MOLECULAR TECHNIQUES IN THE STUDY AND CONTROL OF PORCINE CIRCOVIRUS TYPE 2

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By

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Every day you may make progress.
Every step may be fruitful.
Yet there will stretch out before you an ever-lengthening, ever-ascending, ever-improving path.
You know you will never get to the end of the journey.
But this, so far from discouraging, only adds to the joy and glory of the climb.

Sir Winston Churchill

ABSTRACT

Porcine circovirus type 2 (PCV2) is an emerging virus that may result in devastating disease that affects swine herds worldwide. Only a decade has passed since researchers began to study the characteristics of the virus and the resulting diseases, and limited information was available regarding the long term presence or persistence of the virus in healthy swine herds. Our research contributes to the knowledge of the persistence of the virus in serum and semen, and the PCV2 antibody profile in healthy pigs. In addition, we developed a novel quantitative polymerase chain reaction (PCR) assay and quantified the PCV2-shedding in healthy and PCVD (porcine circovirus disease)-affected pigs. Lastly, we determined the efficacy of a novel vaccine in a subset of pigs from a PCVD-affected herd.

Porcine serum was assayed by two PCR protocols (nested polymerase chain reaction (nPCR) and non-nested PCR) and a competitive enzyme-linked immunosorbent assay (cELISA) to determine when PCV2 viremia and a rise in the serum level of PCV2-specific antibody (Ab) occurred in pigs raised in a large Canadian farrow-to-finish barn. Eight serial blood samples were collected from each of 40 pigs from 5 to 156 (±1.5) days of age; six pigs were removed from the study for various reasons at various times. Viremia was not detected in the samples collected before 72 days of age but was detected in those collected on or after 72 days: of 33 pigs, seven (21%) had only one serum sample positive for PCV2 deoxyribonucleic acid (DNA) by nPCR after day 72; 11 (33%) were intermittently positive by nPCR, non-nested PCR, or both between 72 and 156 days; and the remaining 15 (45%) were repeatedly positive (in two to four samples). The level of serum Ab against PCV2 declined after weaning and increased between 72 and 107 days of age, only after PCV2 was detected in serum. Our results show that PCV2 viremia persists in the presence of elevated levels of PCV2-specific Ab.

In a separate study, we determined the long term presence or persistence of PCV2-shedding in semen from healthy boars and the effects of PCV2 on sperm quality. A nPCR protocol was applied to porcine semen to demonstrate the PCV2-shedding patterns and duration in naturally-infected boars. Sperm morphology analysis was performed on a subset of samples to determine if the presence of PCV2 DNA in semen was associated with reduced semen quality. Semen was collected serially from 43 boars representing six breeds, aged 33.9 to 149.3 weeks. Of the 903 semen samples collected, 30 samples (3.3%) were positive for PCV2 DNA by nPCR from 13 boars. Boars shedding PCV2 DNA in semen ranged between 35.9 and 71.0 weeks of age, and shedding occurred over a period of up to 27.3 weeks. A semen nPCR test was 2.6 times more likely to be positive when collected from pigs that were $\geq$ 52 weeks of age and 3.0 times more likely to be positive when collected from pigs that were $\leq$26 weeks from the time of entry into the stud main unit (Generalized Estimating Equations: P=0.02; 95% confidence interval (CI) of the Odd's ratio (OR) 1.2 to 5.5 and P=0.01; 95% CI of the OR 1.3 to 6.9, respectively). PCV2 DNA was detected in semen from Duroc and Landrace boars only; however, the semen of the Hamline, Large White maternal, Large White paternal, and Meishan-synthetic boars were negative for PCV2 DNA. These results demonstrate a sporadic and long-term shedding pattern of PCV2 DNA in semen from naturally-infected boars. PCV2 DNA in semen did not have detrimental effects on sperm morphology; however, boar age and possibly breed may contribute to the persistence of PCV2-shedding in semen.

To further our studies of PCV2-shedding from pigs, a PCV2 quantitative molecular assay was developed using SYBR green technology. This assay allowed for the simultaneous quantification of all the genotypes of PCV2. The emergence of multiple genotypes of PCV2, as demonstrated by phylogenetic analysis of whole genome or capsid sequences, makes it necessary

to have quantitative diagnostic assays that perform equally well on all strains. The objectives of this study were to develop and validate a novel real-time PCR assay targeting the highly conserved replication-associated gene (*Rep*) open reading frame 1 (ORF1) and investigate the effects of diagnostic specimen choice on its performance. The assay was tested in naturally-infected conventional pigs, experimentally-infected gnotobiotic pigs, and plasmid-spiked negative serum, lung tissue, and feces and found to have a linear detection range of 2.2×10^3 to 2.2×10^{10} copies of PCV2 per mL. The assay successfully detected and quantified PCV2 DNA in serum, buffy coat, feces, and multiple lymphoid (bronchial, mesenteric, and superficial inguinal lymph nodes, thymus, tonsil, ileal Peyer's patches, and spleen) and non-lymphoid (myocardium, lung, kidney, liver, and gluteal muscle) tissues from naturally-infected pigs. Across all tissues and sera of naturally-infected pigs, the mean PCV2 concentration was 3.0 logs higher in pigs that were demonstrating weight loss, as a primary clinical sign of PMWS (postweaning multisystemic wasting syndrome) when compared with PMWS-nonaffected pigs. PCV2 concentration measured by tissue culture and immunohistochemical staining in homogenized liver samples of experimentally-infected gnotobiotic pigs were compared to the concentrations estimated by quantitative PCR. Similar trends were noted with increasing PCV2 concentration detected in subclinically-infected to severely PMWS-affected pigs across all assays. Our diagnostic assay was developed with a conserved target sequence, and performed efficiently in the quantification of PCV2 in a variety of tissues from naturally- and experimentally-infected pigs.

With a quantitative assay, we then determined the amount of PCV2-shedding in feces between healthy and PCVD-affected herds. This study examined if pigs (n=100) in a PCVD-affected herd shed more PCV2 in their feces than pigs in a PCVD-nonaffected herd (n=101), and

if differences in shedding among production stages within and between the herds existed. The PCV2-shedding was quantified by real-time PCR. The highest median PCV2-shedding was found in the nursery stage of the PCVD-affected herd and in the grower stage of the PCVD-nonaffected herd. The PCV2-shedding was significantly higher in earlier stages (newly weaned, nursery, and pregrower) in the PCVD-affected herd (Wilcoxon Rank Sum; $P<0.001$) compared with the PCVD-nonaffected herd. PCV2 DNA was not detected in a significant proportion of lactating sows (parity $\geq$3) in the PCVD -nonaffected herd (Fisher's Exact Test; $P=0.001$) compared with the PCVD-affected herd. The results of this study suggest there may be an association between the presence of PCV2 in the feces of lactating sows and increased PCV2-shedding in younger pigs.

The quantitative PCR assay was used in a field study to determine the efficacy of a novel transdermal immune stimulating complex (ISCOM) technology-based PCV2 virus-like particle (VLP) vaccine candidate administered at one and three weeks of age. Fifty-four pigs (vaccinates (VX) (n=27); non-vaccinated controls (CTRL) (n=27)) with variable levels of maternally-derived antibodies (MDAb) (high (hiMDAb) versus low (loMDAb)) in a PCVD-affected farm were assessed for PCV2-specific Ab levels by cELISA, PCV2 DNA concentration in serum and feces by quantitative PCR, mortality, and average daily gain (ADG) from 1 to 18 weeks of age. A significant reduction in mortality ($P=0.05$) was observed in the VX. PCV2 DNA concentration in serum was lower in the VX at 9 and 11 weeks of age ($P=0.003$ and 0.01, respectively). The VX (both hiMDAb and loMDAb groups) at nine weeks of age had a higher Ab response to natural PCV2 exposure, while the CTRL (both hiMDAb and loMDAb groups) had a significantly reduced Ab response in comparison. This suggests a priming of the immune response against PCV2 infection by use of the vaccine. During this period from 9 to 11 weeks of

age, the loMDAb CTRL were susceptible to PCVD. PCV2-specific Ab in serum was significantly higher in the VX at the onset of PCVD-related mortality (P=0.001); however, no significant difference was observed in the PCV2 DNA concentration shed in feces, or in ADG between VX and CTRL. The benefit of the novel ISCOM vaccine was most evident in the loMDAb VX, as these pigs were protected from a PCVD-related death that was otherwise experienced in the loMDAb CTRL. However, producers and veterinarians using a vaccine against PCV2 would expect a significant improvement in ADG and reduced viral shedding in feces. Although the vaccine prevented a PCVD-related death in the highest risk population, the loMDAb group, modifications regarding the protein or ISCOM concentration, or PCV2 protein construction should be considered to potentially improve the vaccine's efficacy. This study is the first report of the use of an ISCOM matrix (Matrix Q) mixed with PCV2 VLP protein administered transdermally for the prevention of PCVD in swine.

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LIST OF ABBREVIATIONS

ºC	Degrees Celsius
× g	Gravity
aa	Amino acid(s)
AASV	American association of swine veterinarians
Ab	Antibodies
ADG	Average daily gain
ATCC	American type culture collection
B	Boar
BG	Bred gilt
BLN	Bronchial lymph node
bp	Base pair
Cap	Capsid-associated protein
cELISA	Competitive enzyme-linked immunosorbent assay
CI	Confidence interval
cm	Centimetre
CT	Congenital tremors
CTRL	Non-vaccinated controls
DNA	Deoxyribonucleic acid
ds	Double stranded
ELISA	Enzyme-linked immunosorbent assay
FIN	Finisher
g	Gram
GEE	Generalized estimating equations
GLM	Gluteal muscle
GR	Grower
hiMDAb	High maternally-derived antibodies
ID	Identification number
IFA	Immunofluorescence antibody
Ig	Immunoglobulin
IHC	Immunohistochemistry
ILN	Ileal lymph node
ISCOM	Immune stimulating complex
ISH	*In situ* hybridization
kb	Kilobase
kg	Kilogram
LN	Lymph node
loMDAb	Low maternally-derived antibodies
MDAb	Maternally-derived antibodies
mg	Milligram
mL	Millilitre
MLN	Mesenteric lymph node
mM	Millimolar
nm	Nanometre
nM	Nanomolar

nPCR	Nested polymerase chain reaction
NU	Nursery
OR	Odd's ratio
ORF	Open reading frame
ORI	Origin of replication
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PCV2	Porcine circovirus type 2
PCVAD	Porcine circovirus-associated disease
PCVD	Porcine circovirus disease
PDNS	Porcine dermatitis and nephropathy syndrome
PG	Pregrower
PI	Percent inhibition
PK15	Porcine kidney tissue culture cell line 15
PMWS	Postweaning multisystemic wasting syndrome
PNP	Proliferating and necrotizing pneumonia
PP	Peyer's patch
PPV	Porcine parvovirus
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
Rep	Replication-associated protein
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
S ≤2	Lactating sow parity ≤2 (young sow)
S ≥3	Lactating sow parity ≥3 (old sow)
TCID	Tissue culture infective dose
Th1	T-helper cell 1
Th2	T-helper cell 2
TSE	Transmissible spongiform encephalopathy
U	Unit(s)
µg	Microgram
µL	Microlitre
µM	Micromolar
VG	Virgin gilt
VLP	Virus-like particle
VX	Vaccinates

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

We are fortunate to have at the University of Saskatchewan two pioneers in the discovery and research of porcine circovirus type 2 (PCV2) and porcine circovirus disease (PCVD). The practising veterinarian, Dr. John Harding, who assisted in the recognition and description of the first reported PCV2-associated disease named postweaning multisystemic wasting syndrome (PMWS) (89) and Dr. John Ellis, the researcher that lead a group to discover the novel virus associated with the severe wasting disease in pigs (59). Drs. Harding and Ellis have continued to research this emerging virus and the plethora of diseases associated with it. The body of research and publications which study PCV2 has grown substantially over the last 10 years; however, the morbidity and mortality related to PCV2 infection in pigs continues to plague the swine industry.

A resurgence of severe PCVD was recognized in Eastern Canada after the industry believed the worst was over (35), but this only proved that there is more to learn and understand regarding the virus. Vaccines against PCV2 are now available and are efficacious, but researchers continue to study and uncover complex revelations about the virus and its associated diseases.

This thesis is concerned with the development and use of molecular assays for studies that contribute to the knowledge in multiple areas of PCV2 research. Four of the five research articles (chapters three to six) that comprise this thesis have been peer-reviewed and published. The fifth manuscript (chapter seven) has been submitted for publication. Our research began in the year 2002 and many basic questions were not known about the virus prior to this year and

include: are healthy infected pigs persistently infected; what is the PCV2-specific antibody (Ab) status of healthy infected pigs over time; is PCV2 shed in semen from healthy boars; and does the presence of PCV2 affect semen quality? After our research began, real-time quantitative polymerase chain reaction (PCR) technology became widely available and we subsequently developed a novel PCV2 real-time assay. This novel assay enabled us to determine what amount of virus is shed in feces between healthy and PCVD-affected pigs, and among production stages. Additionally, as a contributing member to a European consortium research group with objectives to investigate various aspects of PCV2 and PCVD, we tested the efficacy of a novel vaccine in PCVD-affected pigs using the real-time assay.

Most of the questions we sought answers to were driven by the industry and producers that are working diligently to make whatever changes possible to reduce their pig losses, improve production, and to prevent PCVD from affecting their herds. Our research answered many questions previously unanswered and contributes to the basic understanding of PCV2 epidemiology in pig populations.

1.2 Discovery of Porcine Circovirus

Porcine circovirus (PCV) was first detected in 1974 by Tischer and others as a papovavirus- and picornavirus-like contaminant of a continuous porcine kidney cell line, PK15 (ATCC-CCL31) (215). In 1982, this newly isolated virus was further characterized and reported to be a persistent noncytopathic, noneveloped virus with a capsid size of 17 nm (214). By serologic study, this noncytopathic PCV was found to be present in pig populations, but did not appear to be associated with any clinical disease or pathology (216). It was not realized until 15 years later that another PCV had emerged and was associated with a severe wasting disease in

pigs in Canada. This devastating disease was termed postweaning multisystemic wasting syndrome (PMWS) (47;89).

1.2.1 Postweaning Multisystemic Wasting Syndrome

PMWS was recognized as a widespread disease by 1997 to 1998; however, retrospective data based on necropsies dating back to the early 1990's confirmed that the disease had resulted in pig losses in one farm in 1991 (89). Dr. JCS Harding, a practising veterinarian from Humboldt, Saskatchewan, and Dr. EG Clark, a pathologist at the Western College of Veterinary Medicine together described the clinical and pathological disease that was emerging in Canadian swine (47;88). Affected pigs were primarily 7 to 15 weeks of age and presented with wasting, respiratory distress, enlarged lymph nodes, diarrhea, pallor, and jaundice (89). Subsequently, these wasted pigs were found to have gross and/or microscopic lesions in multiple organs and suffered from an array of potential pathologies. Granulomatous interstitial pneumonia, hepatitis, nephritis, myocarditis, enteritis, pancreatitis, and lymphadenopathy were some of the lesions recognized in pigs suffering with PMWS (11;59).

1.2.2 PCV2 Isolation

In 1998, a PCV was found to be associated with PMWS in affected pigs in Saskatchewan, Alberta, and Manitoba (59). The virus was recognized in lesions from multiple organs using electron microscopy, immunohistochemical staining (IHC), and *in situ* hybridization (ISH). This was the first report that described a PCV associated with clinical disease and pathology in pigs. Additionally, it was recognized that the PCV associated with PMWS was closely related to, but antigenically distinct from the PCV tissue culture contaminant that was discovered in 1974 (11). This observation lead to the terminology (152) of porcine circovirus type 1 (PCV1) to describe the nonpathogenic circovirus first described by Tischer and

others in 1974 (215), and porcine circovirus type 2 (PCV2) to describe the circovirus associated with PMWS in young pigs by Ellis and others (59).

Genomic analysis confirmed that two distinct types of PCV existed, as the approximate sequence identity of the genome between PCV1 and PCV2 was 68% (85). A more comprehensive analysis revealed that the sequence identity among PCV2 isolated from North American and European outbreaks of PMWS was >96% similar, and these PCV2 isolates had <82% sequence identity with PCV1 (152).

1.2.3 PCV2 Classification

The family *Circoviridae* includes two genera: *Circovirus* and *Gyrovirus*. This family of viruses contains small, nonenveloped icosahedral viruses with a circular single stranded DNA genome (214). Within the *Circovirus* genus, many viruses are represented and host species infected include: birds, plants, and mammals (20;218). The most studied virus within the *Circovirus* genus is PCV2; however, the *Circovirus* genus consists of more than 10 pathogenic and nonpathogenic viruses (150;217;219). The *Gyrovirus* genus includes a single virus named the chicken anaemia virus (48). Recently, in the 2009 Virus Taxonomy List (224), the *Anellovirus* genus was removed from the *Circoviridae* family and reclassified as the *Anelloviridae* family and consists of the torque teno viruses found to infect people and mammals (96).

1.2.4 PCV2 Structure and Molecular Characteristics

With a virion size of approximately 17 nm and a genome of 1.7 kb, PCV is the smallest virus known to replicate autonomously (150;214). The genome is a single stranded covalently closed circle and replicates *via* rolling circle replication (39). This replication mechanism involves an intermediate double stranded replicative form of DNA (150). The origin of

replication (ORI) of PCV2 is found adjacent to a stem loop structure (Figure 1.1) which contains a conserved nonamer sequence at the apex and a palindrome of 11 nucleotides producing the stem (143).

The virus contains three expressed open reading frames (ORF) which encode a replication-associated protein *Rep* (ORF1), a capsid protein C*ap* (ORF2), and a viral-induced apoptotic protein (ORF3). An ambisense orientation of the *Rep* and *Cap* genes is observed where ORF1 is located immediately following the ORI and a short intergenic region, and ORF2 is located immediately preceding a short intergenic region and the stem loop structure.

The *Rep* gene produces both a full-length transcript from the viral plus or positive strand (*Rep*: 314 aa) and a truncated form (*Rep'*: 178 aa) and both are necessary for the initiation of replication of the virus (66). The *Cap* gene transcribes the structural capsid protein (233 aa) in a counter clockwise direction from the minus or negative strand, and is the main antigenic determinant of the virus (141). The recently described ORF3 protein (105 aa) is transcribed from the ORF1 region; however, it is transcribed in a counter clockwise direction and is not required for viral replication, but is hypothesized to contribute to apoptotic activity and viral pathogenesis (125).

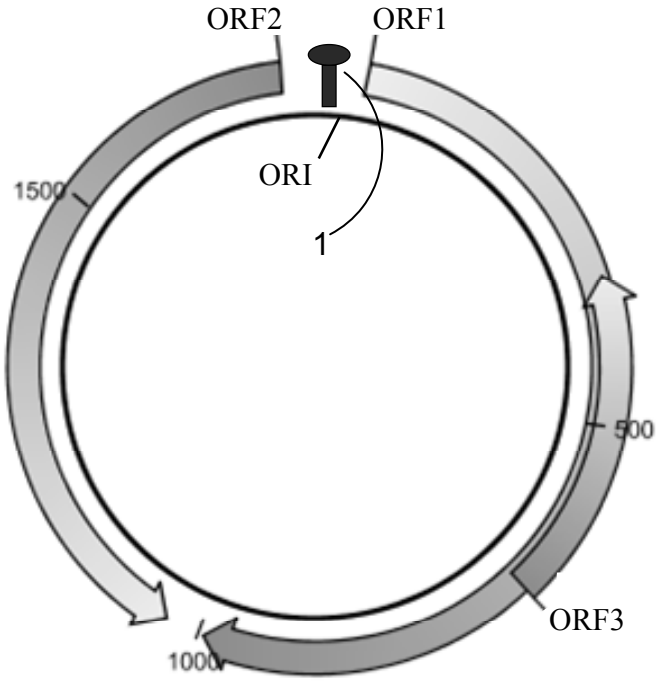

Figure 1.1: PCV2 genomic organization of ORF1 (replication-associated protein; *Rep*), ORF2 (capsid protein; *Cap*), and ORF3 (apoptotic protein). Origin of replication (ORI) is located adjacent to the stem loop structure and precedes ORF1. Ambisense orientation is observed between ORF1 and ORF2, where ORF3 is contained within ORF1 and in a counter clockwise direction. Nucleotide base pair designations for 1, 500, 1000, and 1500 are noted.

1.2.5 PCV2 Virulence and Genotypes

It was recognized shortly after the isolation of PCV2 from PMWS-affected pigs that PCV2 isolates were divergent and comprised genotypes (142). Universal nomenclature to describe the genotypes of PCV2 was proposed in 2008 (202) and PCV2a, PCV2b, and PCV2c are now used by PCV2 researchers in the published literature. Recombination among PCV isolates has been described (40;49;95;122;130); therefore, further designations, such as PCV1/2a, will arise to describe the recombined virus based on the nucleotide sequences of the *Rep* and *Cap* genes of the virus (72).

It wasn't until 2005 when a Canadian report described increased PCVD and PCVD-related mortality associated with a genotype of PCV2 that speculation regarding variable virulence among PCV2 genotypes began (36). A PCR-restriction fragment length polymorphism (RFLP) assay found that PCV2 banding type 321, analogous to PCV2b, a novel genotype to swine in Ontario and Quebec, was associated with the outbreak (35).

Several PCV2 differential molecular diagnostic assays have been reported and include: PCR-RFLP (35), nested PCR (nPCR) (129), non-nested PCR (95), SYBR green real-time quantitative PCR (148;230), and a probe multiplex real-time quantitative PCR (70). Although it is agreed that genotypes of PCV2 exist, primarily described as PCV2a and PCV2b (77), the evidence to support variable virulence among PCV2 isolates is contradictory. There is currently no consensus regarding the association between PCV2 genotype and virulence, as reports both support (71;77;118;174;213) and refute (13;50;144;179) the association.

Lesions of unprecedented severity were found to be associated with genotype PCV2b and a resurgence of PCVD in Canada in field studies (35;71); however, specific pathogen-free pigs infected experimentally with PCV2a or PCV2b had no significant differences in lymphoid

lesions, but significant differences were found between isolates within the same genotype (179). Among the recent controversy regarding the potential variability in PCV2 virulence and its association with PCV2 genotype, one study has emerged in germ-free swine that considers the dual or heterologous infection with PCV2a and/or PCV2b, and the order of infection (i.e. 2a/2a, 2b;2b, 2a/2b, and 2b/2a) (92). It was found that dual heterologous infection with PCV2a given first, followed by PCV2b seven days later, or *vice versa*, induced severe clinical PCVD. Due to the ubiquitous nature of PCV2 in swine populations around the globe and the presence of both PCV2a and PCV2b within populations (77), clarifying the controversy regarding PCV2 genotypes and their relationship to increased or decreased virulence will be difficult in field studies.

1.3 Porcine Circovirus Disease

PCVD (5;199) and porcine circovirus-associated disease (PCVAD) (17) are terms that have been presented to describe the gamut of clinical disease outcomes associated with PCV2 infection. PMWS was the first described clinical syndrome associated with PCV2 infection (59); however, it has been determined that the virus contributes to several other syndromes, disorders, or clinical outcomes.

1.3.1 Spectrum of Diseases

Since the recognition of PMWS in the 1990's (89), PCV2 has been associated with additional disease syndromes including: congenital tremors (CT) (211), fetal myocarditis (28) porcine respiratory disease complex (PRDC) (105), porcine dermatitis and nephropathy syndrome (PDNS) (8), proliferating and necrotizing pneumonia (PNP) (79), abortion (228), and other reproductive disorders (166). Recently, central nervous system lesions in slaughter-weight pigs has been described associated with PCV2 infection (55).

Conflicting reports have been published regarding the association between PCV2 infection and CT (82;103;211). CT is associated with demyelination of the brain and spinal cord, and type A2 specifically refers to a viral cause of the disease (38). One study found no PCV2 in the central nervous system of piglets suffering from CT using ISH and IHC labeling on paraffin-embedded tissues (103), whilst another study found that piglets affected with CT had more PCV-infected cells in the brain and spinal cord than clinically normal piglets using ISH, PCR, and indirect immunofluorescence antibody (IFA) testing on frozen tissue sections (211). Since PCV2 has not been definitively proven or disproven to be associated with CT and is sporadically observed, it is not considered a significant outcome of PCV2 infection.

PRDC is associated with PCV2 infection (105), but the disease has a multifactorial etiology (86). PRDC is characterized by lung lesions, where bronchopneumonia and interstitial pneumonia are often present alone or in combination (86). Vaccination against PCV2 infection can reduce mortality and improve production parameters due to PRDC (86). This observation further supports the hypothesis that PCV2 infection contributes to the manifestation of PRDC.

PDNS primarily affects older pigs and is associated with red to purple lesions on the skin and kidneys accompanied by necrotizing vasculitis, and may be exacerbated by coinfections such as porcine reproductive and respiratory syndrome virus (PRRSV) with PCV2 (8;41). However, it was recently reported that combined experimental infection with torque teno virus genogroup 1 and PRRSV, in the absence of PCV2 infection, produced disease similar to PDNS (116). Further study to reveal the specific cause or factors required to produce PDNS are needed.

PCV2 was the most commonly found pathogen in PNP cases in a retrospective study using ISH (79). PRRSV, swine influenza virus, and Aujeszky's disease virus are also found associated with PNP which causes a severe form of interstitial pneumonia. However, it is

accepted that PCV2 may be the main contributor to PNP in Europe and that PRRSV may further manifest PNP in North America (79).

Reproductive failure as a result of PCV2 infection was recognized shortly after the discovery of PCV2 (151). An increase in abortion (25;228), premature delivery (184), mummified fetuses (190), fetal myocarditis (28;228), and the delivery of stillborn piglets (10;109) have been observed in association with the detection of PCV2 antigen and/or nucleic acid in tissues or sera of affected piglets.

1.3.2 Cofactors Contributing to Disease

Both infectious and noninfectious cofactors may contribute to the development of PCVD in swine. Activation of the immune system appears to be a critical component of the pathogenesis of PCV2 infection in swine that exhibit PMWS (12;114). Gnotobiotic piglets infected experimentally with PCV2 produced only mild asymptomatic disease; however, injection with keyhole limpet hemocyanin in incomplete Freund's adjuvant after PCV2 infection resulted in clinical disease (114). Vaccination may also activate the immune system and perpetuate the progression of PMWS, as seen in experimental infection with PCV2 followed by vaccination with a commercial *Mycoplasma hyopneumoniae* vaccine (113). A modified-live PRRSV vaccine increased PCV2 replication and microscopic lesions in PCV2 infected swine (6). Additionally, the time of vaccination in relation to the time of PCV2 infection may also affect the outcome and severity of lesions in PCVD-affected pigs (172).

The immune status of the sow, specifically the amount of PCV2 antibody (Ab) and PCV2 virus circulating in maternal serum may affect piglet susceptibility to PCVD. Maternally-derived PCV2 Ab passively transferred in colostrum may protect piglets and is Ab titre-dependent (149).

Additionally, PCV2 viremia in sows was significantly related to mortality in PCVD-affected piglets (31).

Numerous studies have determined that experimental coinfection with other swine pathogens exacerbates PCVD. The severe symptoms associated with PMWS do not manifest when PCV2 is inoculated alone experimentally; however, PCV2 coinfection with PRRSV (10), porcine parvovirus (PPV) (7;61), or *M. hyopneumoniae* (181) results in disease.

Intensified production practices have been implicated as a cofactor in the development of PCVD. Early weaning practices or relocating piglets prior to 21 days of age (193) and other considerations including pen size, cross-fostering, and vaccination protocols are risk factors for the development of PCVD (194). It has been hypothesized that geographic areas with PCV2 positive swine that have not progressed to PCVD have more traditional production practices and have not conformed to more intensified animal husbandry (132).

Breed differences in the susceptibility to PCVD have been observed where less mortality is experienced in those pigs of pure or cross-bred Pietrain decent compared with Large White and Duroc crossed pigs (127), and where Landrace are most susceptible when compared with Pietrain, Large White and Duroc bred pigs (171;178). Recently, specific genes have been identified and associated with an increased susceptibility to PCVD (101).

1.3.3 Prevalence and Geography

PCV2 infection and PCVD have been reported in swine populations worldwide: USA (11;64), Canada (59), Great Britain (80), Ireland (210), Europe (14;142), Japan (170), Korea (106), Philippines (139), China (130), South Africa (54), Mexico (220), France (194), New Zealand (162), Australia (167), and Brazil (37). The effect of PCVD in swine populations is variable based on published data. However, morbidity commonly ranges from 4% to 30% of

PCVD-affected pigs but can escalate to 60%, and the mortality among affected pigs can reach 50% (64) to 80% (201).

1.4 Transmission

PCV2 is shed in various secretions including: oronasal (200;207), urine, feces (147), colostrum (206), milk (81), and semen (146). The primary route of PCV2 transmission is theorized to be the oronasal route. An increase in the amount of PCV2 shed in secretions (147;200) and found in tissues (91) is observed in pigs with more severe PCVD when compared with less severe or asymptomatic disease. PCV2 may also be transmitted from the sow to the fetus *via in utero* infection. Additionally, it has been hypothesized that plasma infected with PCV2 used in commercial feed may infect naïve pigs with PCV2; however, contradictory studies both support (185) and refute (205) this hypothesis.

1.4.1 Horizontal Transmission

Oronasal infection with PCV2 occurs *via* contact with contaminated urine and feces (199), and direct nose-to-nose contact with PCV2-infected pigs (26;138). Under experimental conditions, PCV2 negative pigs are infected when allowed contact with PCV2 positive pigs (4;26) and PCV2 negative pigs can develop PCVD by mingling with PCVD-affected pigs (168). Additionally, PCVD can be transmitted to healthy PCV2 positive pigs when allowed to mingle with PCVD-affected pigs (56;117). Aerosol transmission of PCV2 is possible, as concentrations of airborne PCV2 of up to 10^7 copies of PCV2 per cubic metre of air were detected by quantitative PCR (222). PCVD can also be transmitted from affected to unaffected PCV2 positive pigs in an adjacent pen or across an aisle without direct contact between the pens; however, direct contact results in higher numbers of PCVD-affected pigs (117).

1.4.2 Vertical Transmission

Vertical transmission from the pregnant sow to her unborn piglets was demonstrated in experimental infection studies where the pregnant sow was infected with PCV2 *via* the oronasal route (83;184). Additionally, PCV2 has been detected in stillborn or aborted fetuses in reproductive failure studies (25;140;228). Experimental transuterine infection with PCV2 into the peritoneal cavity of fetuses results in infection of untreated fetuses in the uterus (188;197). Oronasal infection is considered the primary route of infection with PCV2, and historically, vertical transmission appeared to be relatively rare (140). However, a recent study detected PCV2 DNA in the pre-suckle serum of 39.9% of neonatal pigs (204); therefore, vertical transmission from the pregnant sow to her fetuses may be a greater risk for PCV2 transmission than originally reported.

1.4.3 PCV2 Shed in Semen

Experimental infection (119;136) and field studies (146;198) have shown that PCV2 is shed in semen. Typically, PCV2 detection studies in semen are based on molecular methods and the infectivity of the virus is not known. It is argued that remnants of the virus or viral DNA may be present, but that infectious virus is not present. Intraperitoneal injection of PCV2 positive raw semen does result in viremia in naïve pigs confirming the potential infectivity of PCV2 in semen; however, artificial insemination did not result in viremia or seroconversion of inseminated naïve females in the same study (137). Semen spiked with PCV2 can infect naïve females when introduced by artificial insemination; therefore, it is not known at this time if PCV2 in naturally-infected semen is sufficient to infect naïve females (135). Additionally, in experimental studies, semen spiked with PCV2 and artificially-inseminated into sows has resulted in both the infection of the sow and of her piglets (134).

Several studies are contradictory in regards to PCV2 and its presence in semen. PCV2 was not found to be shed persistently or intermittently in semen in an experimental infection study (136); however, PCV2 was shed intermittently in semen samples from naturally-infected boars up to 71 weeks of age (146). In addition, contradictory results were found regarding the fractionation of semen and the presence of PCV2 in the various subfractions. PCV2 was more abundant in the seminal fluid and nonsperm cell fraction in one study (108), and in the cellular or sperm fraction in a different study (183). Recently, it has been shown that vaccination against PCV2 decreases the duration of PCV2 viral shedding in semen (3).

1.5 PCV2 Diagnostics

A multitude of assays exist to detect either PCV2 DNA or antigen, or PCV2-specific Ab in serum of live or dead infected pigs. However, the detection of PCV2 does not assume PCVD. Unthriftiness of young pigs aged 7 to 15 weeks of age is the most outstanding clinical sign of PMWS and gross and/or microscopic lesions in multiple organs during post mortem examination suggests PCV2-associated disease; however, additional histopathological findings are necessary to definitively diagnose PMWS and PCVD.

1.5.1 Individual and Herd Differential Diagnosis

A case definition for a PMWS diagnosis was proposed in 2000 by Sorden (209) and in 2007, the American Association of Swine Veterinarians (AASV) Board of Directors approved a case definition with the minimum findings necessary to diagnose PCVD (17). These histopathological findings include: depletion of lymphoid cells in lymphoid tissues; disseminated granulomatous inflammation in one or more tissues; detection of PCV2 within the lesions; and in the case of PCVD in reproductive disease, PCV2 antigen should be present in fetal myocarditis lesions.

Two diagnostic criteria have been proposed for PCVD diagnosis at the herd level. Firstly, a significant increase in the post-weaning mortality and wasting is observed, and secondly, the diagnosis of PCVD based on the individual case definition should occur in a minimum of 20% of pigs during post-mortem examination (76).

1.5.2 PCR Assays

The PCR detection of PCV2 has been used since early in the characterization of the virus and its disease outcomes, as early as 1998 (152). PCR assays have evolved from conventional nested (104;119) and non-nested assays (32;152) to real-time quantitative assays (97;148;183;230). A multiplex real-time quantitative assay is also available to differentiate between genotypes PCV2a and PCV2b of the virus (71).

PCR assays have been used in all types of tissues and excretions from PCV2-infected pigs (119;148;200) and may have applications in the diagnosis of PCVD at the herd level (76). Quantification of PCV2 in tissues (91) is correlated with the severity of lesions and distinguishes wasting from non-wasting pigs. Additionally, a threshold quantitative PCR used on serum or swabs has been proposed as a diagnostic test in live animals suspected of PCVD to confirm the diagnosis (169;200). However, it should be considered that not all PCR assays are equivalent. An inter-laboratory ring trial determined that significant variability in the quantification and detection limit of PCV2 is present across assays (90).

1.5.3 PCV2 Antibody Detection Assays

Various quantitative and semi-quantitative assays have been applied to pig sera to measure the PCV2-specific Ab present. Assays for Ab detection include: ELISA techniques (24;124;225); IFA techniques (10;232); serum neutralization assay (67;154); and the immunoperoxidase monolayer assay (IPMA) (59). Additionally, an enzyme-linked

immunosorbent assay (ELISA) has been developed to differentiate between genotypes PCV2a and PCV2b of the virus (186) and assays exist to differentiate among immunoglobulins (Ig) IgG (225), IgM (33;153;227), and IgA (153) Ab.

PCV2-specific Ab are prevalent in swine herds worldwide and in both the presence and absence of PCVD (15;58;126;145;191;192;225). This observation is consistent with the widespread infection, often subclinically, of PCV2 in pigs. Contradictory studies show that Ab levels may be higher in herds affected with PCVD (191;208), or may be comparable to healthy or unaffected herds (121). Similar to PCR assays, it must be considered that Ab detection assays vary in their ability to quantify PCV2-specific Ab levels in serum accurately.

1.6 PCVD Prevention and Control

Considering the relative novelty of PCV2, several commercial vaccines became available in less than 10 years from the time of discovery of the virus (59). Until the availability of vaccines, control and prevention of PCVD were based on the modification of production practices. The intensification of swine husbandry has been hypothesized to exacerbate PCVD and Dr. Francois Madec presented a 20-point plan that brought humanity, biosecurity, and hygiene back to the practice of raising pigs (131). The 20-point plan is a guide for proper herd management and many of the points, if not followed, have been proven as risk factors for PCVD (133;193;194). Additionally, research has confirmed that controlling concomitant infections reduces PCVD, as PPV (7) and PRRSV (10) contribute to the development of PCVD and the replication of PCV2.

1.6.1 Swine Production Practices: 'Madec's 20-point Plan'

Madec's 20-point plan (131) reduces and prevents the development of PCVD in PCV2-positive herds. The 20-point plan could be applied to other pathogenic infections that may affect

a herd, as its focus is on the management of the herd. There are actions to perform affecting multiple production stages, with the focal point on strict "all-in all-out" procedures where cleaning and disinfection of areas occurs between batches of pigs. Other points of the plan include: small farrowing pens with solid dividers; treatment of sows against parasites prior to farrowing; limited adoptions; suitable animal density in pens; good air quality; suitable room temperature; avoid mixing batches, pens, and animals from different barns; suitable vaccine program; strict hygiene; and the separation of sick animals from the healthy. The implementation of the proper production practices described in the 20-point plan are reported to improve the dire situation in herds affected with PCVD (133;199).

1.6.2 PCV2 Vaccination

There are currently four commercial vaccines available for the prevention of PCVD around the world. Not all of the vaccines are equally available, as licensing and registration vary among countries. In April of 2010, Suvaxyn® PCV2 One Dose was removed voluntarily from the marketplace. It is hypothesized that a recombined PCV1/2a emerged from vaccination with the chimeric killed vaccine strain (72). As of May 2011, the Suvaxyn® PCV2 One Dose vaccine has not been reintroduced to the marketplace.

Two approaches to produce protective immunity against PCV2 exist among the commercial vaccines and include: (1) vaccination of the sow to passively transfer immunity to the neonate; or (2) vaccination of the piglet at or near the period of time when weaning occurs. Details regarding the manufacturer, type of antigen, and approach of the vaccines are outlined in Table 1.1 (76).

Vaccine	Manufacturer	Antigen	Licensed for
Circovac®	Merial	Inactivated PCV2	Females
Ingelvac Circoflex®	Boehringer Ingelheim	PCV2 ORF2 protein	Piglets >2 weeks
Suvaxyn® PCV2 One Dose	Fort Dodge Pfizer	Inactivated PCV1-2 chimera	Piglets >4 weeks
Porcillis® PCV(EU) Circumvent® PCV (US)	Intervet-Schering	PCV2 ORF2 protein	Piglets >3 weeks

Table 1.1: Details of commercial vaccines produced against PCV2 (76).

Vaccination of sows increases serum PCV2-specific Ab that is then delivered passively to the neonatal suckling piglet. Passive immunity has been found to be protective against PCVD (100) and reduces serum viral load (177). In the piglet, maternally-derived Ab begin to wane between three and six weeks of age (145); therefore, vaccination can be effective in eliciting an immune response in the piglet to protect against PCVD. This protective response reduces the morbidity and mortality associated with PCVD during the late nursery and into the finisher period (7 weeks of age and older) (110;203). Piglet vaccination protects against PCV2 viremia, fecal and nasal shedding (68), and the development of microscopic lymphoid lesions (176). All vaccines are effective in improving various outcomes of PCV2 infection and disease in both experimental and field conditions. Reduced mortality and/or PCVD-related morbidity (63;100;110;203), reduced PCV2-associated lymphoid lesions (173;203), reduced incidence or prevalence of coinfections (110), and improved average daily gain (63;98;187) are accomplished by use of commercial PCV2 vaccines.

Maternally-derived Ab specific to PCV2 may interfere with the priming of piglets by vaccination and this is reflected in the published PCV2 vaccine research. Conflicting studies reveal that maternally-derived Ab may (69) or may not (68;110;175) affect vaccine efficacy.

1.6.3 Immune Stimulating Complex

First described in 1984, an immune stimulating complex (ISCOM) matrix consists of purified saponins extracted from the tree bark of *Quillaja saponaria* Molina, then mixed with detergent solubilized cholesterol, and followed by dialysis and ultrafiltration to yield a matrix or adjuvant (158). A stable structure is formed by the addition of a desired antigen and results in both a delivery system and an innate immune stimulant or adjuvant for the antigen (128). The advantages of applying ISCOMs in vaccination are numerous. ISCOM vaccine preparations are

immune modulators which overcome obstacles observed with the use of conventional (killed or non-replicating) vaccines including: route of administration (160), type of immune response elicited (161), and the ability to prime the animal in the presence of maternally or passively-derived Ab (156;159;161).

ISCOMs elicit a more balanced Th1/Th2 or cell-mediated/Ab type response than that observed with typical killed or inactived vaccines (155). ISCOMs have the ability to induce responses that will provide protection against intracellular pathogens by the production of strong T cell responses and enhanced cytokine secretion (18;46). An additional advantage of ISCOMs is that modified-live (attenuated) organisms or adjuvants with toxic or pathological sequellae are not necessary. The ISCOM matrix provides the delivery system and the adjuvant activity necessary to produce a protective and balanced immune response with the use of killed antigens, mimicking the response observed in natural infection but not typically seen with the use of killed or inactived vaccines.

The ability of vaccines to prime the host's immune system in the presence of maternal or passively-derived Ab is currently one of the greatest challenges of vaccine development. Experimental ISCOM vaccines can prime the host's immune system in the presence of maternal Ab (84;157) and experimental vaccines have been tested in multiple animal species for bacteria, viruses, and parasites (1;2;21;22;30;34;164). ISCOMs are currently being used in clinical trial studies for humans and commercial vaccines including an equine influenza vaccine (27) and a feline leukemia virus vaccine (226) are available in veterinary medicine.

CHAPTER 2

HYPOTHESES AND SPECIFIC OBJECTIVES

Five distinct studies or manuscripts comprise this thesis (chapters three to seven). The hypotheses tested in chapters three, four, six, and seven were based on PCV2 infection in naturally-infected pigs and the use of molecular diagnostic tests to detect or quantify PCV2. Each chapter represents an individual study and all studies were performed in different commercial swine facilities located in Saskatchewan.

Our research was possible due to the collaboration of local commercial swine facilities with an interest in the prevention or reduction of PCVD in their swine herds. All facilities observed high biosecurity procedures and had management and production practices consistent with reducing the risk and impact of PCVD. None of the facilities used commercial or experimental PCV2 vaccines, with the exception of our study to determine the efficacy of a novel ISCOM PCV2-VLP vaccine.

Chapter five, describing the development and validation of a novel quantitative PCR assay, allowed us to complete the studies in chapters six and seven. The samples used in the validation of the assay for chapter five were from archived tissues stored by Dr. Steve Krakowka at the Ohio State University (tissue samples from pigs experimentally-infected with PCV2 and non-infected gnotobiotic pigs) and Dr. John Harding at the University of Saskatchewan (tissue samples from PCV2 naturally-infected conventional pigs).

The hypotheses tested are listed in order of the chapters presented in this thesis.

Hypothesis 1:

PCV2 is detected persistently in the serum of naturally-infected healthy pigs and the virus persists in the presence of high levels of PCV2-specific IgG Ab.

Objectives:

(1) To test pig serum by two PCR protocols (nested and non-nested conventional PCR) across all production stages (farrowing crate, nursery, grower area, and finisher area) at 5, 12, 19, 40, 72, 107, 135, and 156 days of age for the presence of PCV2 DNA in each of 40 pigs.

(2) To test pig serum by cELISA across all production stages (farrowing crate, nursery, grower area, and finisher area) at 5, 12, 19, 40, 72, 107, 135, and 156 days of age for the level of PCV2-specific Ab in each of 40 pigs.

(3) To determine when PCV2 DNA is detected in young healthy pigs that are infected naturally with PCV2.

(4) To determine when PCV2-specific maternally-derived Ab in serum begins to wane and the pigs' adaptive IgG Ab response increases following natural infection with PCV2.

(5) To determine if significant relationships exist among measures of serum IgG Ab, viremia, and the effects of sex, breed, or age.

Hypothesis 2:

PCV2 is shed intermittently in boar semen over many months and the virus does not have detrimental effects on semen quality in healthy boars infected naturally with PCV2.

Objectives:

(1) To test semen over a nine month period by conventional nested PCR for the presence of PCV2 DNA from 43 healthy boars infected naturally with PCV2.

(2) To test semen quality by sperm morphological analysis to determine if the presence of PCV2 in semen affects the quality.

(3) To determine the relationship among boar age, time after stud entry, breed, and a semen sample positive with PCV2 DNA.

Hypothesis 3:

A quantitative SYBR green real-time PCR assay can be developed and used to quantify PCV2 in tissues from pigs infected naturally and experimentally with PCV2.

Objectives:

(1) To develop a novel SYBR green quantitative PCR that targets the highly conserved *Rep* gene (ORF1) of PCV2.

(2) To test the novel assay in multiple tissues from pigs infected naturally with PCV2. Tissues to include: serum, buffy coat, feces, and multiple lymphoid (bronchial, mesenteric, and superficial inguinal lymph nodes, thymus, tonsil, ileal Peyer's patches, and spleen) and non-lymphoid (myocardium, lung, kidney, liver, and gluteal muscle) tissues.

(3) To validate the developed PCR using tissues from conventional pigs infected naturally with PCV2, gnotobiotic pigs infected experimentally with PCV2, and plasmid-spiked negative serum, lung, and fecal samples.

(4) To determine the effects of tissue specimen on the performance of the novel PCR assay using samples spiked with PCV2 DNA.

(5) To compare PCV2 concentrations measured by tissue culture and immunohistochemical staining in homogenized liver samples of gnotobiotic pigs infected experimentally with PCV2 with the DNA concentrations estimated by the novel PCR assay.

Hypothesis 4:

Young pigs (<16 weeks of age) in a PCVD-affected commercial herd shed significantly more PCV2 DNA in their feces compared with pigs in a nonaffected herd.

Objectives:

(1) To use the quantitative PCR assay developed in hypothesis three to test 100 pooled fecal samples. "Pooled" refers to multiple samples collected from the floor of a single pen which are subsequently combined into one larger sample or "pool". Ten pooled samples will be collected from age-matched pigs in each of a PCVD-affected and a nonaffected herd and across all production stages (newly weaned, nursery, pregrower, grower, finisher, virgin gilt, bred gilt, lactating sow parity ≤2, lactating sow ≥3, and boar).

(2) To determine which production stage or stages of PCVD-affected and nonaffected pigs have significantly higher amounts of PCV2 DNA shed in feces.

(3) To determine if PCV2 DNA shed in feces is significantly different between stages in a PCVD-affected and a nonaffected herd.

(4) To determine if a higher amount of PCV2 DNA shed in sow feces may result in a higher amount of PCV2 DNA shed in the feces of young pigs.

Hypothesis 5:

A novel transdermal ISCOM technology-based PCV2 VLP vaccine administered at one and three weeks of age does not produce adverse reactions, significantly reduces mortality and the PCV2 DNA concentration in serum and feces, and significantly increases the PCV2 specific IgG Ab and the average daily gain in a naturally-PCVD-affected herd.

Objectives:

(1) To determine if rectal temperature, injection site reactivity, and the activity level of one week old conventional piglets are adversely affected by varied doses of ISCOM Matrix Q injected subcutaneously.

(2) To vaccinate 54 pigs (vaccinates (n=27); non-vaccinated controls (n=27)) with a novel ISCOM technology based PCV2 VLP vaccine at one and three weeks of age.

(3) To collect blood samples from 54 pigs at 1, 3, 7, 9, 11, 13, and 18 weeks of age and rectal fecal samples at 3, 7, 9, 11, 13, and 18 weeks of age. Using these samples, test three non-invasive outcome measures to determine the efficacy of the novel vaccine including: PCV2-specific IgG Ab level in serum and PCV2 DNA concentration in serum and feces.

(4) To weigh pigs at 1 and 18 weeks of age to determine the average daily gain (ADG) of each pig.

(5) To determine if the novel vaccine administered transdermally could successfully prime young pigs against PCV2 in the presence of PCV2 maternally-derived Ab and reduce the mortality caused by PCVD.

(6) To determine if the amount of maternally-derived IgG Ab (high or low based on a median cutoff value) in serum at one week of age has an affect on the development of the PCV2-specific IgG Ab response of pigs in a PCVD-affected herd.

CHAPTER 3

DETECTION OF PCV2 VIREMIA AND SEROCONVERSION IN NATURALLY INFECTED PIGS IN A FARROW-TO-FINISH BARN

Can J Vet Res. 2006. McIntosh KA, Harding JCS, Ellis JA, Appleyard GD.

This research was conducted in 2002-2003.

3.1 Abstract

Porcine serum was assayed by two PCR protocols (nested PCR (nPCR) and non-nested PCR) and a cELISA assay to determine when PCV2 viremia and a rise in the serum level of PCV2-specific Ab occurred in pigs raised in a large Canadian farrow-to-finish barn. Eight serial blood samples were collected from each of 40 pigs from 5 to 156 (±1.5) days of age; six pigs were removed from the study for various reasons and at various times. Viremia was not detected in the samples collected before 72 days of age but was detected in those collected on or after 72 days: of 33 pigs, seven (21%) had only one serum sample positive for PCV2 DNA by nPCR after day 72; 11 (33%) were intermittently positive by nPCR, non-nested PCR, or both between 72 and 156 days; and the remaining 15 (45%) were repeatedly positive (in two to four samples). The level of serum Ab against PCV2 declined after weaning and increased between 72 and 107 days of age, only after PCV2 was detected in serum. Our results show that PCV2 viremia persists in the presence of elevated levels of PCV2-specific Ab.

3.2 Introduction

PCV was identified in 1974 as a picornavirus-like contaminant of a porcine kidney tissue culture cell line (PK15) (215). In 1998, an antigenically and genetically distinct PCV (152) was

isolated from pig tissue and named PCV type 2 (PCV2) (59); PCV2 is associated with clinical disease in pigs (102;151).

PMWS, an emerging disease in swine, is caused by PCV2 (59;60;102). However, evidence suggests that manifestation requires coinfection with a pathogen such as porcine parvovirus (PPV) (61) or a similar immune stimulant (114), stress, or cofactor. This syndrome, first described in 1996 (89), debilitates swine 7 to 15 weeks of age, with wasting, respiratory distress, enlarged lymph nodes, diarrhea, pallor, and jaundice. Gross and histologic lesions affect multiple organ systems and are associated with interstitial pneumonia, lymphadenopathy, hepatitis, nephritis, myocarditis, enteritis, and pancreatitis (11;47).

Ab specific for PCV2 have been retrospectively detected in swine serum dating back to 1973 (225). Diagnosis of PMWS relies on the detection of either PCV2-specific nucleic acid or antigen associated with lesions in affected tissues. The virus has been isolated from heart, lung, liver, kidney, spleen, salivary gland, lymph node, thyroid, thymus, gastrointestinal tract, feces, pancreas, testes, and brain (60;102).

The primary route of transmission is unknown, but evidence suggests that PCV2 can be transmitted both horizontally and vertically. It has been detected in ocular, nasal, and fecal samples from naturally infected swine (207). Isolation of PCV2 from aborted pig fetal tissue (151) suggests vertical transmission. Detection of PCV2 nucleic acid in the semen of naturally (107) and experimentally (120) infected boars suggests transmission from boars to PCV2-naïve gilts and their litters.

Although PMWS is the most commonly recognized disease associated with PCV2, the virus has been implicated in additional diseases, such as congenital tremors (CT) (211), porcine dermatitis and nephropathy syndrome (PDNS), and reproductive disorders (151).

It is hypothesized that swine industry intensification, management and weaning practices, and infectious triggering agents (such as PPV) may have contributed to the emergence of PCV2-associated diseases in swine, as a retrospective serologic study determined that PCV2 has been present in swine populations for 30 years (225).

There has been limited investigation into the spread of PCV2 in breeding herds in which the virus is endemic but the incidence of overt disease is low. We examined the dynamics of PCV2 Ab production in relation to virus circulation within the farm. Epidemiologic studies will contribute to the development of vaccination strategies and understanding of the pathogenesis of PCV2 and PCV2-associated diseases.

3.3 Materials and Methods

3.3.1 Animals and Sample Collection

Forty newborn pigs were randomly chosen at a high-security farrow-to-finish barn in Saskatchewan that housed 14 000 pigs. All 1200 sows in the barn had received vaccine against *Erysipelothrix rhusiopathiae* (Eryshield; Grand Laboratories, Larchwood, IA, USA), PPV (Parvo-Vac; Pfizer, Kirkland, QC, Canada), and *Escherichia coli* (Kolivax; Wyeth, Guelph, ON, Canada), and all pigs were routinely vaccinated at eight weeks of age against *E. rhusiopathiae* (Suvaxyn E-Oral; Wyeth, Markham, ON, Canada).

The chosen pigs were not segregated from other animals in the barn but were ear-tagged to allow for relocation. They were either nursed by their biologic dam or fostered by cohort sows that had farrowed the same day; the piglets were arranged in farrowing crates separated by solid partitions from birth and until weaning at 19 days of age, as was routine in the barn. After weaning, the pigs were moved into the nursery until about 72 days of age, into grower areas until about 135 days of age, and then into finisher areas until slaughter at about 156 days of age. This

management practice, to relocate pigs to different sections of the barn at different growth stages, resulted in the mixing and remixing of pigs. Eight serum samples were serially collected from each of the pigs from the first week after birth and until slaughter, before movement into a new area, and at the midpoint of each stage (Figure 3.1) between October 2002 and March 2003. A minimum of 3 mL of blood was collected from the anterior vena cava into individual sterile serum tubes, transported in a chilled, insulated box, and stored at 4°C for 18 hours before sample preparation.

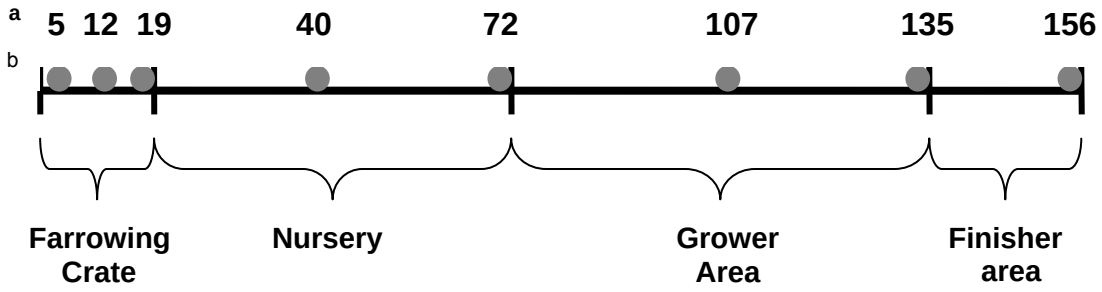

Figure 3.1: Schematic, based on the relocation of pigs within a Saskatchewan farrow-to-finish barn, of the collection of eight blood samples from 40 pigs between October 2002 and March 2003 to determine the percent inhibition (PI) of antibody specific for porcine circovirus type 2 (PCV2) by competitive enzyme-linked immunosorbent assay (cELISA) and to detect PCV2 DNA by nested (n) or non-nested polymerase chain reaction (PCR) at the following stages and average ages (±1.5 days): postpartum (5 days), mid-farrowing (12 days), weaning (19 days), mid-nursery (40 days), end of nursery (72 days), mid-grower (107 days), end of grower (135 days), and slaughter (156 days). Collections were made before relocation.
[a] average age ±1.5 days of pigs at each blood collection
[b] blood collections made prior to relocation

Animals entering the facility were not screened for PCV2-specific Ab or PCV2 in blood or tissues, but the only live animals accepted as replacement stock were those delivered by cesarean section or hysterectomy. Artificial insemination was performed with the use of semen from boars that had been serologically found to be PCV2-positive (unpublished data, 2003) by a cELISA (225). The barn had experienced sporadic cases of PMWS and other PCV2-associated syndromes since 1999 but had no significant losses due to PCV2-related diseases. Diagnosis of suspected PMWS was based on observed jaundice, unthriftiness, and dyspnea, and was confirmed by histopathologic and immunohistochemical study of tissues. Blood samples were centrifuged at 500 × g for 15 minutes at 4ºC. Serum was removed and stored in sterile 1.5 mL microcentrifuge tubes at -70ºC until DNA extraction and PCR or cELISA analysis.

3.3.2 DNA Extraction

For DNA extraction, from 200 µL of each serum sample, a commercial kit (DNeasy Tissue Kit; Qiagen, Mississauga, ON, Canada) was used according to the manufacturer's instructions, except that the DNA was recovered with the use of 100 µL of sterile water. The DNA was stored at -70°C for 24 hours before PCR. All extractions were tested in duplicate by each PCR protocol in a commercial amplification system (PTC-200 DNA Engine; MJ Research, Watertown, MA, USA).

3.3.3 PCR Assays

Two previously described PCR protocols (61;119) were adapted to reduce nonspecific amplification and improve the detection of PCV2 in serum. For nPCR (119), the reaction-mix concentrations were adjusted to 1.6 µM of each primer and 1.25 U of Taq DNA polymerase (Fermentas Taq DNA polymerase; VWR International, Edmonton, AB, Canada); for non-nested PCR (61) the annealing temperature was adjusted to 58°C.

The lowest detection limit of each PCR assay, expressed as the median tissue culture infective dose ($TCID_{50}$/mL), was determined with the use of 10-fold dilutions of PCV2 field isolate Stoon7 (59) spiked into 200 µL aliquots of serum known to be PCV2-negative and DNA extracted as described above. The Stoon7 had been obtained from porcine lymph tissue in 1998 from a PMWS case in Saskatchewan; the concentration of PCV2 in tissue culture was determined by a previously described immunoperoxidase monolayer assay (59) after one passage in PCV-negative PK15 cells. The lowest detection limits of the nPCR and non-nested PCR assays were 9.7×10^{-4} and 9.7×10^{-2} $TCID_{50}$/mL, respectively.

3.3.4 Competitive ELISA Assay

A previously described cELISA (225) was used to detect PCV2-specific Ab in porcine serum with the following adaptations: plates were coated with partially purified PCV2 (225) (French isolate 48285) recovered from a pig with PMWS in 1998 (11) and diluted to achieve a well concentration of 0.2 µg; goat immunoglobulin against mouse antigen, labeled with horseradish peroxidase and containing 4% normal goat serum, was diluted to 1:5000; and the enzyme substrate was incubated for 20 minutes. Percent inhibition (PI) values greater than 44% were considered positive for PCV2-specific Ab on the basis of previously published data (225).

3.3.5 Statistical Analysis

The SAS GENMOD procedure (94) was used to analyze factors influencing Ab PI or persistence of viremia in the tested pigs. Univariate analyses were performed to investigate the possibility of a relationship between the measured variables and breed, sex, or age at the beginning of the experiment. The model used was $Y = \mu + sex_i$ or $breed_j$ or + days of age_k where Y = an observed value for Ab PI or viremia; μ = the population mean for that trait; sex_i = a fixed

effect due to sex, where i = 1 or 2; $breed_j$ = a fixed effect due to breeding line, where j = l, h, or w; and days of age_k = a fixed effect of age on the first bleeding-day group, where k = 3, 4, 5, 6, or 7. Partial data sets were included in this analysis because the procedure accounts for missing values.

3.4 Results

3.4.1 Animals

The piglets used in this study were farrowed between October 3rd and 7th, 2002, and varied by sex (23 males, 17 females) and breed (26 Large White, 10 Landrace, and four Hamline). The first blood collection was made at 5 ± 1.5 (range three to seven) days of age. Six pigs were removed at various times from the study because of missing ear tags (three pigs), chronic lameness (two pigs), and congenital heart disease (one pig).

3.4.2 PCR and Competitive ELISA Assays

As Figure 3.2 shows, the PI of PCV2-specific Ab declined after weaning, and viremia was subsequently detected by nPCR and non-nested PCR. The PI of PCV2-specific Ab rose after the viremia (between 72 and 107 days of age) and remained elevated until the end of the study. The nPCR assay detected PCV2 DNA in more serum samples than did the PCR assay. Of 34 pigs tested, seven (21%) had only one serum sample positive for PCV2 DNA by nPCR after day 72, 11 (33%) were intermittently positive by nPCR, non-nested PCR, or both between 72 and 156 days, and the remaining 15 (45%) were repeatedly positive (in two to four samples).

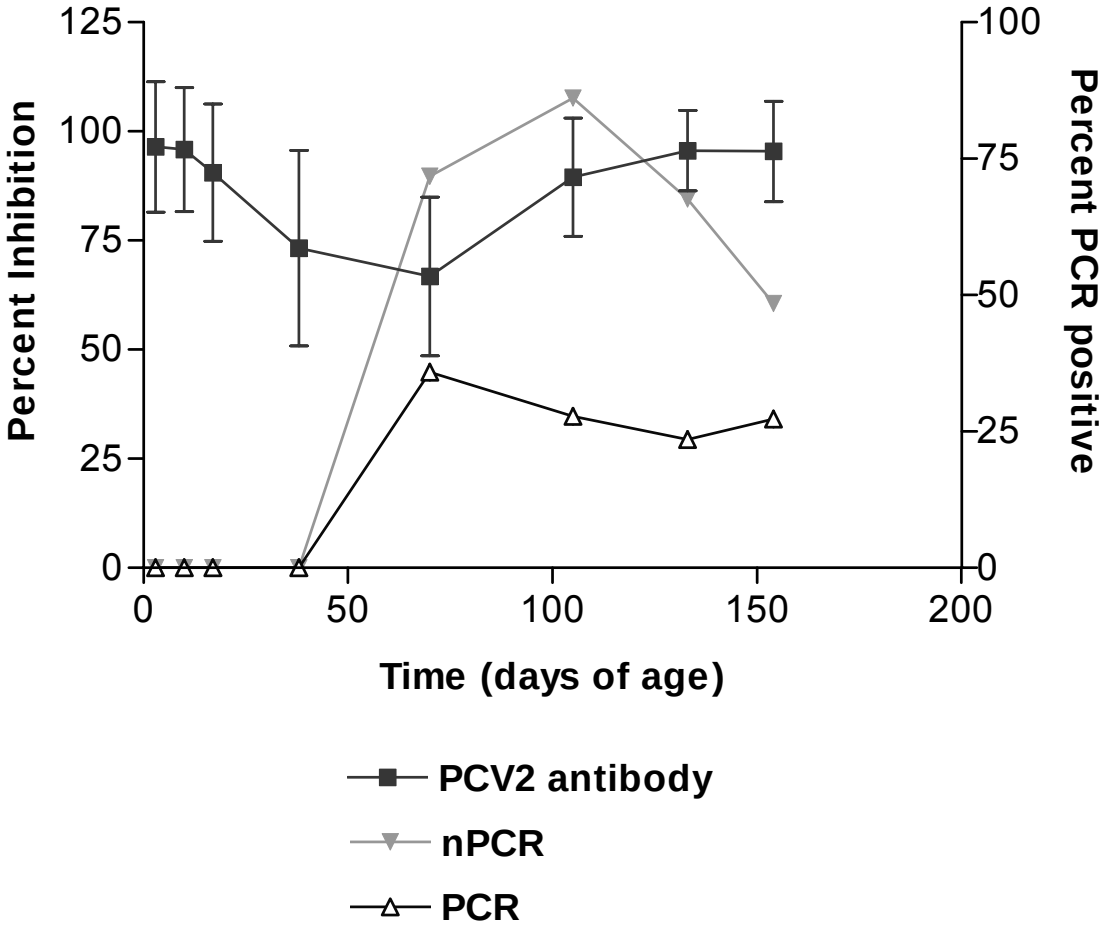

Figure 3.2: Changes in mean percent inhibition (PI) of porcine circovirus type 2 (PCV2) specific antibody (black squares) according to competitive enzyme-linked immunosorbent assay (cELISA); PI values above 44% were considered positive for diagnostic purposes. A rise in PCV2 DNA, an indicator of serum viremia, determined from replicate serum samples, was detected by nPCR (grey triangles) or non-nested polymerase chain reaction (PCR) (white triangles) after a decline in serum antibody PI.

3.4.3 Statistical Analysis

There were no significant correlations between measures of serum Ab or viremia and effects of sex, breed, or age. The pigs in this study belonged to a herd that was endemically infected with PCV2 and since 1999 had experienced occasional confirmed cases of PMWS or PCV2-associated diseases. They were not segregated but were maintained in the barn with the remaining population and relocated to pens according to previously established management practices. There were no recognized cases of PMWS or PCV2-associated diseases during the blood collection period (October 2002 to March 2003) among the study pigs.

3.5 Discussion

To compare PI levels of PCV2-specific serum Ab between pigs and over time, cELISA was used. The piglets had high PIs from the time of first measurement, 5 (±1.5) days after birth and until weaning at 19 days of age, which indicated transfer of maternal Ab to nursing piglets. A further decline in PI was observed at 40 and 72 days of age, the lowest level being observed at 72 days (53 days after weaning), coincident with PCR detection of viremia. The average PI for any collection was above the cutoff for a positive result. However, 10% and 15% of the pigs tested at 40 and 72 days of age, respectively, had PIs below 44%, which suggests wide variation in serum Ab levels among animals. Variation was greater before viremia, owing to passive Ab absorption: the decay of maternally-derived Ab may vary between two weeks and several months (221;223), and maternal Ab may be only partially protective against viral diseases in swine (51). The effect of sow immunity or colostrum on the variation in piglet antibody levels was not investigated in this study but needs to be elucidated.

Viremia was not detected until 72 days of age, which suggests either sufficient protection by maternal PCV2-specific Ab or another immunocellular mechanism before 72 days or the

absence of exposure to infectious PCV2. Since PCV2 was present and has circulated within the population used for this study, it is possible that the pigs had been exposed to the virus before 72 days of age. The fact that PCV2 can be detected in ocular, nasal, and fecal samples from naturally infected swine (207) supports the transmission of virus from sows to nursing piglets and between littermates or penmates, or both. It has been postulated that vertical transmission of PCV2 can occur in fetal pigs, as the virus has been isolated from tissues of aborted and stillborn animals (151). Infectious PCV2 transmission *via* semen or colostrum has not been reported but is a possible means of transmission to fetal or nursing piglets. However, the spread of PCV2 to naïve pigs may be greatest during the mixing and remixing of pigs in the nursery (at 40 to 72 days of age), coincident with declining serum levels of PCV2-specific maternal Ab (Figure 3.2).

The level of PCV2-specific Ab increased after 72 days of age and remained high until the end of the study. The sequence of events suggests that PCV2 viremia is established concurrently with waning Ab levels; however, PCV2 DNA was periodically detected in serum until the time of slaughter in the presence of high levels of Ab, which suggests that the Ab may be non-neutralizing once an infection is established.

The intermittent detection of PCV2 viremia may represent a real phenomenon, but it must be considered to be a possible function of the use of PCR as a method to detect PCV2 DNA in serum as an indicator of viremia. Although PCR is a sensitive molecular diagnostic tool that can detect PCV2 at low concentrations in serum, it does not provide information on quantity of virus or infective capability. The PCR assays used in this study had different detection limits. As expected, more samples were positive for PCV2 DNA in serum with the nPCR protocol than with the non-nested protocol.

Diseases associated with PCV2 have a significant impact on swine populations worldwide. Epidemiologic research on herds with endemic infection and either sporadic cases or a low incidence of overt PCV2-related disease has been limited. This report describes the relationship between PCV2 viremia and PCV2-specific serum Ab formation in pigs housed in a large farrow-to-finish barn in which PCV2 was endemic. Epidemiologic studies of PCV2 infection in this type of pig management facility contribute to the development of vaccination strategies and understanding of the pathogenesis of PMWS and PCV2-associated diseases. The results from this study suggest that PCV2-specific Ab in serum is not sufficient to prevent or resolve PCV2 viremia in pigs. Furthermore, vaccination may be hindered by the presence of maternally derived PCV2-specific Ab in young pigs.

3.6 Acknowledgements

This study was supported by grants from Hypor Inc. and the Saskatchewan Agriculture and Development Fund. We are grateful for the technical assistance of Jill Kingdon, at Hypor, the participating barn staff, and Anita Quon and Carrie Rhodes. We thank Drs. Sarah Parker and Sylvia Checkley for statistical guidance.

CHAPTER 4

NESTED PCR DETECTION AND DURATION OF PCV2 IN SEMEN WITH SPERM MORPHOLOGICAL ANALYSIS FROM NATURALLY INFECTED BOARS

J Vet Diagn Invest. 2006. McIntosh KA, Harding JCS, Parker SE, Ellis JA, Appleyard GD.

This research was conducted in 2002-2003.

4.1 Abstract

A nPCR protocol was applied to porcine semen to demonstrate the PCV2-shedding patterns and duration in naturally infected boars. Sperm morphology analysis was performed on a subset of samples to determine if the presence of PCV2 DNA in semen was associated with reduced semen quality. Semen was collected serially from 43 boars representing six breeds, aged 33.9 to 149.3 weeks. Of the 903 semen samples collected, 30 samples (3.3%) were positive for PCV2 DNA by nPCR from 13 boars. Boars shedding PCV2 DNA in semen ranged between 35.9 and 71.0 weeks of age, and shedding occurred during a period of up to 27.3 weeks. A semen nPCR test was 2.6 times more likely to be positive when collected from pigs that were ≤52 weeks of age, and 3.0 times more likely to be positive when collected from pigs that were ≤26 weeks from time of entry into the stud main unit (generalized estimating equations: P=0.02; 95% confidence interval (CI) of the odds ratio (OR) 1.2 to 5.5, and P=0.01; 95% CI of the OR 1.3 to 6.9, respectively). These results demonstrate a sporadic and long-term shedding pattern of PCV2 DNA in semen from naturally infected boars. PCV2 DNA in semen does not appear to have detrimental effects on sperm morphology; however, boar age and, possibly, breed may contribute to the persistence of PCV2-shedding in semen.

4.2 Introduction

PCV2 was first isolated in 1998 from swine exhibiting PMWS (59). PMWS was reported in 1996 and primarily affects swine 7 to 15 weeks of age with clinical features that include wasting, dyspnea, enlarged lymph nodes, diarrhea, pallor, and jaundice (88). Pathologically, PMWS is associated with gross and histological lesions in multiple organs, interstitial pneumonia, lymphadenopathy, hepatitis, nephritis, myocarditis, enteritis, and pancreatitis (11;59;88).

Since the recognition of PCV2 as the etiological agent of PMWS (59;60;102), the virus has been associated with several porcine circoviral diseases (PCVD) in pigs: porcine dermatitis and nephropathy syndrome (PDNS) (151), congenital tremors (CT) (211), abortion, and reproductive failure (166). In experimental infections, PCV2 is detected in lesions from various tissues including heart, lung, spleen, liver, kidney, lymph node, thymus, thyroid, salivary gland, testes, intestine, pancreas, and brain (60;102).

Although the pathogenesis and primary mode of transmission of PCV2 are not known, there is evidence to support horizontal and vertical transmission. PCV2 was detected in ocular, nasal, and fecal secretions from swine infected experimentally (115) and field cases (207) suggesting horizontal transmission. Recently, PCV2 has been cultured from the feces of swine infected naturally suggesting a potential for fecal–oral transmission (229). Evidence of vertical transmission is supported by the isolation of PCV2 from aborted fetal pig tissues (151) and stillbirth myocardia (166). PCV2 has been detected in semen from boars infected naturally with the virus, but the shedding pattern and duration were not determined (108). In another study, PCV2 DNA was detected intermittently in semen from day 5 to 47 postinfection in specific-pathogen–free boars infected intranasally with PCV2 (119). There is, however, a lack of

information on the long-term shedding patterns of PCV2 in semen from naturally-infected boars. Because artificial insemination is a common practice in swine breeding, these data have significant implications for PCV2 transmission and biosecurity in the industry. Superior animal production and semen quality are required to be competitive and profitable, and it is unknown whether the presence of PCV2 in semen is detrimental to semen quality. In this study, the dynamics of PCV2-shedding in naturally-infected boars maintained in a commercial artificial insemination stud were measured and sperm morphological traits of PCV2-shedding and non-shedding boar were characterized.

4.3 Materials and Methods

4.3.1 Animals and Sample Collection

One-hundred-twenty-five boars, consisting of Duroc, Landrace, Hamline, Large White maternal, Large White paternal, and Meishan-synthetic breeds, were maintained at a biosecure commercial artificial insemination stud in Saskatchewan, Canada, permitted for export sales by the Canadian Food Inspection Agency. Semiannual vaccination for porcine parvovirus (Parvo-vac; Pfizer Inc., Kirkland, QC, Canada) was maintained, and all boars entering the stud were quarantined for 50–60 days and treated with two doses of 25 mg/kg dihydrostreptomycin (Ethamycin; Pfizer Inc., Kirkland, QC, Canada) while in quarantine. Although the stud received replacement boars from both porcine respiratory and reproductive virus (PRRSV) positive and negative herds, there was no evidence of viral circulation in the stud based on quarterly serum (IgG) PRRSV ELISA (HerdChek ELISA; IDEXX Laboratories Inc., Westbrook, ME, USA) testing and weekly PCR (Prairie Diagnostic Services; University of Saskatchewan, Saskatoon, SK, Canada) testing of semen dating back to September 1999. Boars in this study were tested for PCV2 Ab in serum (to confirm exposure) by a cELISA, as previously described (225). During

this study, less than 5% of semen samples collected were unacceptable because of motility or morphological defects, and a conception rate of 88% was observed.

4.3.2 DNA Extraction

Semen collected from individual boars was diluted with extender (Androhep PLUS Extender; Minitube, Verona, WI, USA) to a final concentration of three-billion live sperm cells per 80 mL total volume, stored at 4ºC, and shipped in insulated boxes within 24 hours of collection from February 2002 to September 2003. Because semen collection was dictated by commercial product demand, collections from individual boars were subject to temporal variation. Extended semen was mixed by inversion, transferred to individual sterile 50 mL tubes, centrifuged at 500 × g at 4ºC for 15 minutes and supernatant gently poured off, leaving 5 mL of concentrated cells in residual extender. Samples were vortexed for 15 seconds, and 1 mL was transferred to individual sterile 1.5 mL microcentrifuge tubes and stored at -70ºC pending DNA extraction. DNA was extracted from 100 mL of each thawed and vortexed sample using a commercial kit (DNeasy tissue kit; Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's instructions, except DNA was recovered with 100 mL of sterile water. DNA was stored at -70ºC for 24 hours pending nPCR.

4.3.3 Nested PCR Assay

A previously described nPCR protocol (119) was adapted to eliminate nonspecific amplification. Adaptations for the primary reaction included 1.0 mM $MgCl_2$, 1.6 mM each primer, and 1.25 U Taq DNA polymerase (Fermentas Taq DNA polymerase; VWR International, Edmonton, AB, Canada) using the conditions of 94ºC for 3 minutes, followed by 20 cycles of 94ºC for 1 minute, 65ºC for 1 minute, and 72ºC for 1 minute and 1 final cycle of 72ºC for 7 minutes. Secondary reaction adaptations included 1.25 mM $MgCl_2$, 1.6 mM each primer, and

1.25 U Taq DNA polymerase using the conditions of 94ºC for 3 minutes, followed by 35 cycles of 94ºC for 30 seconds, 65ºC for 30 seconds, and 72ºC for 30 seconds and one final cycle of 72ºC for 7 minutes. Single DNA extractions were tested in duplicate by nPCR in a commercial amplification system (DNA Engine (PTC-200); MJ Research Inc., Watertown, MA, USA). If agreement between duplicates was not observed, DNA extraction and duplicate nPCR was repeated for that sample; all duplicate samples had agreement after re-extraction, when additional testing was necessary. Assay controls included extraction of a negative (100 mL sterile water) and a PCV2 virus–spiked sample using a PCV2 field isolate (Stoon7) at a concentration of 9.7×10^{-2} $TCID_{50}$/mL in 100 mL of boar semen per extraction event. Stoon7 was obtained from porcine lymph tissue in 1998 from a PMWS case in Saskatchewan (59). One negative control was used per 10 samples, per PCR event. The lowest detection limit of the nPCR assay was determined by 10-fold dilutions of Stoon7 spiked into pre-tested 100 mL aliquots of PCV2-negative semen. The concentration of Stoon7 was determined by an immunoperoxidase monolayer assay following one passage in PCV negative PK15 cells, as previously described (59).

4.3.4 Sperm Morphological Analysis

Spermiograms were prepared by mixing approximately 20 mL of eosin nigrosin sperm morphology stain (5.0 g nigrosin, 0.83 g eosin Y, 75 mL sterile distilled H_2O with pH adjusted to 6.8) with 20 mL of semen in extender, as previously described (19). One slide was prepared per boar per collection, and slides were stored at room temperature until examination of 200 cells per slide under 1000^3 oil emersion magnification to determine the ratio of live to dead sperm cells and specific morphological defects. Defects were categorized as: head (pyriform, tapered, microcephalic, macrocephalic, teratoid); midpiece (mitochondrial sheath defect, distal midpiece

reflex); principle piece (multiple tails, tail stump defect, coiled principle piece, accessory tails); detached normal heads; detached abnormal heads; proximal cytoplasmic droplet; knobbed acrosome; and the proportion of normal cells.

4.3.5 Statistical Analysis

The proportion of boars that had at least one positive PCV2-positive semen sample in each breed was compared with the Fisher's Exact Chi-Square Test. Generalized Estimating Equations (52) (GEE) were used to evaluate the individual effect of age, time after stud entry, number of samples, and breed on the semen nPCR results in boars with at least one PCV2-positive test. The effect of clustering was accounted for at the individual boar level.

To determine the effect of seminal PCV2 status on semen quality, the mean percentage of live and normal sperm from nPCR positive and negative samples were compared using data from boars with both positive and negative samples. Since the data were normally distributed, the significance of observed differences was evaluated with the Student's *t*-test. All statistical tests were conducted using statistical software (Stata Statistical Software 9.0; StataCorp LP, College Station, TX, USA).

4.4 Results

4.4.1 Animals

All boars were positive for PCV2 Ab in serum by cELISA prior to initiating semen collections. No boars within the facility presented with PMWS or PCVD during the study. Because boars were sampled only during commercial production phases, the average time between individual collections for all boars was 11 ± 8.9 days (range 2 to 81). Of the 903 semen samples collected from 43 boars, serial collections were analyzed over 7.4 ± 1.1 months (range 6.5 to 9).

4.4.2 nPCR Assay

PCV2 DNA was detected by nPCR in 30 of 903 semen samples (3.3%) from 13 (30.2%) boars tested (Figure 4.1). PCV2 DNA was only detected in Duroc and Landrace boars. While the study was not balanced by breed, three of five Duroc boars (60%) and 10 of 23 (43.5%) Landrace boars had at least one PCV2-positive semen sample. The remaining 30 boars composed of Hamline (five), Large White maternal (six), Large White paternal (two), and Meishan-synthetic (two) breeds, did not shed PCV2 in semen, as detected by nPCR (33.9 to 49.3 weeks of age at collection). The lowest detection limit of the modified nPCR was 9.7×10^{-4} $TCID_{50}$/mL.

Pig[a]	Total[b]	32[c]	36	40	44	48	52	56	60	64	68	72	76	80	84	88	92	94
DU1	**1/27**						1	2	1/3	2	2	2	1	2	3	4	2	3
DU2	**1/10**	1	1/2		1	1	1	1	3									
DU3	**4/9**		1/1		1/2	1/1	1		1/4									
LR1	**1/22**								2	1/4	3	3	3	2	2	2	1	
LR2	**1/27**		1	4	4	4	3	1/4	3	4								
LR3	**2/36**							1	1/4	3	1/4	5	4	5	4	4	2	
LR4	**3/23**	1		3	1/4	4	2	2/4	4	1								
LR5	**1/16**				1/2	3	1	1	3	4	1		1					
LR6	**5/23**		1/1	1/4	1		2	1/3	2/3	3	3	3						
LR7	**8/27**			1	3/3	2/4	2/4	1/3	3	3	3	3						
LR8	**1/24**		3	1/4	4	3	3	4	3									
LR9	**1/16**	1/1	2	2	1	2	3	2	3									
LR10	**1/24**	1	4	3	1/3	3	4	3	3									
Pos	**30**	1	3	2	7	3	2	5	5	1	1	0	0	0	0	0	0	0
Total	**284**	4	14	21	25	25	25	28	41	24	16	16	9	9	9	10	5	3

Figure 4.1: Results of nPCR to detect PCV2 DNA in semen from 13 boars with a positive sample from February 2002 to September 2003 (n=43 boars tested).
From 13 boars with a positive semen sample:
- ☐ 254 semen samples were negative for PCV2 DNA;
- ▒ 30 semen samples were positive for PCV2 DNA (n=903 semen samples tested);
- ■ no semen collected.

The number of positive samples of the total is shown as a fraction.
a. Pig identification based on breed: DU=Duroc; LR=Landrace.
b. Number of positive semen samples collected of total tested by nPCR for each boar.
c. Age of boar in weeks; 4 week intervals.

4.4.3 Statistical Analysis

No PCV2-positive semen samples were detected after 71 weeks of age, and there were a number of boars from each breed with no sample collected before 71 weeks of age. When those boars were omitted from the analyses, only two boars remained in each of those breed groups that had no boars with a PCV2-positive semen sample. There was no significant difference in the proportion of positive Duroc (2/3) or Landrace (10/20) boars with tests taken before 71 weeks of age (P=1).

Because no positive PCV2 nPCR results were obtained after 71 weeks of age, samples collected after 71 weeks were excluded from the analysis of the effect of age on the PCV2 status of the sample. Only samples from boars with at least one positive PCV2 semen nPCR result were included. This made the interpretation of the effect of age more conservative. A model that included all of the negative samples after 71 weeks of age would increase the calculated odds of a sample being nPCR-negative with increasing age. Breed and the number of samples analyzed per boar were not significant predictors of the PCV2 semen status. Semen nPCR test results were less likely to be positive as age and the time after entry increased; however, these variables could not be evaluated together in a single model because they increase consistently together and their effects would be additive in the model (colinearity). With age as a continuous predictor, the effect on the PCV2 semen nPCR result was not linear. The final model evaluating the effect of age used age dichotomized as ≤52 weeks or >52 weeks and an exchangeable correlation structure (correlation between results from the same animal is the same at different time points). A semen nPCR test was 2.6 times more likely to be positive when collected from boars that were ≤52 weeks of age (P=0.02; 95% CI of the OR 1.2 to 5.5). The final model evaluating the effect of the time from entry used the time from entry dichotomized as ≤26 weeks after entry or >26 weeks

after entry and an exchangeable correlation structure. A semen nPCR test was 3.0 times more likely to be positive when collected from boars that were ≤26 weeks from entry (P=0.01; CI of the OR 1.3 to 6.9).

Sperm morphology analysis was performed on a subset of boars in this study: 10 PCV2-shedding boars (199 smears; boar ID: DU2, DU3, LR2, LR4–LR10) and eight boars that did not shed PCV2 in semen (115 smears: data not shown) to determine the effect of PCV2 semen status on semen quality (based on the proportion of live and morphological normal cells). There was no significant difference between the proportion of live sperm (P=0.16) or morphologically normal sperm (P=0.54) between positive and negative samples. This did not change if a more complex analysis (GEE) was used to account for the effect of results of sperm analysis clustering on an individual animal basis (analysis not included).

4.5 Discussion

PCV2 DNA was detected in semen from 30.2% of naturally-infected boars and in 3.3% of semen samples collected from a commercial artificial insemination stud. PCV2-shedding in semen was sporadic in frequency and was detected over a period of up to 27.3 weeks. Of the 43 boars tested in this study, 73.3% of the PCV2-positive semen tests came from five boars, and we observed marked variation in the duration and number of individual PCV2-positive semen samples in each boar.

Boars ranged from 33.9 to 149.3 weeks of age for the period of this study; however, semen samples were more likely to be PCV2 DNA positive when collected from younger boars. The observation that no PCV2 positive semen samples were collected after 71 weeks of age and that younger boars were more likely to have PCV2-positive semen may be of importance to the swine industry. However, care must be taken in the interpretation of this result, as the number of

animals in this study was small and may not be representative of the population. Semen quality (proportion of live and morphologically normal cells) was not affected by the presence of PCV2 DNA in semen when PCV2-shedding and non-shedding boars were compared. Because a small number of animals were evaluated and the within and between animal variability was high, differences attributed to the presence of PCV2 DNA in semen would be difficult to detect.

All boars were positive for PCV2 Ab in serum before collection, but did not exhibit any PCVD; therefore, PCV2 seminal shedding may occur in the absence of clinical disease. It is not known if PCV2 detected by nPCR in semen was infectious or the quantity of virus present. Molecular assays are an efficient detective method to determine the presence of the virus; however, PCV2 virus isolation from semen has not been reported. Similarly, PRRSV RNA is detected in semen by PCR (44); however, transmission studies have proven that exposure of gilts to PRRSV-infected semen results in seroconversion and isolation of PRRSV from tissues in infected gilts (189). PCV2 shed in semen may contribute to the infection of susceptible sows and fetuses upon breeding; however, PCV2 isolation from semen or a bioassay using PCV2-susceptible animals is required to confirm the potential infectivity of PCV2 in semen.

Observations regarding PRRSV may be analogous to PCV2, as PRRSV causes a persistent infection in boars (43). Landrace, Yorkshire, and Hampshire boars infected experimentally with PRRSV demonstrate variability in the duration of PRRSV seminal shedding among breeds (42).

The observed trend of differences in breed susceptibility to PRRSV shedding in semen requires further study of variable host factors in PRRSV persistence and resistance. PCV2 DNA was detected in semen from Duroc and Landrace boars only; however, the semen of the Hamline, Large White maternal, Large White paternal, and Meishan-synthetic boars were

negative for PCV2 DNA. Boars were not tested over a constant range in age or time after entry into the stud, and the total number of individual samples or breeds was not equal among boars. Whereas no interpretation regarding the susceptibility of shedding of PCV2 DNA with respect to breed can be made from this study, these observations warrant further study.

Further investigation into the effects of breed and age on the shedding characteristics of PCV2 in semen is needed. The frequency of PCV2 DNA detected in semen in this study was low and sporadic, and regularly scheduled collections over time would further discern the temporal dynamics of viral shedding. It is not known if nPCR of raw semen *versus* extended semen would improve the detection of PCV2; however, it has been reported that PCV DNA is more often detected in the seminal fluid and nonsperm cell fractions of boar semen (108). It must be considered that boars seropositive for PCV2 may have persistent shedding in semen, and testing for PCV2 in semen may be required for commercial artificial insemination studs supplying semen to PCV2-susceptible sows. The significance of PCV2 DNA in semen as it relates to the transmission of the virus and its effects on reproductive success is currently unknown; however, it was determined in this study that PCV2 DNA in semen does not appear to adversely affect semen quality. In addition, the results presented in this study confirm the sporadic and long-term shedding of PCV2 DNA in semen from naturally-infected boars and suggest that boar age and possibly breed may contribute to the persistence of PCV2-shedding in semen.

4.6 Acknowledgements

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CHAPTER 5

DEVELOPMENT AND VALIDATION OF A SYBR GREEN REAL-TIME PCR FOR THE QUANTIFICATION OF PCV2 IN SERUM, BUFFY COAT, FECES, AND MULTIPLE TISSUES

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This research was conducted in 2005 to 2006.

5.1 Abstract

The emergence of multiple genotypes of PCV2, as demonstrated by phylogenetic analysis of whole genome or capsid sequences, makes it necessary to have quantitative diagnostic assays that perform equally well on all strains. The objectives of this study were to develop and validate a novel real-time PCR assay targeting the highly conserved *Rep* gene (ORF1) and investigate the effects of diagnostic specimen choice on its performance. The assay was tested in naturally-infected conventional pigs, experimentally-infected gnotobiotic pigs, and plasmid-spiked negative serum, lung tissue, and feces and found to have a linear detection range of 2.2×10^3 to 2.2×10^{10} copies of PCV2 per mL. The assay successfully detected and quantified PCV2 DNA in serum, buffy coat, feces, and multiple lymphoid (bronchial, mesenteric, and superficial inguinal lymph nodes, thymus, tonsil, ileal Peyer's patches, and spleen) and non-lymphoid (myocardium, lung, kidney, liver, and gluteal muscle) tissues from naturally-infected pigs. Across all tissues and sera of naturally-infected pigs, the mean PCV2 concentration was 3.0 logs higher in wasting *versus* non-wasting pigs. PCV2 concentration measured by tissue culture and immunohistochemical staining in homogenized liver samples of experimentally infected gnotobiotic pigs were compared to the concentrations estimated by quantitative PCR. Similar

trends were noted with increasing PCV2 concentration detected in subclinically-infected to severely PMWS-affected pigs across all assays. Our diagnostic assay was developed with a conserved target sequence, and performed efficiently in the quantification of PCV2 in a variety of tissues from pigs infected naturally and experimentally.

5.2 Introduction

Non-pathogenic PCV was discovered in 1974, as a picornavirus-like contaminant of a porcine kidney tissue culture cell line (PK15) (215). PCV consists of a single stranded circular DNA genome of 1.7 kb, is non-enveloped, and is approximately 17 nm in diameter. In the mid-1990s, PMWS was recognized and found to affect primarily pigs 6 to 15 weeks of age (47;89).

A genetically (152) and antigenically (11) distinct PCV was recovered from a PMWS-affected pig in 1996 (59). The pathogenic PCV was subsequently named porcine circovirus type 2. PMWS is characterized by wasting or unthrifty pigs, jaundice and respiratory distress, accompanied by gross and/or histopathological lesions affecting multiple organs and tissues (11;59;88). Pathologically, PCV2 is associated with interstitial pneumonia, hepatitis, lymphadenopathy, pancreatitis, myocarditis, nephritis, and enteritis (11;47). PCV2 is the etiological agent of PMWS and is found associated with additional disease syndromes including: congenital tremors (CT) (211), porcine respiratory disease complex (PRDC) (105), porcine dermatitis and nephropathy syndrome (PDNS) (8), abortion (228), and reproductive disorders (166). Diseases associated with PCV2 are now collectively termed porcine circovirus diseases (PCVD), or porcine circovirus associated disease (PCVAD). PCV2 nucleic acid or antigen associated with lesions in the tissues of affected pigs is the primary target for the diagnosis of PCVD in pigs.

An increasing number of herds globally are infected with PCV2; however, the infection remains subclinical in the majority of pigs. Progression to overt disease, or PCVD, requires coinfection with another pathogen (53) such as porcine respiratory and reproductive syndrome virus (PRRSV) (10), porcine parvovirus (PPV) (61;115), *Mycoplasma hyopneumoniae* (180), an immune stimulus (113;114), or cofactor which may include early weaning or inappropriate management practices (194).

Recently, viral load has been associated with PCVD clinical severity in pigs (112) and it has become more desirable to use quantitative methods to determine the magnitude of PCV2 infection. In addition, real-time PCR is generally more rapid, less labour intensive, less expensive, and may be more reliable than conventional PCR. Several previously published assays exist using either a probe type (29;45;169;231), or a SYBR green (230) real-time PCR methodology. PCV2 differential diagnostic assays are now available due to the increasing concern regarding the spread of potentially more virulent genotypes of PCV2 (57); however, there is conflicting evidence (13;71).

The objectives of this study were to develop and validate a novel SYBR green real-time PCR assay targeting ORF1 (*Rep*) exploiting oligonucleotide primer binding sites conserved across 244 characterized PCV2 genomes, and to investigate the effects of diagnostic specimen choice on its performance. The assay was designed to target all genotypes of PCV2 (PCV2a/2b) (71), or genogroups (PCV2-G1/G2) (13), and was tested to determine its versatility and performance in multiple tissues using naturally-infected conventional pigs, experimentally-infected gnotobiotic pigs, and plasmid-spiked PCV2-negative serum, lung tissue, and feces. Historically, homogenized and serially diluted pig tissues or serum have been applied to PK15 cells to determine the tissue culture infective dose ($TCID_{50}$) or concentration of PCV2. This

method of quantification is labor intensive, expensive, and extremely time consuming as it requires the maintenance of tissue culture, followed by fixing and immunostaining techniques. In this study, serum and homogenized liver samples from experimentally-infected gnotobiotic pigs were tested to determine the PCV2 $TCID_{50}$ and were compared with the SYBR green real-time PCR assay described here.

5.3 Materials and Methods

5.3.1 Oligonucleotide Primer Design

Complete genome sequences of PCV2 (199 sequences) and PCV1 (23 sequences) were retrieved from Genbank. Additional unpublished PCV2 genome sequences derived from Canadian PCV2 strains (45 sequences) were received from Dr. Carl Gagnon (Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, QC, Canada). Signature Oligo software (LifeIntel, Port Moody, BC, Canada) was used to identify PCV2-specific sequences. Output from Signature Oligo was used to guide oligonucleotide primer selection with Primer3 (196). Specificity of primer sequences was confirmed by comparison to a local database of PCV sequences and to the GenBank DNA database using BLASTn (16) configured for short, nearly exact matches (word size seven, expect value 1000).

5.3.2 Conventional PCR

The conventional PCR reaction mixture for each of the assays PCV2-492 bp and PCV2-83 bp was 1× PCR buffer (75 mM Tris–HCl (pH 8.8), 20 mM $(NH_4)2SO_4$, 0.01% (v/v) Tween 20), 2 mM $MgCl_2$, 0.8 mM each primer, 0.25 mM each dNTP, 1.25 U Taq DNA polymerase (Fermentas Taq DNA polymerase; Fermentas, Mississauga, ON, Canada), 2 µL template, and was made up to 50 µL with sterile water. Amplification of DNA for PCV2-492 bp was achieved by 3 minutes at 94ºC, followed by 40 cycles of 30 seconds at 94ºC, 1 minute at 60 ºC, and 1

minute at 72ºC, and a final extension of 5 minutes at 72ºC. Amplification for PCV2-83 bp was performed similarly, but with an annealing temperature of 59ºC. PCR reactions were performed in a DNA Engine (PTC-200) thermal cycler (MJ Research Inc., Watertown, MA, USA), and amplicons were visualized by ethidium bromide staining on a 1.5% agarose gel with a DNA ladder (GeneRuler DNA Ladder Mix; Fermentas, Burlington, ON, Canada).

5.3.3 Standard Curve

A region of the PCV2 genome corresponding to nucleotides 179-670 of GenBank Accession DQ220739 was PCR amplified from extracted (DNeasy Tissue Kit; Qiagen Inc., Mississauga, ON, Canada) porcine lymph node using primers PCV2 492F (5'-TATTGTTGGCGAGGAGGGTA-3') and PCV2 492R (5'-TGGTAACCATCCCACCACTT-3'). The resulting amplicon of 492 bp (GenBank Accession EU126886) was ligated into a cloning vector (pCR 2.1-TOPO; Invitrogen, Burlington, ON, Canada) and used to transform competent *E. coli*. Plasmid DNA was recovered from an overnight culture (Wizard Plus SV Minipreps DNA purification System; Fisher Scientific Ltd., Nepean, ON, Canada) and quantified by a dsDNA assay kit (Quant-iT PicoGreen; Invitrogen, Burlington, ON, Canada). Fluorescence intensity was measured by a microplate reader (NOVOstar; BMG Lab technologies Ltd.; Durham, NC, USA). The plasmid concentration was converted from mass to copy number based on a plasmid size of 4423 bp (492 bp insert, 3931 bp vector) and assuming 650 g mol^{-1} for each bp of DNA, and was calculated to be 2.2×10^9 per µL. Conventional PCR reactions for development of the plasmid with PCV2-492 bp insert (pl-492) were conducted using reaction components and conditions described above. A 10-fold dilution series of pl-492 was used to generate the real-time PCR standard curve.

5.3.4 PCV2 Naturally-Infected Pigs and Clinical Specimen Collection

All procedures were conducted in accordance with the University of Saskatchewan's Committee for Animal Care and Supply (permit #20060004). In October 2006, naturally PCV2 infected wasting (n=3) and age-matched non-wasting (n=3) pigs originating from a biosecure conventional commercial farrow-to-finish facility, containing 1200 sows, were selected for this study (Table 5.1). The selected pigs were tagged, bled *via* the jugular vein, then transported to the Western College of Veterinary Medicine, Saskatoon, Saskatchewan, and euthanized by cranial captive bolt. The postweaning mortality on the farm was approximately 15.9% for the period immediately preceding this study and PCVD had been diagnosed based on unthriftiness, dyspnea, diarrhea and jaundice, and was confirmed by PCV2 immunohistochemical staining and the presence of typical histological lesions in the tissues of selected pigs (Prairie Diagnostic Services, Saskatoon, SK, Canada). Serum, buffy coat, lymphoid (bronchial, mesenteric, and superficial inguinal lymph nodes, thymus, tonsil, ileal Peyer's patches, and spleen) and non-lymphoid (myocardium, lung, kidney, liver, and gluteal muscle) tissues and feces were collected from each pig.

Approximate age (in weeks)	Wasting pigs		Non-wasting pigs	
	ID	Gross lesions	ID	Gross lesions
9	21	Poor body condition; enlarged abdomen; few small areas of congestion on pole of spleen; pale kidneys; depleted thymus; non-collapsed lungs with prominent lobular pattern; anteroventral consolidation (20%); dilated and fluid-filled spiral colon	36	Slightly enlarged systemic LN
9	26	Poor body condition; very enlarged systemic LN; non-collapsed caudal lung lobes; cranioventral bronchopneumonia	30	No abnormal findings
13	3	Poor body condition; enlarged inguinal LN; multiple diffuse white pinpoint foci on kidneys; depleted thymus & Peyer's Patch; non-collapsed lungs; excess straw colored fluid in abdomen; full stomach	44	Enlarged LN systemically; few small foci bilaterally

Table 5.1: Gross pathological lesions of age-matched conventional pigs tested by a SYBR green real-time PCR for PCV2 in serum, buffy coat, feces, and multiple tissues.
ID=pig identification number; LN=lymph node.

5.3.5 PCV2 Experimentally-Infected Gnotobiotic Pigs and Clinical Specimen Collection

In addition to samples from PCV2 naturally-infected conventional pigs, terminal serum and liver samples were collected and processed from PCV2 experimentally-infected and non-infected gnotobiotic pigs in accordance with The Institutional Laboratory Animal Care and Use Committee of The Ohio State University protocols #2000A0174, 2002A0046, and 2005A0195. Briefly, gnotobiotic piglets were infected with 1.0 mL PCV2 OSUp3 (5.0×10^8 $TCID_{50}$/mL) intranasally at 3 days of age and terminated when moribund or at 35 days of age, as previously described (60;111;115). PCV2 viral titers from homogenized liver samples were determined for PCV2-infected and non-infected gnotobiotes (114). Previously published criteria were used to determine the clinical status of the infected gnotobiotic pigs (112).

5.3.6 Template DNA Preparation

Clotted and EDTA-treated blood samples were centrifuged at 500 × g for 15 minutes, and serum and buffy coat were removed, respectively, and stored in sterile 1.5 mL tubes. All porcine samples were stored at -70ºC until DNA extraction. DNA was isolated from samples including terminal serum, buffy coat, lymphoid (bronchial, mesenteric, and superficial inguinal lymph nodes, thymus, tonsil, ileal Peyer's patches, and spleen) and non-lymphoid (myocardium, lung, kidney, liver or liver homogenate, and gluteal muscle) tissues using a commercial DNA extraction kit (DNeasy Tissue Kit; Qiagen Inc., Mississauga, ON, Canada), according to the manufacturer's instructions. Sample volumes for serum and buffy coat were 80 and 50 µL, respectively, and were eluted in 100 µL of buffer. The final elution volume for tissue samples (sample size 15 to 26 mg or 80 µL of liver homogenate) was 200 µL. DNA was extracted from fecal samples (190 to 221 mg) using a commercial kit (Qiamp DNA Stool Mini Kit; Qiagen Inc.,

Mississauga, ON, Canada), according to the manufacturer's instructions, and eluted in 200 µL of buffer. All extracted samples were stored at -70ºC until real-time PCR testing.

5.3.7 Real-time PCR

Each quantitative real-time PCR reaction was performed using 12.5 µL of SYBR green reaction mix (Brilliant SYBR Green QPCR Master Mix; Stratagene, La Jolla, CA, USA), 150 nM of each primer PCV2-83F and PCV2-83R, 30 nM reference dye (ROX; Stratagene, La Jolla, CA, USA), 3 µL of template, and made up to 25 µL with sterile water. All real-time reactions (standards, unknown samples, and controls) were performed in duplicate in neighboring wells on the sample plate. Results reported are an average of the duplicates. Real-time PCR reactions were performed in a thermal cycler and fluorescence detection system (Mx3005P; Stratagene, La Jolla, CA, USA). Cycling conditions were 10 minutes at 95ºC, followed by 45 cycles of 30 seconds at 95ºC, 1 minute at 59ºC, and 30 seconds at 72ºC. A dissociation curve was performed after amplification by a gradual rise in temperature from 55 to 95ºC, and the fluorescence signal was measured every 0.5ºC. Fluorescence normalization and data analysis was performed by thermal cycler program software (MxPro-Mx3005P v.3.00; Stratagene, La Jolla, CA, USA).

5.3.8 Sensitivity and Assay Cutoff Determination

Serum, lung tissue, and feces from a PCV2-negative pig, as determined by conventional PCR with primers PCV2 492F and PCV2 492R, were spiked with known concentrations of pl-492 in a serial 10-fold dilution series. DNA extraction and real-time PCR were performed on each sample, as described above.

5.4 Results

5.4.1 PCV2-specific Oligonucleotides

Potential primer sequences were identified by determination of sequence regions conserved across all available PCV2 genome sequences and not found in PCV1 genomes. Primers were required to be closely matched in predicted annealing temperature and show little potential for dimerization and self-interaction. Based on these criteria, oligonucleotides PCV2-83F (5'-AAAAGCAAATGGGCTGCTAA-3') and PCV2-83R (5'-TGGTAACCATCCCACCA CTT-3') were selected for real-time PCR protocol development. These primers amplify an 83 bp region of the *Rep* gene (ORF1) of PCV2 corresponding to nucleotides 588 to 670 on DQ220739.

5.4.2 PCR Optimization and Specificity

A series of PCR amplifications across an annealing temperature gradient indicated that the optimal annealing temperature for PCV2-83F and PCV2-83R was 59ºC. Primers were tested in conventional PCR reactions against one PCV1 and one each of PCV2a and 2b templates and amplification of the 83 bp product was observed only with PCV2 templates (data not shown). Products of three independent amplifications with PCV2-83F and PCV2-83R were sequenced to confirm the specificity of the target (data not shown).

5.4.3 Real-time PCR Performance

A plasmid standard curve was conducted over a range of target DNA concentrations (6.6×10^{8} to 6.6×10^{-1} copies of plasmid pl-492 per 25 µL reaction; Figure 5.1). The linear portion of the standard curve was found to span 6.6×10^{7} to 6.6×10^{0}; therefore, a lower detection limit (or cutoff) of 6.6 copies per 25 µL reaction, equivalent to 2.2×10^{3} copies per µL was established. This cutoff corresponded to a threshold cycle (C_t) of 35.47 and was applied to tested samples. Samples with a $C_t > 35.47$ were reported as not quantifiable (i.e. below the detection limit of the

test) for PCV2 DNA. Extracted samples with a copy number exceeding 6.6×10^7 per 25 µL reaction, or a $C_t < 10.81$ were diluted 1:10 using sterile water and re-tested. The dilution factor was taken into consideration when calculating copies of PCV2 in the original sample. A dissociation curve (Figure 5.2) using pl-492 (6.6×10^8 to 6.6×10^{-1} copies per 25 µL reaction) and serum, buffy coat, multiple tissues, and feces from PCV2-infected wasting pig #3 (Table 5.2) was made. Dissociation, or the melting temperature (T_m) of the 83 bp PCR product occurred between 77.1 and 77.8ºC. Extraction and PCR negative controls consisting of sterile water and reaction mix did not produce any amplification product; therefore, no peaks were observed (Figure 5.2).

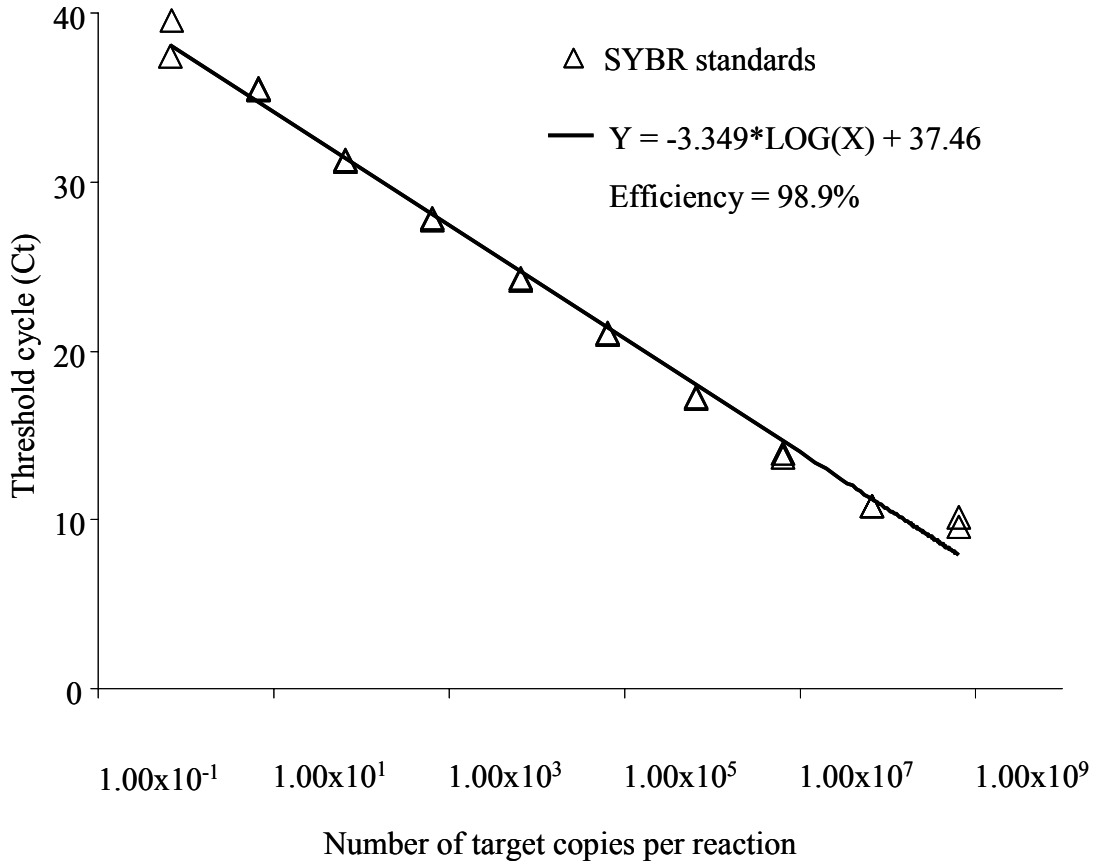

Figure 5.1: Standard curve plot (copy number vs. threshold cycle (C_t) with regression and efficiency) for PCV2 DNA in plasmid (pl-492) detected by SYBR green real-time PCR. Individual data points corresponding to duplicate wells containing 25 µL reactions are plotted.

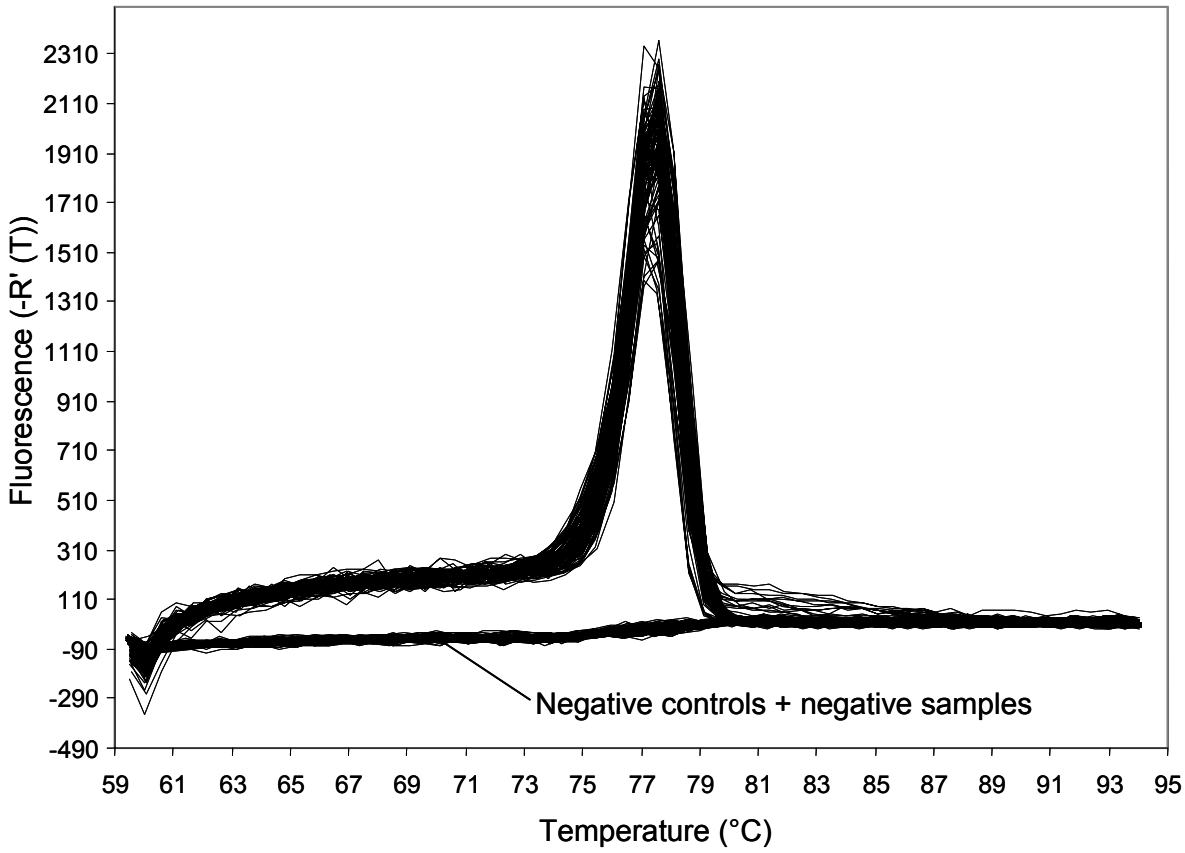

Figure 5.2: Typical dissociation curve of PCV2 PCR product following 45 cycles of amplification of plasmid positive control pl-492 and porcine samples. Samples tested include serum, buffy coat, feces, lymphoid (bronchial, mesenteric, and superficial inguinal lymph nodes, thymus, tonsil, ileal Peyer's patches, and spleen) and non-lymphoid (myocardium, lung, kidney, liver, and gluteal muscle) tissues. Dissociation of PCR product occurred at 77.8ºC and no amplification was seen in negative controls and negative samples, as indicated.

Tissue	Wasting[a]				Non-wasting[a]		
	Pig 3	**Pig 21**	**Pig 26**		**Pig 30**	**Pig 36**	**Pig 44**
Serum	1.23×10^{9}	2.91×10^{10}	3.30×10^{8}		0	1.51×10^{5}	9.79×10^{6}
Buffy coat	3.69×10^{6}	8.11×10^{7}	8.13×10^{7}		8.22×10^{3}	0	2.55×10^{6}
Feces	2.26×10^{9}	7.04×10^{10}	1.57×10^{9}		1.84×10^{7}	5.99×10^{7}	1.74×10^{8}
BLN	0	1.11×10^{9}	1.82×10^{8}		0	0	2.05×10^{6}
MLN	1.17×10^{10}	1.15×10^{11}	1.83×10^{9}		0	0	1.43×10^{5}
ILN	3.03×10^{7}	7.99×10^{8}	9.24×10^{8}		0	0	9.91×10^{5}
Tonsil	6.07×10^{6}	8.04×10^{10}	3.89×10^{8}		2.38×10^{4}	0	0
Thymus	0	4.35×10^{10}	5.81×10^{8}		5.32×10^{5}	0	0
PP	3.21×10^{7}	1.02×10^{4}	8.26×10^{8}		4.82×10^{5}	0	3.22×10^{3}
Spleen	4.33×10^{7}	2.98×10^{9}	2.18×10^{8}		0	0	0
Heart	4.03×10^{7}	2.23×10^{7}	2.22×10^{8}		1.43×10^{4}	0	6.26×10^{5}
Lung	1.11×10^{9}	1.79×10^{8}	4.75×10^{6}		2.51×10^{4}	0	8.37×10^{4}
Kidney	7.65×10^{8}	5.32×10^{8}	4.04×10^{7}		0	0	6.33×10^{4}
Liver	1.86×10^{9}	5.52×10^{8}	4.80×10^{7}		0	0	6.33×10^{4}
GLM	1.13×10^{8}	4.67×10^{7}	4.75×10^{8}		0	6.96×10^{5}	8.76×10^{5}

Table 5.2: Copy number of PCV2 genomes detected in serum, buffy coat, feces, and multiple tissues for 3 wasting (3, 21, 26) and 3 non-wasting (30, 36, 44) age-matched pigs. Values reported were calculated from the average of samples tested in duplicate.
BLN=bronchial lymph node; MLN=mesenteric lymph node; ILN=ileal lymph node; PP=Peyer's patch; GLM=gluteal muscle.
[a] Copies per mL or g

5.4.4 Effects of Template Source on Sensitivity

The copies of PCV2 genomes detected in serum, lung, and feces were affected variably by inhibitors and the DNA extraction process (Figure 5.3). Of the three tissue types, the number of copies detected in serum was the most accurate until approaching the lower detection limit where a 2 log difference in expected *versus* detected copies was observed. In both lung and feces, the target copy number measured was less than the expected copy number by approximately 2 logs. A 1:10 dilution of extracted lung tissue improved detection to within a log (Figure 5.4), and subsequent dilution to 1:100, 1:500 and 1:1000 gave no further improvement. Dilution did not improve detection in feces (data not shown). A lower detection limit or cutoff value was assigned based on the plasmid standard curve, as described above.

5.4.5 Quantification of PCV2 in Naturally- and Experimentally-Infected Gnotobiotic Pigs

All samples were measured (mL) or weighed (mg) prior to extraction. All samples were expressed as the number of copies of PCV2 genomes per gram or mL of original sample from either naturally-infected conventional pigs (Table 5.2), or experimentally-infected gnotobiotic pigs (Figure 5.5). Although the data set is small and representing only three pigs per group, the concentrations of PCV2 DNA were consistently higher in the wasting *versus* non-wasting pigs (Table 5.2). In experimentally infected gnotobiotes, a similar trend of increasing viral load with increasing severity of the observed clinical outcome was observed for both serum and homogenized liver (Figure 5.5). In addition, homogenized liver samples tested by tissue culture and immunohistochemical staining (expressed as $TCID_{50}$) and real-time PCR tested samples (Figure 5.5) illustrate similar trends in increasing viral load from subclinical to severely PMWS-affected status.

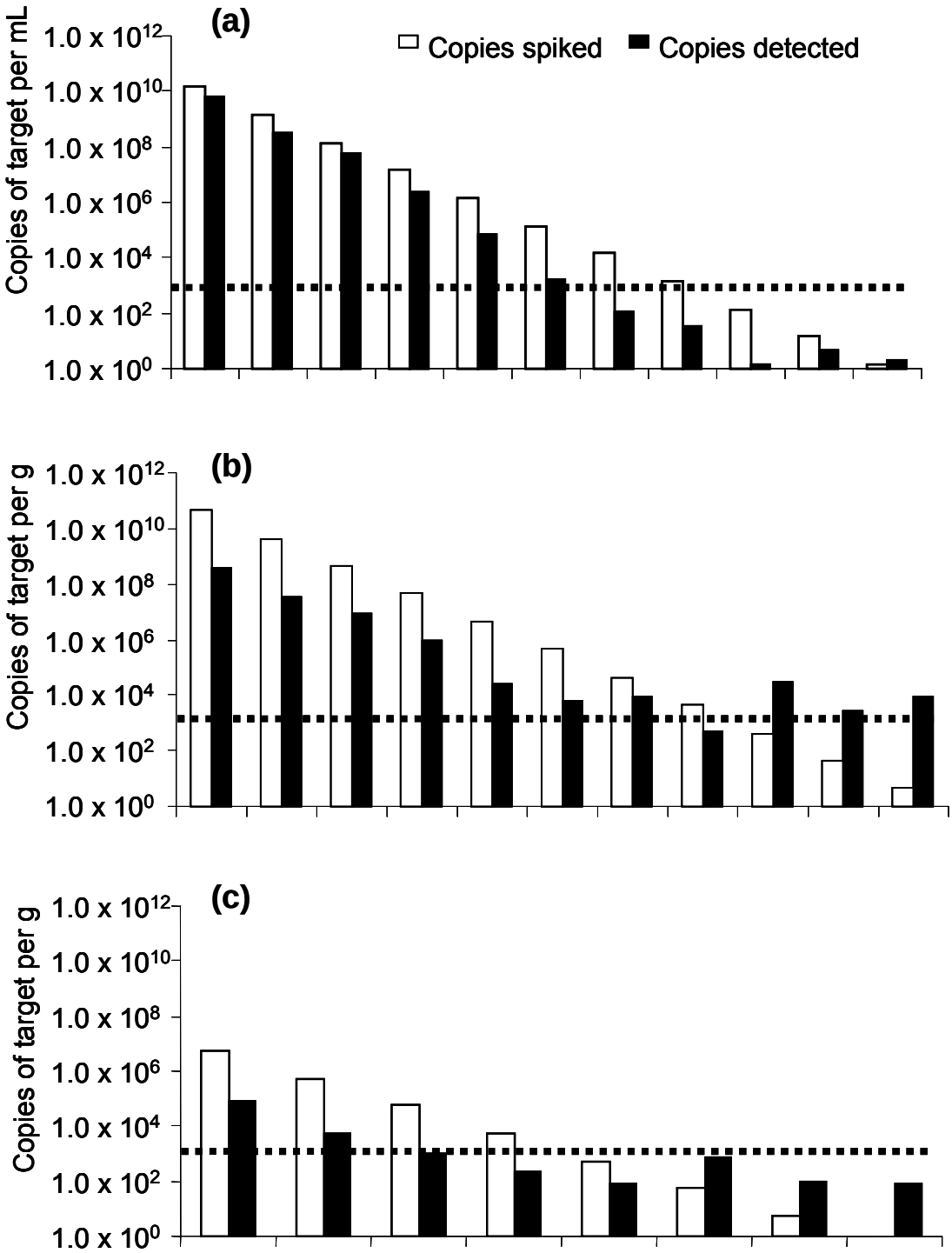

Figure 5.3: Copies of plasmid DNA spiked into (a) serum, (b) lung, and (c) feces followed by DNA extraction and real-time PCR to determine the effect of extraction and tissue type on the quantification of PCV2. The lower detection limit (broken line) is based on the standard curve (Figure 5.1), and is indicated (broken line).

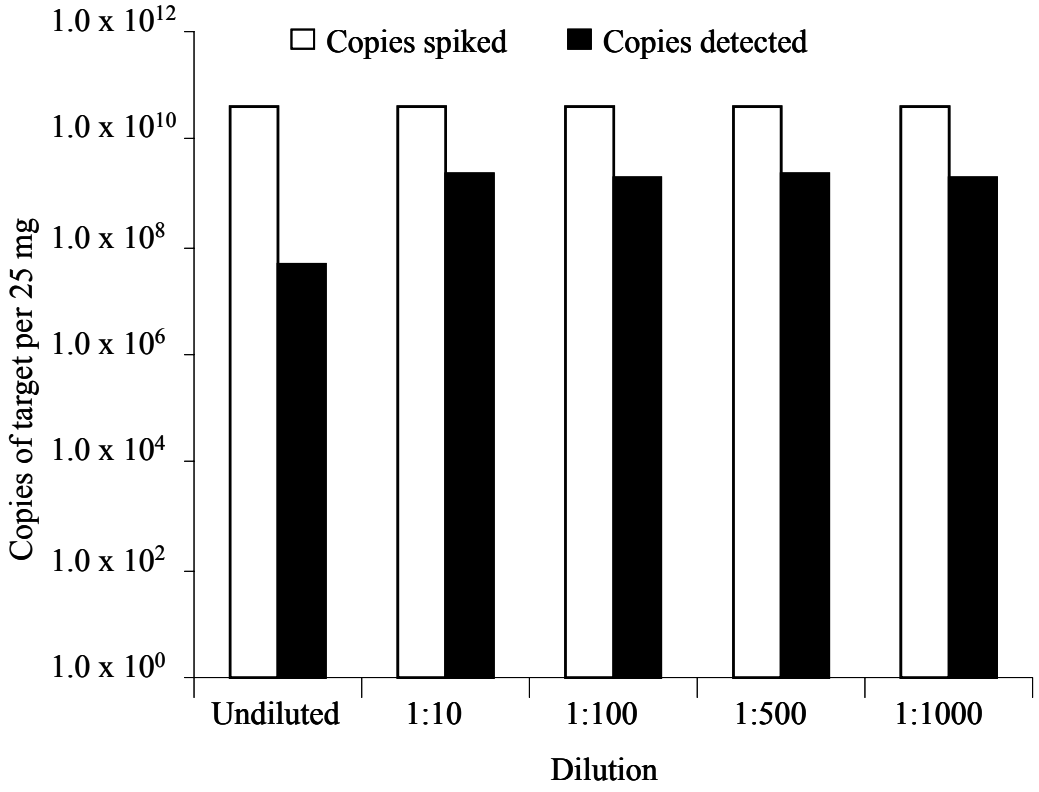

Figure 5.4: Determination of the effects of template dilution on quantification of plasmid DNA in lung tissue.

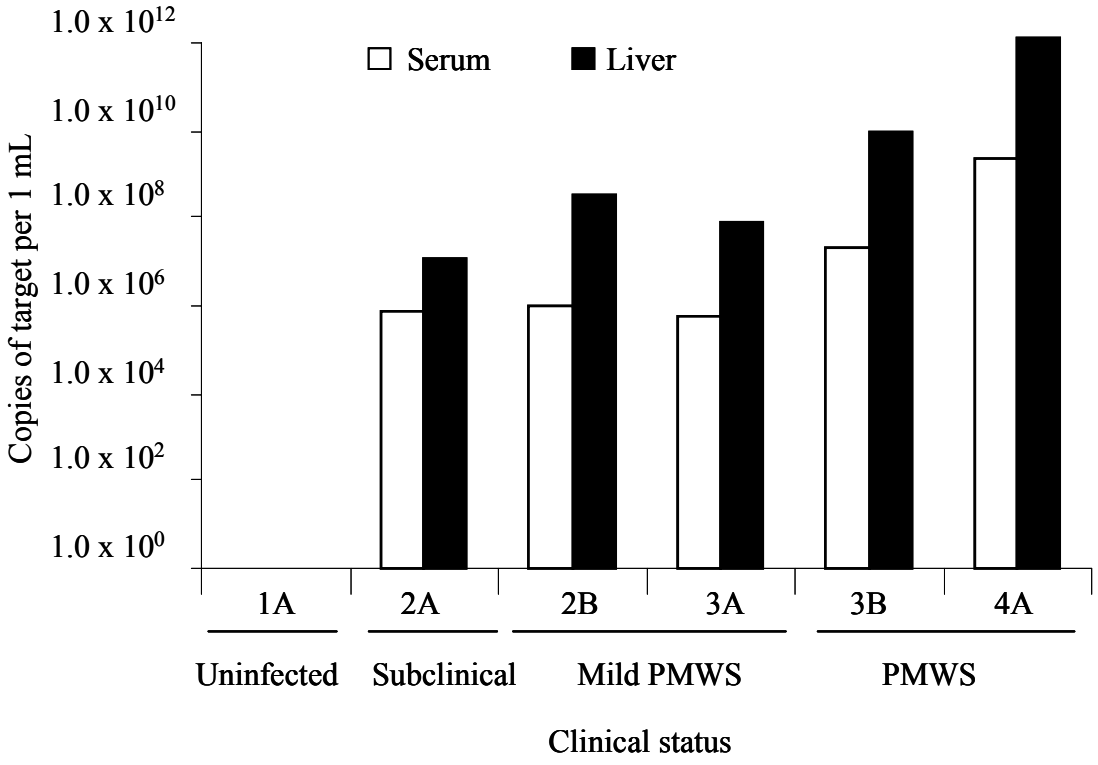

Figure 5.5: Quantification of PCV2 in serum and liver homogenate derived from experimentally infected gnotobiotic pigs. Titers (based on tissue culture infective dose 50 ($TCID_{50}$)) from homogenized liver samples with clinical characteristics (status/outcome based on previously published criteria by Krakowka et al., 2005) are indicated.

5.5 Discussion

The SYBR green real-time PCR described here was developed to target the replication-associated gene (ORF1) of PCV2. The majority of published real-time PCR assay primers, including both probe and SYBR green-based assays, target the capsid gene (ORF2) (29;45;73;169;230). The efficiency of a probe or a primer in a quantitative PCR assay will be variably affected by mismatches between the oligonucleotide and the desired target sequence. Depending on the degree of mismatch, this inefficiency will reduce the accuracy of quantification or, in more extreme cases, result in false-negatives. Thus it is desirable to choose target sequences that are strongly conserved within the target population. Upon analysis of whole PCV2 genome sequences, it is recognized that ORF1 is more conserved across all PCV2 sequences than ORF2 (50). In addition, ORF1-based primer targets are conserved across genotypes 2a and 2b of PCV2 when compared with the ORF2, encoding the capsid protein. Since ORF2 is not as well conserved, a more reliable and quantifiable real-time assay would target the ORF1 region of the PCV2 genome which is a considerable advantage of the assay described here over previously reported diagnostic tests.

Serum, lung tissue, and feces from a PCV2 negative pig were spiked with known quantities of a plasmid containing a PCV2 DNA target. These samples were used to determine what effect, if any, resulted from inherent PCR inhibitors, or the DNA extraction process on the assay sensitivity and the quantification of PCV2. In lung tissue and feces, the detected copies of PCV2 genomes were consistently 1 to 2 logs lower than the expected value; however, in serum, expected and observed quantities were in good agreement. A 10-fold dilution of the extraction material improved quantification in lung tissue (Figure 5.4), but did not improve the quantification in feces (data not shown). This suggests that in lung samples and in samples

exceeding the detectable range of the assay (>6.6×10^7 copies per 25 µL reaction), a dilution series of the extraction volume in random samples is recommended to determine the ideal dilution for the data set; therefore, one can achieve the most accurate quantification of PCV2 in the original sample.

A very low concentration of PCV2 DNA was detected in samples of spiked lung tissue that were below the calculated lower detection limit of the assay (i.e. not quantifiable and below the broken line; Figure 5.3b). The pig used as the source of this tissue was determined to be PCV2 negative using a conventional PCR assay. However, it is possible that PCV2 was present at a level below the detection limit of conventional PCR; therefore, a positive result could occur from the additive effect of spiking the samples with the plasmid PCV2 target DNA. In future studies, PCV2-negative gnotobiotic pig tissues should be used to further clarify this issue.

Serum, buffy coat, feces, and various tissues were tested from non-wasting and PCVD-affected or wasting conventional pigs infected with PCV2 (Table 5.2). PCV2 was detected in all tissue types in wasting pigs with the exception of the bronchial lymph node (LN) and the thymus from pig #3. In contrast, several tissues in one or more of the non-wasting pigs (serum, buffy coat, bronchial LN, mesenteric LN, ileal LN, tonsil, thymus, Peyer's patch, spleen, heart, kidney, liver, and gluteal muscle) did not have detectable or quantifiable levels of PCV2 DNA. The assay was able to quantify a range of PCV2-positive samples from 3.22×10^3 to 1.15×10^{11} per mL or g of sample, and a trend was observed where virus load was higher in the majority of tissues of PCVD-affected pigs. This is consistent with observations made elsewhere (29;112;200). However, the variability of PCV2 concentration should be investigated in larger data sets to determine if it is real, and of any biological importance.

The quantity of PCV2 detected by real-time PCR in serum and homogenized liver samples from experimentally-infected gnotobiotic pigs increased with the severity of clinical disease. This trend was analogous to the calculated tissue culture infective dose when compared with the severity of disease. This suggests that a relationship may exist between the quantity of PCV2 DNA in serum and liver, and the severity of PCVD, as previously reported (112;200).

In conclusion, the SYBR green real-time PCR assay described here was tested in naturally-infected conventional pigs, experimentally-infected gnotobiotic pigs, and plasmid-spiked negative serum, lung tissue, and feces and found to have a linear detection range of 2.2×10^3 to 2.2×10^{10} copies of PCV2 per mL. The assay successfully detected and quantified PCV2 DNA in serum, buffy coat, feces, and multiple lymphoid (bronchial, mesenteric, and superficial inguinal lymph nodes, thymus, tonsil, ileal Peyer's patches, and spleen) and non-lymphoid (myocardium, lung, kidney, liver, and gluteal muscle) tissues. With the emergence of genotypes of PCV2, it is necessary to use a quantitative assay that is equally efficient across multiple isolates to maintain accurate quantification of PCV2, and therefore, possibly resolve the current issues related to viral load in tissues and associated disease state. The assay described in this study was developed with a conserved target sequence across 244 PCV2 sequences, and was successfully tested in multiple tissues, naturally-infected conventional pigs, and experimentally-infected gnotobiotic pigs. As with any PCR-based diagnostic, especially those for virus targets, a periodic re-evaluation of the assay in the context of recently emerged strains and genotypes is essential to maintain the validity of the test.

5.6 Acknowledgements

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CHAPTER 6

QUANTITATIVE PCR FOR PCV2 IN SWINE FECES IN A PCVD-AFFECTED COMMERCIAL HERD AND A NON-AFFECTED COMMERCIAL HERD

Can Vet J. 2008. McIntosh KA, Harding JCS, Parker SE, Krakowka S, Allan G, Ellis JA.

This research was conduction in 2006.

6.1 Abstract

This study examined if pigs in a PCVD-affected herd (n=100) had shed more PCV2 in their feces than pigs in a PCVD-nonaffected herd (n=101), and if differences in shedding among production stages within and between the herds existed. The PCV2-shedding was quantified by real-time PCR. The highest median PCV2-shedding was found in the nursery of the PCVD-affected herd and in the grower of the PCVD-nonaffected herd. The PCV2-shedding was significantly higher in earlier stages (newly weaned, nursery, and pregrower) in the PCVD-affected herd (Wilcoxon Rank Sum; $P<0.001$) compared with the PCVD-nonaffected herd. PCV2 DNA was not detected in a significant proportion of lactating sows (parity ≥3) in the PCVD-nonaffected herd (Fisher's Exact Test; $P=0.001$). The results of this study suggest there may be an association between the presence of PCV2 in the feces of lactating sows and increased PCV2-shedding in younger pigs.

6.2 Introduction

First described in the mid-1990's (88), PMWS was found to clinically affect pigs 7 to 15 weeks of age with wasting, enlarged lymph nodes, dyspnea, diarrhea, pallor, and jaundice. In 1998, PCV2 was isolated from a pig in Saskatchewan showing clinical signs of PMWS (59). In the last decade, other clinical syndromes have been recognized in association with PCV2

infection; these include the porcine dermatitis and nephropathy syndrome (PDNS) (151;195), congenital tremors (CT) (211), abortion (166), and reproductive disorders (109;228). For simplicity, diseases associated with PCV2 infection are now collectively termed porcine circovirus diseases (PCVD) (5;199), or porcine circovirus associated disease (PCVAD) (17).

The general consensus among scientists is that PCV2 is the etiological agent of PCVD; however, other infectious agents (53), stressors, or cofactors are required concurrently to exacerbate PCVD in pigs infected with PCV2. Coinfection with porcine reproductive and respiratory syndrome virus (PRRSV) (10;93), *Mycoplasma hyopneumoniae* (180), or porcine parvovirus (PPV) (61;115); immune stimulation (113;114); or production and weaning practices (194) can contribute to, or exacerbate, PCVD in PCV2-infected pigs. The PCV2 nucleic acid, or antigen, or both can be found in, and PMWS is characterized by, gross or microscopic lesions in multiple organs and tissues of infected pigs (11;59;88). PCV2 is shed in various secretions from both naturally- and experimentally-infected pigs, and in healthy *versus* PCVD-affected pigs *via* nasal, tonsillar, tracheobronchial, oropharyngeal, fecal, and urinary routes (200;207). Additionally, PCV2 DNA is detected in semen from experimentally- (119) and naturally- (146) infected boars. An association exists between the severity of clinical disease, or PCVD, in affected pigs and the PCV2 viral load that is found in tissues from affected animals (29;112); however, PCV2 quantified from fecal samples has not been reported in PCVD-affected *versus* - nonaffected pigs, or across production stages. The objectives of this study were as follows: 1) to determine if pigs in a PCVD-affected herd shed more PCV2 in their feces than did pigs in a PCVD-nonaffected herd; and 2) to determine if there are differences in the amount of PCV2 shed in feces among production stages within and between a PCVD-affected and a PCVD-nonaffected commercial swine herd.

6.3 Materials and Methods

6.3.1 Animals and sample collection

Approximately 100 pooled fecal samples were collected from each of two commercial swine facilities in Saskatchewan practicing high-biosecurity procedures. Both facilities were farrow-to-finish operations housing Landrace and Large White sows, or their respective crosses. PCV2 vaccines were not used in either facility prior to collecting samples for this study. Both barns were infected endemically with PCV2; however, the nonaffected herd (300 sows) had no clinical evidence or diagnosis of PCVD and a long-standing postweaning mortality of <2%, while the PCVD-affected herd (1200 sows) was experiencing a PCVD-related mortality of 14.5% during the period of sampling.

Based on prior testing of lymphoid tissue, the PCV2 genotypes present in each barn were determined, as per a previously described method (87). In limited testing of lymphoid tissue, concurrent PCV2a and PCV2b were detected in the PCVD-nonaffected herd, whereas PCV2b alone was detected in the PCVD-affected herd. PMWS was recognized as the primary clinical manifestation of PCVD in the affected herd; the diagnosis was based on observed jaundice, unthriftiness, and dyspnea, and confirmed by PCV2 immunohistochemical staining and the presence of typical histological lesions in the tissues of selected pigs (Prairie Diagnostic Services, Saskatoon, SK, Canada). Based on clinical records and serologic testing, there was no evidence of PRRSV, *Mycoplasma hyopneumoniae*, swine influenza virus H1N1, *Actinobacillus pleuropneumoniae*, or *Haemophilus parasuis* in either herd; however, the PCVD-affected herd had experienced sporadic disease associated with *A. suis* and *Streptococcus suis*.

Each barn was sampled in a cross-sectional manner with approximately 50 fecal samples being collected from each of the feeding and breeding herds. From the feeding herd,

approximately 10 samples (range=10 to 11) were collected from each of five evenly spaced production weeks between weaning and market age (newly weaned (NW), nursery (NU), pregrower (PG), grower (GR), and finisher (FIN)) (Table 6.1). From the breeding herd, sampling was stratified by age with approximately 10 samples (range=7 to 14) collected from each of virgin (VG) and bred (BG) gilts, young (S ≤2) and old (S ≥3) sows (based on a parity of ≤2 or ≥3), and breeding boars (B) (Table 6.1).

Production stage	Age of pigs	Number of pens	
		PCVD-nonaffected	PCVD-affected
Newly weaned (NW)	3-4 weeks	10	11
Nursery (NU)	7-8 weeks	10	10
Pregrower (PG)	11-12 weeks	10	10
Grower (GR)	15-16 weeks	10	10
Finisher (FIN)	22-24 weeks	10	11
Virgin gilt (VG)	6 months	10	10
Bred gilt (BG)	8-10 months	10	10
Lactating sow parity ≤2 (S ≤2)	11-15 months	10	10
Lactating sow parity ≥3 (S ≥3)	15+ months	14	11
Boar (B)	12-24 months	7	7
Total number of pooled fecal samples by barn		**n=101**	**n=100**

Table 6.1: The representative ages and the number of pens tested for each production stage in two commercial swine facilities (PCVD-nonaffected herd; PCVD-affected herd) tested for PCV2 in pooled fecal samples by quantitative real-time PCR.
S ≤2=young sow; S ≥3=old sow.

The barns were sampled on different days: PCVD-nonaffected herd (n=101 samples; 18 Sept 2006) and PCVD-affected herd (n=100 samples; 17 May 2006). However, all samples within a barn were collected on the same day. The bred gilts, sows, and boars were housed in crates or stalls; therefore, the fecal samples collected from these animals represented the individual animal. By contrast, all other animals were housed in pens containing a variable number of pigs (Table 6.2). Fecal samples obtained from pens comprised multiple individual fecal samples and were considered pools. By using sterile gloves for each collection, samples representing separate defecation events from multiple pigs in a single pen were pooled into a sterile bag and transported in an insulated box to the laboratory for processing on the same day. Upon arrival, pooled samples were homogenized, and 1 g of feces from each bag was stored in individual sterile 15 mL conical tubes and frozen at -70°C until DNA extraction.

Barn	NW	NU	PG	GR	FIN	VG	BG	S≤2	S≥3	B
PCVD-nonaffected	12	12	5-6	5-6	12	20	1	1	1	1
PCVD-affected	20-25	20-25	20-25	20-25	20-25	4-5	1	1	1	1

Table 6.2: The number of pigs housed per pen for each production stage in a PCVD-nonaffected herd and a PCVD-affected herd. Pooled fecal samples from each production stage in each barn were tested for PCV2 by quantitative real-time PCR.
NW=newly weaned; NU=nursery; PG=pregrower; GR=grower; FIN=finisher; VG=virgin gilt; BG=bred gilt; S ≤2=lactating sow parity ≤2; S ≥3=lactating sow parity S ≥3; B=boar.

6.3.2 DNA Extraction and Quantitative PCR

Fecal samples were extracted for DNA by using a commercial kit (QIAamp DNA stool mini kit; Qiagen, Mississauga, ON, Canada), following the manufacturer's instructions for DNA isolation from larger amounts of stool. Samples consisting of 1 g of homogenized feces were removed from -70°C and 10 mL of stool lysis buffer (ASL Buffer, QIAamp DNA stool mini kit; Qiagen, Mississauga, On, Canada) was added immediately, prior to thawing. DNA extraction modifications included pipetting 1.5 mL, instead of 2 mL, of lysate from 10 mL of homogenized sample in stool lysis buffer. Extracted DNA was stored at -70°C until quantitative real-time PCR was performed.

A previously described (148), quantitative SYBR green (DNA binding dye) real-time PCR assay and plasmid standard curve (Genbank accession EU126886) for PCV2 was used to determine the viral copy number in pooled fecal samples; it was reported as copy number of PCV2 in 1 g of feces. The assay was developed to detect the current genotypes of PCV2 with equal efficiency; it does not differentiate between genotypes within PCV2. Each extracted fecal sample was tested in duplicate, and each quantitative PCR 96-well plate contained a plasmid standard curve in duplicate (range 6.60×10^{6} to 6.60×10^{-1} copies per well). Each plate contained a negative control in duplicate, and duplicate values were averaged for all samples only when the difference between samples had a threshold cycle (C_t) value <1. If the C_t was >1, the extracted sample was repeated in duplicate. A cutoff value representing the detection limit of the assay was extrapolated from a plasmid standard curve, as previously described (148), and was set at 1 to 10 copies of PCV2 per gram of homogenized feces.

6.3.3 Statistical Analysis

Since PCV2-shedding in feces was non-normally distributed, nonparametric tests were used for statistical comparison. The Wilcoxon Rank Sum Test was used to compare production stage group median PCV2-shedding between PCVD-nonaffected and PCVD-affected pigs. To control for multiple comparisons, a Bonferroni correction was applied to adjust the *P*-value at which comparisons were considered significantly different (P=0.005 for 10 comparisons).

Within each barn, the median PCV2-shedding in feces for the production stage with the highest observed load was compared with that of the preceding production stage, using a Wilcoxon Rank Sum Test. For groups where there was an observed difference in the presence of pigs not shedding detectable virus in the feces, the numbers of shedders and non-shedders were compared, using a Fisher's Exact Test. Statistical analysis was performed, using a commercial statistics package (Statistix 8; Analytical Software, Tallahassee, FL, USA). The box and whisker plots in Figure 6.1 were created by using a separate statistics package (Stata 10; StataCorp LP; College Station, TX, USA).

6.4 Results

Since an interaction was present between production stage and barn with respect to the PCV2 shed in feces, separate barn comparisons were made for each production stage. The observed median PCV2 shed in feces was higher in the PCVD-affected herd compared with the PCVD-nonaffected herd in the NW, NU, PG, GR, S $\leq$2, S $\geq$3, and B stages, but it was significantly higher only in the three youngest (NW, NU, PG) stages (P<0.001; Figure 6.1). Conversely, the observed median PCV2 shed in feces was higher in the PCVD-nonaffected herd in the FIN, VG, and BG stages, but it was significantly higher only in the VG stage (P=0.0002; Figure 6.1). The highest observed PCV2 shed in feces occurred in a later production stage in the

PCVD-nonaffected herd (GR) than in the PCVD-affected herd (NU), and the PCV2 shed was significantly higher than in the immediately preceding stages ($P \leq 0.0001$) for each herd. For most production stages, there was detectable viral DNA in all or almost all fecal samples. More sows (S $\leq$2 and S $\geq$3) in the PCVD-nonaffected herd did not shed PCV2 in feces, or the level of PCV2-shedding was below the detection limit of the quantitative PCR assay used in this study, when compared with the PCVD-affected herd (Figure 6.2). However, this difference was significant only for the older sow group (S $\geq$3; P=0.001).

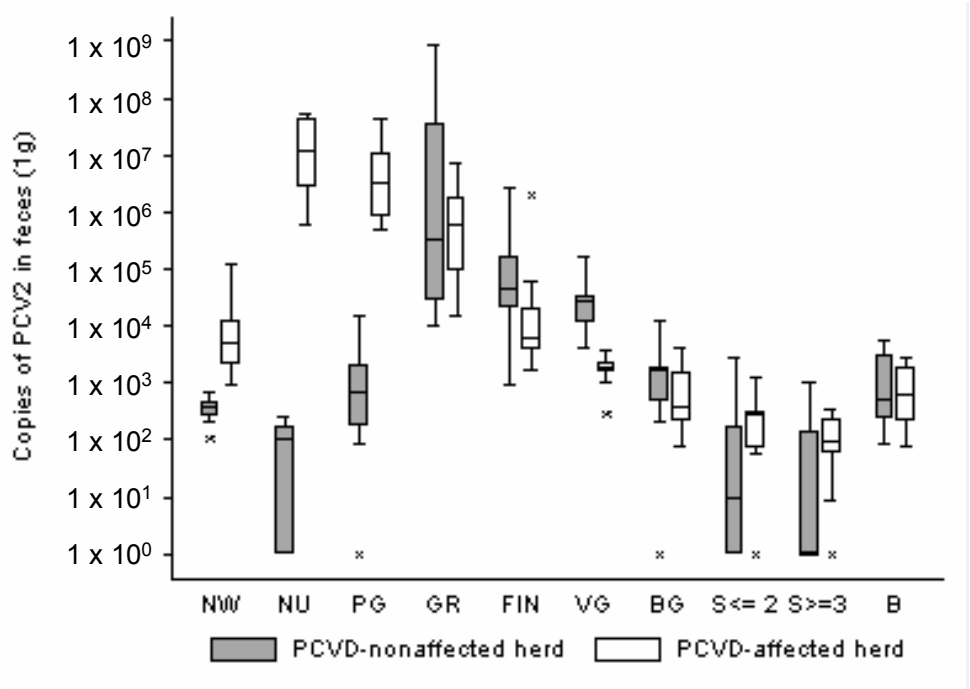

Figure 6.1: Box and whisker plots of PCV2 shed in feces by pen quantified by real-time PCR (approximately 10 pooled pens per stage; range=7 to 14) for each production stage in a PCVD-nonaffected and a PCVD-affected commercial swine herd. Production stages significantly different between barns were NW, NU, and PG (P<0.001), and VG (P=0.0002). (Horizontal lines represent the median; upper and lower edges of the boxes represent the 75th and 25th percentile, respectively; and lines (whiskers) attached to the top and bottom of the boxes extend to the maximum and minimum data points that are up to 1.5 times the interquartile range (1.5 IQR) from the 75th or 25th percentile, respectively. Outliers are data points that are more than 1.5 IQR from either the 25th or 75th percentile and are denoted as 'x').
NW=newly weaned; NU=nursery; PG=pregrower; GR=grower; FIN=finisher; VG=virgin gilt; BG=bred gilt; S ≤2=lactating sow parity ≤2; S ≥3=lactating sow parity S ≥3; B=boar.

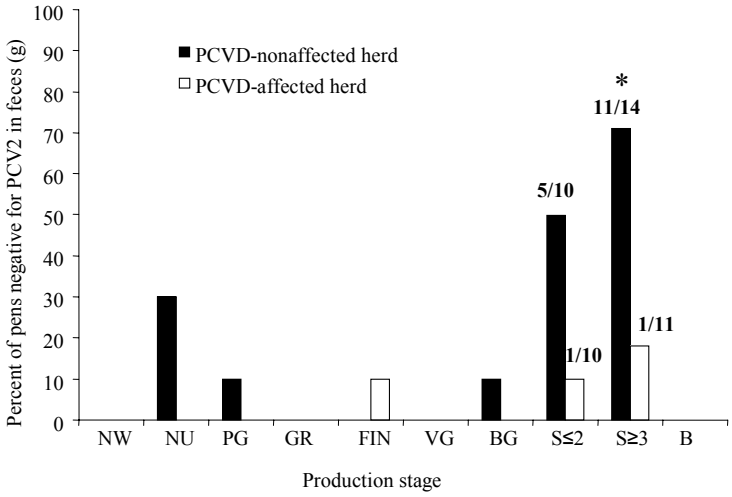

Figure 6.2: The percent of pooled fecal samples that were negative for PCV2 by quantitative real-time PCR for each production stage in a PCVD-nonaffected and a PCVD-affected commercial swine herd. The number of sows that were negative for PCV2 in feces of the total tested is shown. Production stage significantly different between barns (S ≥3; *P*=0.001) is denoted with an asterisk (*).
NW=newly weaned; NU=nursery; PG=pregrower; GR=grower; FIN=finisher; VG=virgin gilt; BG=bred gilt; S ≤2=lactating sow parity ≤2; S ≥3=lactating sow parity S ≥3; B=boar.

6.5 Discussion

This study evaluated the PCV2 shed in pig feces among production stages in a PCVD-affected compared with a PCVD-nonaffected herd, using quantitative real-time PCR. Quantities of PCV2 DNA were significantly higher in younger production stages in the PCVD-affected herd (NW, NU, and PG) than in the PCVD-nonaffected herd; however, gestational sows and boars (BG, S, and B) had similar median PCV2 shed in feces between herds. Possible explanations for the differences in viral shedding profiles of young pigs between these herds may be associated with early PCV2 exposure or the infectious dose, or both, to which piglets are exposed in the farrowing crate by sows shedding PCV2. Sows shedding PCV2 in feces into the surrounding environment may expose piglets to the virus *via* the oronasal route (207). In addition, vertical transmission of PCV2 from the sow to piglets can occur *in utero* (151;228), and PCV2 is shed in colostrum (206). It is feasible that sows with a higher systemic PCV2 viral load may expose piglets to a higher infectious dose of PCV2, either *in utero* or during the neonatal period, but this has not been reported.

Maternal Ab protection provided to piglets in the neonatal period may be extremely variable among sows (149;182). The amount or concentration and the virus neutralizing capability of the Ab provided to piglets from the sow affect both the duration of immunity in the young pig and the ability to reduce or prevent viral replication. Both of these conditions contribute to effectively reduce the PCV2 viral load in the piglet; however, the immune status of the sows or their respective piglets in this study was not known. Passive Ab provide protection in early life (NW, NU, and, possibly, PG); when maternal Ab wane, PCV2 viremia is detected (145). The report that the amount of PCV2 shed in feces is correlated with the systemic or tissue PCV2 viral load (200) supports the observation that a significant increase in the PCV2 shed in

feces occurred in the GR stage in the PCVD-nonaffected herd, after protective maternal Ab had waned. This suggests that sufficient protection was afforded to the piglet until degradation of maternal Ab at approximately 10 weeks of age, at which time, PCV2-shedding in feces was observed to increase significantly. It can only be postulated that passive protection afforded to the piglet by maternal Ab, the dose of PCV2 that piglets are exposed to in early life, or both contribute to the resulting viral load in the pig once it reaches the GR stage.

In the PCVD-affected herd, the PCV2 shed in feces was highest in the NU stage. The PCV2-specific immunity of the sows, or their respective progeny, was not tested in this study; however, by pooling fecal samples representing each production stage within a PCVD-affected and a PCVD-nonaffected herd, it was determined that significantly more sows in the PCVD-affected herd shed detectable levels of PCV2 in their feces. This observation suggests that sows shedding PCV2 in their feces may contribute to the earlier exposure and infection of piglets in the farrowing crate, as seen by the PCV2 fecal-shedding profile of young pigs in the PCVD-affected herd (Figure 6.1). The corollary is that the majority of sows in a PCVD-nonaffected herd may not shed detectable levels of PCV2 in their feces; therefore, piglet exposure is reduced and delayed until the mixing of pigs in the NU stage. A significant increase in PCV2 DNA is then observed in the feces of pigs in the GR stage, as a result of this delay in exposure in the PCVD-nonaffected herd.

To substantiate the effect of sows shedding PCV2 in their feces on piglets in the farrowing crate, future studies, using serially collected samples of blood and feces from individual animals, are underway. Quantifying PCV2 in the feces of piglets from sows with known immune and fecal-shedding status would further elucidate this association, and piglets from individual sows in a population should be followed through NW, NU, PG, GR, and FIN

production stages. Additionally, it is not known if pen density (Table 6.2) and the resulting increase in the mixing of pigs contributed to the increase in PCV2 fecal-shedding in younger pigs in the PCVD-affected herd in this study. Future studies should include herds consisting of similar pen densities by production stage of the pigs. The PCV2 shed in feces across production stages in PCVD-affected and PCVD-nonaffected herds have not been reported previously. Based on the shedding profile between pigs in a PCVD-affected and a PCVD-nonaffected herd, quantitative real-time PCR of PCV2 in feces may be a useful tool to evaluate viral load in pigs. Future applications for PCV2 quantitative PCR in pig feces may include measures for vaccine efficacy, treatment of PCVD, or changes in production and management practices. More pigs, particularly lactating sows, in the PCVD-nonaffected herd had no PCV2 DNA detected in feces by quantitative real-time PCR when compared with the PCVD-affected herd. As a result, there may be an association between the presence of PCV2 in the feces of lactating sows, increased quantity of the virus in the feces of younger pigs, and mortality in PCVD-affected herds. Further study with serial sampling in PCVD-affected and PCVD-nonaffected farms is required to elucidate this potential relationship.

6.6 Acknowledgements

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CHAPTER 7

REDUCTION IN MORTALITY BY TRANSDERMAL VACCINATION OF PIGLETS WITH ISCOM MATRIX Q-ADJUVANTED PCV2 VIRUS-LIKE PARTICLES IN A PCVD-AFFECTED COMMERCIAL SWINE HERD

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This research was conducted in 2006 to 2007.

7.1 Abstract

Our objective was to determine the efficacy of a novel transdermal ISCOM technology based PCV2 virus-like particle (VLP) vaccine candidate administered at one and three weeks of age. Fifty-four pigs (vaccinates (n=27); non-vaccinated controls (n=27)) with variable levels of maternally-derived PCV2-specific antibodies (MDAb) (high (hiMDAb) *versus* low (loMDAb)) in a PCVD-affected farm were assessed for PCV2-specific Ab levels by cELISA, PCV2 DNA concentration in serum and feces by quantitative PCR, mortality, and average daily gain (ADG) from 1 to 18 weeks of age. A significant reduction in mortality (P=0.05) was observed. PCV2 DNA concentration in serum was lower in the vaccinated pigs at 9 and 11 weeks of age (P=0.003 and 0.01, respectively). In the vaccinates (both hiMDAb and loMDAb groups) at nine weeks of age had a higher Ab response to the challenge, while the non-vaccinated controls (both hiMDAb and loMDAb groups) had a significantly reduced Ab response in comparison. During this period of 9 to 11 weeks of age, the loMDAb CTRL became susceptible to PCVD. PCV2-specific Ab in serum was significantly higher in vaccinated pigs at the onset of PCVD-related mortality (P=0.001); however, no significant difference was observed in the PCV2 DNA concentration

shed in feces, or in ADG. This study is the first report of the use of an ISCOM matrix (Matrix Q) mixed with PCV2 VLP protein administered transdermally for the prevention of PCVD in swine.

7.2 Introduction

Isolated in 1998 (59), PCV2 is a small non-enveloped single stranded circular DNA virus that is the etiological agent of PMWS (9). First recognized in the early to mid 1990's, PMWS typically affects pigs from 7 to 15 weeks of age with jaundice, pallor, dyspnea, diarrhea, enlarged lymph nodes, wasting, and increased postweaning mortality (47;88). A diagnosis of PMWS is based on the presence of clinical signs, in addition to characteristic histological lesions associated with multiple organs and/or lymphatic tissue, and the detection of PCV2-specific antigen or DNA in the affected tissues (201).

PCV2 is associated with additional disease syndromes including porcine dermatitis and nephropathy syndrome (PDNS) (151), porcine respiratory disease complex (PRDC) (105), reproductive disorders (166), and abortion (228). Recently, diseases associated with PCV2 infection were collectively termed porcine circovirus diseases (PCVD) (5) or porcine circovirus-associated disease (PCVAD) (17). It is recognized that both infectious and non-infectious cofactors contribute to the exacerbation of PCVD in pigs and these may include porcine parvovirus (PPV) (115), porcine reproductive and respiratory syndrome virus (PRRSV) (10), *Mycoplasma hyopneumoniae* (180), immune stimulation (114), early weaning (193), and poor production practices (194).

In 2004, an increase in the severity of PCVD was observed in Canada and was proposed to be associated with a particular genotype of PCV2, described as PCV2b (71). This proposition that a specific genotype of PCV2 is related to more severe PCVD remains controversial, as subsequent research has both supported (77;123) and contradicted (13;179) this theory.

However, it is recognized that PCV2 is ubiquitous in swine populations globally, and PCVD contributes to excessive animal and monetary losses in the swine industry.

The PCV2 viral load or DNA concentration in tissues has been reported to be associated with the severity of PCVD in swine (91;112) and recently, the quantification of PCV2 DNA in feces was found to be significantly higher in young pigs (3 to 12 weeks of age) in a PCVD-affected herd when compared with an unaffected herd (147). Additionally, PCV2 DNA concentration in serum, as measured by quantitative real-time PCR (qPCR), is associated with PCVD severity (29). Therefore, PCV2 DNA concentration in serum and feces may be an appropriate and non-invasive way to determine the efficacy and ability of a vaccine against PCV2 to reduce PCV2 infection, replication, and ultimately death due to PCVD in swine.

Due to the ubiquitous nature of PCV2, implementing vaccination protocols may be the best solution for the reduction and prevention of PCVD. Commercial vaccines against PCV2 have been effective in reducing the viral load, morbidity, and/or mortality associated with PCVD in experimental (173) and natural (110;203) PCV2 infections. Additionally, several experimental PCV2 vaccines have improved the immunity to PCV2 infection in swine (23;62;65). Protocol variations include the vaccination of dams, their offspring, or both (187); however, MDAb may (69) or may not (175) interfere with a piglet's ability to mount their own response to PCV2 vaccination during the first three weeks of life. To circumvent the potential effects of MDAb, target antigen (such as VLP, as described in this study) can be formulated with an ISCOM technology based Matrix Q adjuvant. Traditional ISCOMs have successfully been used in animals with MDAb (156). Furthermore, inactivated adjuvanted vaccines may have a propensity toward Ab (Th2) responses, as opposed to a more balanced Ab (Th2) and cell-mediated (Th1) response which is optimal for protection against viral infections and can be achieved by the use

of an ISCOM technology based adjuvant (157). The objectives of this study were as follows: 1) to determine if rectal temperature, injection site reactivity, and the activity level of 1 week old conventional piglets were adversely affected by varied doses of Matrix Q injected subcutaneously; 2) determine if a novel Matrix Q PCV2 VLP vaccine administered transdermally could successfully prime young pigs to PCV2 in the presence of PCV2 MDAb; 3) use four non-invasive outcome measures of PCV2-specific Ab level in serum, PCV2 DNA concentration in serum and feces, and average daily gain (ADG) to determine the efficacy of the vaccine; and 4) to determine if the use of the novel vaccine reduced mortality caused by PCVD in a commercial farm experiencing elevated mortality associated with PCVD.

7.3 Materials and Methods

7.3.1 Matrix Q testing for adverse reactions

The potential for adverse reactions of the Matrix Q (the ISCOM matrix particles with no added protein) used in this study was tested by the subcutaneous injection of variable doses of the matrix into seven day old piglets. Piglets were injected with either 75 µg (n=3), 100 µg (n=3), or 150 µg (n=3) of the Matrix Q diluted in sterilized 0.01 M phosphate buffered saline (PBS) to a final volume of 200 µL using a 1 mL-25 gauge needle. A single injection was administered subcutaneously 1 cm off the dorsal midline between the shoulders and piglets were observed seven times over a period of 30 hours for rectal temperature, injection site reactivity (swelling, redness, or pain), and activity level (active or lethargic). Piglets used for the testing of Matrix Q adverse reactions were not used in the vaccine study described here.

7.3.2 Animals

Fifty-four PIC-crossbred piglets (six piglets from each of nine litters; 27 male; 27 female) were grouped by litter and matched by weight within each litter and randomly assigned to either

a vaccinate group (VX) or a non-vaccinated control (CTRL) group. Within litter, each of the six piglets were assigned to a weight classification of small, medium, or large; therefore, two piglets per weight classification per litter could be randomized into either treatment group (VX or CTRL), and ear-tagged. Litter and weight at one week of age were considered the most important variables biasing the outcome measures of vaccine efficacy, and the randomization resulted in 14 females and 13 males in the CTRL and 13 females and 14 males in the VX. The median weight for VX and CTRL at one week of age was 2.5 +/- 0.56 kg and 2.5 +/- 0.76 kg, respectively. Pigs used in this study were born between October 17th and 20th, 2006, were not segregated from the barn population, but moved through the production stages and mixed with other similarly aged pigs, as would normally occur. The piglets remained with their biological or fostered dam in solid partitioned farrowing crates from birth and until three weeks of age, moved to the nursery at three weeks and stayed there until 11 weeks of age, and then into the grow-finish rooms from 11 weeks of age and until dispatch for slaughter.

The pigs were housed in a 600 sow commercial swine farrow-to-finish facility in Saskatchewan practicing high-biosecurity procedures. Except as described in this study, commercial or experimental porcine circovirus type 2 vaccines were not used in this facility prior to or during this study. Sows were routinely vaccinated for porcine parvovirus (PPV), *Erysipelothrix rhusiopathiae*, *Leptospira bratislava*, *L. canicola*, *L. grippotyphosa*, *L. hardjo*, *L. icterohaemorrhagiae*, and *L. pomona* using a commercially available product (FarrowSure B; Pfizer Animal Health, Kirkland, QC, Canada). The facility experienced a 10.1% postweaning mortality in the two months preceding this study and 40% of the postweaning mortality was attributed to PCVD based on the clinical observations (unthriftiness, dyspnea, and diarrhea) by farm technicians. Additionally, the presence of characteristic lymphoid lesions of PCVD and

typical PCV2 immunohistological lesions based on PCV2 capsid staining were observed in the tissues of selected pigs submitted for diagnostics (Prairie Diagnostic Services, Saskatoon, SK, Canada). All procedures were conducted in accordance with the University of Saskatchewan's Committee for Animal Care and Supply (permit #20060004).

7.3.3 Sample collection

All blood samples were collected *via* the jugular vein into individual sterile serum tubes and the clotted blood was transported from the barn to the Western College of Veterinary Medicine in a chilled and insulated box and stored at 4ºC for 18 hours. Serum was then removed after centrifugation at 500 g × 15 minutes at 4ºC and aliquots from individual pigs were stored in sterile 1.5 mL microcentrifuge tubes at -70ºC until DNA extraction or ELISA analysis. Serial serum samples were collected at 1, 3, 7, 9, 11, 13, and 18 weeks of age. This collection schedule was based on the results of a previously described study that used conventional PCR and an ELISA to detect PCV2 in a naturally-infected herd (145). Rectal fecal samples were collected at 3, 7, 9, 11, 13, and 18 weeks from individual pigs into sterile 2.0 mL microcentrifuge tubes, transported as described above, and stored at -70ºC until analyses. Fecal samples were not collected at one week of age, as piglets were too small to retrieve a rectal sample. Sporadically, rectal fecal samples were not collected from all pigs at each time point, as a sample may not have been available. Pigs were weighed at 1 and 18 weeks of age and the ADG of each pig calculated.

7.3.4 Matrix Q preparation

The adjuvant component Matrix Q (AbISCO-300) was prepared according to standard procedures and supplied by Isconova AB, Uppsala, Sweden. In brief, it was prepared from semipurified saponins originating from *Quillaja saponaria* Molina and mixed with detergent solubilized cholesterol (C8503, Sigma-Aldrich, USA) and egg derived phosphatidyl choline (E-

PC S; Lipoid GmbH Products, Germany). After 6 to 18 hours of incubation at room temperature, matrix particles were formed by removal of the detergent by ultrafiltration. The final product was filtered through a 0.22 μm filter and the saponin concentration was 3.12 mg/mL, as determined by reverse phase high performance liquid chromatography (RP-HPLC). All components of animal origin used for the process were issued a TSE certificate and the matrix was shipped from Sweden to the Western College of Veterinary Medicine in Saskatoon, Canada and stored at 4ºC. The matrix preparation was dissolved to a final concentration of 75 μg in 200 μL of either PCV2 VLPs (VX) or 0.01 M PBS (CTRL).

7.3.5 PCV2 VLP preparation

Sf9 cells cultured in spinner flasks were infected with recombinant baculovirus expressing the complete ORF2 gene of PCV2 at a multiplicity of infection of one and at a cell concentration of 2×10^6 cells/mL. Cell culture was harvested at 72 hours post-infection. The cell culture was centrifuged at 4000 × g for 10 minutes at 4°C and VLPs extracted from the resulting cell pellet. Briefly, cells were lysed in lysis buffer (0.2% NP40, 50mM tris-HCl, 200 mM NaCl), and the resulting suspension clarified for 15 minutes at 10,000 × g. The supernatant containing the VLPs was further concentrated through a 30% sucrose cushion by ultracentrifugation at 100,000 g × 3 hours at 4°C. The resulting VLP pellet was resuspended in PBS and PCV2 capsid expression was confirmed by western blot. The characteristic morphology of the PCV2-like virions and the integrity of the VLPs were confirmed by electronic microscopy and the amount of VLPs was estimated to be 10^{12} VLPs/mL. Total protein quantification was 50 mg/mL, as estimated by a Bicinchoninic Acid (BCA) Protein Assay.

7.3.6 Vaccination

Piglets were vaccinated at one and three weeks of age using a transdermal needle-free injection device (Biojector 2000; Bioject Medical Technologies, Tualatin, Oregon, USA). A total of 200 µL was injected into the neck of each piglet and contained either 75 µg of Matrix Q and 1.66×10^{11} VLPs (or 8.3 mg of total VLP protein) for the VX or 75 µg of Matrix Q only made up to 200 µL with sterile 0.01 M PBS for the CTRL. Blood was collected from each piglet immediately prior to each vaccination at one and three weeks of age.

7.3.7 Postmortem analyses

The carcasses of pigs that were part of this study that either died or were euthanized because moribund between 1 and 18 weeks of age were submitted for necropsy and histopathology (Prairie Diagnostic Services, Saskatoon, SK, Canada). Additionally, 13 tissue samples (inguinal lymph node, mesenteric lymph node, spleen, liver, kidney, ileal Peyer's patch, thymus, lung, bronchial lymph node, heart, tonsil, and gluteal muscle) were collected separately at the time of necropsy from each pig and submitted for IHC for PCV2 capsid antigen (Prairie Diagnostic Services, Saskatoon, SK, Canada). Pathologists and technicians were blinded from the treatment group of the animals submitted for necropsy, histopathology, bacteriology, and IHC, and a cause of death was ascertained based on the pathologic and bacteriologic results.

7.3.8 DNA extraction

Serum samples (n=345) were thawed and DNA extracted using a commercial DNA extraction kit (DNeasy tissue kit; Qiagen Inc., Mississauga, ON, Canada), as previously described (148). Fecal samples (n=286) were removed from storage, immediately weighed to allow for quantification per gram of feces, and DNA extracted using a commercial DNA extraction kit (Qiamp DNA stool mini kit; Qiagen, Mississauga, ON, Canada), as previously

described (148). One template blank or negative control sample was extracted with every set of 24 samples. Extracted negative control, serum, and fecal samples were stored at -70ºC until PCR analysis.

7.3.9 Quantitative real-time PCR

A previously described qPCR assay (148) was used to determine the amount of PCV2 virus present in the serum and feces of both VX and CTRL for all samples collected during this study. The qPCR assay applied targets the highly conserved *Rep* gene (ORF1) and does not differentiate between genotypes PCV2a and PCV2b of the virus. Primers, reaction mix, template volume, cycling conditions, dilutions, plasmid standard curve development and extrapolation, and equipment were as previously described (148). All extracted samples and negative controls were tested in duplicate on 96-well plates and included a plasmid standard curve on each plate in duplicate over a range of 6.6×10^{8} to 6.6×10^{-1} copies of plasmid per reaction (or 2.2×10^{11} to 2.2×10^{2} copies per mL). A dissociation curve or melting temperature of between 77.1ºC and 77.8ºC was observed for each PCV2 positive sample and a cutoff value corresponding to a threshold cycle (C_t) of 35.47 (6.6 copies per reaction or 2.2×10^{3} copies per mL) was applied, as previously described (148). Duplicate samples with C_t values >1 were repeated in duplicate. Individual sample results were reported as an average of the duplicate. All serum samples were measured (µL) and fecal samples weighed (mg) prior to DNA extraction. The amount of PCV2 virus (copy number of PCV2 genomes) was adjusted mathematically by taking into consideration the starting volume or weight of each individual sample, the final volume of elution, and the volume of template added to the real-time PCR reaction mix, as previously described (148). Serum and fecal samples were expressed as the number of copies of PCV2 genomes in the original sample per 1 mL or 1 g, respectively.

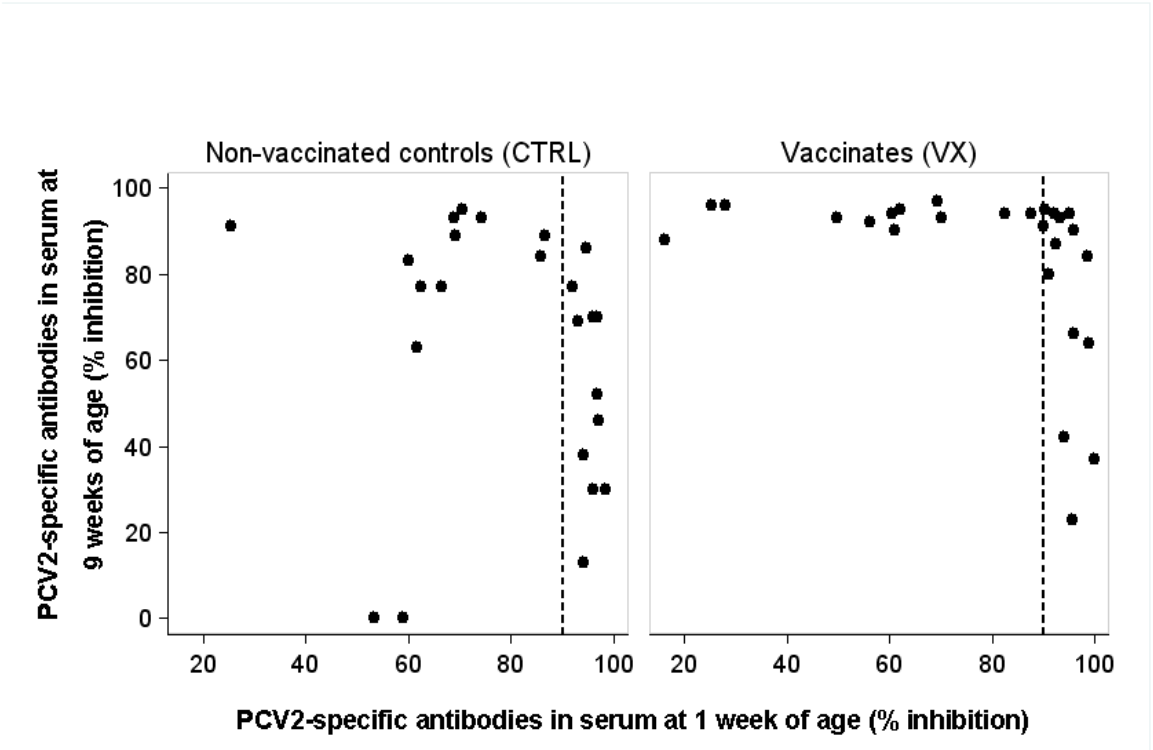
7.3.10 Competitive ELISA

An aliquot from the same serum samples (n=345) used for DNA extraction was used to measure PCV2-specific Ab level by cELISA. The percent inhibition (PI) of a sample was determined using a previously described cELISA (225) with modifications (145). One additional adaptation was made where each original serum sample was diluted a further 1:2 to a final concentration of 1:500 in each sample well. Samples were tested in duplicate on 96-well microtiter plates and results reported as an average of the duplicate. Each 96-well plate included a pig serum PCV2-positive and –negative sample, and a no serum blank control.

7.3.11 Statistical Analyses

PCV2-specific MDAb can affect vaccine performance in young pigs (69); therefore, PCV2-specific Ab measured at one week of age were taken to be representative of MDAb. The effect of MDAb on the relationship between vaccine status and outcome measures was accounted for in the analysis. Because the period from 9 to 11 weeks of age was identified as a biologically important period in this study due to the onset of PCVD-related mortality and increased variability in PCV2-specific Ab and PCV2 DNA concentration in serum, analyses and discussion were primarily limited to this period. The relationship between MDAb (measured at one week of age) and Ab (measured at nine weeks of age and concomitant with the onset of PCVD-related mortality) was not linear (Figure 1), and animals were categorized as having either loMDAb or hiMDAb for the analysis. The median PI or Ab level at one week of age (prior to vaccination) for all animals was 90.0 and was used as the cutoff point for hiMDAb (≥90.0 PI) *versus* loMDAb (<90.0 PI). Figures presented represent the effect of VX *versus* CTRL within

MDAb status group; however, statistical analysis was also performed for VX *versus* CTRL, irrespective of MDAb (figures and data not shown).



(Dashed reference line – Median week 1 PCV2-specific antibody 90.0)

Figure 7.1: Scatter plot for PCV2-specific antibodies in serum measured by competitive ELISA (% inhibition) from non-vaccinated control (CTRL) *versus* vaccinated (VX) pigs in a PCVD-affected barn at one *versus* nine weeks of age. Dashed reference line is the median PCV2-specific antibodies measured at one week of age and determined to be 90.0% inhibition. The median value of 90.0% represents the cutoff value for low PCV2-specific maternally-derived antibodies (loMDAb) (<90.0%) *versus* high PCV2-specific maternally-derived antibodies (hiMDAb) (≥90.0%).

VX and CTRL, and VX and CTRL within MDAb status group (hiMDAb and loMDAb) were compared based on five outcome measures: PCV2-specific Ab levels in serum; PCV2 DNA concentration in serum; PCV2 DNA concentration in feces; mortality; and ADG. Continuous outcomes (PCV2-specific Ab in serum, copies of PCV2 in serum and feces, and ADG) were compared using the Student's *t*-test for the comparison of two groups or a two-way Analysis of Variance (ANOVA) where multiple factors were considered. Results were considered significant where $P \leq 0.05$.

For outcomes that were not normally distributed, a Wilcoxon Rank-Sum Test was used. The Bonferroni correction was applied to adjust for the *P* value at which a significant difference was detected (*P* was significant where $P \leq 0.05$/k comparisons, where multiple comparisons were made for an outcome) (165). *P* values were rounded up to two decimal places.

Proportional mortality due to PCVD was compared using the Fisher's Exact Test and was significant where $P \leq 0.05$. Pigs that died or were euthanized from causes other than PCVD (non-PCVD related deaths such as: meningoencephalitis (n=2); septicemia (n=1); bloated abdomen (n=1)) were not included in the comparison of survival between VX and CTRL (Table 1). ADG was calculated from the difference in weight between the beginning and end of the study (1 and 18 weeks of age; duration=124 days) and divided by the duration.

Statistical analysis was performed, and scatter plots and line graphs were created using a commercial statistics package (Stata 11; StataCorp LP, College Station, Texas, USA).

Pig	Group[a]	Antibody Group[b]	Age[c]	Cause of Death[d, e]	Necropsy Observations Associated with the Pathological and Histological Diagnosis of PCVD		
					Gross Pathology	Lymph Node Pathology	Immunohistochemistry
11	CTRL	hiMDAb	2	Bloated abdomen[f]			
13	VX	hiMDAb	9	Meningoencephalitis (*S. suis*)			
53	CTRL	hiMDAb	9	Meningoencephalitis (*S. suis*)			
38	CTRL	loMDAb	9	PCVD	small, long-haired, wasted, pale kidneys, depleted thymus, generalized enlargement of LN	depleted follicles, epithelioid macrophage infiltration	abundant in tonsils, intestine, kidney
34	VX	hiMDAb	10	Septicemia (*S. suis*)			
23	CTRL	loMDAb	10	PCVD	small, long-haired, wasted, depleted thymus, generalized enlargement of LN	lymphoid atrophy, scattered multinucleated cells	abundant in tonsils, lung, intestine
20	CTRL	loMDAb	11	PCVD	small, wasted, pale kidneys, small cobblestone liver, generalized enlargement of LN	pathognomonic intracellular inclusions typical of PCV2 infection	abundant in tonsils, lung, intestine, kidney
47	CTRL	loMDAb	11	PCVD	small, long-haired, thin, depleted thymus, finely pink cobblestone lungs failed to collapse	pathognomonic intracellular inclusions typical of PCV2 infection	abundant in tonsils, lung, intestine, kidney
43	CTRL	loMDAb	11	PCVD	small, long-haired, wasted, generalized enlargement of LN	characteristic lymphoid lesions of PCVD; lymphocyte depletion	abundant in tonsils, lung, intestine

Table 7.1: Results of pathologic and bacteriologic assessment of pigs vaccinated with an immune stimulating complex (ISCOM) Matrix Q adjuvanted porcine circovirus type 2 (PCV2) virus-like particle (VLP) transdermal vaccine that either died or were euthanized between 1 and 18 weeks of age.

[a] Groups are vaccinated (VX) (n=27) or non-vaccinated controls (CTRL) (n=27). CTRL received ISCOM Matrix Q only which contained no PCV2 VLP protein.
[b] Antibody group includes the following designations based on the PCV2-specific antibody measured at 1 week of age by competitive ELISA and were considered representative of the PCV2-specific maternally-derived antibodies (MDAb) in serum: low maternally-derived antibodies (loMDAb; <90.0% inhibition) or high maternally-derived antibodies (hiMDAb; ≥90.0% inhibition).
[c] Age in weeks.
[d] *Streptococcus suis*, as determined by bacteriologic assessment of brain, kidney, liver, lung, and spleen.
[e] Porcine circovirus disease (PCVD), as determined by the case definition (gross observations, lymphoid depletion, and PCV2 antigen associated with lesions in immunohistochemical analysis).
[f] Death was a result of a non-infectious cause (i.e. bloated abdomen or Atresia ani).

7.4 Results

7.4.1 Matrix Q testing for adverse reactions

No adverse observations were made regarding the injection site or activity level of any piglet during the 30 hour period after injection with the Matrix Q. The expected normal rectal temperature of an unweaned piglet >24 hours of age is 39.2 ± 0.3ºC (212). Taking into consideration the baseline rectal temperature of each piglet (temperature prior to injection), only one piglet at 30 hours post-injection was observed to have a potential low grade fever of 39.6ºC (a temperature greater than 0.3ºC above its baseline temperature and greater than 39.5ºC). This piglet received the lowest dose of Matrix Q of 75 µg.

7.4.2 PCV2-specific antibody in serum

At nine weeks of age, overall Ab levels were significantly higher in the VX than compared with the CTRL when not considering MDAb status (Wilcoxon Rank Sum Test; median Ab: VX=92.5%, CTRL=73.5%; P=0.001).

PCV2-specific Ab levels in serum for each treatment group and MDAb status over the sampling time period are shown in Figures 2a-2b. In the hiMDAb animals (Figure 2a), VX had higher Ab at week nine (median Ab=96%) than CTRL (median Ab=51%) (Wilcoxon Rank Sum Test; P=0.03). In the loMDAb pigs (Figure 2b), VX had higher Ab at week nine (median Ab=94%) than CTRL (median Ab=84%) (Wilcoxon Rank Sum Test; P=0.002). Differences are considered significant at $P \leq 0.03$ after applying a Bonferroni correction for two comparisons.

7.4.3 PCV2 DNA concentration in serum and feces

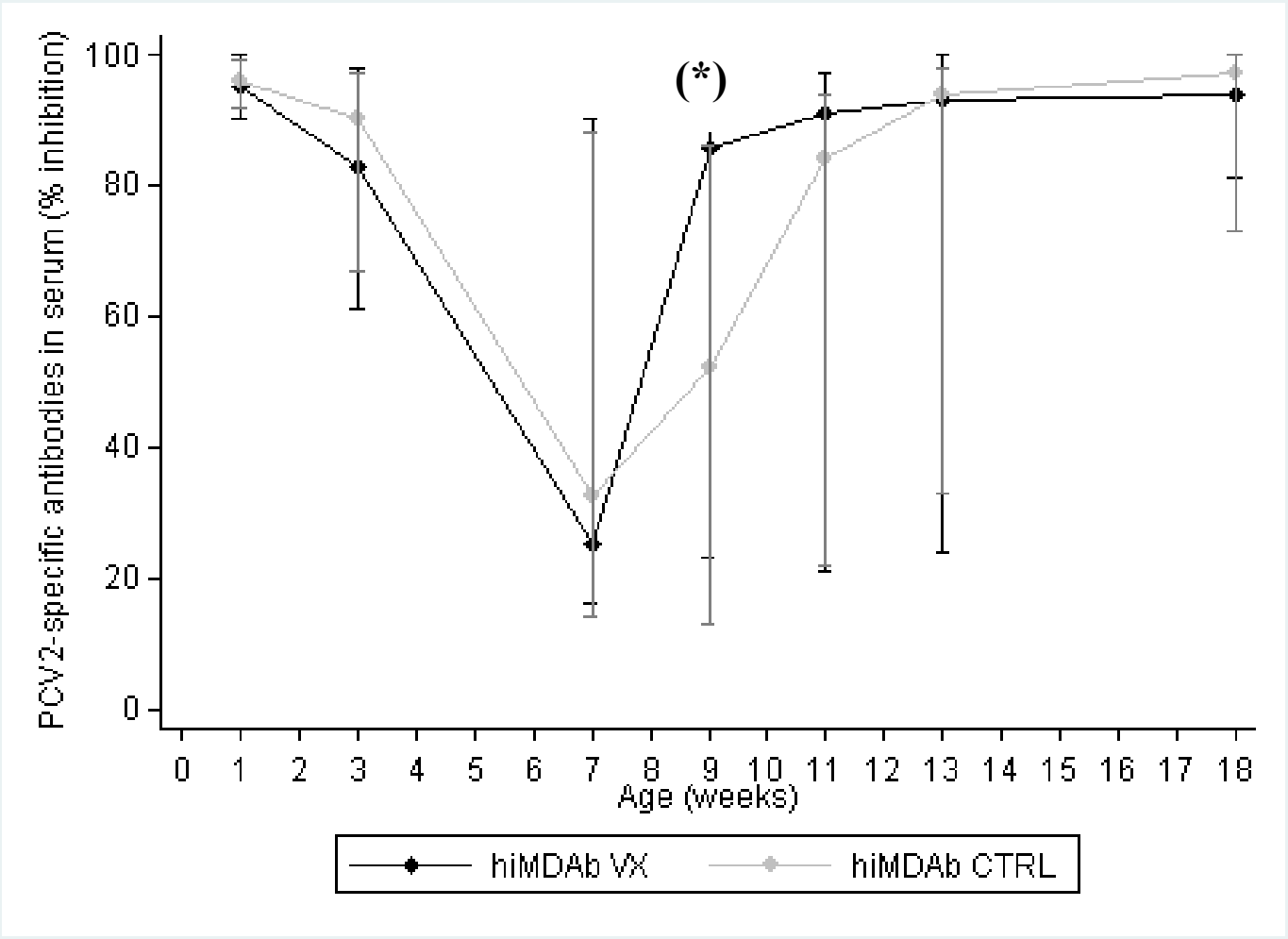
Overall, VX had significantly lower PCV2 DNA concentration in serum at 9 and 11 weeks of age, (median copies per mL: VX=4.89×10^5, CTRL=2.31×10^6; P=0.003 and VX=1.12×10^5, CTRL=8.72×10^5; P=0.01, respectively) when compared with the CTRL. At 7,

13, and 18 weeks of age, PCV2 DNA concentration in serum was not significantly different between VX and CTRL (P=0.20, P=0.12 and P=0.02, respectively). Differences are considered significant at $P \leq 0.01$ after applying a Bonferroni correction for five comparisons.

PCV2 DNA was not detected in the serum of any pig by qPCR at one and three weeks of age (Figures 2c-2d). PCV2 DNA concentration in the hiMDAb pigs (Figure 2c) was not significantly different in serum at nine weeks of age between the VX and CTRL (Wilcoxin Rank Sum Test; P=0.14). However, in the loMDAb pigs (Figure 2d), VX had lower PCV2 DNA concentration at week nine (median copies per mL=6.73×10^5) than CTRL (median copies per mL=3.87×10^6) (Wilcoxon Rank Sum Test; P=0.01). Differences are considered significant at $P \leq 0.03$ after applying a Bonferroni correction for two comparisons.

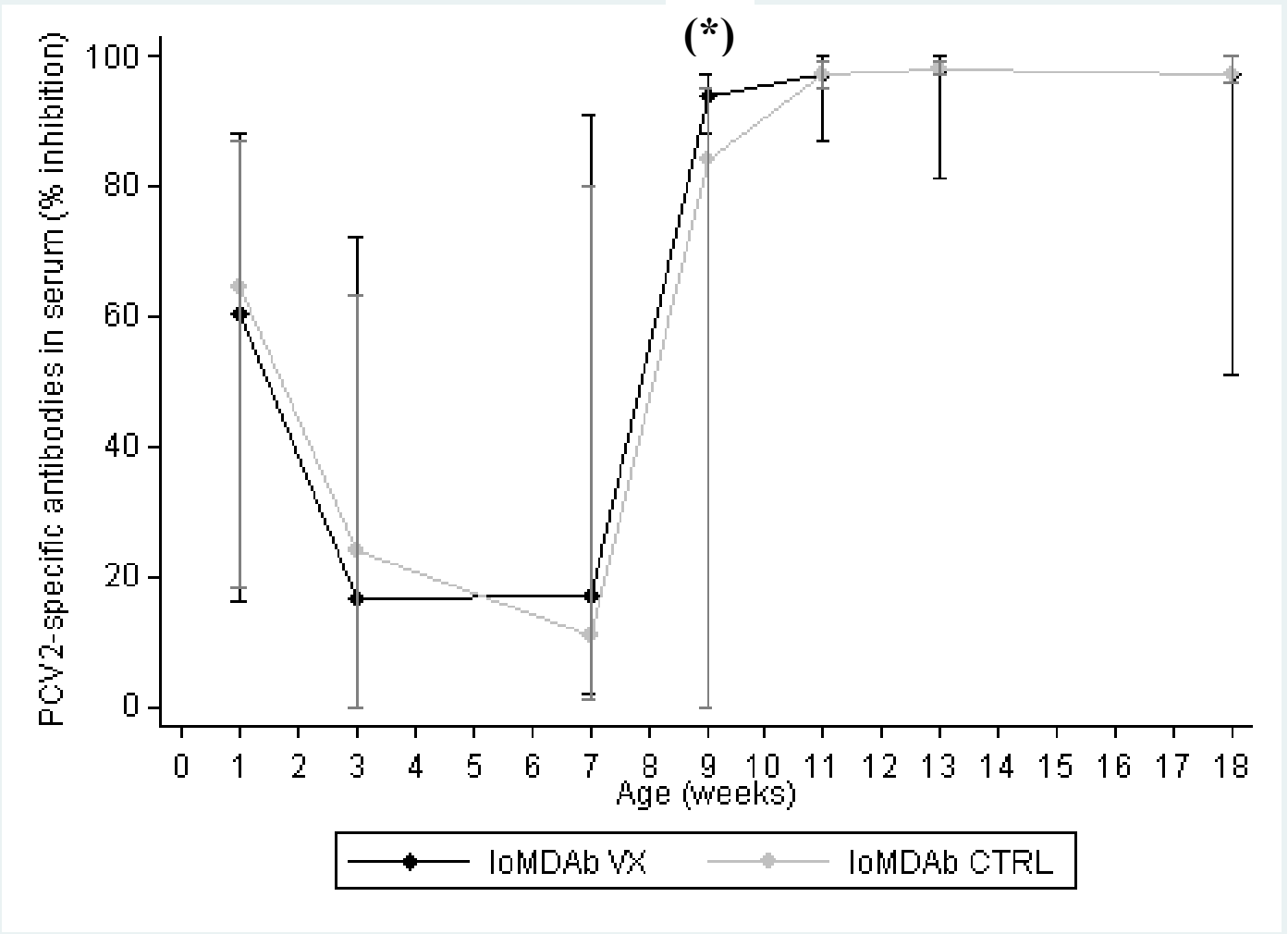
PCV2 DNA was not detected in the feces of any pig by qPCR at three weeks of age (Figures 2e-2f). PCV2 DNA concentration in feces did not differ significantly between VX and CTRL in any of the sampling periods (P>0.05 for all time periods) (data not shown) and this did not change when MDAb were considered (Figures 2e-2f).

(a)



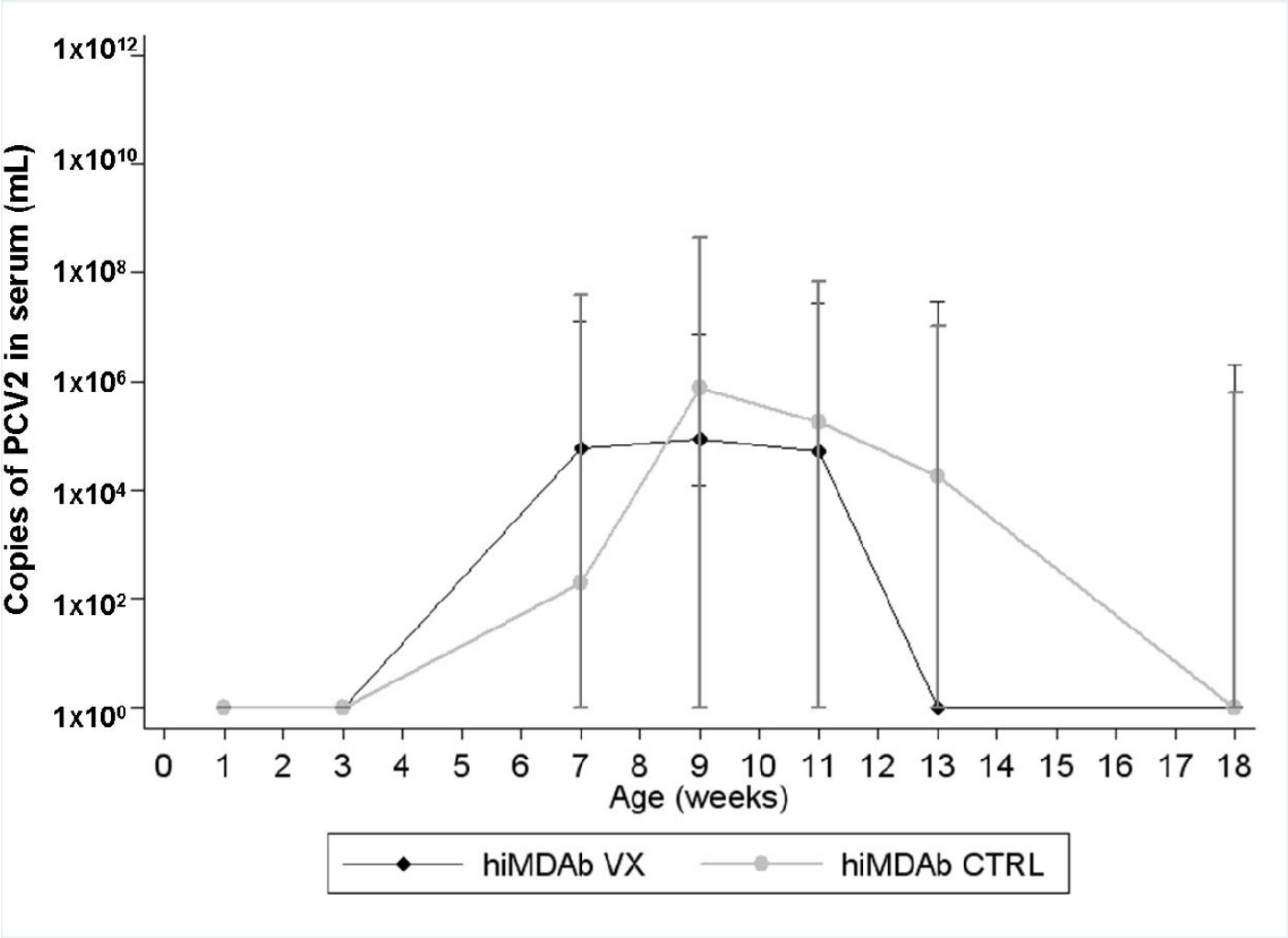
Figures 7.2a – 7.2f: Line graphs presenting PCV2-specific antibody levels (% inhibition), as determined by competitive ELISA, in pigs with (a) high maternally-derived PCV2-specific antibodies (hiMDAb) and (b) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. PCV2 viral load in serum and feces (copies of PCV2 DNA per mL or g, respectively), as determined by quantitative real-time PCR, in pigs with (c) and (e) high maternally-derived PCV2-specific antibodies (hiMDAb), and (d) and (f) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. Median values are plotted and whiskers attached to the top and bottom of the median extend to the maximum and minimum data points. Asterisks (*) denote comparisons between VX and CTRL that are statistically significant within MDAb group.

(b)



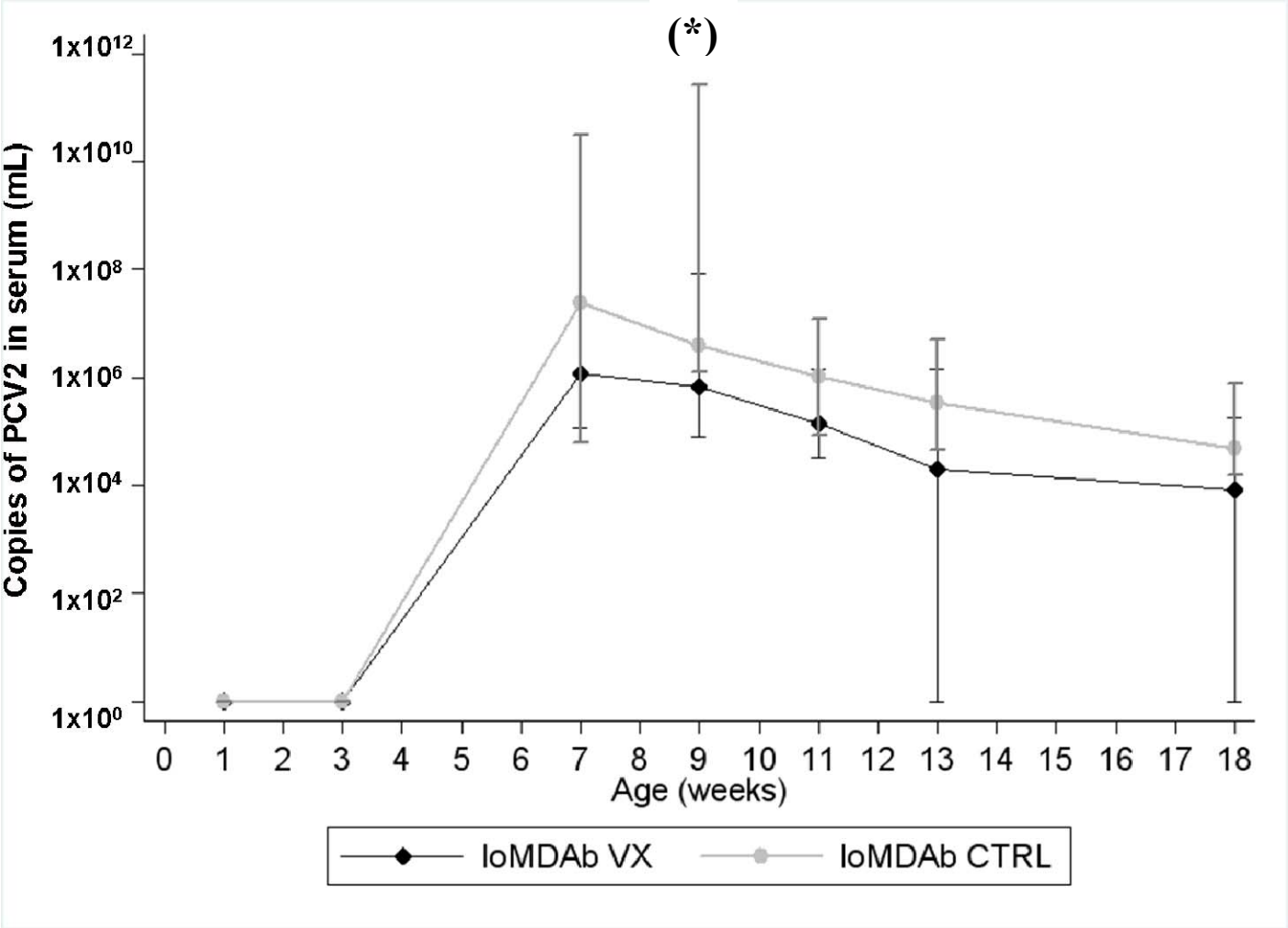
Figures 7.2a – 7.2f: Line graphs presenting PCV2-specific antibody levels (% inhibition), as determined by competitive ELISA, in pigs with (a) high maternally-derived PCV2-specific antibodies (hiMDAb) and (b) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. PCV2 viral load in serum and feces (copies of PCV2 DNA per mL or g, respectively), as determined by quantitative real-time PCR, in pigs with (c) and (e) high maternally-derived PCV2-specific antibodies (hiMDAb), and (d) and (f) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. Median values are plotted and whiskers attached to the top and bottom of the median extend to the maximum and minimum data points. Asterisks (*) denote comparisons between VX and CTRL that are statistically significant within MDAb group.

c)



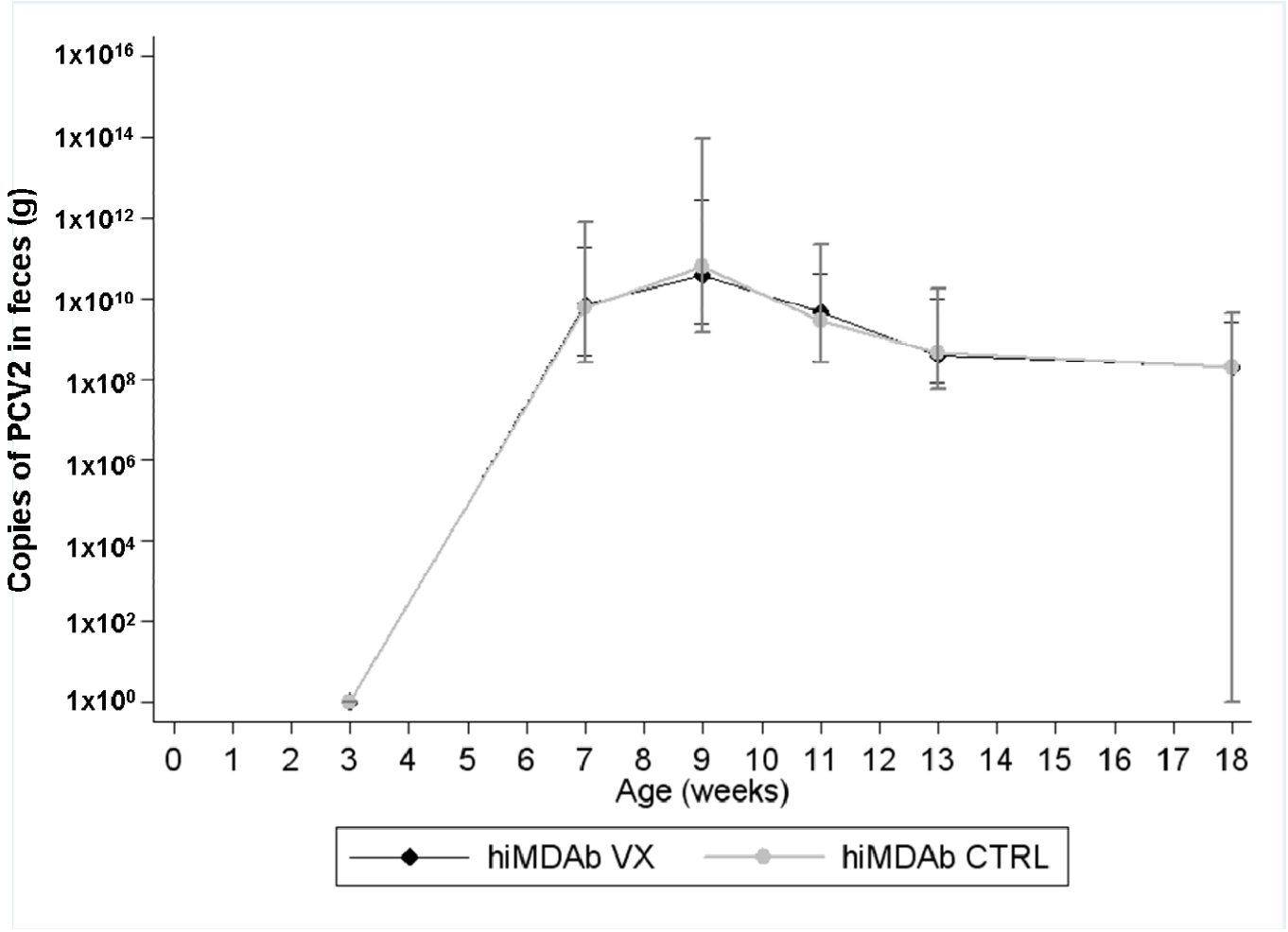
Figures 7.2a – 7.2f: Line graphs presenting PCV2-specific antibody levels (% inhibition), as determined by competitive ELISA, in pigs with (a) high maternally-derived PCV2-specific antibodies (hiMDAb) and (b) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. PCV2 viral load in serum and feces (copies of PCV2 DNA per mL or g, respectively), as determined by quantitative real-time PCR, in pigs with (c) and (e) high maternally-derived PCV2-specific antibodies (hiMDAb), and (d) and (f) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. Median values are plotted and whiskers attached to the top and bottom of the median extend to the maximum and minimum data points. Asterisks (*) denote comparisons between VX and CTRL that are statistically significant within MDAb group.

(d)



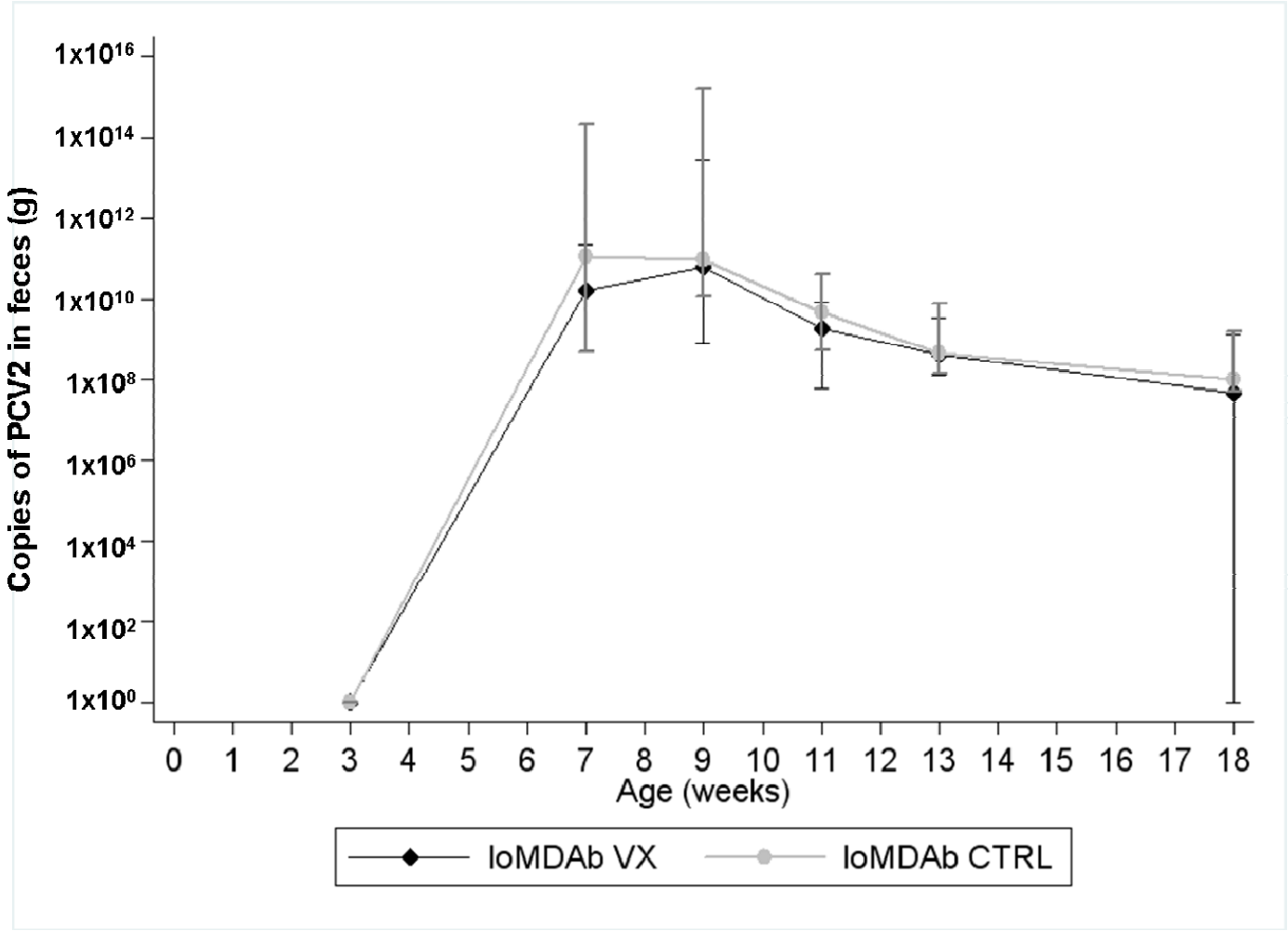
Figures 7.2a – 7.2f: Line graphs presenting PCV2-specific antibody levels (% inhibition), as determined by competitive ELISA, in pigs with (a) high maternally-derived PCV2-specific antibodies (hiMDAb) and (b) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. PCV2 viral load in serum and feces (copies of PCV2 DNA per mL or g, respectively), as determined by quantitative real-time PCR, in pigs with (c) and (e) high maternally-derived PCV2-specific antibodies (hiMDAb), and (d) and (f) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. Median values are plotted and whiskers attached to the top and bottom of the median extend to the maximum and minimum data points. Asterisks (*) denote comparisons between VX and CTRL that are statistically significant within MDAb group.

(e)



Figures 7.2a – 7.2f: Line graphs presenting PCV2-specific antibody levels (% inhibition), as determined by competitive ELISA, in pigs with (a) high maternally-derived PCV2-specific antibodies (hiMDAb) and (b) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. PCV2 viral load in serum and feces (copies of PCV2 DNA per mL or g, respectively), as determined by quantitative real-time PCR, in pigs with (c) and (e) high maternally-derived PCV2-specific antibodies (hiMDAb), and (d) and (f) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. Median values are plotted and whiskers attached to the top and bottom of the median extend to the maximum and minimum data points. Asterisks (*) denote comparisons between VX and CTRL that are statistically significant within MDAb group.

(f)



Figures 7.2a – 7.2f: Line graphs presenting PCV2-specific antibody levels (% inhibition), as determined by competitive ELISA, in pigs with (a) high maternally-derived PCV2-specific antibodies (hiMDAb) and (b) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. PCV2 viral load in serum and feces (copies of PCV2 DNA per mL or g, respectively), as determined by quantitative real-time PCR, in pigs with (c) and (e) high maternally-derived PCV2-specific antibodies (hiMDAb), and (d) and (f) low maternally-derived PCV2-specific antibodies (loMDAb) in vaccinated (VX) *versus* non-vaccinated control (CTRL) groups. Median values are plotted and whiskers attached to the top and bottom of the median extend to the maximum and minimum data points. Asterisks (*) denote comparisons between VX and CTRL that are statistically significant within MDAb group.

7.4.4 Mortality and postmortem analyses

Mortality attributed to PCVD was significantly higher in CTRL (5/25) when compared with VX (0/25) (Fisher's Exact Test; *P*=0.05) and all animals that died due to PCVD were in the loMDAb CTRL group. The primary clinical features observed by farm technicians in the PCVD-affected pigs in this study prior to their death were wasting, dyspnea, and diarrhea and all PCVD-related deaths occurred between 9 and 11 weeks of age. Nine of 54 pigs died or were euthanized during this study and of those deaths, eight were attributed to an infectious cause (five to PCVD; three to *S. suis*) and one to a congenital defect (Atresia ani). Details regarding the age, treatment group, MDAb status, cause of death, and the gross and microscopic observations associated with the case definition of PCVD of all deceased pigs during this study are summarized in Table 1. A PCVD diagnosis is based on the PCVD case definition of observed clinical and histological findings detailed by the American Association of Swine Veterinarians (AASV) (17). Those pigs with a diagnosis associated with *Streptococcus suis* infection were serotyped by slide agglutination with antiserum for those routinely typed (serotypes 1-9) (Prairie Diagnostic Services, Saskatoon, Canada) and all pigs were found to have a serotype outside that of 1 to 9.

7.4.5 Average daily gain (ADG)

Between 1 and 18 weeks of age, the ADG in VX was 0.7630 +/- 0.0747 kg/day and in CTRL was 0.7465 +/- 0.0771 kg/day and no significant difference in ADG was found between groups (Two-sample *t*-test; *P*=0.47). However, within treatment group, MDAb status at one week of age appeared to have some effect on ADG. In VX, pigs with hiMDAb had a tendency toward a greater ADG (0.7868 +/- 0.0797 kg/day) when compared to pigs with loMDAb (0.7373 +/- 0.0619 kg/day) (Two-sample *t*-test; *P*=0.10). In the CTRL, no difference between hiMDAb

(0.7569 +/- 0.0881 kg/day) and loMDAb (0.7379 +/- 0.0639 kg/day) was detected (Two-sample *t*-test; *P*=0.52).

7.5 Discussion

Commercial vaccines are now available for the prevention of PCV2-associated diseases or PCVD (110;173;203); however, the ability of these vaccines to 'override' maternal Ab and immunologically prime passively immune piglets has been conflicting (69;110). Our objective was to determine the efficacy of a novel vaccine candidate in a subset of pigs with variable levels of MDAb in a PCVD-affected farm by assessing PCV2-specific Ab levels, PCV2 DNA concentration in serum and feces, mortality, and ADG. This study is the first report of the use of an ISCOM technology based Matrix Q adjuvant mixed with PCV2 VLP protein administered transdermally for the prevention of PCVD in swine.

In VX, significantly higher PCV2-specific Ab in serum at nine weeks was observed. This elevated level of Ab may have assisted in preventing pigs in the VX from progressing to PCVD, as all deaths resulting from PCVD during this study occurred between 9 and 11 weeks of age. The occurrence of increased Ab levels at nine weeks of age when compared with seven weeks of age (Figures 2a-2b) suggests a PCV2 natural challenge occurred. Upon further investigation, the VX (both hiMDAb and loMDAb groups) at nine weeks of age had a higher Ab response to the challenge, while the CTRL (both hiMDAb and loMDAb groups) had a significantly reduced Ab response in comparison. During this period of 9 to 11 weeks of age, the loMDAb CTRL became susceptible to PCVD. After 11 weeks of age, Ab levels were high in the majority of pigs in both the VX and CTRL signifying a response to a considerable natural PCV2 challenge immediately preceding and during the period of 9 to 11 weeks of age.

Due to the ability of ISCOMs to facilitate the priming of animals to antigen in the presence of MDAb (156), piglets were vaccinated at one and three weeks of age. Serum collected at one week of age and immediately prior to vaccination with the Matrix Q PCV2 VLP transdermal vaccine confirms that MDAb were present in the piglets based on the elevated PI of PCV2-specific Ab (Figure 1c). When considering MDAb and vaccine status at nine weeks of age (at the onset of PCVD-related deaths), hiMDAb CTRL pigs had a delayed Ab response to natural challenge (Figure 2a); however, none of these pigs succumbed to PCVD. A significant difference was observed in the Ab levels of loMDAb VX *versus* loMDAb CTRL pigs, where all pigs dying due to PCVD occurred in loMDAb CTRL group. It appears that vaccination and/or the presence of high MDAb may have protected the pigs from a PCVD-associated death. Additionally, the novel transdermal Matrix Q formulated PCV2 VLP vaccine appeared to have successfully primed young pigs in the presence of high MDAb, as there was a significantly higher Ab response at nine weeks of age in hiMDAb VX pigs when compared to hiMDAb CTRL (Figure 2a). The observation that mortality due to PCVD was clustered in the loMDAb group implies that vaccination and hiMDAb are both equally protective, and that the benefit of Matrix Q/PCV2 VLP vaccination would be most apparent in pigs with loMDAb.

PCV2 DNA in serum was not detected in one and three week old piglets, suggesting that exposure to PCV2 in the farrowing crate or during gestation was minimal, MDAb were very effective at protecting piglets from PCV2 infection, and/or the PCV2 DNA concentration was below the detection limit of the assay. After the pigs were mixed and weaned into the nursery at three weeks of age, observed median PCV2 DNA concentrations in sera were lower in the VX compared with the CTRL for all subsequent sampling periods, with the exception of the hiMDAb VX group at seven weeks of age (Figure 2c), although these differences were not

statistically significant at all time periods (Figures 2c-2d). It is biologically important that the VX had significantly lower PCV2 DNA in serum at 9 and 11 weeks of age when compared with CTRL, as this was the period of time where animal deaths attributed to PCVD occurred. When considering MDAb status, a significant difference in PCV2 DNA concentration in serum was only observed in the loMDAb group and the vaccine did not appear to decrease serum viral load in the hiMDAb group.

In a previous study, PCV2 DNA concentration in feces was found be significantly different between aged matched pigs in a PCVD-affected and a non-affected herd (147). Additionally, fecal swabs have been used by other researchers to determine the ability of a vaccine to reduce viral shedding, as a measure of vaccine efficacy (69). By contrast, PCV2 DNA concentration in feces did not differ between VX and CTRL in this study, irrespective of MDAb status. Our method included weighing individual rectal fecal samples and mathematically adjusting for the amount of feces tested, as opposed to a taking a rectal swab, which may contain a variable mass or volume of fecal material. PCV2 DNA quantification from a measured amount of feces is a more accurate method of quantifying DNA; however, a very small sample (<250 mg) is used and the virus may not be homogeneously shed in feces. In the previously described study that determined significant differences between aged matched PCVD-affected and non-affected pigs (147), samples were pooled and a 1 g sample was extracted for quantification, as opposed to the individual 250 mg samples tested in this study. It is possible that a larger sample of 1 g would offset the problem of a non-homogeneous sample in animals shedding higher levels of PCV2 in feces. Alternatively, although the Matrix Q PCV2 VLP vaccine significantly reduced PCV2 DNA concentration in serum at 9 and 11 weeks of age, it did not appear to reduce the PCV2 shed in feces at any time point.

The herd chosen for this study was experiencing elevated mortality due to PCVD. From 9 and 11 weeks of age, five deaths attributed to PCVD occurred in the CTRL, exclusively the loMDAb CTRL group. Importantly, no PCVD-associated deaths were observed in the VX for the duration of the study (Table 1); thus, the Matrix Q PCV2 VLP vaccine was concluded to significantly reduce death due to PCVD.

S. suis infection was the only other infectious cause of death in the pigs and was responsible for the only two deaths in the VX and contributed to one death in the CTRL. *S. suis* is a common pathogen of pigs with meningitis being an important clinical presentation (75). *S. suis* serotype 2 is the most commonly isolated and frequently reported serotype from pigs with severe disease; however, more than 30 serotypes have been reported with variable disease association. The *S. suis* associated with mortality in this study was not associated with serotypes 1-9.

ADG is an important production measure and has been used to assist researchers in determining the benefits of vaccines against PCV2 infection (110). In this study, pigs with hiMDAb had numerically higher ADGs when compared with the loMDAb pigs within each treatment group; however, only within the VX did pigs with hiMDAb approach a significant improvement in ADG over the loMDAb pigs. A larger sample size may have elucidated this association, but it appears that the vaccine did not appreciably improve ADG.

Considerations for future studies testing vaccines based on ISCOM technology would include measures for cell-mediated immunity and IgG Ab isotypes. These parameters, albeit not the focus of this study, would have contributed to a greater understanding of the immune response invoked by the novel vaccine used here.

In conclusion, our objectives were to assess the ability of a novel Matrix Q PCV2 VLP transdermal vaccine to successfully prime passively immune young pigs to PCV2 in a PCVD-affected herd. Four non-invasive outcome measures along with mortality were used to assess the efficacy of the novel vaccine. The vaccine did not appear to reduce PCV2 DNA concentration in feces nor significantly improve ADG. However, a significant reduction in mortality and PCV2 DNA concentration in serum in the VX was observed during 9 to 11 weeks of age, the period of time when CTRL pigs were dying due to PCVD and PCV2-specific Ab in serum was significantly higher in VX at the onset of PCVD-related mortality. High PCV2-specific MDAb was also associated with protection from PCVD-related death, as observed in the hiMDAb CTRL group and the vaccine was able to increase the PCV2-specific Ab response in the presence of elevated PCV2-specific MDAb, as evidenced in the hiMDAb VX group. However, viremia and fecal shedding were not significantly reduced in the hiMDAb VX group.

The benefit of the ISCOM vaccine was most evident in the loMDAb VX group, as these pigs were protected from a PCVD-related death, unlike the loMDAb CTRL group. In addition to a reduction in mortality, producers and veterinarians would expect a significant improvement in ADG and reduced viral shedding in feces prior to implementing a PCV2 vaccine in a PCVD-affected herd. Therefore, these results indicate that the Matrix Q PCV2 VLP transdermal vaccine described here prevented PCVD-related death in the highest risk population (loMDAb group), but requires further modification in either ISCOM matrix dose or PCV2 VLP protein construction or concentration in future study.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The studies that comprise this thesis are divergent; however, all sought to discover novel information using molecular techniques for the detection or quantification of PCV2 in pigs infected naturally with the virus. In addition, Ab detection and quantification was valuable to describe the passive transfer of PCV2-specific Ab to newborn piglets and to determine when pigs were challenged naturally with PCV2 in the barn setting. It is also important that pigs were not segregated from the population in any of our studies, but maintained in the barn according to previously established industry standard production practices. This allowed us to determine our results in a true barn scenario directly applicable to commercial farms. Our research began in PCV2 positive healthy herds and progressed to studies in PCVD-affected herds. This progression was shaped by the current events of the local swine industry in Saskatchewan.

At the start of this research in 2002, PCVD was present in the herds in Saskatchewan, but was sporadic. PCVD was found to affect random individual pigs, but severe losses were rarely recognized at the herd level in most facilities with high biosecurity and proper production and management practices. This situation changed in approximately 2005. Previously healthy herds, albeit positive for PCV2, started to experience increased losses due to PCVD and were diagnosed at the herd level. This change in the PCV2 dynamic offered us an opportunity to study PCV2-fecal shedding in a healthy herd *versus* a PCVD-affected herd and to test a novel vaccine in a PCVD-affected herd that was not using commercial PCV2 vaccines.

The first study was concerned with the detection of PCV2 in serum by two PCR methods and the PCV2-specific Ab profile of pigs. The hypothesis – PCV2 is persistently present in the

serum of healthy pigs with high PCV2-specific Ab – was supported by testing pigs from five days of age and until slaughter at 156 days of age. We chose to collect blood samples at the beginning, midpoint, and end of each production stage in the barn for a total of eight successive collections. At the time, there were no published reports on the persistence of viremia and the Ab profiles of healthy pigs infected naturally with PCV2 that spanned all production stages.

Three previously described assays were modified and used to complete the objectives for this study. A conventional nested and a non-nested PCR were used to detect PCV2 DNA in serum and a competitive ELISA measured the PCV2-specific Ab. The PCR assays had different detection limits and as expected, and a greater percentage of samples were positive for PCV2 DNA in serum using the nested PCR method than with the non-nested assay. At the time of this study (2002) real-time quantitative PCR was not readily available.

Piglets had very high levels of PCV2-specific Ab at five days of age; however, these Ab declined after weaning at 40 days of age, and viremia was detected by both PCR methods at the subsequent blood collection (72 days of age). After PCV2 DNA was detected in the serum of the young pigs, the PCV2-specific Ab rose and remained elevated until the end of the study (156 days of age). After the detection of PCV2 DNA in serum at 72 days of age, blood was collected at 107, 135, and 156 days of age and 45% of pigs were repeatedly positive in a minimum of two to a maximum of four samples. Based on these observations, several conclusions can be made about the passive transfer of PCV2-specific Ab and the young pig's immune response to PCV2 natural challenge.

Based on the very high levels of PCV2-specific Ab at five days of age, passive transfer of PCV2-specific Ab to the nursing piglets was successful; however, 10% and 15% of the pigs tested at 40 and 72 days of age had Ab levels below the cutoff for a positive result. This suggests

a wide variation in the Ab passively transferred to the piglets. Viremia was not detected until 72 days of age, which suggests sufficient protection by maternal PCV2-specific Ab or other immunocellular protective mechanisms, or the absence of exposure to PCV2 in the first weeks of life. PCV2 is shed in the secretions of healthy PCV2-positive pigs (147;200), so it is likely that piglets were exposed to PCV2 in the farrowing crate by the sow. However, a PCV2 natural challenge may be greatest during the mixing and remixing of pigs in the nursery stage, after weaning. The mixing of pigs in the nursery is concomitant with the waning of maternally-derived PCV2-specific Ab and the subsequent detection of PCV2 DNA in serum. At 107 days of age, following the detection of PCV2 DNA serum at 72 days of age, an observed increase in the PCV2-specific Ab in the pigs occurred indicating a response to natural challenge with PCV2 in the nursery. This sequence of events suggests that PCV2 viremia is established when young pigs are mixed in the nursery.

In conclusion, no correlations existed among measures of serum Ab, viremia, and the effects of sex, breed, or age. However, the results from this study suggest that the PCV2-specific Ab in serum is not sufficient to prevent or resolve PCV2 viremia in pigs, as the virus persists in the presence of elevated levels of PCV2-specific Ab. Recent research confirms that it is not the presence of PCV2 in the serum, but the PCV2 viral load in serum and/or tissues of pigs that are associated with the disease potential or outcome of PCV2 infection (78;91;169;200). Additionally, a 'litter effect' has been described, where the sow PCV2 viral load and PCV2-specific Ab level in serum has a significant effect on litter mortality attributable to PCVD (31).

To re-emphasize the importance of passively transferred maternally-derived PCV2-specific Ab in the protection of the piglet from PCVD, the commercial vaccine produced by Merial is administered prior to farrowing (177). The vaccine produces elevated PCV2-specific

Ab in the serum of sows resulting in elevated Ab transferred to the suckling piglets through colostrum. The elevated PCV2-specific Ab in the young pig due to vaccination of the sow is protective against PCVD during the period of susceptibility following weaning (100). PCV2 viral load is kept below a level that contributes to the development of PCVD.

The second study was concerned with the detection of PCV2 in semen by nested PCR and the effects of the presence of the virus on semen quality. The hypothesis – PCV2 is intermittently-shed in the semen of healthy pigs and does not affect semen quality – was supported by testing 43 boars aged 33.9 to 149.3 weeks for a period of up to nine months. A total of 903 semen samples were collected representing Duroc, Landrace, Hamline, Large White maternal, Large White paternal and Meishan-synthetic breeds. Semen collection was dictated by commercial product demand; therefore, variation between collections and among boars occurred and could not be controlled. The average time between individual collections was 11 ± 8.9 days with a range of 2 to 81 days.

At the time, two studies with a focus on the detection of PCV2 in semen were published. PCV2 had been detected in semen from boars infected naturally with the virus, but the shedding pattern and duration were not determined (108). In another study, PCV2 DNA was detected intermittently in semen from day 5 to 47 postinfection in boars infected experimentally with PCV2 (119). However, there was a lack of information regarding the long-term shedding patterns of PCV2 in semen from naturally-infected boars, and there were no reports regarding the potential effects of the presence of PCV2 in semen on semen quality.

PCV2 DNA was detected in 30 of 903 semen samples and from 13 of the 43 boars tested. While the study was not balanced by breed, PCV2 DNA was only detected in the Duroc and Landrace breeds. In another study, a host genetic difference among breeds suggests that

Landrace pigs may have a predisposition to PCV2-induced disease and lesions (171). Studies regarding PRRSV in swine produce similar observations to PCV2 infection, as PRRSV causes a persistent infection, and variability in the duration and presence PRRSV shed in semen exists among Landrace, Yorkshire, and Hampshire breeds (42). These observations suggest that breed susceptibility to PCV2-shedding in semen may exist.

When considering boar age and time after entry into the boar stud, semen samples were less likely to be positive for PCV2 DNA with an increase in time. Boars ranged from 33.9 to 149.3 weeks of age for the period of this study; however, no PCV2 positive semen sample was found in any boar after 71 weeks of age. A semen sample was 2.6 times more likely to be positive when collected from boars that were ≤52 weeks of age, and 3.0 times more likely to be positive when collected from boars that were ≤26 weeks from the time of entry into the stud main unit. It appears that younger boars are more susceptible to shedding PCV2 in semen.

Spermiograms were prepared with each semen sample submission for a subset of boars, and the ratio of live to dead sperm cells and specific morphological defects were determined, as a measure of semen quality. The effect of PCV2 semen status on semen quality, based on the proportion of live and morphologically normal cells was determined from 314 smears: 199 smears from 10 PCV2-shedding boars; and 115 smears from eight boars that did not shed PCV2 in semen. There was no association found between PCV2 DNA detection in semen and a reduction in semen quality.

In conclusion, PCV2-shedding in semen occurred during a period of up to 27.3 weeks and samples were more likely to be PCV2 positive when collected from younger boars. In addition to boar age, particular breeds may be more susceptible to PCV2-shedding in semen. PCV2-shedding in semen was sporadic in frequency and occurred in the absence of clinical

disease, as no PCVD was observed in the herd during this study. An elevated level of PCV2 in serum or tissues may not be necessary to result in the shedding of the virus in semen, as elevated levels of virus are typically associated with PCVD, and there was no observed clinical disease in the herd.

Semen quality, measured as the proportion of live and morphologically normal cells, was not affected by the presence of PCV2 DNA in semen when PCV2-shedding and non-shedding boars were compared. However, it must be considered that boars infected naturally with PCV2 may have sporadic and long-term shedding in semen, and testing for PCV2 in semen may be required for commercial studs supplying semen for artificial insemination. In a recent study, semen spiked with infectious PCV2 used for artificial insemination of sows resulted in viremia of the sow and PCV2 infection of piglets *in utero* (134). Future considerations regarding semen used in commercial swine facilities or for export may include a requirement for PCV2 testing in semen.

The third study was concerned with the development and validation of a novel quantitative PCR assay for PCV2. The hypothesis – a SYBR green real-time PCR assay can accurately quantify PCV2 in multiple tissues from PCVD-affected and nonaffected pigs – was supported by exploiting oligonucleotide primer binding sites conserved across 244 characterized PCV2 genomes. The assay targets the highly conserved ORF1 or *Rep* gene of PCV2 and was designed to target all genotypes (PCV2a/2b) (71) or genogroups (PCV2-G1/G2) (13) of PCV2.

The assay was tested in multiple tissues from pigs: PCV2 naturally-infected conventional pigs (serum, buffy coat, feces, and multiple lymphoid (bronchial, mesenteric, and superficial inguinal lymph nodes, thymus, tonsil, ileal Peyer's patches, and spleen) and non-lymphoid (myocardium, lung, kidney, liver, and gluteal muscle) tissues; PCV2 experimentally-infected and

non-infected gnotobiotic pigs (terminal serum and liver tissue); and plasmid-spiked PCV2-negative serum, lung tissue, and feces. In addition, homogenized liver samples from the experimentally-infected gnotobiotes were tested by tissue culture and immunohistochemical staining, and expressed as $TCID_{50}$.

Several quantitative assays were published at the time of the development of our assay (29;45;73;169;230); however, the majority of the assays target the less conserved capsid gene (ORF2). It is most desirable to choose target sequences that are strongly conserved, as the efficiency and ability to accurately quantify the target will be variably affected by mismatches between the oligonucleotide and the target sequence. The *Rep* gene (ORF1) is more conserved than the *Cap* gene (ORF2) (50) and the *Rep* gene is conserved among genotypes of PCV2. A more reliable quantitative PCR assay is an advantage of the assay described here.

A plasmid (pl-492) and 10-fold dilution series was prepared for use in a standard curve to allow for the quantification of unknown samples by extrapolation. A dissociation curve was performed after amplification by a gradual rise in temperature from 55ºC to 95ºC, and the fluorescence signal was measured every 0.5ºC. A typical dissociation curve observed for PCV2 infected tissues and the plasmid standards consisted of a single peak at 77.8ºC with no amplification in negative controls and negative samples (Figure 5.2).

To determine the cutoff or detection limit of the assay, a known amount of plasmid (pl-492) was spiked into the serum, lung tissue, and feces from a PCV2-negative pig. The linear portion of the standard curve was found to span 6.6×10^7 to 6.6×10^0 copies of plasmid per 25 µL reaction and was equivalent to 2.2×10^3 copies per mL. The detection limit corresponded to a threshold cycle (C_t) of 35.47 and was applied to each sample. By spiking known concentrations of plasmid at the beginning of the extraction process into the tissues from a PCV2-negative pig,

we were able to determine the effects of potential inhibitors and the extraction process (Figure 5.3). Detection in serum was the most accurate until approaching the lower detection limit; however, in both lung and feces, the target copy number measured was less than the expected by approximately 2 logs. A 1:10 dilution of extracted lung tissue improved detection to within a log (Figure 5.4); however, dilution did not improve detection in feces.

To validate the assay, PCV2 naturally-infected wasting (n=3) and age-matched non-wasting (n=3) pigs were selected and serum, buffy coat, feces, and 12 additional tissues were tested by the novel assay. Although the data set was small and represented only three pigs per group, the concentrations of PCV2 DNA were consistently higher in the wasting *versus* non-wasting pigs (Table 5.2). In addition to samples from PCV2 naturally-infected conventional pigs, liver samples were collected and processed from PCV2 experimentally-infected and non-infected gnotobiotic pigs. PCV2 titers from the homogenized liver samples were determined for the gnotobiotes based on $TCID_{50}$ and immunohistochemical staining titration analysis (Figure 5.5). Increasing viral load from subclinical to severely PCVD-affected pigs was observed and found to be analogous to the observed results by the titration analysis. This suggests a relationship between the quantity of PCV2 DNA in tissue and the severity of PCVD, as reported by others (87;112;200).

In conclusion, the assay developed in this study with a conserved target sequence performed efficiently in the quantification of PCV2 from a variety of tissues from naturally- and experimentally-infected pigs. In testing these tissues, we confirmed the results of published studies where PCVD-affected pigs have increased PCV2 viral load in tissues when compared with non-affected pigs. Due to the conserved target of this assay, as PCV2 continues to evolve in

the field with a potential for new genotypes, this assay will out-perform existing or future assays with less conserved targets.

The fourth study was concerned with the quantification of PCV2 in the feces of PCVD-affected pigs compared with nonaffected pigs. The hypothesis – young pigs (<16 weeks of age) in a PCVD-affected commercial herd shed significantly more PCV2 in their feces compared with pigs in a nonaffected commercial herd – was supported by quantitative PCR of approximately 200 pooled fecal samples. Publications regarding PCV2 shed in feces have focused on quantification of the virus in pigs infected experimentally with PCV2 (32;68) or pigs naturally infected by the categories of subclinical, PMWS-affected, and nonaffected pigs (200). No previous reports have quantified PCV2 in feces by production stage in PCVD-affected and nonaffected herds.

The quantitative real-time SYBR green assay developed in the third study presented in this thesis was used to test approximately 100 pooled fecal samples from each of the PCVD-affected and nonaffected herds. Each barn was sampled in a cross-sectional manner with approximately 50 fecal samples collected from each of the feeding and breeding herds. Fecal samples comprised multiple individual fecal samples from pigs housed in a pen with a variable number of pigs (Table 6.2) resulting in pooled samples. Each of 10 production stages was represented by 10 pooled samples: newly weaned; nursery; pregrower; grower; finisher; virgin gilt; bred gilt; lactating sow parity ≤2; lactating sow ≥3; and boar (Table 6.1).

The observed median PCV2 shed in feces was higher in the PCVD-affected pigs for all production stages, with the exception of older pigs in the finisher, virgin gilt, and bred gilt stages. However, the amount of PCV2 shed was only significantly higher in the younger pigs in the PCVD-affected herd representing the newly weaned, nursery, and pregrower stages of 12 weeks

of age or younger. Of particular interest, the grower stage pigs (15 to 16 weeks of age) had significantly higher PCV2 in feces in the nonaffected herd. This suggests that PCVD-affected pigs may be exposed to PCV2 earlier or experience a higher infectious dose, or both.

Exposure to PCV2 in the first three weeks of life would be primarily dependent on the sow and her shedding status in the farrowing crate. Our research found that the majority of lactating sows in the nonaffected healthy herd, in particular higher parity sows, did not have detectable levels of PCV2 in feces. In contrast, most lactating sows in the PCVD-affected herd shed PCV2 in feces (Figure 6.2). Sows shedding PCV2 in feces into the surrounding environment within the farrowing crate may expose piglets to the virus earlier and with a higher infectious dose. This early exposure in the farrowing crate would explain the increased PCV2 shed in the feces of the younger pigs (from 3 to 12 weeks of age) in PCVD-affected herds. Comparatively, pigs in the nonaffected herd had increased PCV2-shedding three weeks later in the grower stage at 15 to 16 weeks of age. The observed delay in PCV2 fecal shedding in the nonaffected healthy herd would support the proposal that it is the mixing and remixing of pigs postweaning that contributes to and spreads PCV2 among the pigs, and not exposure to the virus from the sow in the farrowing crate.

In addition, it must be considered that sows with increased PCV2 shed in feces have a higher systemic PCV2 viral load (200) and may be more likely to expose piglets to PCV2 *via* colostrum (206), or infect the fetus *in utero* (151;228). However, there have been no reports that correlate serum PCV2 viral load in the sow to infection of the piglet *via* colostrum or the fetus *in utero*.

Lastly, maternal Ab protection provided to suckling piglets may be extremely variable among sows (149;182), but can be protective against PCVD and reduce the PCV2 viral load in

serum. This is evidenced by immunization of the sow prior to farrowing to increase serum PCV2-specific Ab, reduce serum PCV2 viral load (177), and protect young pigs from PCVD *via* passive immunity (100). The PCV2 immune status of the sows in this study was not known. Therefore, passive immunity to the piglets is another variable to consider when speculating as to why young pigs in the PCVD-affected herd succumbed to disease.

In conclusion, more pigs in the nonaffected healthy herd, particularly lactating sows, did not shed PCV2 in feces. As a result, there may be an association between the presence of PCV2 in the feces of lactating sows while in the farrowing crate with their piglets, increased quantity of the virus in the feces of pigs 3 to 12 weeks of age, and the morality associated with PCVD. Additional studies are warranted as these results from obtained from only one healthy and one PCVD-affected herd.

The fifth and final study evolved from an opportunity provided by collaboration with the European Union sixth framework programme; Control of Porcine Circovirus Diseases (PCVDs): Towards Improved Food Quality and Safety. This group consisted of researchers and veterinarians from varied disciplines and countries focused on PCV2 research from the years 2004-2008. This collaboration granted us access to ISCOM matrix technology and recombinant PCV2 protein for use in vaccination. During the four year research project, the PCVD status changed in parts of Canada, including a marked increase in PCVD mortality in Saskatchewan herds. The timing was ideal, as most of the producers in Saskatchewan were not using commercial PCV2 vaccines, yet, and we had a novel PCV2 vaccine candidate to test.

This final study was concerned with determining the efficacy of a novel vaccine to prevent PCVD in pigs based on reduced mortality and four non-invasive outcome measures. The hypothesis – the use of a novel ISCOM based PCV2 VLP vaccine administered transdermally to

one and three week old piglets can reduce PCVD-related mortality - was supported by vaccinating pigs in a PCVD-affected herd with the novel vaccine (n=27), or ISCOM matrix alone without PCV2 VLP protein (n=27). To determine the efficacy of the vaccine, mortality and four non-invasive outcome measures were used: PCV2-specific Ab level in serum; PCV2 DNA concentration in serum and feces; and average daily gain (ADG). Taking into consideration the potential interference of maternally-derived Ab, it was imperative to learn if the vaccine could prime young pigs against PCV2 in the presence of PCV2 maternally-derived Ab and result in a reduction of mortality caused by PCVD in the affected herd.

Prior to this study, several experimental PCV2 vaccine candidates had been reported to improve the immunity to PCV2 infection in swine (23;62;65). However, the use of ISCOM matrix technology in conventional pigs was not previously reported. Several research studies have been performed and published using ISCOMs and human rotavirus protein in gnotobiotic pig studies (74;99;163), but our study was the first to target PCV2 and test the matrix in conventional pigs.

A collection schedule was made based on the results of our first study that determined the PCV2 Ab profile and viremia status in healthy conventional pigs infected naturally with PCV2 (145). The observations made suggested to focus the collection of samples during the late nursery and pregrower stages from 7 to 13 weeks of age; therefore, samples were collected every two weeks during this period. Blood and rectal fecal samples (with the exception of week one for fecal samples) were collected at seven consecutive time points between 1 and 18 weeks of age from each of the 54 pigs in the study. Study pigs were weighed at 1 and 18 weeks of age to determine ADG over the study period. Pigs that were part of this study that either died or were euthanized because moribund were submitted for necropsy and histopathology to determine

cause of death or morbidity. Proportional mortality due to PCVD was calculated based on the results of the necropsy.

Two previously described assays were used to measure the non-invasive outcomes and determine the efficacy of the novel vaccine in this study. The quantitative PCR assay developed in the third study in this thesis was used to quantify PCV2 in the serum and feces of the vaccinated and control pigs. The previously described competitive ELISA used in the first two studies in this thesis was also applied in this last study to measure PCV2-specific Ab.

PCV2 viral load in tissues has become a measure of the infection status or potential disease state of pigs in studies of PCV2 and PCVD. The PCV2 viral load or DNA concentration in tissues has been reported to be associated with the severity of PCVD in swine (87;112). Furthermore, in the fourth study described in this thesis, the quantification of PCV2 DNA in feces was found to be significantly higher in young pigs in a PCVD-affected herd when compared with a nonaffected herd (147). Additionally, PCV2 DNA concentration in serum, as measured by quantitative real-time PCR, is associated with PCVD severity (29;200). Therefore, PCV2 DNA concentration in serum and feces are appropriate matrices to measure vaccine efficacy, as they are correlated with a reduction in PCV2 replication and viral load. Additionally, these outcome measures have the benefit of being non-invasive. Previously described studies show that quantitative PCR is a valuable tool to determine the efficacy of vaccination against PCV2, as evidenced by a reduction of the viral load in serum and tissues (69;110;176).

An important consideration in determining vaccine efficacy is the production of PCV2-specific Ab that are protective against the development of PCVD. However, maternally-derived Ab can affect vaccine performance in young pigs (69); therefore, the use of ISCOM matrix technology was employed to attempt to circumvent this problem. ISCOMs have the ability to

prime animals against specific antigens in the presence maternally-derived Ab (84). We were able to determine the effects of maternally-derived Ab on our novel vaccine by categorizing our study pigs into either high (hiMDAb) or low maternally-derived Ab (loMDAb) groups within the vaccinated and control groups.

In the analysis of this study, vaccine efficacy, based on the mortality and outcome measures described, was made between vaccinated and control pigs. Additionally, the analysis went further by examining the effect of maternally-derived Ab status within and between the vaccinated and control pigs. Based on the high level of PCV2-specific maternally-derived Ab that were present in the one week old pigs in this study, analysis of the effects of the maternally-derived Ab on the ability of the novel vaccine to prime the pigs were made by categorizing the pigs as having either high or low maternally-derived Ab. This was achieved by using the PCV2-specific Ab median value among the study pigs as the cutoff point, as determined by competitive ELISA. Using this approach, the effect of the level of maternally-derived Ab on the ability of the novel vaccine to prime the young pigs could be determined.

Using mortality as an outcome measure of vaccine efficacy was encouraging. Mortality attributed to PCVD was significantly higher in the control group (five pigs) compared with the vaccinated group (none of the pigs). An additional observation made was that all of the control pigs that died due to PCVD were categorized in the loMDAb group. This observation suggests that the vaccine was successful in priming and protecting pigs with loMDAb against a PCVD-related death; however, control pigs with hiMDAb appeared to be protected by innate factors against PCVD and did not require vaccination to prevent a PCVD-related death. This is supported by the observation that none of the control pigs with hiMDAb died due to PCVD.

PCV2-specific Ab level in serum was found to vary significantly among the groups immediately prior to the period when the study pigs experienced PCVD-related deaths. All deaths resulting from PCVD occurred between 9 and 11 weeks of age, and significantly higher levels of PCV2-specific Ab were observed in the vaccinated group at nine weeks of age. Upon further analysis, both hiMDAb and loMDAb groups within the vaccinated pigs at nine weeks of age had significantly higher PCV2-specific Ab when compared to the hiMDAb and loMDAb groups within the control pigs. Based on these observations, the vaccine successfully primed and elicited a PCV2-specific Ab response in the vaccinated pigs despite high levels of maternally-derived PCV2-specific Ab in the hiMDAb group.

PCV2 DNA concentration in serum demonstrated that the vaccine significantly reduced the PCV2 viral load in the vaccinated pigs during the period of PCVD-related deaths. However, when considering MDAb status within the vaccinated pigs, a significant difference in PCV2 DNA concentration in serum was only observed in the loMDAb group and the vaccine did not appear to decrease serum viral load in the hiMDAb group. Based on these observations, the vaccine was not as efficient in the hiMDAb at stimulating a response to neutralize or prevent PCV2 replication. Since the previous outcome measure of PCV2-specific Ab was significantly higher in both the loMDAb and hiMDAb groups within the vaccinated pigs, it can be hypothesized that other immune factors contributing to the prevention of PCV2 replication, such as cell-mediated responses, were not as effectively stimulated or primed in the hiMDAb group.

PCV2 DNA concentration in feces did not differ between the vaccinated and control pigs in this study, irrespective of MDAb status. Fecal swabs have been used by other researchers to determine the ability of a vaccine to reduce viral shedding, as a measure of vaccine efficacy (69). Additionally, our research from the fourth study described in this thesis found significant

differences in the amount of PCV2 shed in feces between a PCVD-affected and a nonaffected herd (147). However, the fecal extraction methodology between the fourth study and the vaccine efficacy study differed, and this difference may have contributed to the negative outcome observed here. Alternatively, although the vaccine significantly reduced the PCV2 concentration in serum in the vaccinated pigs during the period of PCVD-related deaths, the vaccine did not reduce the amount of virus shed in feces.

Average daily weight gain was not significantly different among the groups in this study. However, pigs with hiMDAb had numerically higher ADG compared with loMDAb pigs within the vaccinated group. The difference in ADG between vaccinated and control pigs and among MDAb status groups may have been significant during the nursery and pregrower period when PCVD-related deaths occurred; however, weights were not taken at additional time points during the study. ADG is an important production measure and has been used to assist researchers in determining the benefits of vaccines against PCV2 (38). It appears that the vaccine in our study did not appreciably improve ADG; however, additional measurements during the study may have elucidated a benefit during the period of time when pigs succumbed to PCVD.

In conclusion, the benefit of the novel ISCOM vaccine was most evident in the loMDAb VX, as these pigs were protected from a PCVD-related death that was otherwise experienced in the loMDAb CTRL. However, producers and veterinarians using a vaccine against PCV2 would expect a significant improvement in ADG, viral load in serum, and reduced viral shedding in feces. Although the vaccine prevented a PCVD-related death in the highest risk population, the loMDAb group, modifications regarding the protein or ISCOM concentration, or PCV2 protein construction should be considered to potentially improve the vaccine's efficacy.

Upon completion of the five studies that comprise this thesis, a general observation or conclusion can be made. Molecular diagnostic assays, in particular quantitative assays, are extremely useful tools that have evolved over the last 10 years in the study of PCV2 infection and PCVD. All of the studies presented here share this diagnostic tool and allowed us to discover previously unknown information regarding PCV2 in both healthy and PCVD-affected pigs. The quantification of PCV2 in serum and tissues has a direct relationship to the PCVD status of affected pigs (87;112;200). The ability to determine the PCV2 status of a herd that may progress to increased losses due to PCVD is an important element in the prevention of disease. The importance of non-invasive screening tools to ascertain herd health will give producers and veterinarians the information needed to decide on the implementation of vaccines against PCV2 or to make other changes in the management of their herd. Additionally, with the use of quantitative molecular assays, vaccine efficacy can continue to be tested and improved upon.

Vaccination programs have proven to be effective against the development of PCVD; however, due to the ubiquitous PCV2 infection of pig herds around the world, this is not a virus that will be eradicated. Rather, research will continue to discover answers regarding the transmission and pathogenesis of the virus, and vaccine use will reduce pig losses due to the development of PCVD. PCV2 infection and pathogenesis is complex. Producers and veterinarians can not become complacent regarding the threat of PCV2 and PCVD, as evidenced by the resurgence of severe disease in Canada in the last five years (71). Most aspects of PCV2 infection including the cofactors/triggers, transmission, effect of nutrition, sites of replication, pathogenesis, interaction with host immune response, role of porcine genetics, molecular processes of replication, and potential control of PCVD have been studied. Hundreds of research articles revealing details of PCV2 and PCVD have been published over the last decade.

However, we remain decades away from a complete understanding of the complexity of PCV2 infection and pathogenesis, and the development of PCVD.

I like pigs.
Dogs look up to us. Cats look down on us.
Pigs treat us as equals.

Sir Winston Churchill

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