that over many years and generations lose infectiousness and the ability to replicate in the host. There are no known exogenous retroviruses in pigs. In recent years pig PERV's have been extensively studied out of interest to use the pig as a potential xenotransplant organ donor for humans. When certain pig cell culture lines are in direct contact with human cell culture lines, porcine endogenous retroviruses have the ability to infect the human cells. This raised fears that if pig organs were used in immune suppressed humans the pig viruses could jump species and become infectious and contagious in the human recipient and contact persons. PERV-A and a recombinant PERV-A/C which appears to regain the ability to replicate were infectious in the cell culture studies. Although these viruses have been extensively investigated in human xenotransplant studies, these endogenous viruses have not been examined as a potential health risk for the commercial pig. All pigs carry PERV-A and PERV-B in their genome but only a limited number are also carriers of the PERV-C. If it could be determined that the PERV-A/C acts in association with other agents leading to greater pathogenesis, the developed RT-PCR assays from this study could be used to eliminate the PERV-C from the gene pool since it is only present in a relatively small percentage of pigs.

The goal of this study was to develop several real-time RT-PCR assays capable to detection PERV-ABC, PERV-C and recombinant PERV-A/C. Once test capabilities were developed the further goal was to establish the level of PERV-C in commercial pigs in Iowa and the U.S. in a cross-sectional study. The final goal was to investigate the presence of PERV-A/C and determine any association with apparent disease. Once the assays were developed and validated, we surveyed several hundred commercial pigs of different ages and different sites to determine the relative levels of the three classes of PERV. As in other published reports, all the study pigs were positive to PERV-A and PERV-B. Only 24% of those sampled were positive to PERV-C while 18% were also positive to the PERV-A/C.

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

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The spontaneous recombination of PERV-A and PERV-C is thought to be associated with immune stimulation. To evaluate the pathogenic role of PERV-A/C in pigs suffering from disease outbreak situations, sites were identified and paired samples from affected and non-affected pigs were collected. Disease outbreaks associated with several agents were identified and paired samples were collected for assay. This final component of the study is still underway as we continue to identify disease outbreaks in various parts of the U.S. A second paper will be submitted for publication once the final analysis is completed.

Key words: Porcine Endogenous Retrovirus, exogenous retrovirus, PERV-A, PERV-B, PERV-C, PERV-A/C, xenotransplant, real-time RT-PCR.

Abstract:

The objective of this study was to determine the prevalence of porcine endogenous retrovirus (PERV) A/C recombinants and their association with disease expression in three commercial swine operations in the United States. To accomplish this goal, a general real-time reverse transcriptase (RT)-PCR assay specific for PERV-A, B, and C (PERV-ABC) and a real-time RT-PCR assay specific for PERV-C were utilized. In addition, a quantitative real-time RT-PCR assay for the detection of PERV-A/C was developed. The real-time RT-PCR was able to detect as low as 5 fg/ reaction of the pJET PERV-A/C clone corresponding to 3,600 copies/reaction or 144 copies/ μ l. The diagnostic specificity was found to be 100% with an intra-run coefficient of variance (CV) of 0.23%. The three assays were then used to screen pig serum samples obtained from three to 25 week old pigs (n=204 pigs and 369 samples) from three commercial swine operations in the U.S. While all 369 samples were found to be positive for PERV-ABC RNA, PERV-C RNA and PERV-A/C RNA were detected in 24.1% (89/369) and 18.7% (69/369) of the samples respectively. Twenty percent (43/215) of the samples collected from nursery pigs (3-9 weeks of age) were found to be positive for PERV-A/C RNA compared to 16.9% (26/154) of the samples collected from grow-finisher pigs (12-25 weeks of age). On two of the farms, serum was collected from healthy appearing pigs (n=60 pigs) and from their pen-mates suffering from various clinical conditions including diarrhea, wasting and respiratory disease (n=60 pigs). When clinically affected pigs were compared to unaffected pigs, 25% (15/60) of the samples from affected pigs were found to be positive for PERV-A/C RNA, whereas in clinically healthy pigs 8.5% (5/60) of the samples were found to be PERV-A/C positive. Interestingly, it was possible to identify PERV-A/C in the same pigs on more than one consecutive bleeding indicating PERV-A/C viremia. The tools developed in the course of this study are ideal for rapid and easy screening of large numbers of pig serum samples for presence of PERV-ABC, PERV-C or PERV-A/C. The obtained results indicate that PERV-A/C is present in the U.S. pig population and associated with continuous viremia in selected pigs. More studies using larger numbers of samples are warranted.

Introduction:

Retroviruses are RNA viruses that exist in two main groups: endogenous or exogenous retroviruses. Endogenous retroviruses are present in all vertebrates accounting for 5% of their genomes (Tucker et al., 2006). Pigs carry porcine endogenous retroviruses (PERV) in their genome (Langford et al., 2001) and three PERV subtypes have been identified to date. PERV-A and PERV-B are present in genomes of all pigs, and immortalized pig cell lines were able to produce virus capable of infection of certain pig and human cell lines. In contrast, PERV-C is absent from many pigs and this virus with its host range mostly restricted to porcine cells, is not infectious to human cells (Takeuchi et al., 1998). In order to look at the infectivity of the virus *in vivo*, immunosuppressed newborn rats and minks were inoculated with PERV (Specke et al., 2002). After inoculation, the animals were screened for a period of three months and monitored for antibody reaction against PERV and presence of proviral DNA. Over the observation period no antibody reaction was observed.

Furthermore, in the same study, non-human primates were inoculated with PERV after immunosuppression. Although a primary cell line originating from the same animals used was susceptible to PERV *in vitro* no evidence of *in vivo* infection was detected (Specke et al., 2002). More recently, the possible existence of exogenous porcine retrovirus as opposed to endogenous retroviruses in pigs was proposed (Scobie et al., 2004; Wood et al., 2004). It was demonstrated that human-tropic recombination between PERV-A and PERV C (designated as PERV A/C) was not a product of *in vitro* recombination; instead PERV-A/C appeared to exist *in vivo* as an exogenous virus (Wood et al., 2004). Furthermore, a PERV-A/C recombinant was isolated from porcine peripheral blood mononuclear cells that was not present in a proviral form in the miniature swine genome (Scobie et al., 2004; Wood et al., 2004). The recombinant virus was found to be infectious to human cells *in vitro*, such as the kidney epithelial 293 cell line (Bartosch et al., 2002; Krach et al., 2001; Martin et al., 1998; Martin et al., 2000; Oldmixon et al., 2002; Patience et al., 1997; Takeuchi et al., 1998; Wilson et al., 2000).

The role of endogenous retroviruses in mammalian hosts remains unclear, but evolutionary, physiological and pathological actions have been described (O'Neill et al., 2001; Ryan, 2004). Human endogenous retroviruses have been associated with a broad range of pathologies, including neoplasia, congenital defects, and autoimmune diseases (Deininger and Batzer, 1999; Portis, 2002). Although much is known about the three classes of PERV present within the pig genome, little work has been done on the potential of these viruses to be involved in the pathological processes of commercial growing pigs. A recent study conducted in the United Kingdom showed the presence of high levels of PERV RNA in serum samples obtained from a Large-White crossbred herd that suffered from porcine circovirus associated

disease (PCVAD) and porcine respiratory and reproductive syndrome (PRRS) (Tucker et al., 2006). This study also indicated the existence of retroviremia and the existence of PERV-A/C recombinants in pigs (Tucker et al., 2006). However, additional studies are necessary and required to confirm and investigate more closely the possible association of PERV-A/C retroviremia with porcine endemic diseases such as pneumonia, diarrhea, PRRS and PCVAD. The objective of this study was to determine the prevalence of porcine endogenous retrovirus (PERV) A/C recombinants and their association with disease expression in three commercial swine operations in the United States by using a quantitative real-time PERV-A/C reverse transcriptase (RT)-PCR assay, a PERV-C RT real time assay, and a PERV-ABC RT real time assay.

Objectives:

A recent pilot trial by the co-investigators confirmed the existence of a recombinant exogenous retrovirus in pigs from four European herds. High virus titers were found in grow-finish age pigs from two farms with high disease exposure and high death loss (10-20%). Low virus titers were seen in barrier-raised minipigs and conventional pigs from farms that were negative for porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2-associated diseases (PCVAD). This proposal will extend the pilot study to include US herds and generate additional data to support the hypothesis that retroviremia could be directly responsible for, or indirectly driving (via immunosuppression) poor health in growing pigs. The overall aim of this study is to investigate prevalence and degree of retroviremia in US herds and its association with pig health. To achieve this aim, three specific objectives are proposed:

1. Cross sectional sampling of defined US pig populations
2. Quantitative survey and sequence analysis of serum retroviremia in US pig populations
3. Epidemiological investigation of associations between retroviremia and endemic disease

Materials and Methods:

1. Samples origin

All serum samples were obtained from conventional crossbred pigs (varying proportions of Duroc, Landrace, Yorkshire and Large White genetics) from three different swine operations in the U.S located in Iowa, Kansas and North Carolina. The samples were collected during 2006 and 2007. A total of 204 pigs were sampled and a total of 369 serum

samples were analyzed. The blood was collected in 8.5 ml serum separator tubes (Fisher Scientific Inc.), immediately centrifuged at 2000 $\times g$ for 10 min at 4°C and stored at -80°C until use. All farms were multisite productions; other farm characteristics are summarized in Table 2. The age of the pigs sampled differed from farm to farm and pigs ranged from three to 25 weeks of age. This corresponds to the following swine production stages: Nursery pigs (3-9 weeks of age; n=215 samples) and finisher pigs (12-25 weeks of age; n=154 samples).

1.1. *Farm 1 samples.* Three different sites (A, B and C) were sampled. All three sites were wean-to-finish operations with subclinical PCVAD in the finisher pigs. Selected pigs on sites A and B were individually ear-tagged at three weeks of age and blood was collected from the same pig over time. A total of 35 pigs were sampled from site A where consecutive blood samples were collected at 3, 6, 9, 12 and 15 weeks of age (n=53 samples). Site B blood samples were obtained from 16 pigs at four consecutive bleedings at 6,9,14 and 25 weeks of age (n=163 samples). Site C samples consisted of 33 blood samples collected from 33 three-week-old pigs.

1.2. *Farm 2 samples.* The sample set from Farm 2 was obtained from 60 three-week-old pigs. Thirty of the 60 pigs were clinically healthy and the remaining 30 pigs suffered clinically from weight loss and diarrhea. Healthy and clinically affected pigs were housed within the same pens and barn. Fecal samples were cultured for bacteria and *Salmonella typhimurium* and *Escherichia coli* were isolated.

1.3. *Farm 3 samples.* Samples collected on Farm 3 were obtained from 60 25-week-old pigs on a farm with a recent PCVAD outbreak resulting in 30% mortality. As with Farm 2, at the time of blood collection, 30 pigs were clinically healthy and 30 showed clinical signs consistent with lethargy, decreased weight gain or weight loss and respiratory disease. Healthy and clinically affected pigs were housed within the same pens and barn. The presence of porcine reproductive and respiratory syndrome virus (PRRSV), PCV2, swine influenza virus (SIV), rotavirus type A, *Pasteurella multocida*, *Mycoplasma hyopneumoniae*, *Lawsonia intracellularis*, *Cryptosporidium*, *Streptococcus suis*, *Salmonella typhimurium*, and *Haemophilus parasuis* were confirmed on Farm 3.

2. RNA Extraction and general real-time RT conditions

RNA was extracted from 140 μ l of each serum sample using the QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Extracted RNA was stored at -20°C for further use. Each sample was run in triplicate for each PCR method (PERV-ABC, PERV-C and PERV-A/C). C_T readings throughout the paper represent the mean

of each sample.

3. PCR system and software information.

All of the reactions were carried out in a 7500 Fast Real-Time PCR system (Applied Biosystems™). The results were analyzed with 7500 Fast System Software Version 1.4.0 (Applied Biosystems™). From each amplification plot a C_T value was calculated representing the cycle number at which the reporter dye fluorescence was detectable above an arbitrary threshold. The threshold was set at a level that was significantly higher than the background, for all the detectors in all the PCR runs. Samples were considered positive when the C_T value was less than 45 and negative when there was no observed C_T value during the 45 amplification cycles. The detection software calculated the correlation coefficient (r^2) of the standard curve, standard deviation of triplicates and copy number of samples based on standard curve. Standard curves were accepted when r^2 was >0.99 and the reaction efficiency, measured as the slope of the standard curve, was between -3.2 and -3.9. Only reactions with a standard deviation under 0.31 were accepted. Quantification of the copy number was done by comparing the mean C_T values of different dilutions of the standard curve. The copy numbers were then multiplied by a dilution factor of 1:1.25 to obtain copy number/ml serum.

4. Development of a real-time RT-PCR for simultaneous detection of endogenous retrovirus (PERV) A, B and C classes (PERV-ABC).

4.1. *Primer and probe design.* The primers and probe (Table 1) for the detection of all three classes (A, B and C) of PERV were designed from the Gag protein coding sequence region 1332–1430, which is highly conserved in all the three classes of PERV. The probe was labeled with 6-carboxy fluorescein (FAM) reporter at the 5' end and Black Hole Quencher at the 3' end.

4.2. *PCR conditions.* The detection of PERV-ABC in pig sera was done by performing single step real-time RT-PCR assay using the TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems; ABI, Foster City, California, USA). The total reaction volume was 25µl and consisted of 12.5 µl 2X master mix without AmpErase® UNG, 0.625 µl of 40× multiscribe buffer, 20 µM of each primer (ABC-F and ABC-R), 5 µM of the ABC probe and 5 µl RNA. The thermocycler conditions consisted of 30 min at 48°C, 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Refer to

section 5.0 for machine info.

4.3. *Positive control.* As a positive control, RNA was extracted from the PK-15 ATCC CCL-33 cell line (American type culture collection, Manassas, Virginia, USA) infected with PERV A, B, and C. RNA was extracted from 200 µl of the cell line by using TRIzol® (Invitrogen Life Technologies, Baltimore, Maryland, USA). The RNA pellet was dissolved in 30 µl of DEPC treated water (Ambion, Austin, Texas, USA). Positive control RNA was used at a volume of 2.5 µl of RNA in the real-time RT-PCR reaction.

5. Development of a real-time RT-PCR for PERV-C

5.1. *Primer and probe design.* The primers and probe (Table 1) for the detection of PERV-C were designed from envelope protein coding sequence region 7483-7643. The reverse primer and the probe were found to be highly divergent from PERV-A in this region and cross-reaction with PERV-A or PERV-B was not expected. Due to a homologous region in the envelope protein with PERV-C, the PCR reaction was expected to detect PERV-A/C recombinants.

5.2. *PCR conditions.* Detection of PERV-C RNA was carried out using conditions described for PERV-ABC with the following modified thermocycler conditions: 40 cycles at 95°C for 15 s, at 55°C for 30 s followed by 60°C for 1 min.

5.3. *Positive Control.* As positive control for the PERV-C real-time RT-PCR assay, genomic DNA from a mini-pig from the United Kingdom (supplied by Dr. Linda Scobie) was used.

6. Development of a quantitative real-time RT-PCR for detection recombinant PERV A/C

6.1. *Primer and probe design.* The primers and probe specific for the detection of PERV A/C recombinants (Table 1) were designed from the variable and transmembrane region of the envelope protein coding sequence. Since PERV-A and PERV-C possess sequence dissimilarity in these regions, their detection with the A/C-F, A/C-R primers and the A/C probe was not expected.

6.2. *PCR conditions.* Real-time RT-PCR was performed on extracted RNA by using the OneStep RT-PCR Kit (Qiagen, Valencia, California, USA). The total reaction volume was 25 µl which consisted of the 0.2 mM dNTP mix, 0.8 µM of each primer (A/C-F, A/C-R), 0.2 µM of A/C probe labeled with CAL fluor® Orange 560 reporter dye at the 5' end and Black Hole Quencher™ dye at the 3' end, 0.5 µl of 1:10 diluted ROX™ passive reference dye (Invitrogen Life Technologies, Baltimore, Maryland, USA), 1.2 mM MgCl₂, 5 µl RNA and 1 µl RT enzyme mix. The single step real-time PCR

cycling conditions consisted of 30 min at 50°C, 15 min at 95°C followed by 45 cycles of 95°C for 15 s, 55°C for 30 s and 60°C for 2 min.

6.3. *Positive control.* RNA was extracted from the 13910 cell pellet (human 293 cells infected with a wild type isolate of PERV recombinant A/C; courtesy: Dr. Linda Scobie) by using TRIzol® (Invitrogen Life Technologies). The RNA pellet was dissolved in 50 µl DEPC treated water (Applied Biosystems™, Foster City, California, USA) and 5 µl of RNA was then used in the real-time RT-PCR reaction.

6.4. *Negative controls.* DNA from ST-IOWA cells (PERV-A positive only) and genomic DNA from a United Kingdom mini-pig (PERV-C positive only; courtesy of Dr. Linda Scobie) were used as negative controls.

6.5. *Quantification.* The standard curve was generated by using 1:10 serial dilutions of a pJET PERV A/C plasmid clone. A 1260 bp recombinant A/C fragment was amplified with the primer pair A/C-F and A/C-R using DNA extracted from the 13910 cell pellet. The amplified product was purified using the QIAquick PCR Purification Kit (Qiagen). The PCR product was blunted with a thermostable DNA blunting enzyme provided in CloneJET™ PCR Cloning Kit (Fermentas Inc., Glen Burnie, Maryland, USA) prior to ligation. Ligation and transformation was performed according to kit instructions. Presence of inserts in the clones was confirmed by PCR reaction using primer pairs A/C-F and A/C-R and sequencing.

6.6. *Sensitivity.* The sensitivity of the assay was evaluated by testing 1:10 serial dilutions of pJET plasmid clone containing the PERV-A/C recombinant insert.

6.7. *Specificity.* The specificity of the real-time RT-PCR assay was evaluated by performing the assay on known PERV-A/C positive control; 13910 cell pellet (human 293 cells infected with a wild type isolate of PERV-A/C recombinant), PERV-A positive control; ST-IOWA cells (PERV A positive only) and PERV-C positive control; UK mini-pig (PERV C positive only).

6.8. *Reproducibility.* The reproducibility of the assay was evaluated by testing all the PERV-A/C positive serum samples including the 1:10 serial dilutions of the pJET PERV A/C clone and the 13910 PERV-A/C positive cell pellet in triplicate and in three consecutive runs.

6.9. *Sequencing.* Sequencing of the pJET PERV A/C plasmid clone and PERV-A/C real-time RT-PCR positive serum samples was done by using 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA) at the DNA facility at Iowa State University (Ames, Iowa, USA).

7. Statistical Analysis

Statistical analysis was done using JMP software version 8.0.1. A student t-test was done in order to compare values of clinically healthy pigs versus clinically affected pigs. Differences in incidence were evaluated by using Fisher's exact test. A *P* value < 0.05 was considered significant.

Results:

1. Detection of PERV-ABC RNA

The detection rate with the PERV-ABC real-time RT PCR assay was 100% (204/204 pigs and 369/369 samples). In Farm 1, site A pigs had C_T values ranging from 24.9 to 34.8 with a mean C_T value (\pm SE) of 29.9 ± 0.35 , site B pigs ranged from 22.7 to 33.5 with a mean of 29.5 ± 0.15 and site C pigs ranged from 35.5 to 36.0 with a mean of 30.9 ± 0.41 (Table 3). For Farm 2, C_T values ranged from 22.3 to 38.1 with a mean C_T value of 32.2 ± 0.45 for clinically healthy pigs versus 30.2 ± 0.46 in clinically affected pigs which was statistically significant ($P = 0.003$). For Farm 3, C_T values ranged from 23.6 to 31.8 with mean C_T values of 32.2 ± 0.32 for clinically healthy pigs compared to clinically affected pigs that had mean C_T values of 30.2 ± 0.32 .

2. Detection of PERV C RNA

PERV-C RNA was found to be present in all farms with a prevalence ranging from 9.1% to 54.3% in the pigs sampled. From the 369 serum samples tested, 24.5% (89/369) were found to be positive for PERV-C across all farms which corresponded to 23.5% (48/204) pigs found to be positive for PERV-C. For Farm 1, on site A 5/16 pigs were found to be positive for PERV-C (Table 3), on site B 19/35 pigs were found to be positive for PERV-C (Table 3), and on site C 3/33 pigs were found to be positive for PERV-C. On Farm 2, PERV-C was detected in 0/30 healthy pigs and in 8/30 clinically affected pigs. On Farm 3, PERV-C was detected in 5/30 healthy pigs and in 8/30 clinically affected pigs.

3. Sensitivity, Specificity and reproducibility of the real-time RT-PCR assay for detection of PERV A/C RNA

3.1 Sensitivity. The sensitivity of the assay was evaluated by testing 1:10 serial dilutions of DNA (50 ng to .05 fg with copy number 36×10^9 to 36 copies/reaction) from the pJET plasmid clone with a PERV A/C recombinant sequence (Table 4). The real-time RT-PCR assay was able to detect as low as 5 fg/reaction of the pJET PERV-A/C clone corresponding to 3,600 copies/reaction or 144 copies/ μ l. The sensitivity was further compared with already published

(Scobie et al., 2004) gel-based RT-PCR for detection of PERV A/C recombinants. The gel based RT-PCR assay using same amount of RNA could detect only up to 5 pg/reaction corresponding 3,600,000 copies/reaction or 144,000 copies/ μ l. The real-time RT-PCR assay was found to be 1,000 times more sensitive than a gel-based RT-PCR (data not shown). The diagnostic sensitivity was found to be 30% (three more positive PCR reactions out of a total of 10 dilutions tested) greater for the real-time RT-PCR assay compared to the gel-based method.

3.2 Specificity. The specificity of the real-time RT-PCR assay was evaluated by performing the assay on known PERV-A/C positive (13,910 cell pellet) or negative (ST-IOWA, UK mini-pig) controls. No C_T readings (Table 4) were obtained in samples ST-IOWA and UK mini-pig (PERV-A only and PERV-C only positive controls), while fluorescence was detected using the PERV-A/C positive control and all of its dilutions indicating a diagnostic specificity of 100%. The specificity of the primers and probe used in the assay was also evaluated by testing DNA from HeLa cells (human cervical cancer epithelial cell line) known to have endogenous retroviruses. No C_T readings were obtained indicating absence of non-specific cross-reaction.

3.3. Reproducibility. The reproducibility of the assay was evaluated by testing all the PERV-A/C positive serum samples in triplicates and in three consecutive runs. The intra-run CV as determined from the mean C_T readings of the three replicates per sample was 0.23% while the inter-run CV determined from the mean C_T readings for a sample in three consecutive PCR runs was 0.52%. The standard deviation for the mean C_T values of the triplicates for the samples tested ranged from 0.064 to 0.162.

4. Detection of PERV A/C recombinants

Recombinant PERV-A/C RNA was present on all three farms with similar percentages as reported for PERV-C. From the 369 serum samples tested, 18.7% (69/369) were found to be positive for PERV-A/C across all farms which corresponded to 22.0% (45/204) pigs found to be positive for PERV-A/C. All PERV-A/C positives pigs were detected between 6 and 14 weeks of age, which roughly corresponds to the age at which pigs are in the late nursery and early finisher stages of production.

The mean \log_{10} load of PERV-A/C (\pm SE) in PCR positive animals on Farm 1, site A was 5.4 ± 0.31 , for site B it was 5.3 ± 0.32 and for site C it was 5.2 ± 0.14 (Table 3). For Farm 1, site A where four consecutive samples were taken over

time, 3/16 pigs were positive for PERV-A/C. The incidence of PERV-A/C in the three PCR positive pigs was as follows: 2/3 pigs were positive for 1 of the 4 collections and 1/3 pigs was positive for 2 of the 4 collections on consecutive blood collections. For Farm 1, site B where five consecutive samples from each pig were taken over time, 19/35 pigs were positive for PERV-A/C. The incidence of PERV-A/C in the 19 PCR positive pigs was as follows: 8/17 were positive for 1 of the 5 collections, 5/17 were positive for 2 of 5 collections (3/5 intermittent; 2/5 consecutive), 2/17 pigs was positive for 3 of 5 collections (1/2 intermittent; 1/2 consecutive), 2/17 were positive for 4 of 5 collections (1/2 intermittent; 1/2 consecutive), and 2/17 were positive during all five collections.

In Farm 2 all pigs determined to be positive for PERV-A/C retroviremia were found in the clinically affected group where 7/30 pigs were found positive for PERV-A/C. The mean \log_{10} load of PERV-A/C (\pm SE) in PCR positive animals was 5.7 ± 0.48 genomic copies /ml and the incidence of PERV-A/C was significantly ($P = 0.01$) higher compared to clinically healthy pigs.

In Farm 3, 5/30 unaffected pigs and 8/30 clinically affected pigs were found to be positive for PERV-A/C. The incidence of PERV-A/C positive pigs was not significant ($P = 0.53$) between groups. The mean \log_{10} load of PERV-A/C (\pm SE) in PCR positive clinically healthy pigs was 4.6 ± 0.10 and for clinically affected pigs it was 4.8 ± 0.12 .

5. Sequencing

Sequencing analysis of four PERV-A/C positive RT-PCR products (two from Farm 1 and two from Farm 2) revealed 100% homology with the previously described (Scobie et al., 2004) PERV-A/C recombinant retrovirus sequence.

Results by objective:

Objective 1 and Objective two: Cross sectional sampling of defined US pig populations and Quantitative survey and sequence analysis of serum retroviremia in US pig populations

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3.3. Reproducibility. The reproducibility of the assay was evaluated by testing all the PERV-A/C positive serum samples in triplicates and in three consecutive runs. The intra-run CV as determined from the mean C_T readings of the three replicates per sample was 0.23% while the inter-run CV determined from the mean C_T readings for a sample in three consecutive PCR runs was 0.52%. The standard deviation for the mean C_T values of the triplicates for the samples tested ranged from 0.064 to 0.162.

Objective three: Epidemiological investigation of associations between retroviremia and endemic disease

Detection of PERV A/C recombinants

Recombinant PERV-A/C RNA was present on all three farms with similar percentages as reported for PERV-C. From the 369 serum samples tested, 18.7% (69/369) were found to be positive for PERV-A/C across all farms which corresponded to 22.0% (45/204) pigs found to be positive for PERV-A/C. All PERV-A/C positives pigs were detected between 6 and 14 weeks of age, which roughly corresponds to the age at which pigs are in the late nursery and early finisher stages of production.

The mean \log_{10} load of PERV-A/C (\pm SE) in PCR positive animals on Farm 1, site A was 5.4 ± 0.31 , for site B it was 5.3 ± 0.32 and for site C it was 5.2 ± 0.14 (Table 3). For Farm 1, site A where four consecutive samples were taken over time, 3/16 pigs were positive for PERV-A/C. The incidence of PERV-A/C in the three PCR positive pigs was as follows: 2/3 pigs were positive for 1 of the 4 collections and 1/3 pigs was positive for 2 of the 4 collections on consecutive blood collections. For Farm 1, site B where five consecutive samples from each pig were taken over time, 19/35 pigs were positive for PERV-A/C. The incidence of PERV-A/C in the 19 PCR positive pigs was as follows: 8/17 were positive for 1 of the 5 collections, 5/17 were positive for 2 of 5 collections (3/5 intermittent; 2/5 consecutive), 2/17 pigs was positive for 3 of 5 collections (1/2 intermittent; 1/2 consecutive), 2/17 were positive for 4 of 5 collections (1/2 intermittent; 1/2 consecutive), and 2/17 were positive during all five collections.

In Farm 2 all pigs determined to be positive for PERV-A/C retroviremia were found in the clinically affected group where 7/30 pigs were found positive for PERV-A/C. The mean \log_{10} load of PERV-A/C (\pm SE) in PCR positive animals was 5.7 ± 0.48 genomic copies /ml and the incidence of PERV-A/C was significantly ($P = 0.01$) higher compared to clinically

healthy pigs.

In Farm 3, 5/30 unaffected pigs and 8/30 clinically affected pigs were found to be positive for PERV-A/C. The incidence of PERV-A/C positive pigs was not significant ($P = 0.53$) between groups. The mean \log_{10} load of PERV-A/C (\pm SE) in PCR positive clinically healthy pigs was 4.6 ± 0.10 and for clinically affected pigs it was 4.8 ± 0.12 .

Sequencing

Sequencing analysis of four PERV-A/C positive RT-PCR products (two from Farm 1 and two from Farm 2) revealed 100% homology with the previously described (Scobie et al., 2004) PERV-A/C recombinant retrovirus sequence.

Discussion:

The goals of the study were to develop several real-time RT-PCR assays capable of detecting PERV-ABC, PERV-C and recombinant PERV-A/C and then use the assays to determine the prevalence of PERV viremia (ABC, C, and A/C) in U.S. pigs. In addition, it should be determined if pigs remain PERV-A/C positive over time and if there was a potential association between retroviremia and disease. A total of 369 serum samples from three different farms were analyzed by real-time RT-PCR assays and 100% if the samples were found to be positive for PERV-ABC, 24.1% (89/369) were found to be positive for PERV-C, and 18.7% (69/369) of the samples were found to be positive for PERV-A/C.

In the present study, PERV-C retroviremia was found to vary from 9.1% to 54.30% on the investigated farms and sites. This is supported by previous studies which have shown the PERV-C subgroup to be either absent or represented in only a limited number of pigs in most of breeds (Li et al., 2006; Scobie et al., 2004; Tucker et al., 2006; Wu et al., 2008). The level of PERV-C in commercial swine is unknown; however, a large scale survey using samples from seven different Chinese miniature pig breeds conducted in China found 30.46% of the pigs to be positive for PERV-C (Wu et al., 2008).

PERV-A/C recombinants were detected in 9.1% to 54.3% of the pigs on the different farms. This was very similar to what was observed with PERV-C. Previous studies have suggested the level of activity of one or more PERV-C loci to drive the production of PERV-A/C recombinants (Hector et al., 2007). Further, in this study, the majority of the PERV-C positive samples were found to be also positive for PERV-A/C. In fact, 77.5% (69/89) of the PERV-C positive samples were also positive for PERV-A/C. The higher level of PERV-C viremia compared to PERV-A/C viremia can be explained from previous studies that have demonstrated that not all the type C PERVs recombine with PERV-A (Wood et al., 2004).

It has been suggested that higher levels of retroviral RNA are most prominent in pigs less than two months of

age (Tucker et al., 2006). Similarly, our study also found that PERV A/C recombinants were more prevalent in nursery pigs. When pigs were separated into nursery pigs (0-9 weeks of age) and grow-finish pigs (10-25 weeks of age), PERV A/C was detected in 20% of the samples (43/215) collected during the nursery stages and in 16.9% of the samples (26/154) collected during the grow-finish period. In pigs where multiple blood samples were collected from the same animals (Farm1, sites A and B), 37.3% (19/51) of the pigs were found to be positive for PERV-A/C with 57.9% of the pigs (11/19 pigs) being positive more than one time. Moreover, in 54.5% of those pigs (6/11) the presence of PERV-A/C was found to be consecutive rather than intermittent (45.5%; 5/11 pigs). To the author's knowledge, this represents the first characterization of PERV-A/C viremia in commercial pigs.

Finally, when we compared clinically healthy and diseased pigs (Farms 2 and 3), evidence was found that the prevalence of PERV-A/C was higher in clinically affected pigs (25%) compared to clinically healthy pen-mates (8.3%). This trend was especially evident on Farm 2 where PERV-A/C was only detected in clinically affected animals. Since the current study was limited to selected farms, limited pig numbers, limited age groups and limited disease etiologies further studies are warranted in this area as this may be a potential important finding. We are continuing to evaluate other sites with clinically affected and unaffected in the same barns and pens. An additional paper will be forthcoming.

The tools developed in the course of this study are fast, easy to perform and reliable and therefore ideal for rapid and easy screening of large numbers of pig serum samples for presence of PERV-ABC, PERV-C or PERV-A/C. Moreover, the assays could and should be used for future assessment of retroviremia levels in diseased and non-diseased pigs from both, commercial and experimental settings. The importance of ubiquitous viruses such as the different PERV types may be currently underestimated and especially PERV-A/C may play an important role in multi-factorial disease in pigs which gain more and more importance with wide-spread usage of vaccination and medication protocols and intense housing conditions on today's pig operations.

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