RESEARCH REPORT
SWINE HEALTH

Title: The role of boar semen in porcine circovirus type 2 (PCV2) transmission: Validation of diagnostic tools and determination of infectivity of PCV2 positive semen samples – **NPB #06-080**

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II. Industry Summary

Within the past 3 years, North American has seen a marked increase in porcine circovirus type 2 (PCV2) associated disease (PCVAD). The rapid spread of PCVAD has raised important questions about transmission of PCV2. To date, transmission of PCV2 is not well understood; however, PCV2 DNA has been detected in multiple body tissues and most body fluids of the pig. It is generally believed that the main route of transmission is the fecal-oral route. However, due to the rapid spread of PCVAD and the extensive use of artificial insemination in the swine industry, semen transmission has been suggested as a potentially important route of dissemination of PCV2. If that is found to be the case, testing and control measures could be initiated to minimize the risk of spread in semen.

It is essential that the industry has a highly sensitive and specific test to detect PCV2 in biological samples such as semen. Through this project we were successful in developing a polymerase chain reaction (PCR) test to detect PCV2 in semen, blood and serum. This is a new, highly sensitive and quantitative test with built-in internal controls for quality assurance. This procedure has now been published and is being adapted for use in several private and university diagnostic laboratories.

Since two main types of PCV2 (PCV2a and PCV2b) are now circulating in North America, it was important to use both types in our boar inoculation studies. Mature boars that were PCV2 naive were experimentally infected with either PCV2a or PCV2b. The boars were sampled (semen, blood swabs, and serum collected) 20 times over a 90 day period. A portion of the boars were euthanized at different time points post infection to determine the distribution and amount of PCV2 in reproductive organs and other tissues. Results indicated that both the PCV2a and PCV2b inoculated boars became subclinically-infected, seroconverted, and shed low quantities of PCV2 DNA in semen as determined by quantitative real-time PCR. PCV2 infection had no affect on semen quality. PCV2 was detected earlier in serum than it was in semen and blood swabs. Shedding of PCV2 peaked at 2-3 weeks post infection and in some boars persisted for the duration of the study.

A swine bioassay was used to evaluate if the PCV2 (PCV2a and PCV2b) DNA detected in semen is infectious. PCV2-naïve, 4 week old pigs were intraperitoneally inoculated with semen from the experimentally-infected boars. Serum samples were collected and all pigs inoculated intraperitoneally with PCV2 PCR positive semen became viremic and developed anti-PCV2 antibodies. This indicates that PCV2 virus in semen is infectious and breeding or insemination with semen is a potential route of PCV2 transmission.

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Finally, to evaluate if extended semen is a potential point source of infection, PCV2 positive semen was used to artificially inseminate PCV2-naive gilts. Gilts used for this portion of the study became pregnant and carried the pregnancy until termination of the study at 105 days of gestation. None of the gilts had evidence of PCV2-associated reproductive failure. Weekly blood samples from the inseminated gilts were negative for anti-PCV2 antibodies and serum samples from all fetuses were negative for PCV2 antibodies or virus.

In summary, a new, highly sensitive and quantitative PCR for detection of both PCV2a and PCV2b in semen and blood and serum was developed and is now available. PCV2a and PCV2b are shed in low amounts in semen of experimentally-inoculated boars. The PCV2 present in semen is infectious in a swine bioassay model; however, under the conditions of this study we were not able to transmit PCV2 or induce PCV2-associated reproductive failure by artificial insemination using PCV2-positive semen. The current state of knowledge does not support routine testing of semen from AI centers for PCV2 as we do for PRRSV.

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III. Scientific Abstract

Porcine circovirus type 2 (PCV2) is an economically important pathogen. It has been demonstrated that PCV2 DNA can be detected in boar semen by PCR; however, the biological relevance of this is unknown. Currently two distinct genotypes of PCV2 (PCV2a and PCV2b) are circulating in North American swine herds. The objectives of the study were to (1) develop and validate a quantitative PCR (qPCR) assay with an exogenous internal positive control (IPC) that can be utilized for the detection of PCV2 in semen samples, (2) evaluate and compare the amount of PCV2 DNA present in semen over time in PCV2a- and PCV2b-infected boars and correlate incidence and amount of PCV2 present in semen to that in serum and blood swabs, and (3) determine if semen positive for PCV2 DNA is infectious in a swine bioassay or when used for artificial insemination of gilts.

For the first objective, a duplex qPCR method for the simultaneous detection of porcine PCV2 and an exogenous IPC in semen samples was developed. The purpose of the IPC was to monitor DNA extraction and PCR inhibition. The IPC consisted of a mutated PCV2 plasmid clone which differed from the target PCV2 in the probe binding region and thus was detected by the use of a second probe with different end-labeling. Validation of the duplex qPCR was accomplished by testing semen samples from 12 boars experimentally-inoculated with PCV2, 10 boars naturally infected with PCV2, and 3 PCV2 negative control boars. To assess the second objective, fifteen 7-month-old PCV2 naïve Landrace boars were randomly allocated to three treatment groups: Group 1 (n = 3) served as negative controls, and groups 2 (n = 6) and 3 (n = 6) were intranasally and intramuscularly inoculated with PCV2a and PCV2b, respectively. Semen, serum, and blood swab samples were collected for up to 90 days post inoculation (DPI) and necropsies were performed on DPI 23, 48 or 90.

The final objective was completed by intraperitoneal inoculation of 4-week-old pigs with PCV2 DNA-negative (bioassay-control; n = 4), PCV2a DNA-positive (bioassay-PCV2a; n = 4), or PCV2b DNA-positive (bioassay-PCV2b; n = 4) raw semen, or PCV2 live virus (bioassay-positive; n = 4), respectively. Landrace gilts were used for the artificial insemination portion of the study and were inseminated with PCV2 DNA-negative semen (gilts-controls; n=3) from experimentally-infected boars, and six gilts were artificially inseminated with semen positive for PCV2a DNA (gilts-PCV2a; n = 3) or PCV2b DNA (gilts-PCV2b; n = 3).

We found that the duplex qPCR assay was more sensitive, specific, rapid, and repeatable than nested PCR (nPCR) methods for the detection of PCV2 DNA in semen. PCV2 DNA was found to be present mainly in the cell fraction portion of semen. Larger quantities of both PCV2a- and PCV2b- DNA were detected earlier in serum and blood swab samples than in raw semen. The incidence and duration of presence of PCV2 DNA in semen varied among individual boars. In all sex glands, PCV2 DNA was detected by PCR; however, PCV2 antigen was not detected by immunohistochemistry and PCV2 had no effect on sperm morphology. Differences in shedding patterns between PCV2a and PCV2b were not observed. In the final part of this project, pigs inoculated intraperitoneally with PCV2 DNA-positive semen and PCV2 live virus became viremic and developed anti-PCV2 antibodies indicating that the PCV2 DNA present in semen was infectious; however

serum samples collected from artificially inseminated gilts in all groups remained negative for anti-PCV2 antibodies for the duration of the experiment. In addition, fetal serum samples from all 105-day-gestation fetuses were negative for anti-PCV2 antibodies or PCV2 DNA.

In summary, under the conditions of this study, the duplex qPCR assay was found to be a valuable tool for accurate and quantitative detection of PCV2 DNA in boar semen. Boars shed low amounts of both PCV2a and PCV2b in semen without clinical signs and some of the boars had an extended period of shedding of PCV2 DNA in semen. In addition, PCV2 DNA-positive semen is infectious; however transmission to via artificial insemination may be dose related.

IV. Introduction

A marked increase of PCV2 associated disease (PCVAD) was observed in North America in 2005. In many of the cases, diagnostic investigations confirmed that these recent severe PCVAD outbreaks were associated with the apparent introduction of a new PCV2 genotype (PCV2b). The reports of rapid regional spread of PCV2b and PCVAD have raised important questions on transmission of PCV2. Early PCVAD research supports the idea that PCV2 transmission occurs through direct contact via oronasal, fecal, and urinary routes (Magar *et al.*, 2000; Bolin *et al.*, 2001).

In previous studies, PCV2 has been detected in semen samples using nested PCR techniques (Larochelle *et al.*, 2000; Kim *et al.*, 2001; McIntosh *et al.*, 2006) and virus isolation (Kim *et al.*, 2001). The amount of PCV2 DNA shed in semen is generally thought to be low. Larochelle *et al*. (2000) were the first to identify PCV2 viral DNA in semen after experimental inoculation of PCV2-naïve boars. PCV2 DNA was detected at the first collection of semen at 5 days post inoculation and intermittently thereafter until termination of semen collection on Day 47 at which time half of the boars were still positive using nested PCR (Larochelle *et al.*, 2000). Kim *et al.* (2001) also used nested PCR to detect PCV2 viral DNA in semen and found that all seminal plasma fractions (28/28 samples) were positive. More recently, McIntosh *et al*. (2006) associated extended shedding in semen of naturally infected boars with age and breed as younger boars were more likely to shed PCV2 for extended periods and higher incidence of shedding was observed in some breeds including Duroc and Landrace. Detection of PCV2 antigen in testes and accessory sex glands of the boar was reported by our group in a naturally-infected and clinically affected boar (Opriessnig *et al.*, 2006). However, the current body of evidence has still not clarified the role of boar studs in transmission of PCV2, namely, whether PCV2 positive semen can infect sows through artificial insemination.

It has been shown that PCV2 can infect fetuses and be associated with reproductive failure. Vertical transmission of PCV2 has been demonstrated in individual sows in the field (West *et. al*. 1999; O'Connor et al., 2001; Ladekjaer-Mikkelsen et al., 2001) and experimentally (Carlolet et al. 2002; Park et. al 2005). Fetal infection results in viremia, antibody production and/or fetal death (West et al., 1999; Sanchez et al., 2001). Park *et al.* (2005) inoculated six pregnant sows intranasally 3 weeks prior to expected farrowing and observed abortion and premature farrowing. Moreover, gilts inoculated oronasally two months before insemination and by the intra-uterine route at insemination by using 2ml of PCV2 inoculum at each time point had litters with mummified fetuses and PCV2-associated myocarditis (Rose *et al.*, 2007).

V. Objectives

<u>Part 1: To validate diagnostic and monitoring tools for porcine circovirus type 2 (PCV2) in boar semen and studs by:</u>

- Testing of serum by PCR
- Testing of semen by PCR
- Comparison of quantitative PCR versus nested PCR
- Characterization of the serological response by ELISA and IFA

<u>Part 2: To determine the role of boar studs in PCV2 transmission by:</u>

- Determination of the levels of PCV2 shed in semen over time
- Determination if the PCV2 in semen is infectious

VI. Materials and Methods

Part 1: To validate diagnostic and monitoring tools for porcine circovirus type 2 (PCV2) in boar semen and studs:

Source of samples

Semen samples for testing were obtained from twelve, 8 month old purebred Landrace boars experimentally inoculated with PCV2. The semen samples (n=36) tested were collected at 16, 20, and 23 days post inoculation (DPI). Serum samples collected from experimentally inoculated boars were positive for PCV2 DNA as determined by qPCR (Opriessnig et al., 2003) on or before DPI 16 and the boars developed anti-PCV2 antibodies post inoculation as determined by ELISA (Nawagitgal et al., 2002). Samples from 3 PCV2-naive control boars (n=9) were also tested. In addition, semen samples from boars in 4 different commercial boars studs with natural exposure to PCV2 (n=10) were tested. Naturally exposed boars ranged from 9 to 38 months of age.

Sample handling

Raw semen samples from the experimentally-infected boars were immediately aliquoted in 1.5 ml vials and stored at -80°C after collection and until the time of DNA extraction. Cell and seminal plasma semen fractions were separated following centrifugation of raw semen at 800 × g for 20 min and were also stored at -80°C until further processing. Raw semen samples collected from boar studs were shipped on ice overnight from the boar studs to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) and stored at -80°C on arrival.

Development of the Internal Positive Control (IPC) for the PCR

The exogenous IPC was a mutated PCV2 molecular DNA clone constructed by mutating a stretch of 25 bases at nucleotide position 1582 in the published sequence (GenBank accession no. **AF264042**) of the original single-copy PCV2 clone (Fenaux et al., 2002). Since four restriction sites, MluI, HpaI, BglII and AvrII, are absent in both the PCV2 genome and the backbone vector pBluescript SK II, the sequences of them were used to substitute the 25-bp probe sequence in the order. A fusion PCR technique was performed to generate a fusion fragment containing the mutated probe sequence. In the first-round PCR, the upstream fragment was amplified using the original PCV2 plasmid with a forward primer XO5 and a reverse primer MP3 while the downstream fragment was amplified with a forward primer MP5 and a reverse primer EC3. The two fragments were purified and used as the templates for the second-round PCR with primers XO5 and EC3. *Pfu* Ultra high-fidelity DNA polymerase (Stratagene, La Jolla, California) was used in PCR amplification to minimize unwanted mutations. The second-round PCR product (~1 Kb) was double-digested with XhoI and EcoRI restriction enzymes and ligated into the original PCV2 plasmid that was digested with the same restriction enzymes. One of the obtained mutated clones, verified by sequencing, was used as the exogenous IPC.

DNA extraction

Each semen sample was extracted twice, without (200 µl semen sample) and with inclusion of the IPC (200 µl semen sample and 10 µl of the IPC at a concentration of 0.02 pg or 2045 genomic copies). DNA was extracted using a commercial kit (QIAamp® DNA Mini Kit, Qiagen Inc., Valencia, California) with the following modifications: For complete and efficient lysis of the cells and to decrease the lysis time the incubation time was extended to 1.45 hr at 65°C with brief vigorous vortexing every 20 min. For proper solubilization and homogeneity and to ensure optimal binding of the DNA to the column membrane, the amount of ethanol and buffer AL was increased from 200 µl to 400 µl each. To increase the final DNA concentration, the final DNA elution volume was 50 µl and the incubation time after adding the elution buffer was increased to 10 min. The DNA was used directly or stored at -20°C for future use.

qPCR

The sequence of the primers and probes used for detection of the target PCV2 DNA and the exogenous IPC are shown in Table 1. The primer set and P1591 probe were designed from the highly conserved region of

the PCV2 genome (Opriessnig et al., 2003) using computer software (ABI Prism Primer Express, Version 1.5, PE Applied Biosystems, Foster City, California) according to the manufacturer's suggestions. The P1591M probe was complementary to the modified nucleotide positions 1582 to 1606 in the mutant PCV2-plasmid. While the primer set was the same for both the target PCV2 DNA and the IPC, the two designed probes differed in specificity as well as labeling. The P1591 probe was specific for the target PCV2 DNA and labeled with fluorescent reporter dye 6-carboxyfluorescein (6FAM) at the 5' end and quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. The P1591M probe was specific for the IPC as it was designed from the mutated PCV2 sequence present only in the internal control and lacking in the target PCV2 DNA. It was labeled with a different reporter dye (VIC) at the 5' end but same quencher (TAMRA) at the 3' end.

Table 1. Primers and probes used in the generation of the exogenous IPC and duplex qPCR assay for detection of the target PCV2 DNA and the exogenous IPC.

Name		*Sequence (5'-3')*	*Nucleotide Position*
XO5	Forward primer	AACTCGAGCTGCAGCCC	N/A (complementary to the vector sequence)
MP3	Reverse primer	GGAGATCTGTTAACACGCGTTAATCAGAATACT G	1568-1601
MP5	Forward primer	TGTTAACAGATCTCCTAGGCGTACTCCTCAACTG	1587-1620
EC3	Reverse primer	TAAGGTTGAATTCTGGCCCTGC	415-436
P1570	Forward primer	TGGCCCGCAGTATTCTGATT	1561-1580
P1642	Reverse primer	CAGCTGGGACAGCAGTTGAG	1613-1632
P1591	Probe	*6FAM*-CCAGCAATCAGACCCCGTTGGAATG-*TAMRA*	1582-1606
P1591M	Probe	*VIC*-ACGCGGTTAACAGATCTCCTAGGC-*TAMRA*	1582-1606

In order to develop and validate the IPC and the duplex qPCR assay for PCV2 the we did the following steps: (a) The optimal concentration for the IPC was determined; (b) the two probes were checked for cross-reactivity; (c) the qPCR assay was tested as single and as (d) duplex qPCR assays on experimentally- and on naturally-PCV2-infected boars as wells as on negative control boars, and then compared the results from (c) and (d) to each other and also to results obtained by using (e) nPCR assays.

a. Optimal IPC concentration. To avoid any interference in the amplification and detection of target PCV2 DNA by the IPC, it was necessary to determine the amount of IPC being spiked in each semen sample. To identify the IPC amount with the least inference, the threshold cycle (C_T) readings for the PCV2 target DNA in the duplex PCR was compared with the corresponding C_T reading obtained in the single PCR. IPC 1:10 serial dilutions including concentration ranging from 0.2 ng (20,440,000 copies) to 0.002 pg (204 copies) were added to each semen sample.

b. Cross-reactivity of the probes. The probes were checked for possible cross-reactivity by serial diluting (1:10 to 1:10,000) the two standards (PCV2 plasmid; mutated PCV2 plasmid) and by testing both standards separately or combined in single (P1591 probe or P1591M probe) and duplex (P1591 and PM1591M probes) PCR assays.

c. Single qPCR without IPC. The single qPCR reaction consisted of a total volume of 25 µl containing 12.5 µl of the commercially available master mix (TaqMan Universal PCR Master Mix, PE Applied Biosystems), 5 µl semen DNA (extracted without spiking IPC), 400 nM of each primer and 200 nM of the P1591 probe specific for the target PCV2 DNA or 200 nM of the P1591M probe specific for the mutant PCV2 plasmid. All the reactions were carried out in triplicates. Five progressive 1:10 dilutions of a known copy

number of PCV2 genomic DNA excised from a purified PCV2 plasmid or of mutant PCV2 DNA excised from a purified mutant PCV2 plasmid were used to generate the standard curves. The reactions were carried out in a 7500 Fast Real-Time PCR System (ABI, Foster City, California). The PCR cycling parameters were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The results were analyzed with 7500 Fast System Software version 1.4.0 (ABI, Foster city, California). From each amplification plot, a C_T value was calculated representing the PCR 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. Samples were considered to be positive when the C_T value was equal or less than 40.

d. Duplex qPCR with IPC. A duplex PCR reaction for simultaneous detection of both target PCV2 DNA and the IPC in a single tube was conducted for each semen sample and the semen fractions. The duplex PCR reaction mixture consisted of a total volume of 25 µl containing 12.5 µl of the commercially available master mix (TaqMan Universal PCR Master Mix, PE Applied Biosystems), 5 µl DNA, 800 nM of each primer, 200 nM of the P1591 probe and 200 nM of the P1591M probe. The PCR cycling conditions and data analysis were same as described above.

e. nPCR. The 36 raw semen samples from boars experimentally inoculated with PCV2 and the 9 samples from the negative control boars were assayed for detection of PCV2 DNA using two different previously published nPCR protocols and primer sets [nPCR protocol A, (Larochelle et al., 2000); nPCR protocol B, (Kim et al., 2003)]. Briefly, 5 µl of the extracted semen DNA (without IPC) was used for amplification of outer PCR products in a 50 µl reaction and 10 µl of the outer PCR product was then used as template for nPCR reaction. The nPCR products were run on 1.3% agarose (Amresco, Solon, Ohio) gel and bands were visualized with ultraviolet light after staining with ethidium bromide.

Statistical analysis

The coefficient of variation (CV) was calculated by using the following equation: standard deviation/mean*100, where the standard deviation is the root mean square deviation of the values from their arithmetic mean.

Part 2a: Boar inoculation part of the study:

Animals and housing

Fifteen male, purebred Landrace pigs were segregated early weaned from a single farm and brought to the Iowa State University Livestock Infectious Disease Isolation Facility at approximately 3 weeks of age. The source farm was known to be free of porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV) by frequent serologic surveillance of the herd. Animals were group housed until 6 months of age and then were individually penned.

Experimental design

At 7 months of age, boars were randomly allocated into three groups. Group 1 (n = 3) served as negative controls, and group 2 (n = 6) and 3 (n = 6) boars were inoculated with PCV2a or PCV2b, respectively. Randomly selected boars from each group were euthanized at three different time points during the study by lethal overdose of intravenous pentobarbital. Necropsies were performed on one boar from group 1 and two boars from each of groups 2 and 3 at DPI 23, DPI 48, and DPI 90. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee.

Inoculation

Group 2 boars were inoculated intranasally (3mL) and intramuscularly (2mL) with a stock of PCV2a isolate ISU 40895 with an infectious titer of $10^{4.5}$ $TCID_{50}$ per ml. The PCV2a isolate was originally obtained from a clinical PCVAD affected pig in a western Iowa herd in 1998 and has been shown to cause PCVAD in previous experimental studies using growing pigs. Group 3 boars were inoculated intranasally (3mL) and intramuscularly (2mL) with a stock of PCV2b isolate NC 16845 with an infectious titer of $10^{4.3}$ $TCID_{50}$ per ml. The PCV2b isolate was acquired from a finishing pig affected with severe PCVAD in North Carolina in 2006. Both PCV2a and PCV2b infectious virus stocks were generated similarly by transfecting PK-15 cells with

respective PCV2a and PCV2b infectious DNA clones. The PCV2a and PCV2b isolates shared 95.7% nucleic acid homology. The virus inocula were stored at -80°C until experimental inoculation.

Sample Collection

Semen and serum samples were collected from all boars prior to PCV2 inoculation on DPI -3. Semen, serum, and blood swabs were collected on DPI 2, 4, 6, 9, 13, 16, 20, 23, 27 and every week thereafter until DPI 90. In addition, serum samples were collected on DPI 1 from all boars. For sample collection, individual animals were moved into the collection pen, and once mounted on the collection apparatus, gloved-hand semen collection was initiated using an insulated mug lined by a 2 liter disposable plastic bag with tear-away filter. Post ejaculation, the filter portion of the collection bag containing the gel fraction was torn off and discarded. Bags containing raw semen were sealed and placed immediately into a thermal container and transported to the laboratory for processing.

Concurrently, during semen collection, serum and blood swabs were obtained while the boar was mounted on the collection apparatus. Whole blood was collected by venapuncture of an ear vein and collected into a 300 µL serum gel capillary tube. Similarly, blood swabs were obtained using individual packaged sterile swabs and placed into a sterile 5mL polystyrene round-bottom tube containing 0.5mL sterile saline. After capillary tubes were centrifuged, the serum and blood swabs were stored at -80°C until tested.

Semen sample processing

Collection bags containing raw semen samples were gently agitated by inverting the sealed bag to re-suspend spermatozoa equally after transportation. Raw semen was carefully allocated into a sterile 1.5mL centrifuge vial using a sterile 10mL graduated serology pipette and stored at -80°C until testing.

Semen morphology

For morphology analysis, well-mixed raw semen from each ejaculate was initially diluted at a rate of 1:10 to 1:20 with pre-warmed extender. Subsequently, the diluted semen was mixed with an equal volume of buffered formal saline for preservation. A full morphology differential was performed on each sample with wet mounts and a phase contrast microscope at 1,000× under oil.

Serology

Serum samples were tested for the presence of anti-PCV2 IgG antibodies using an ELISA based on PCV2 recombinant major capsid protein (ORF2) as previously described. Samples with a sample-to-positive ratio equal or greater than 0.2 were considered positive.

PCR

Serum, blood swab samples, and raw semen were tested for the presence and amount of PCV2 DNA by quantitative real-time PCR as previously described. An internal positive control composed of the PCV2 genome clone with 25 nucleotide substitution beginning at position 1582 (mutated PCV2) was added to the raw semen samples prior to DNA extraction for quality control as previously described. In addition, selected tissue samples from each boar (lymph node, testes, epididymis, bulbourethral gland, seminal vesicle, and prostate) collected at necropsy were also tested for PCV2 DNA. Briefly, DNA was extracted from 0.25g tissue using a commercially available isolation kit following the manufactures protocol and recommendation. The PCR parameters used in this study were essentially the same as previously described.

Necropsy

At necropsy, lungs were evaluated for the presence of pneumonia lesions (0-100% of the lung affected by lesions), lymph nodes were examined for evidence of enlargement, and additional gross observations in other organ systems were recorded. Sections of all major organ systems were collected in 10% buffered formalin. Tissue sections collected included testes, epididymis, seminal vesicles, bulbourethral gland, prostate, penile tissue, four lung sections (cranial, middle, caudal and accessory lobes), liver, spleen, kidney, tonsil,

ileum, colon, thymus, lymph nodes (tracheobronchial, mediastinal, mesenteric, iliac, superficial inguinal), myocardium, and urinary bladder.

Microscopic evaluation and immunohistochemistry

Formalin-fixed tissue samples were routinely processed, stained with hematoxylin and eosin and evaluated in a blinded fashion by a veterinary pathologist. Microscopic lesions characteristic of PCV2-infection (depletion of germinal center lymphocytes and histiocytic replacement or infiltration) in lymphoid tissues (lymph nodes, thymus, tonsil, and spleen) were scored as previously described. IHC staining using rabbit polyclonal antisera for PCV2 antigen detection was performed on lymphoid tissues, testes, epididymis, seminal vesicles, bulbourethral gland, prostate, and penile tissue. The presence and amount of PCV2 antigen was scored as previously described.

Statistical analysis

Data was assessed for overall quality, including normality. Differences in amount of PCV2 DNA in serum, semen, and blood swab samples were analyzed using repeated measures analysis of variance (MANOVA) with DPI as fixed factor followed by non-parametric Kruskal-Wallis one-way ANOVA. Data until DPI 48 were included for the analysis. Differences in amount of PCV2 DNA in tissues (lymph node, testes, epididymis, seminal vesicles, bulbourethral gland, and prostate) and normal sperm morphology were assessed using non-parametric Kruskal-Wallis one-way ANOVA. Calculations were done using statistical software, SAS®. Outliers were excluded from the analysis.

Part 2b: Swine Bioassay and Artificial Insemination Portion of the Study:

Animals

Swine bioassay study. Twelve, four-week-old mixed breed pigs were purchased from a commercial farm negative for antibodies to PCV2, PRRSV, and swine influenza virus (SIV).

Artificial insemination study. Nine, three-week-old purebred Landrace gilts were purchased from a farm negative for antibodies to PRRSV and SIV and positive for antibodies to PCV2.

Semen. Raw semen negative for PCV2 DNA, raw semen positive for PCV2a DNA, and raw semen positive for PCV2b DNA collected from a previous PCV2 experimental inoculation trial were used in this study. The raw semen samples were tested and found to be negative for the presence of porcine circovirus type 1 (PCV1) DNA, porcine parvovirus (PPV) DNA, or PRRSV RNA by PCR. Aliquots of fresh raw semen were saved and placed into sterile 5ml polystyrene round-bottom tubes and stored at -80°C. The remaining raw semen was extended at a 1:1 dilution with a commercially available semen extender, which was warmed to match semen temperature, and the extended semen was then cooled to approximately 17°C. A portion of each semen sample was further diluted with semen extender to approximately 5 billion sperm per 80ml insemination dose. The extended semen was stored at 17°C for up to 96 hrs and rotated twice daily.

Animals, housing, experimental design, and inoculation

Swine bioassay study. The pigs were randomly divided into 4 groups and each group was housed in a separate room on arrival at the isolation facility. Each room was equipped with an ante-room where personnel protective equipment (face masks, boots, gloves, and disposable covers) were supplied for room entrance by animal care takers, and each room was ventilated separately. Animals were contained in a 1.5 × 2.5 m pen supplied with a nipple drinker and a self feeder. Animals were fed *ad libidum* a balanced, pelleted, complete feed ration free of animal proteins and antibiotics. Inoculation was done intraperitoneally for all groups using an aseptic technique and a 24 gauge, ½ inch hypodermic needle as described. The bioassay-control group received 7ml of PCV2 DNA-negative raw semen, the bioassay-PCV2a group received 7ml PCV2a DNA-positive raw semen, and the bioassay-PCV2b group received 7ml PCV2b DNA-positive raw semen. The number of PCV2 genomic copy numbers/ml of raw semen was $10^{5.5}$ and $10^{5.3}$ for PCV2a and PCV2b, respectively. The PCV2a and PCV2b isolates shared 95.7% nucleotide sequence identity. The bioassay-positive group received 3ml of PCV2a live virus (ISU-40895) generated by transfection of PK-15 cells with the PCV2a

ISU-40895 infectious DNA clone at a dose of $10^{4.5}$ $TCID_{50}$ combined with 4 mLs of sterile saline. Serum samples were collect weekly, starting on the day of inoculation (Day 0 post inoculation; DPI 0). On DPI 49, all pigs were humanely euthanized by lethal overdose of intravenous (auricular vein) pentobarbital. At necropsy, gross lesions were assessed on each pig and tissues from all major organ systems were collected for microscopic examination and immunohistochemistry (IHC).

Artificial insemination study. The gilts were randomly divided into 3 treatment groups and isolation rooms at 8 months of age. The general room set-up was similar to that described for the bioassay pigs. The gilts were housed in a 2 × 4 m pen supplied with a nipple drinker. Animals were fed daily approximately 2.5 kg of a balanced ground corn-soy based complete feed ration free of animal proteins and antibiotics. Prior to artificial insemination, gilts were individually synchronized for estrus detection for 17 consecutive days using a commercially available liquid product, that was added to the daily feeding at the recommended dosage. Twenty-four hours after termination of the treatment, each animal received a 5ml intramuscular injection of gonadotropin and was then bred by artificial insemination upon estrus detection. Each gilt was inseminated up to three times during the same estrus period in 24 hour intervals. Initially, 80 ml of 1:1 extended raw semen (collected on the same day) was used. For second and third artificial insemination matings, semen extended to approximately 5 billion sperm per 80ml dose was used. To evaluate a potential negative effect of the semen extender used on infectivity of the PCV2, an equal amount of extender was added to PCV2 live virus in a 1:1 dilution. In addition and as a control, PCV2 live virus was diluted with minimum essential medium (MEM) in a dilution of 1:1. The PCV2 titer was estimated by immunofluorescence assay (IFA) with PCV2 a specific antibody as described at 0, 8, 24, 48, and 96 hours post dilution. Serum samples from all gilts were collected prior to insemination and weekly thereafter. At approximately 5 and 8 weeks post artificial insemination, ultrasonography was used to confirm pregnancy. Necropsy was performed at 105 days of gestation and gilts were humanely euthanized by intravenous (auricular vein) overdose of pentobarbital. All fetuses were immediately extracted and fetal serum was collected.

Serology

All weekly collected serum samples were analyzed for presence of anti-PCV2 IgG antibodies with an opening reading frame 2 (ORF2) based PCV2 ELISA as previously described. A serum with a sample to positive ratio (s/p) equal or greater than 0.2 was considered to be positive.

Quantitative real-time polymerase chain reaction

Serum samples collected weekly from the bioassay pigs and individual fetal serum samples were tested for the presence and amount of PCV2 DNA by quantitative real-time PCR as described.

Sequencing

PCR products from the PCV2a or PCV2b positive semen, the PCV2 inoculum, and serum from one randomly selected pig at 49 DPI in each of the bioassay groups (bioassay-PCV2a, bioassay-PCV2b, and bioassay-positive) was sequenced and compared to each other. A nested PCR was used to amplify the entire ORF2 gene for sequencing and subsequent sequence comparison as previously described.

Necropsy

At necropsy, the percentage of lungs affected by lesions ranging from 0-100% and presence and degree of lymphadenopathy were evaluated. Both fresh tissues and tissues fixed in 10% buffered formalin were collected. Tissues included lung (all lobes), liver, spleen, kidney, tonsil, ileum, colon, thymus, multiple lymph nodes (tracheobronchial, mediastinal, mesenteric, iliac, superficial inguinal) and myocardium. In addition, uterus, ovary and placenta were collected from the breeding animals.

Microscopic evaluation

The formalin fixed tissue samples were routinely processed and stained with hematoxylin and eosin for microscopic evaluation. Lungs, lymph nodes, and other lymphoid tissues (tonsil and spleen) were scored for the

presence and severity of PCV2-associated lesions as previously described by a veterinary pathologist blinded to the treatment group.

Immunohistochemistry

Immunohistochemical (IHC) staining for detection of PCV2-antigen in formalin fixed, paraffin embedded tissue was performed on selected lymphoid tissues (tracheobronchial, mediastinal, mesenteric, iliac, superficial inguinal thymus, tonsil, spleen and Peyer's patches) collected from all bioassay pigs as described. The distribution and amount of PCV2 antigen in individually examined tissue sections were scored ranging from 0 (no signal) to 3 (strong signaling) as previously described.

VII. Results

Part 1

Amount of IPC used in the semen DNA extraction

Except for 0.02 pg and 0.002 pg, the spiked exogenous IPC being more abundant than the target, interfered in the amplification of the target thereby affecting its C_T readings in all the semen samples tested. The C_T readings for the target PCV2 DNA from the duplex qPCR did not match the C_T values for the target in the single qPCR . This indicated that the IPC was too dominant and prevented target PCV2 DNA from being amplified efficiently. Since the duplex qPCR assay used the same primer set for amplification of both the target and the IPC, the concentration of each of the primers was increased to 800 nM in the duplex assay to avoid any competition between the target and the IPC. The C_T readings for the target in the duplex qPCR are very similar to the corresponding C_T readings in the single qPCR when the semen samples were spiked with 0.002 pg of the IPC. However, the IPC could not be detected in most of the spiked samples as reflected by the lack of VIC fluorescence in the duplex qPCR. A concentration of 0.02 pg of the IPC was found to be most suitable for spiking semen samples as the target C_T readings from single and duplex qPCR assays closely matched without compromising amplification of the target PCV2 DNA and the detection of the spiked IPC.

Lack of cross-reactivity between probes

The single qPCR using the PCV2 plasmid as template did not show any fluorescence (C_T reading ≥ 40) with the P1591M probe. Similarly, when the mutated PCV2 plasmid was used as a template there was no fluorescence (C_T reading ≥ 40) with the P1591 probe. The duplex qPCR assay with both the probes in the PCR reaction mixture using equal amounts of PCV2 and mutated PCV2 plasmids as template gave C_T readings for both similar to what was observed in the single qPCR.

Duplex q-PCR assay results on experimentally- and naturally-PCV- infected boar semen samples

All the negative semen samples from the three PCV2 negative boars had $C_T \geq 40$ cycles. In contrast, the semen samples from the experimentally PCV2-inoculated boars as well as the semen samples from the naturally infected boars contained different amounts of PCV2 DNA as indicated by different C_T reading. The C_T readings in single and duplex qPCR for the target PCV2 DNA were similar indicating lack of interference of the target PCV2 DNA and the IPC DNA in the duplex qPCR assay. In all samples tested, the performance of the duplex qPCR assay was equivalent to that of single qPCR. Furthermore, the C_T readings for the IPC in the duplex qPCR assay were consistent within as well as between consecutive PCR runs. Lack of PCR inhibition was confirmed by the consistent C_T reading for the IPC in the duplex qPCR assay in samples where the C_T reading for the target PCV2 DNA was ≥ 40 cycles. The mean C_T reading for the IPC within a qPCR run ranged between 32.1 and 32.3. The CV for the mean IPC C_T reading between different consecutive qPCR runs was found to be 0.32%.

Comparison of incidence and amount of PCV2 DNA in different semen fractions

Comparison of C_T readings obtained on the different semen fractions showed that the C_T readings were lower in the cell fractions than in the corresponding plasma fractions suggesting that most of the PCV2 DNA was associated with the cell fractions rather than the plasma fractions. Furthermore, the amount of PCV2 in the

cell fraction was comparable to the amount of PCV2 found in the corresponding raw semen. The CV between C_T readings of raw semen and their cell fractions was 2.3% whereas that between raw semen and their plasma fractions was 6.3%.

Sensitivity, specificity and reproducibility of the duplex qPCR assays

The sensitivity of the duplex qPCR assay was evaluated by testing a total of 36 raw semen samples from boars experimentally inoculated with PCV2. The duplex qPCR assay was able to detect as low as 2790 PCV2 target genome copies/ml of semen sample and detected 80% (29/36) of the semen samples as being positive. In contrast, nPCR protocol A detected only 66% (24/36) and the detection limit for this assay was determined to be 17,521 PCV2 target genome copies/ml of raw semen. The nPCR protocol B detected 0% (0/36) of semen samples as being positive; under the conditions of this study this assay failed to show discrete bands suggesting its sensitivity to be much lower than in the two other assays. The specificity of the duplex qPCR assay was evaluated by conducting the qPCR assay on raw semen samples from boars known to have a negative PCV2 status (n=9). PCV2 DNA was not detected in any of the control boars tested. Similar results were obtained with nPCR protocols A and B. The reproducibility of the qPCR assay was evaluated by testing all the semen samples in triplicates and in three consecutive qPCR runs. The intra-run CV as determined from the C_T readings of the replicates per semen sample was found to be 0.2% while the inter-run CV determined from C_T readings for a semen sample in three consecutive PCR runs was 3.94%.

Part 2a:

Serology

All boars had passively acquired anti-PCV2 antibodies at weaning which decayed as expected. By 4 months of age, all 15 boars were negative for anti-PCV2 antibodies. Group 1 boars (negative controls) remained negative for anti-PCV2 antibodies for the duration of the study. After experimental inoculation at approximately 7 months of age, anti-PCV2-specific IgG antibodies were first detected on DPI 13 in 3/6 group 2 boars (PCV2a-inoculated) and all boars in this group had detectable anti-PCV2 antibodies by DPI 23. Five of six boars in group 3 (PCV2b-inoculated) developed anti-PCV2-specific antibodies between DPI 16 and 27. The PCV2b boar that did not develop anti-PCV2 antibodies was euthanized at 23 DPI as predetermined by the experimental design.

Quantitative real-time PCR

Serum samples. PCV2 DNA was not detected in serum samples of group 1 boars during the study. PCV2 DNA in serum samples was detected in all group 2 boars by DPI 6 with the earliest detection on DPI 2 in 3/6 boars. Length of viremia ranged from 18 to 43 days between individual group 2 boars with the last detection of PCV2 DNA in serum samples on DPI 48. PCV2 DNA in serum samples of group 3 boars was first detected on DPI 6 in 3/6 boars and all boars in this group became viremic by DPI 16. Length of viremia ranged from 8 to 84 days between individual group 3 boars and PCV2 DNA was still present in serum samples of one of the boars at the termination of the study on DPI 90.

Semen samples. Inhibition of PCV2 DNA detection in semen samples was not observed in any of the semen samples as noted by consistent internal positive control levels. PCV2 DNA was not detected in raw semen samples collected from group 1 boars during the study. The amount of detectable PCV2 DNA in semen varied among individual group 2 boars from 221 to 103,336 genomic copies/mL raw semen with peak shedding between 16 and 23 DPI. All group 2 boars were PCR positive on raw semen with shedding intervals ranging from 1 collection point to 11 consecutive collections (49 days). Intermittent shedding was not observed. In group 3, 5/6 boars shed PCV2 DNA in raw semen with first and last detection on DPI 9 and 90, respectively. The amount of detectable PCV2 DNA in positive semen samples was similar to that of group 2 boars and shedding intervals ranged from 0 to 77 days.

Blood swab samples. Group 1 blood swab samples were negative for PCV2 DNA at all collection time points tested. PCV2 DNA in blood swab samples of group 2 boars was first detected on DPI 2, and all boars

were positive for PCV2 DNA by DPI 9 and remained positive until termination of the study. Earliest detection of PCV2b DNA in group 3 boars was on DPI 9, PCV2 DNA was present in all blood swab samples by DPI 13, and PCV2 DNA was still present in blood swab samples on DPI 90. PCV2 DNA was not detected in any semen samples on DPI 2 (0/12) and 4 (0/12). For DPI 6 and 9, 1/12 and 3/12 animals were PCV2 PCR positive on semen and only data starting from DPI 13 were used for statistical analysis. PCV2 DNA was detected in 2/12 blood swab samples from 2 of 12 boars on DPI 6 and data prior to DPI 9 was excluded from the statistical analysis. The amount of PCV2 DNA in serum samples was significantly ($P < 0.05$) higher than in semen from DPI 13 to 20. Similarly, the amount of PCV2 DNA in serum samples was significantly ($P < 0.05$) higher than in blood swabs from DPI 13 to 20 ($P < 0.05$) and PCV2 DNA was detected earlier in serum samples than in semen samples or blood swabs.

Tissue samples. Group 1 boar tissues (lymph nodes, testes, epididymis, bulbourethral gland, seminal vesicle, and prostate) were negative for PCV2 DNA on DPI 23, 48, and 90. In contrast, lymph nodes and sex glands tested from all group 2 and 3 boars were positive for PCV2 DNA on each necropsy day. The group mean log transformed PCV2 genomic copy numbers in 0.25g of lymph node tissue for group 2 and 3 boars was 10^{6}±0.15 and $10^{6.1}$±0.62, respectively. Significant differences in the amount of PCV2 DNA present in tissues were not observed between groups 2 and 3 or when compare by DPI ($P > 0.05$).

Sperm Morphology

Mean percent normal sperm morphology prior to experimental inoculation was 79%, 70%, and 68% for group 1, group 2, and group 3 boars, respectively. Sperm morphology was not significantly different between PCV2 inoculated and non-inoculated boars or PCV2a and PCV2b groups during weekly morphology evaluations during the study (data not shown).

Macroscopic and microscopic evaluation

At DPI 23, no macroscopic or microscopic lesions were present in the group 1 boar. Macroscopic lesions in group 2 boars included moderately enlarged lymph nodes (2/2) and mottled-tan lungs (2/2) with 18-29% of the lungs grossly affected by lesions. Microscopically, 2/2 group 2 boars had mild to moderate nonsuppurative interstitial pneumonia, 1/2 had mild lymphoid depletion, and 1/2 had mild multifocal lymphocytic interstitial nephritis. Group 3 macroscopic lesions were limited to mottled-tan lungs in 2/2 boars with 4-9 % of the lungs grossly affected by lesions. Microscopic lesions in group 3 boars included mild multifocal nonsuppurative interstitial pneumonia in 2/2 boars, and mild multifocal lymphocytic interstitial nephritis in 1/2 boars.

At DPI 48, no macroscopic lesions were present in group 1 or 2 boars, and the group 1 boar had no microscopic lesions. Microscopically, 1/2 group 2 boars had mild lymphoid depletion and multifocal mineralization in the prostrate gland. In group 3, 1/2 boars had mild lymphadenopathy, 2/2 had moderate nonsuppurative interstitial pneumonia, 2/2 had mild lymphoid depletion, 2/2 had mild multifocal lymphocytic interstitial nephritis, and 1/2 had accumulation of low numbers of macrophages within the testicular interstitium.

On DPI 90, the euthanized group 1 boar had mild multifocal bronchiolar associated lymphoid hyperplasia. Microscopically, 2/2 group 2 boars had mild to moderate nonsuppurative interstitial pneumonia, 1/2 had mild lymphoid depletion and 1/2 had mild bronchiolar associated lymphoid hyperplasia. Macroscopic lesions in group 3 boars included mild lymphadenopathy and mild interstitial pneumonia with 6% of the lungs grossly affected by lesions in 1/2 boars. Microscopically, 2/2 group 3 boars had mild multifocal nonsuppurative interstitial pneumonia, and 1/2 had mild lymphoid depletion.

IHC

PCV2 antigen was not detected in any of the control boars. Low amounts of PCV2 antigen was detected in lymphoid tissues (lymph nodes, tonsil, spleen) in 2/2 group 2 and 2/2 group 3 boars on DPI 23. One of two boars in group 2 and group 3 were PCV2 IHC positive in lymph nodes and tonsil on DPI 48. No IHC signal

was observed in lymphoid tissues of group 2 and 3 boars on DPI 90. PCV2 antigen was not detected in testes, epididymis, seminal vesicles, bulbourethral gland, prostate or penile tissue of necropsied boars in the study.

Part 2b

Swine bioassay study.

All DPI 0 serum samples were negative for anti-PCV2 antibodies. Furthermore, the bioassay-control pigs remained free of anti-PCV2 IgG antibodies for the duration of the study. One of three bioassay-PCV2a pigs had detectable anti-PCV2 antibodies from DPI 28 onwards. The remaining two bioassay-PCV2a pigs developed anti-PCV2 IgG antibodies by DPI 49. Two of three bioassay-PCV2b pigs developed detectable anti-PCV2 antibodies by DPI 49. All three of the bioassay-positive pigs had detectable anti-PCV2 IgG antibodies by DPI 14 and remained positive for the duration of the trial.

PCV2 DNA was not detected in the weekly collected bioassay-control serum samples for the entire duration of the study. PCV2 DNA was detected in 1/3 bioassay-PCV2a pigs at DPI 21, 2/3 at DPI 28, and 3/3 at DPI 35 (Table 3). All pigs remained PCV2 viremic until termination of the study. Two of three bioassay-PCV2b pigs were positive for PCV2 DNA in serum on DPI 21, and 3/3 pigs were PCV2 DNA positive from DPI 35 until DPI 49. PCV2 DNA was detected in 3/3 serum samples from bioassay-positive pigs on DPI 7, and all pigs in this group remained viremic until DPI 49. The sequence of the entire PCV2 ORF2 PCR product collected in each treatment group on DPI 49 was 99.9% similar to the original inoculum.

Macroscopic lesions were not present in the bioassay-control animals. Gross lesions in the bioassay-PCV2a animals included mild fibrinous peritonitis with mild lymphadenopathy (2/3), diffuse non-collapsing lungs (1/3), and mild liver abscessation (1/3). Bioassay-PCV2b gross lesions included fibrinous peritonitis (2/3) and mild lymphadenopathy (1/3). All bioassay-positive animals had diffuse non-collapsing lungs with mild-to-moderate lymphadenopathy in 2/3 pigs. Microscopically, 1/3 bioassay-control pigs had mild multifocal lymphocytic inflammation expanding interstitial lung parenchyma. The microscopic lesions in the bioassay-PCV2a animals included moderate multifocal lymphomacrophagic interstitial pneumonia (3/3), mild multifocal lymphocytic interstitial nephritis (1/3), and mild multifocal lymphocytic myocarditis (1/3). Microscopic lesions in the bioassay-PCV2b pigs included mild-to-moderate multifocal lymphomacrophagic interstitial pneumonia in all pigs. Lesions in the bioassay-positive group included mild to moderate multifocal lymphomacrophagic interstitial pneumonia (3/3), mild multifocal lymphocytic interstitial nephritis (3/3) and mild lymphoid depletion (1/3).

Low amounts of PCV2 antigen were detected in lymph node sections of 2/3 bioassay-positive pigs. PCV2 antigen was not detected in tissue sections of bioassay-control pigs, bioassay-PCV2a pigs, or bioassay-PCV2b pigs.

Artificial insemination study.

In the extender-dilution-*in vivo*-assay, the starting titer of the PCV2 live virus diluted with semen extender was determined to be $10^{4.2}$ $TCID_{50}$ per ml while the control virus stock diluted with MEM had a titer of $10^{4.5}$ $TCID_{50}$ per ml. The PCV2 titers slightly decreased after dilution, and the final titer of PCV2 at 96 hours post dilution was $10^{4.0}$ $TCID_{50}$ per ml and $10^{3.66}$ $TCID_{50}$ per ml for the MEM diluted PCV2 and the extender diluted PCV2, respectively. Three of 3 control gilts, 2/3 gilts-PCV2a, and 3/3 gilts-PCV2b were confirmed pregnant by ultrasonography at 35 and 56 days post insemination. One animal in the gilt-PCV2a group was breed on two consecutive estrus cycles, but failed to become pregnant. The total number of fetuses retrieved from the gilts-control group was 25 with litter sizes of 4, 7, and 14 fetuses (+1 mummy). The total number of fetuses retrieved from the gilts-PCV2a group was 16 with litters of 5 and 11 fetuses. The total number of fetuses retrieved from gilts-PCV2b group was 40 with litters of 9, 13, and 18 fetuses (+1 mummy). Gross lesions were not observed in any of the fetuses. At the time of artificial insemination, all gilts were negative for anti-PCV2 antibodies as determined by ELISA (s/p ratio < 0.2). None of the gilts developed anti-PCV2 IgG antibodies during the 105 days post artificial insemination. No gross lesions were observed during necropsy and no microscopic lesions were present in examined gilt tissues. All 79 fetal serum samples collected at 105 days of gestation were negative for anti-PCV2 antibodies and for PCV2 DNA.

VIII. Discussion

Part 1

The duplex qPCR assay described herein is specific for the detection of PCV2 DNA in semen. The duplex qPCR assay has several advantages to traditionally used diagnostic methods (Garkavenko et al., 2005; Kim et al., 2001; Kim et al., 2003; Larochelle et al., 2000; McIntosh et al., 2006) including rapidness, high repeatability, decreased risk of cross-contamination through absence of post-PCR handling, precise quantitative detection of viral loads, automated product detection, and improved specificity and sensitivity. One of the problems reported with PCR on semen samples is the decrease in extraction efficiency and PCR detection sensitivity because of the presence of PCR-inhibiting substances (St-Laurent et al., 1994; Wiedmann et al., 1993). These semen-specific inhibitors complicate the molecular detection process by contributing to increased false negative results. Thus, monitoring of PCR-inhibition in semen samples is very crucial. One of the methods to monitor PCR inhibition is the inclusion of an IPC in the samples. To our knowledge, reports of a qPCR assay or application of an exogenous IPC for the detection of PCV2 DNA in porcine semen are lacking in the literature. Our newly developed duplex PCV2-specific qPCR assay outlined in this report is the first to utilize an exogenous IPC for monitoring both the process of DNA extraction and PCR inhibition.

For maximum sensitivity and to avoid interference with amplification of the target in the qPCR assay, it was necessary to limit the concentration of internal positive control. Comparison of C_T readings for target PCV2 from duplex and single qPCR showed that the 1:10,000 dilution (concentration 0.02 pg, copy number 2044 per semen sample) of the IPC was most appropriate. At this concentration there was no detectable competition or noticeable interference with the amplification of the target PCV2. Inhibition of amplification of the target PCV2 at all other tested dilutions in the duplex qPCR was not unexpected. At higher concentrations, the IPC being more abundant competed with the target PCV2 for reagents and prevented the target PCV2 from being amplified efficiently or inhibiting its detection completely. Adding 10 µl of 1:10,000 dilution of IPC plasmid resulted in a good correlation with target PCV2 C_T readings between single qPCR and duplex qPCR and the performance (sensitivity, specificity, reproducibility) of duplex qPCR was found to be as good as the single qPCR.

Analysis of cell and seminal plasma fractions by single and duplex qPCR demonstrated that PCV2 DNA was present in both of the semen fractions. This is in agreement with previous findings where PCV2 DNA was found to be mainly present in the seminal fluid and non-sperm cell fractions by qualitative multiplex semi-nested PCR assay (Kim et al., 2003). However, in contrast to the previous study, our duplex qPCR assay showed that the amount of PCV2 DNA was higher in the cell fraction of semen as compared to the seminal plasma fraction and this was true for all semen samples tested. The cell fraction used in our study had both sperm and non-sperm cells present in the cell fraction unlike the study by Kim et al. where sperm head fractions were separated from the non-sperm cells (Kim et al., 2003). We can rule out any type of PCR inhibition in seminal plasma in our study due to consistency of C_T reading for the IPC in the duplex qPCR assays. The C_T readings for raw semen demonstrated a good correlation with the C_T readings of the corresponding cell fractions for all the samples tested suggesting that raw semen is similar to the cell fraction and either are appropriate samples for diagnostic purposes.

The sensitivity of the duplex qPCR assay was compared with that of gel-based nPCR (Kim et al., 2003; Larochelle et al., 2000) assays. There was a good agreement of the duplex qPCR and nPCR protocol with the duplex qPCR detecting 5 additional positive samples (29/36 versus 24/36). These samples were low positives with PCV2 genomic copy numbers below the determined detection limit (17521 copies/ml of semen) for nPCR protocol A and it was determined that the detection limit for the duplex qPCR was 6.3 times higher. Nested PCR protocol B did not have visible bands on the agarose gel suggesting lower sensitivity and performance of this assay under the study conditions.

The specificity of the qPCR was determined by absence of fluorescence in any of the semen samples obtained from negative control boars. The presence of false negative results due to PCR inhibition can be ruled out due to the inclusion of the IPC in all samples tested. Specificity was further confirmed by ruling out any cross-reactivity between the two differently labeled probes.

Consistency of C_T readings for the IPC within and between consecutive PCR runs indicated high repeatability and further suggested lack of DNA extraction inefficiency or PCR inhibition. The DNA extraction efficiency as indicated by the IPC was found to be 97% (i.e. from 2044 spiked copies of the internal positive control, 1984 copies were actually extracted per semen sample).

In summary, the newly developed duplex qPCR assay is a simple, rapid, reproducible, sensitive and specific assay for quantitative detection of PCV2 in porcine semen. The transmission of PCV2 via semen may constitute a major biosecurity risk for swine herds. The application of the assay can be further extended to other types of clinical samples, such as swabs, blood, tissues, etc. The newly developed assay will be extremely valuable for PCV2 detection and quantification for the purpose of disease transmission studies and diagnostic assays.

Part 2a:

Currently, there is little information available in the peer-reviewed literature on shedding of PCV2 in semen. With other swine viruses such as PRRSV, extensive testing of serum, semen or blood swabs is done on a routine basis to monitor and decrease the risk of transmission. The objectives of this portion of the study were to determine if there is a difference in shedding pattern or quantity of virus in semen of distinct PCV2 genotypes and to correlate the incidence and amount of PCV2 within serum and blood swabs to that in semen.

In a previous PCV2 experimental boar inoculation model, detection of PCV2 DNA in semen was intermittent throughout the 47 days post-infection after intranasal inoculation. In the current study, the results indicate that shedding of PCV2 DNA in boar semen was continuous rather than intermittent. Differences in PCV2 DNA shedding between studies could be due to the low number of inoculated boars in both experiments, route of infection, differences in assays used for PCV2 detection, and differences in isolates used for inoculation. In the current study, the boars were inoculated intranasally and intramuscularly to assure that all boars became infected at the same time and with a similar dose. This is not always possible by the intranasal route alone. In addition, it has been determined that real-time PCR has slightly better detection limits compared to nested PCR and there are differences in virulence among PCV2 isolates.

This is the first study to evaluate the amount of PCV2 DNA shed in semen of infected boars, and the results indicate that both PCV2a- and PCV2b- DNA are shed in low quantities in raw semen (221 to 103,336 PCV2 genomic copy numbers/mL). Moreover, we demonstrated that the amount of PCV2 in semen is lower than in serum.

The inocula used in this study had an infectious titer of $10^{4.3}$ $TCID_{50}$ for PCV2b versus $10^{4.5}$ $TCID_{50}$ for PCV2a. PCV2b inoculated boars became viremic 3-7 days after viremia started in the PCV2a boars. This difference in time until detection of viremia onset could be due to the difference in amount of virus given at challenge; nevertheless, the inocula titer differences were minimal and both groups shed PCV2 DNA in semen at similar low quantities.

Length of PCV2 shedding in semen varied among inoculated boars in this study. In PCV2a-inoculated (group 2) boars, the narrowest incidence of detectable PCV2 DNA was 1 collection point with the longest incidence of detection being 11 consecutive collections (49 days). It should be noted that 2 boars within this group were shedding PCV2 DNA in semen but were randomly selected for necropsies on DPI 23 and 48, thus it is unknown how long shedding would have persisted in these boars. In the PCV2b-inoculated group (group 3), 1 boar never shed detectable amounts of PCV2 DNA in semen. However, PCV2 DNA in serum was first detected on DPI 16 and the boar was then euthanized on DPI 23 as predetermined by the experimental design. In all other inoculated boars, serum viremia preceded semen PCV2 DNA shedding. The range in days was 4-14 post serum detection. In addition, the boar that did not shed detectable amounts of PCV2 DNA in semen did however, have detectable levels of PCV2 DNA in tissues (lymph nodes, testes, epididymis, prostate, seminal vesicles, and bulbourethral gland) at necropsy. Only 1 boar in group 3 stopped shedding prior to being euthanized. All other boars had detectable PCV2 DNA in semen at necropsy or until termination of the study. These results are supportive of similar observations in naturally infected boars where extended shedding of PCV2 DNA was observed in semen of young Landrace boars. In addition, the presence of PCV2 DNA in semen had no affect on morphology, which was further confirmed in this study.

In the field, PCVAD is commonly associated with coinfections with other swine pathogens. In experimental PCV2 models, it has been confirmed that coinfections with *Mycoplasma hyopneumoniae*, PPV, and PRRS increase the incidence and severity of PCV2-associated lesions and disease. In the current study, rooms were cleaned daily and there were no new animal introductions. These conditions are considerably different than commercial boar studs and breeding herds. It can be hypothesized that environmental factors, introduction of naïve animals to the population, and coinfections may exacerbate the length of PCV2 shedding in semen which would further support observation of up 27.3 weeks of PCV2 shedding in semen of naturally infected boars.

Recently, it has been shown that serum PCR is more sensitive than semen PCR for the detection of PRRSV in experimentally challenged boars early in the disease phase. Moreover, there was agreement between blood swab samples and serum samples when used for the detection of PRRSV RNA in the first 6 days post-challenge in boars. In the current study, PCV2 DNA was detected in serum prior to and in higher amounts than in semen or blood swab samples. In the later course of infection, blood swab samples remained positive for PCV2 DNA longer than semen and serum samples. One group 2 boar did not have detectable PCV2 in serum after DPI 34, yet quantitative real-time PCR detected PCV2 DNA in blood swab samples at all collection points thereafter including at termination of the study on DPI 90. Previously, in experimentally PCV2 inoculated gilts, plasma viremia was observed up to 21 days post-inoculation. However, peripheral blood mononuclear cells (PBMC) were positive out to 49 DPI in the same animals. Since blood swab samples contain erythrocytes, plasma and PBMC, it is reasonable to conclude that blood swab samples in the current study were positive due to cell-associated PCV2. It is possible that the lack of PCR positive blood swab samples in the early stages of infection were influenced by the low numbers of boars used. Cell associated PCV2 infection may not necessarily be associated with viremia or PCV2 DNA shedding in semen.

Nine out of twelve PCV2a and PCV2b inoculated boars had detectable amounts of PCV2 DNA in serum at necropsy. One PCV2b and two PCV2a inoculated boars necropsied at DPI 90 were not viremic; however, blood swab samples and tissues (lymph nodes and sex glands) were positive for PCV2 DNA. These results indicate and support that PCV2 DNA is cell associated in the later stages of infection. However, it is unknown how long PCV2 remains in the cell associated state in the post viremic phase of infection.

PCV2 antigen was detected in sex glands of a naturally infected diseased boar. In contrast, in the current study no boar was clinically affected and PCV2 antigen was not observed in sex glands of inoculated boars. PCV2 DNA levels in all sex glands ranged from $10^{3.3}$ to $10^{7.3}$ PCV2 copy numbers per 0.25g tissue. In contrast, IHC staining for PCV2 antigen was observed in lymphoid tissues at different necropsy points and this correlated with a minimum of $10^{7.5}$ PCV2 DNA copies in 0.25g of tissue. Previously, it was estimated that 10^{8} genomic copies of PCV2 are needed to detect viral antigen in tissues by IHC. It needs to be taken into account that the previously published PCR and IHC assays and the PCR and IHC assays used in this study are different and this might explain the slight discrepancies in detection limits between studies.

In summary, the results indicate that under the conditions of this study both PCV2a- and PCV2b- DNA were present in semen and all sex glands of experimentally inoculated boars. The incidence of PCV2 DNA in semen and length of shedding may vary amongst individual boars. Furthermore, this study indicates that PCV2 DNA shedding in semen may be continuous rather than intermittent. For boars in the early stages of infection with PCV2, serum appears to be the preferred sample for diagnostic testing. It remains to be determined if infectious PCV2 is shed in semen and a possible route of transmission and dissemination of PCV2 among swine populations. Furthermore, the effect of using PCV2-positive semen on herd reproductive performance needs to be further evaluated to make appropriate decisions on the amount of testing that is justifiable in boar studs.

Part 2b

Semen transmission of certain viruses has been documented and is considered economically important for the swine industry, especially with the wide usage of artificial insemination. Most commercial boar stud operations have some sort of surveillance testing program for the presence of PRRSV, however; testing for the presence of other viruses associated with reproductive failure including PCV2 is infrequent and if positive tests are found interpretation of those results and what action is appropriate to take or not to take is unclear.

Due to the cytotoxicity of semen and the non-cytopathic effect of PCV2 in cell culture, virus isolation techniques can be misleading and unrewarding. Previously, a swine bioassay model was developed to confirm the infectivity of PRRSV in semen. In this study, a similar bioassay model was used to test the infectivity of PCV2 DNA present in semen. The results of the PCV2 swine bioassay confirm that PCV2a and PCV2b present in semen are infectious as evidenced by the detection of PCV2 viremia and seroconversion in the recipients after inoculation. In the experimental PCV2 pig model using intranasal and/or intramuscular inoculations, anti-PCV2 antibodies can be detected between 21-28 DPI. The delayed antibody response to PCV2 observed in pigs intraperitoneally injected with PCV2 DNA-positive semen when compared to pigs inoculated with PCV2 live virus may be dose related. Additional considerations for delayed response in antibody production may be due to natural constituents present in semen or different antigens present competing for an immunological response.

Artificial insemination with semen containing PCV2 DNA did not result in dam or fetal infection in this study. However, PCV2 has the ability to infect and replicate in different stages (zona pellucida free morulae, early blastocysts and hatched blastocysts) of *in vivo* produced embryonic cells with increasing susceptibility of infection in the later stages. It has been shown that PCV2 with an approximate diameter of 17nm in size did not infect intact zona pellucida morulae, although 20nm diameter fluorescent beads could pass through zona pellucida pores. Due to the difficulty to inactivate the virus, PCV2 present in semen may be a risk for infection. PCV2 is associated with the cell fraction portion of semen. This may indicate that there is a higher risk of PCV2 infection during fertilization because spermatozoa may be associated with PCV2 DNA.

Differences in virulence and tropism of PCV2 isolates have been suggested as a possible reason for PCV2-associated reproductive failure when compared to PCV2-associated diseases in growing swine. Two PCV2 isolates were used in this study and both originated from growing pigs with PCVAD. However, it is unknown whether these isolates were also associated with reproductive failure in the field.

In swine, the route of PCV2 exposure is likely fecal-oral and fetal infection has been associated with viremia of the dam. It was recently reported that reproductive failure was associated with insemination of PCV2 spiked semen in PCV2 positive dams. The dams were intranasally inoculated 60 days prior to insemination, and it was unknown whether dams were still viremic at the time of insemination with PCV2 spiked semen. The dams may have been viremic which could have contributed to the reproductive failure. In support of this, in a different study it has been shown that PCV2 experimentally inoculated gilts had detectable PCV2 DNA in peripheral blood mononuclear cells at 63 DPI.

The results of our study indicate that PCV2 DNA present in semen ranging from $10^{5.6}$ to $10^{5.8}$ genomic copies/ml did not cause reproductive failure, development of dam associated anti-PCV2 antibodies, or fetal infection. In addition, the semen extender used in the study was found to have only a minimal effect on the viability and infectivity of PCV2.

The amount of PCV2 present in semen likely affects the outcome. Previously, non-extended raw semen has been shown to infect PRRS negative gilts, but extended semen, though causing a reduction of pregnancy rates, did not cause seroconversion or infection. This indicates that the dose of viruses in semen plays a major role in transmissibility. In further support of this, only 20% of negative gilts inseminated with PRRSV at doses of 2,000 and 20,000 $TCID_{50}$/50ml semen seroconverted while semen containing PRRSV at $\geq$ 200,000 $TCID_{50}$/50ml semen was infectious in 100%.

In summary, we determined that PCV2a and PCV2b stains shed in semen were infectious in a swine bioassay model. We also determined that low doses of PCV2 ($10^{5.6}$ -$10^{5.8}$ PCV2 genomic copies per ml) in extended semen when used for artificial insemination does not cause reproductive failure, seroconversion, or PCV2 viremia in naïve gilts and their offspring. The authors acknowledge that the amount of PCV2 present in semen is a possible contributing factor in transmission of PCV2 via semen to swine herds that utilize artificial insemination. We also are aware of potential PCV2 isolate differences in association with reproductive failure. Further investigations are needed and currently under way.

References are available upon request.