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# Characterization of Neutralizing and Non-neutralizing Epitopes of Porcine Circovirus 2 Capsid Protein

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UNIVERSITY OF CALGARY

Characterization of Neutralizing and Non-neutralizing Epitopes of  
Porcine Circovirus 2 Capsid Protein

by

Narges Nourozieh

A THESIS

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## **Abstract**

Vaccination is the most efficacious way to prevent Porcine circovirus 2 (PCV2)-associated diseases. In experimental pig studies, vaccine-induced neutralizing antibodies (NAs) appear to play a major role in protection from PCV2 infection. The immune response to PCV2 vaccination of farmed pigs has not been studied in detail. I hypothesize that NAs target conformational epitope(s) present on the surface of PCV2 particles. Highly purified PCV2 particles were found to expose only conformational but not linear epitopes on their surface. The screening 160 sera from farmed pigs showed that reactivity of sera with PCV2 particles correlated positively with the level of NA titer, suggesting that NAs recognize surface-exposed conformational epitope(s). This finding was supported by depleting antibodies reacting with linear epitopes of PCV2. No significant change was observed in the level of NA titer after antibody depletion. Altogether, these data suggest that NAs target conformational epitope(s) on the surface of PCV2 particles.

## PREFACE

The study described in this thesis was carried out at the Department of Comparative Biology and Experimental Medicine, University of Calgary, Calgary, Alberta, Canada. This study was performed by myself, Narges Nourozieh, under the supervision of Dr. Markus Czub. The data for the neutralizing antibody titer of the swine sera, used in this thesis, was already published in Journal of General Virology.

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Narges Nourozieh

## **Dedication**

*This thesis is gratefully dedicated to:*

*My beloved parents*

*My amazing husband, Hossein*

*My dearest grandparants and aunts*

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## List of Symbols and Abbreviations

Symbol	Definition
μ	Micro (10 <sup>-6</sup> )
°C	Degree celcius
Aa	Amino acid
×g	g force
AASV	American association of swine veterinarians
ABTS	2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
APC	Antigen presenting cell
APS	Ammonium persulfate
BFDV	Psittacine beak and feather disease virus
BI	Before infection
BME	2-Mercaptoethanol
Bp	Base pair
BSA	Bovine serum albumin
Cap	Capsid protein
Cm	Centimeter
CO <sub>2</sub>	Carbon dioxide
CTL	Cytotoxic T-cells
CV	Coefficient of variability
DAPI	4, 6-Diamidino-2-phenylindole
DC	Dendritic cell
dH <sub>2</sub> O	Distilled water
DNA	Deoxyribonucleic acid
DPI	Days post infection
dsDNA	Double-stranded DNA
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
G	Gram
GAG	Glycosaminoglycans

GFP	Green fluorescent protein
H	Hour
His	Histidine
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HS	Heparan sulphate
IFA	Immunofluorescence assay
IFN- $\gamma$	Interferon gamma
IFN- $\gamma$ -SC	Interferon-gamma secreting cells
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMAC	Immobilized metal affinity chromatography
IPMA	Immunoperoxidase monolayer assay
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside
kDa	Kilodalton
LB	Luria broth
Log	Logarithm
M	Molecular weight marker
mAb	Mouse monoclonal antibody
MEM	Minimum essential media
MHC	Major histocompatibility complex
Min	Minute
mL	Milliliter
mRNA	Messenger RNA
N	Nano ( $10^{-9}$ )
NA	Neutralizing antibody
NAs	Neutralizing antibodies
Ni	Nickel
NK	Natural killer cell
NLS	Nuclear localization signal
Nt	Nucleotides
OD	Optical density

ORF	Open reading frame
Ori	Origin of replication
P	Probability occurring by chance
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PCV1	Porcine circovirus 1
PCV2	Porcine circovirus 2
PCVAD	Porcine circovirus associated disease
PDNS	Porcine dermatitis and nephropathy syndrome
PEG	Polyethylene glycol
PFA	Paraformaldehyde
pH	Potential hydrogen
PK-15	Porcine kidney epithelial cell line
PMWS	Postweaning multisystemic wasting syndrome
PPV	Porcine parvovirus
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
PVDF	Polyvinylidene fluoride
rCap	Recombinant capsid protein
rGFP	Recombinant green fluorescent protein
SAMS	Southern Alberta mas spectroscopy
SD	Standard deviation
SDS	Sodium dodecyl sulfite
SE	Standard error
S/P	Sample to positive ratio
SPF	Specific pathogen free
ssDNA	Single-stranded DNA
TCID	Tissue culture infectious dose
TEMED	N,N,N',N'-Tetramethylethylenediamine

TEM	Transmission electron microscopy
T <sub>H</sub>	T-helper
Ub	Ubiquitin
V	Volts
VP	Virus particle
vs.	Versus

## Chapter One: Introduction

Porcine circovirus (PCV) belongs to the *Circoviridae* family (Tischer et al., 1982), which comprises the genera *Gyrovirus*, *Circovirus*, and the recently-proposed *Cyclovirus* (Li et al., 2010). *Gyrovirus* includes chicken anemia virus and human gyrovirus (Sauvage et al., 2011). *Cycloviruses* have been identified in dragonflies (Rosario et al., 2011), stool samples from humans, chimpanzee, and bats (Li et al., 2010; Ge et al., 2011), and in muscle tissue samples from farmed animals (Li et al., 2010). *Circoviruses* are animal viruses that infect mostly birds (Ritchie et al., 1989), fish (Lorincz et al., 2011), dogs (Li et al., 2013), and domestic and wild boars (Ellis et al., 2003). Twelve different viruses have been described so far; these are circoviruses in dog (Li et al., 2013), pigeon (Woods et al., 1993), duck, canary (Phenix et al., 2001), goose (Todd et al., 2001), gull, finch (Todd et al., 2007), starling (Johne et al., 2006), swan (Halami et al., 2008), psittacine beak and feather disease virus (BFDV) (Ritchie et al., 1989), and PCV1 and PCV2 in pigs (Tischer et al., 1986; Harding & Clark, 1997).

### 1.1 Porcine Circoviruses

PCV is the smallest animal virus that replicates in mammalian cells, which was first discovered as a contaminant of the porcine kidney cell line (PK-15) in 1974 (Tischer et al., 1986).

PCV has two distinct genotypes: the non-pathogenic PCV1 and the pathogenic PCV2. PCV2 was first isolated from tissues of postweaning multisystemic wasting syndrome (PMWS)-affected pigs in Canada in 1991 (Harding & Clark, 1997), and then was isolated in the United States and Europe (Allan et al., 1998). PCV2 is a ubiquitous virus in pig herds due to its relative

stability in the environment and its resistance to disinfectants (Welch et al., 2006; O'Dea et al., 2008).

Genomic alignments revealed that PCV2 has 5 genotypes: PCV2a (genotype 2), PCV2b (genotype 1) (Cheung et al., 2007; Gagnon et al., 2007), PCV2c (Dupont et al., 2008), PCV2d, and PCV2e (Wang et al., 2009). PCV2a and PCV2b have been identified in cases globally; while PCV2c has only been detected in samples from Denmark (Dupont et al. 2008). PCV2d and PCV2e, two rare genotypes, have been described only in China (Wang et al., 2009). PCV2a and PCV2b genotypes show approximately 95% identity; however, their genomes have different sizes. PCV2a and PCV2b genomes have 1768 and 1767 nucleotides, respectively (Olvera et al., 2007). The genome sequence of PCV2b at position 1486-1472 is different from PCV2a at position 1487-1473 and this variation allows the differentiation between PCV2a and PCV2b in diagnostic tests. However, it remains unknown whether these differences affect the virulence of the two genotypes (Cheung et al., 2007).

A genotype shift from PCV2a to PCV2b has been reported in several studies. Dupont et al. (2008) analyzed 218 full-length PCV2 genome sequences, and the results demonstrate that a major shift occurred in year 2003. In North America, evidence demonstrates a high prevalence of PCV2b in or after 2005 (Gagnon et al., 2007). Distribution of PCV2 genotypes were analysed by PCR in feces and tissue samples taken from PMWS-affected and non-affected farms in Ireland over a 9 year period from 1997-2006. The results indicated that all four PMWS-affected farms had PCV2a during the period of 1997-2000; three out of four PMWS-affected farms had PCV2b and one farm had PCV2a in 2003. From 2004-2006, all the isolates from the four farms were PCV2b (Allan et al., 2007). A similar study was conducted in the United States, and PCV2 genotypes were compared between animals of diseased and non-diseased animals. Before 2003,

62% of the sequences submitted to the National Center for Biotechnology Information belonged to PCV2a. After 2003, 92% of the sequences were PCV2b (Cheung et al., 2007). Therefore, PCV2b is a more recently emerging virus than PCV2a.

PCV2b was demonstrated to be more virulent than PCV2a under field conditions (Gagnon et al., 2007). In the province of Quebec in 2004, the isolation of PCV2b and high mortality rates in PMWS-affected pigs were reported (Gagnon et al., 2007). Sequencing of PCV2 isolates collected from PMWS and non-PMWS affected farms showed the association between PMWS and PCV2b (Grau-Roma et al., 2008). In contrast to these preceding reports, experimental studies were unable to confirm the difference in virulence between PCV2b and PCV2a (Fort et al., 2008; Opriessnig et al., 2008c; Harding et al., 2010).

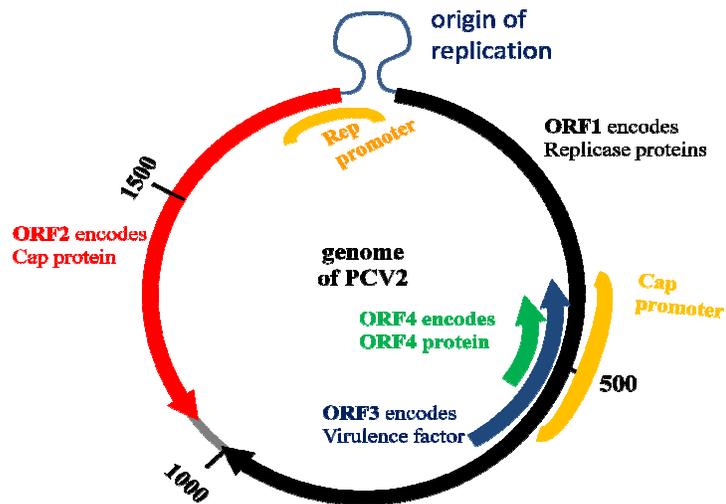
### 1.1.1 PCV2 Genome

PCV is a small non-enveloped virus (17nm) with a single-stranded circular DNA (ssDNA) genome of 1,766-1,768 nucleotides (nt), which is surrounded by an icosahedral capsid protein (Cap) (Tischer et al., 1982). Its genome has been predicted to consist of 11 potential open reading frames (ORFs), but only four have been identified. ORF1 is located on the viral plus-strand and encodes two proteins, Rep [314 amino acids (aa), 35.7 kilodaltons (kDa)] and the spliced Rep' (178 aa); both are necessary for the initiation of PCV2 replication (Mankertz, 2008). ORF2 encodes the Cap protein (233 aa, 27.8 kDa), which is the only known structural protein and the major immunogenic protein of PCV (Mankertz, 2008). The third ORF (ORF3) might be involved in virus-induced apoptosis in PCV2-infected cells (104 aa, 11.9 kDa) (Liu et al., 2005). A recent study has characterized the ORF4 protein (59 aa protein with a 6.5 kDa

molecular mass) (He et al., 2013), and which is believed to prevent virus-induced apoptosis by restricting ORF3 transcription (Gao et al., 2014).

PCV2 has an ambi-sense genome, meaning that Rep and Cap genes are oriented in opposite directions. The origin of replication (Ori) is located between Rep and Cap genes. Ori has a stem-loop structure, where the initiation of replication occurs (Mankertz, 2008). The stem loop comprises a stem, that is a palindrome sequence of 11 base pairs (bp), and a loop of 10-12 nucleotides including a conserved octanucleotide motif sequence (Mankertz, 2008; Faurez et al., 2009). A six-nucleotide sequences called H1, H2, and H3 serves as a binding site for the Rep and Rep' proteins and is located downstream from the stem loop (Mankertz, 2008).

Figure 1–1 shows the schematic diagram of the PCV2 genome.



**Figure 1-1: Schematic diagram of PCV2 genome.**

The PCV2 genome (GenBank accession no.EF39477) is a circular single-stranded DNA genome with ambi-sense orientation of ORF1 and ORF2; ORF3 is located within ORF1. Black arrow: ORF1 gene position at 51-995 nt encodes Rep and Rep' proteins. Red arrow: ORF2 gene position at 1034-1735 nt encodes Cap protein. Blue arrow: ORF3 gene position at 357-671 nt encodes an apoptotic protein. ORF4 gene position at 386-565 nt encodes anti-apoptotic protein. Origin of replication is located between the start of ORF1 and ORF2 and adjacent to the stem loop structure, position 1736-1768 nt and 1-50 nt, where PCV2 replication is initiated. Orange arrows: Rep and Cap promoter are located in at 1627-1759/1-24 nt, and 329-581 nt, respectively.

### 1.1.2 Life Cycle of PCV2

In pigs, the target cells for PCV2 replication are epithelial, endothelial (Steiner et al., 2008), and lymphocytic cells (Yu et al., 2007). In addition, PCV2 infects the PK-15 cells (Meerts et al., 2005), porcine monocytic (Misinzo et al., 2005), and fetal cardiomyocyte cell lines (Meerts et al., 2005). It is possible for PCV2 to infect monocyte/macrophages (Yu et al., 2007) and dendritic cells (DCs) (Vincent et al., 2003; Steiner et al., 2008); however, viral replication appears to be inefficient in these cells. Since DCs have migratory capacity, infection of DCs provides a vehicle by which PCV2 could be trafficked through the host, thereby promoting viral spread (Vincent et al., 2003). The presence of PCV2 in macrophages and DCs is suggested to result from phagocytosis or endocytosis of the infected cells (Vincent et al., 2003; Steiner et al., 2008).

Virus replication has five steps: attachment, entry, replication of viral genome, assembly of virus particles, and egress. The first step of the PCV2 life cycle (attachment) is its binding to host cell receptors. Glycosaminoglycans (GAG) like heparan sulfate (HS) and chondroitin sulfate B are binding receptors for PCV2 (Marsh & Helenius, 2006; Misinzo et al., 2006).

After the target cell takes up PCV2 particles by endocytosis (second step of viral life cycle), PCV2 localizes in endosomes. Release of PCV2 from the endosome requires serine protease and a low (in monocytic 3D4/31 cells) (Misinzo et al., 2005) or neutral pH (in epithelial cells) (Misinzo et al., 2008); how PCV2 transports to the nucleus remains unknown.

The third step of the PCV2 life cycle is viral genome replication. Since PCV2 is a small DNA virus and does not have its own DNA polymerase, the viral genome relies on host DNA polymerases for replication. Therefore, PCV2-ssDNA uses host cell DNA polymerase to make a double-stranded DNA (dsDNA). The dsDNA is transcribed by host RNA polymerase II into

mRNAs. The Rep proteins (Rep and Rep') are synthesized from mRNA in the cytoplasm. The replication proteins contain nuclear localization signals (NLS), which re-import the replication proteins to the nucleus (Mankertz et al., 2008). Binding of the replication proteins to the Ori of the viral genome makes the dsDNA unstable, which generates a nick and a free 3'-OH. Although Rep proteins bind to the dsDNA, the nick is observed in just one of the DNA strands (Mankertz et al., 2008). Subsequently, the DNA polymerase initiates DNA replication from the nick. Rep proteins close the loop by ligating the 5'-phosphate to the 3'-OH of the newly created strand and releasing the positive circular ssDNA. Therefore, Rep proteins are involved in both initiating and terminating of PCV2 genome replication (Mankertz et al., 2008). Further, replication of the PCV2 genome occurs via a rolling circle. After viral genome replication, the Cap protein is produced in the cytoplasm and imported to the nucleus to encapsulate the newly synthesized viral genomes (Faurez et al., 2009). The PCV2-ORF2 encodes an NLS, the first 41 amino acids of Cap, which induces transport of Cap from the cytoplasm to the nucleus (Liu et al., 2001).

The mechanism leading to the fourth and fifth steps of the PCV2 life cycle, virus assembly and egress, have not been studied in detail. PCV2 replication produces massive lymphocyte cell death in infected animals, suggesting that viral egress might occur through cell lysis.

### **1.1.3 PCV2 Transmission**

Both horizontal and vertical routes are important for PCV2 transmission. The presence of PCV2 in secretions and excretions for long periods of time facilitates its horizontal and vertical transmission, which leads to high prevalence of PCV2 infection in swine populations. PCV2-DNA has been isolated from whole blood, feces, oral, and nasal swabs under experimental and

natural conditions (Shibata et al., 2003) and the amount of PCV2-DNA was the same in different sample types over time (Patterson et al., 2011a; Patterson et al., 2011b).

In naturally and experimentally infected pigs, PCV2 is shed in several ways, including oral, nasal, and fecal routes (Shibata et al., 2003; Patterson et al., 2011a; Patterson et al., 2011b). The nasal route might be more effective than other transmission routes (Patterson et al., 2011b). PCV2 shedding has been detected up to 69 days post-infection (DPI) in experimentally-infected pigs (Patterson et al., 2011b), and from 28 until 209 days post farrowing in four naturally-PCV2 infected pigs (Patterson et al., 2011a). Although in these studies, PCV2-DNA was detected by PCR and quantified using real-time quantitative PCR, no information is available about the infectivity of the PCV2-DNA.

PCV2-DNA shedding in semen is important for vertical transmission; the presence of PCV2 has been detected in testes, accessory glands (Opriessnig et al., 2006), and semen of naturally- and experimentally-infected boars (McIntosh et al., 2006; Madson et al., 2008; Schmoll et al., 2008). After experimental intranasal and intramuscular inoculation of 12 boars with PCV2a and PCV2b, viral DNA was detectable first in serum and then in semen for a total of 56 and 81 days, respectively (Madson et al., 2008). PCV2 shedding patterns and duration have been demonstrated in 43 boars, ages 33.9-149.3 weeks under natural conditions. Of the 903 semen samples collected from the boars, 3.3% were positive for PCV2-DNA. The age of shedding was between 35.9 and 71.0 weeks of age, which confirmed a long-term PCV2 shedding in semen (McIntosh et al., 2006). However, whether semen has enough PCV2-DNA to infect fetuses remains uncertain; some evidence indicates that it is rare under natural conditions (Ladekjaer-Mikkelsen et al., 2002; Maldonado et al., 2005).

Subclinical infection with PCV2 has been demonstrated in pregnant sows (Madson & Opriessnig, 2011). Interestingly, experimentally-infected pregnant sows have high numbers of still-born and mummified fetuses (Park et al., 2005). In addition, PCV2 shedding has been detected in milk under experimental (Ha et al, 2009) and field conditions (Shibata et al., 2006). After inoculating PCV2 intranasally to six sows at 93 days of gestation; the virus was detected in milk at the first day of lactation in all animals (Ha et al., 2009).

## **1.2 Porcine Circovirus Associated Diseases**

Pork meat exports play a vital role in the swine industry; for the first half of 2013, 587,000 tonnes of pork were exported from Canada to over 100 countries, with a value of \$1.523 billion ([http://www.susanevans.ca/newsletters/cpi\\_newsletter\\_09-2013/en/main.htm](http://www.susanevans.ca/newsletters/cpi_newsletter_09-2013/en/main.htm)). Data from 2011 indicated that the pork industry was the fourth largest agricultural industry in Canada ([www.statcan.gc.ca](http://www.statcan.gc.ca)). The province of Alberta was recognized as the fourth largest producer of pork, with 1.42 million heads in 2011 ([www.statcan.gc.ca](http://www.statcan.gc.ca)). Therefore, herd health is always an important topic for pork industries. Among all swine viruses, PCV2 infection is one of the biggest problems in pig herds. The mortality rate from PCV2 infection is 4.2-22.5% (Horlen et al., 2008), but can be accelerate to more than 50% in some affected herds (D'Allaire et al., 2007). The data from 185 farms in the USA and 4147 samples showed that 82.6% and 78.8% of sera were positive for PCV2 by PCR and ELISA, respectively (Puvanendiran et al., 2011).

PCV2 infection has been associated with several syndromes including porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, porcine respiratory disease complex (PRDC), granulomatous enteritis, necrotizing lymphadenitis, exudative epidermitis, congenital tremor, and PMWS. In March 2006, the American Association of Swine Veterinarians (AASV)

recommended a term that describes different diseases attributed to PCV2: Porcine Circovirus Associated Diseases (PCVAD) (<http://www.aasv.org/>).

PMWS is the most serious and significant clinical presentation of PCV2 infection and leads to several direct and indirect losses (Opriessnig et al., 2007).

### 1.2.1 Postweaning Multisystemic Wasting Syndrome (PMWS)

PCV2 has been identified as the causative agent of PMWS, which mainly affects 7 to 15 week-old pigs (Harding et al., 1998). Clinical signs attributed to PMWS are weight loss, dyspnea, enlarged lymph nodes, watery diarrhea, pallor, jaundice, and general immune-suppression due to lymphoid depletion (Segales et al., 2004). Figure 1-2 shows a picture of a pig with PMWS.



**Figure 1-2: The pig highlighted by the red arrow exhibits weight loss typical of PMWS in contrast to an un-diseased age-matched pigs (picture from Czub lab photo collection).**

Depletion of lymphocytes, the hallmark of PMWS in PCV2-infected pigs, usually occurs in lymphoid organs such as lymph nodes, tonsils, Peyer's patches, and spleen (Rosell et al., 1999). The depletion affects natural killer cells (NK), B-cells, and T-cells (Shibahara et al., 2000; Nielsen et al., 2003). T-cell depletion is observed in both T-helpers ( $T_H$ ) and cytotoxic T-cells

(CTL) in PMWS-affected animals, but not in healthy. A decrease in leukocyte level has been reported 7 to 10 DPI in specific pathogen-free (SPF) pigs infected with PCV2 alone or PCV2/porcine parvovirus (PPV) dual infection (Steiner et al., 2008).

Apoptosis was first suggested as a reason for lymphoid depletion (Shibahara et al., 2000); however, two papers published in 2004 did not find any association between apoptosis and lymphoid depletion (Mandrioli et al., 2004; Resendes et al., 2004). The lack of T- and B-cells proliferation was therefore proposed as a contribution factor to lymphoid depletion (Mandrioli et al., 2004). Severe T-cells depletion is also observed in a related virus of the same family, the chicken anemia virus (Adair, 2000). Lymphoid depletion may induce immune system deficiency, which enables co-infection with other viral and bacterial pathogens.

### 1.2.2 Co-factors Contributing to PMWS

Although PCV2 is the causative agent of PMWS, not all PCV2-infected pigs develop disease (Ladekjaer-Mikkelsen et al., 2002). Most studies consider PCV2 as an essential but not sufficient agent to cause PMWS (Krakowka et al., 2000; Kim et al., 2001). Environmental factors, immunostimulatory compounds such as vaccination, immunosuppression, and co-infection with other pathogens are co-factors for developing PMWS disease. Co-infection with other swine pathogens including porcine reproductive and respiratory syndrome virus (PRRSV) (Rovira et al., 2002), PPV (Ellis et al., 1999), *Mycoplasma hyopneumonia* (Opriessnig et al., 2004a), *Actinobacillus pleuropneumoniae*, *Pasturella multocida*, and *Streptococcus suis* (Opriessnig et al., 2007) have been identified contributing factors in PMWS development. PRRSV, *Mycoplasma hyopneumonia*, and simian immunodeficiency virus are reported to be the most common co-infection agents with PCV2 (Dorr et al., 2007).

### 1.2.3 Control and Prevention of PMWS

PCV2 targets the animal immune defense mechanisms, leading to PMWS. Importantly, no effective treatments exist for PMWS-affected pigs; therefore, the control and prevention of PCV2 infection is vital for swine herds.

PCV2 infection can be partially controlled by good hygiene/husbandry management and vaccination against PCV2 (Horlen et al., 2008; Martelli et al., 2011). A list of management practices was proposed by Madec et al. (2001) in a 20-point plan to minimize PCV2 infection and to control other pathogens. The keys of that plan are as follows: a) reducing contact between piglets from different litters; b) reducing stressors on the farm; c) providing proper nutrition and d) providing good hygiene. Good hygiene on the farm requires the use of appropriate disinfectants. PCV2 is stable in the environment but proper disinfectant can render it non-infectious (Martin et al., 2008; Kim et al., 2009). Efficacy of different disinfectants was tested *in vitro*, and Virkon S, Clorox bleach, and sodium hydroxide are recognized as the most effective virucidal agents against PCV2 (Royer et al., 2001; Poonsuk et al., 2014). In addition to implementing the 20-point plan, vaccination against PCV2 is important to control PCV2 infection.

### 1.3 Adaptive Immune Response towards PCV2

Both humoral and cell-mediated immunity are essential for full protection against PCV2 infection (Fort et al., 2009a; Fort et al., 2009b; Martelli et al., 2011). Humoral response is an immunity mediated by antibodies, which are present in blood and extracellular spaces. Those antibodies that bind to the pathogens or their products and block the access of pathogens to the cells are referred to us as neutralizing antibodies (NAs). The neutralized pathogens typically are

phagocytosed by macrophages (Murphy, 2011). Antibodies cannot access intracellular pathogens; the destruction of intracellular pathogens is the function of another branch of adaptive immunity called cell-mediated immune responses. Cell-mediated immunity occurs through two main classes of cells, CTL ( $CD8^+$ ) and  $T_H$  ( $CD4^+$ ) cells. Naïve CTL cells have  $CD8^+$  transmembrane glycoprotein and are activated by the antigen presenting cells (APC) and cytokines produced by  $T_H$ . APC include macrophages, DCs, and B-lymphocytes (Murphy, 2011; Owen et al., 2013).  $T_H$  cells are involved in the activation of both B-cells (humoral immunity) and CTL cells (cellular immunity). After the initial activation by the antigen, naïve  $T_H$  cells can differentiate into effector T-cells such as  $T_{H1}$  and  $T_{H2}$ .  $T_{H1}$  cells are directed against intracellular pathogens (viruses and intracellular bacteria) and leads mainly to cell-mediated immunity.  $T_{H2}$  cells are directed against extracellular pathogens (parasites) by inducing humoral response (Murphy, 2011).

A critical cytokine for cell-mediated immunity activation against viral infection is gamma interferon ( $IFN-\gamma$ ) (Steiner et al., 2009).  $IFN-\gamma$  inhibits viral replication, activates macrophages, and increases the expression of major histocompatibility complex (MHC) class 1 molecules in infected cells (Murphy, 2011). High expression of MHC I molecules increases the chance that infected cells will be recognized by CTL cells (Murphy, 2011).  $IFN-\gamma$  enhances the differentiation of naïve  $T_H$  cells into  $T_{H1}$  (Steiner et al., 2009), which release different cytokines ( $IL-2$ ,  $IFN-\gamma$ ,  $TNF\beta$ ) leading to the induction of cellular immunity (Murphy, 2011).  $IFN-\gamma$  is produced by a wide range of innate immune cells, B-cells,  $T_{H1}$ , and CTL of adaptive immune cells (Harris et al., 2000; Frucht et al., 2001). Low level of  $IFN-\gamma$  has been reported in peripheral blood mononuclear cells (PBMC) (Darwich et al., 2003a) and in lymphoid tissues from pigs with PMWS (Darwich et al., 2003b).

### 1.3.1 Cell-Mediated Immune Response towards PCV2

Pigs with low levels of antibody when challenged with PCV2 still elicit protective immunity, this is an indication for the presence of cellular immunity (Fenaux et al., 2004a). The role of cellular immunity in providing protection against PMWS has been elucidated by measuring the number of PCV2-specific IFN- $\gamma$  secreting cells (IFN- $\gamma$ -SC) (Fort et al., 2009a; Fort et al., 2009b; Steiner et al., 2009; Martelli et al., 2011). IFN- $\gamma$ -SC develop between 14 and 21 DPI in subclinically infected pigs (Fort et al., 2009a). A negative correlation exists between viral load and IFN- $\gamma$ -SC, suggesting the role of cellular immunity following PCV2 infection (Fort et al., 2009a). Since treatment with anti-CD8<sup>+</sup> and anti-CD4<sup>+</sup> antibodies reduce the number of IFN- $\gamma$ -SC, it was suggested that both CD4<sup>+</sup> and CD8<sup>+</sup> cells play a role in PCV2 infection (Steiner et al., 2009).

### 1.3.2 Humoral Immune Response towards PCV2

B-lymphocytes produce antibodies, which are the main component of the humoral immune response. B-cells are activated by cytokines and direct interaction between B-cell receptor and antigen (Owen et al., 2013). In response to an infection, immunoglobulin (Ig) M antibody is the first antibody produced. It is detectable 7-14 days after PCV2 infection, reaches a peak at 21 days after infection, and persists until 49 days under experimental conditions (Fort et al., 2007). Activated B-cells undergo isotype switching and start producing other antibody isotypes such as IgG antibodies due to down-regulation of IgM production (Owen et al., 2013). Under experimental conditions, anti-PCV2 IgG antibodies are detected around 14 and 28 DPI (Fort et al., 2007) and persist at least 140 DPI (Opriessnig et al., 2010b). Under field conditions, anti-PCV2 IgG antibodies are detected between 7-15 weeks of age when the maternal antibodies

decline (Rodriguez-Arriola et al., 2002). There are PMWS-affected animals with low level of NA titers, while their PCV2 antibody titer is high (Fort et al., 2007; Fort et al., 2008), suggesting that NA but not total antibody is an indicator of protection from PMWS (Trible et al., 2011).

### ***1.3.2.1 Effect of Neutralizing Antibody on PCV2 Infection***

NA binds to and neutralizes the virus and protects against the infection of new cells, preventing further spread of the virus in the body (Meerts et al., 2006; Reading & Dimmock, 2007). Several mechanisms are involved in virus neutralization, which includes a virus aggregation, interfering with virus-receptor interaction and attachment, and other unknown mechanisms (Klasse & Sattentau, 2002; Reading & Dimmock, 2007). Blocking the virus attachment to the cells is important for naked and enveloped virus neutralization (Klasse & Sattentau, 2002). For example, blocking attachment of vaccinia virus to target cells (Law & Smith, 2001), or prevent attachment of the human immunodeficiency virus (HIV) to CD4<sup>+</sup> cells by binding of NA to an outer envelope protein of HIV (gp120) (Ugolini et al., 1997).

The average of NA titer in PMWS pigs is lower than in non-PMWS and PCV2-negative animals (Fort et al., 2007). High PCV2 replication and the absence of NA correlate with the occurrence of PMWS under both experimental (Meerts et al., 2006) and field conditions (Fort et al., 2007). The kinetics of NA shows that all experimentally-inoculated piglets are positive for NA at 0 DPI due to the maternal antibody transfer. The NA steadily decreases over time and then increases at 14 to 21 DPI (Fort et al., 2007). NA is principally IgG rather than IgM (Fort et al., 2007), IgM antibody is also capable of neutralizing the PCV2 infection (Meerts et al., 2006). In a mouse model, NA is associated mainly with IgG2a isotype rather than IgG1 (Shen et al., 2008).

### ***1.3.2.2 Effect of Maternal Antibody on PCV2 Infection***

Passively acquired antibodies generally play a crucial role in protecting newborn piglets from virus infection (Ward et al., 1996; Suradhat & Damrongwatanapokin, 2003). The presence of maternal antibodies in newborns reduces the severity of clinical disease following group A porcine rotavirus infection (Ward et al., 1996). The majority of newborns piglets have variable levels of PCV2 maternal antibodies due to the ubiquitous nature of the virus. These maternally-derived antibodies have been demonstrated to protect piglets from PMWS (Ostanello et al., 2005). A low level of viremia against experimental PCV2 infection occurs in piglets with maternal antibodies; however, the infection is not completely prevented (McKeown et al., 2005; Opriessnig et al., 2008b). Depending on the initial concentration, maternal antibodies decayed over a period of time (Opriessnig et al., 2004b). Longer period of passive immunity in piglets made them resistant to PCV2 infection and less likely to exhibit PMWS symptoms.

## **1.4 PCV2 Vaccination**

The first commercial vaccine against PCV2 was used in 2004 in France and Germany, thirteen years after the first report of PMWS in Canada (Charreyre et al., 2005). In North America, PCV2 vaccines have been available since 2006. PCV2 vaccines are efficacious in reducing morbidity, mortality, improving growth performance, and shortening the time period to market (Horlen et al., 2008; Kixmoller et al., 2008; Lyoo et al., 2011; Martelli et al., 2011). A study from 21 different farms including 35,000 pigs showed that the use of PCV2 vaccine reduced the mortality by 77.5% as compared to the non-vaccinated animals (de Grau et al., 2007).

PCV2-Cap protein is the most immunogenic protein of PCV2 and the main target for vaccine development (Nawagitgul et al., 2000). There are five commercially available vaccines, including Circovac<sup>®</sup>(Merial), Foster<sup>™</sup> formerly known as Suvaxyn<sup>®</sup>(Fort Dodge Zoetis), Circoflex<sup>®</sup>(Boehringer Ingelheim), Porcilis<sup>®</sup>, and Circumvent<sup>®</sup>(Intervet-Schering). The first vaccine was Circovac, which was developed based on an inactivated PCV2a. Circovac is the only vaccine that can be used in weaning-age piglets and breeding dams. Foster is based on an inactivated chimeric virus and contains PCV2-Cap gene cloned into the backbone of PCV1 (Fenaux et al., 2004a). The three other vaccines are based on PCV2-Cap gene expressed in a baculovirus vector system using an insect cell line (Beach & Meng, 2012). All these commercial available vaccines are used in animals 3 to 4 weeks of age (Beach & Meng, 2012). They are based on the PCV2a genotype, which provides a cross-protective immunity against PCV2b in both sows and piglets (Fort et al., 2008; Opriessnig et al., 2008c).

#### **1.4.1 Vaccination-Induced Adaptive Immunity**

Vaccination against PCV2 induces both humoral and cellular immunity (Fort et al., 2008; Fort et al., 2009b). In terms of humoral immunity, single-dose vaccine in sows is sufficient to induce a high titer of antibodies, which are passively transferred to piglets (Fraile et al., 2012). Seroconversion to PCV2 following vaccination is observed at 21 days (Fort et al., 2009a; Fort et al., 2009b). Several experimental studies demonstrated that vaccination against PCV2 induces NA (Opriessnig et al., 2008a; Fort et al., 2009b; Martelli et al., 2011). Higher NA is observed after chimeric PCV1/2 vaccination as compared to subunit ones (Opriessnig et al., 2009). However, the NA titer does not differ between subunit and inactivated vaccines (Opriessnig et

al., 2010a). Moreover, two-dose vaccinations induce a significantly higher NA titer than a single-dose (Fort et al., 2009b).

Development of cellular immunity against PCV2 after a single vaccination occurs under experimental (Fort et al., 2009a; Fort et al., 2009b), and field conditions (Martelli et al., 2011). Pigs vaccinated with PCV2 could increase in CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> memory T-cells; CD8 $\alpha$  is a cell surface glycoprotein that can be found on activated, antigen experience cytotoxic and memory T-cells, but not naïve T-cells (Ferrari et al., 2014). In addition, vaccination of experimentally-infected piglets induced an increase in the number of CD3<sup>+</sup>, a T-cell co-receptor, and CD4<sup>+</sup> cells (Seo et al., 2012).

#### **1.4.2 Problems with Current PCV2 Vaccines**

In general, vaccines act by either preventing infection or preventing disease. While the prevention of infection is mediated by humoral response, cellular immunity (CD8<sup>+</sup> cells) kills the infected cells and prevents further spread of the infection (Seder & Hill, 2000). Thus, cellular immunity does not directly prevent viral infection, but depending on how efficient CD8<sup>+</sup> cells are in killing the infected cells, the cellular immunity has crucial rule in limiting or preventing disease (Seder & Hill, 2000; Plotkin, 2001). For example, small pox vaccine prevents from infection by humoral immunity and protects against disseminated disease by both humoral and cellular responses mediated by CD4<sup>+</sup> and CD8<sup>+</sup> cells (Slifka, 2004).

All current PCV2 vaccines are killed or recombinant subunit vaccines that generate the humoral immunity (Opriessnig et al., 2008a; Fort et al., 2009b; Martelli et al., 2011) and prevent cells from viral infection. In general, live attenuated vaccines elicit superior protection compared to the other type of vaccines by stimulating both cellular and humoral immunity. It is possible to

attenuate the wildtype PCV2 through serial passages in cell culture. There is no commercial modified live PCV2 vaccine in the market due to potential reversion of live-attenuated to a pathogenic phenotype. It was shown that after 120 passages, there were two nucleotides mutations in the capsid gene, which enhanced the growth ability of PCV2 *in vitro* and attenuated the virus *in vivo* (Fenaux et al., 2004b).

While various vaccine efficacy studies under controlled experimental conditions have clearly proven to decrease PCV2 viral load in serum (Martelli et al., 2011; Seo et al., 2014), PCV2 viral load was neither prevented nor reduced in swine sera collected from vaccinated farmed pigs (Gerber et al., 2012; Solis Worsfold et al., 2015). Therefore, infected animals still shed the virus without showing disease. It remains unclear how vaccination elicits immune responses and controls the PMWS without achieving sterilizing immunity. This discrepancy between experimental and field studies could be due to higher PCV2 infectivity and/or multiple exposures to PCV2, and higher level of immunosuppression in farmed pigs than in experimentally infected pigs. A vaccine which can induce strong cellular and humoral immunity might help PCV2 clearance in infected animals under natural condition. Although current PCV2a-genotype based vaccines are effective, future vaccines should be based on PCV2b genotype due to high prevalence of PCV2b in the field.

### **1.5 Capsid Protein of PCV2**

ORF2 encodes the 233 aa Cap protein, which is a major immunogenic protein of PCV2 and the target for vaccine development (Nawagitgul et al., 2000). The ORF2 genes of certain PCV2 isolates share only 90% Cap sequence identity, indicating that Cap protein is variable (Fenaux et al., 2000). Although the PCV2-Cap protein is variable, the N-terminal 41 amino acids

are conserved among all PCVs. This sequence is rich in arginine and contains NLS (Liu et al., 2001). Importantly, the NLS has rare codons for *Escherichia coli* (*E. coli*), which may prevent proper Cap protein expression in a prokaryotic expression system. Most studies have used a truncated Cap without the NLS to generate recombinant proteins (Wu et al., 2008; Shang et al., 2009; Sun et al., 2010).

The Cap protein contains two kinds of antigenic epitopes that are recognized by the immune system: linear and conformational epitopes. A linear epitope is a sequence of amino acids placed next to each other. A conformational epitope is a sequence of amino acids located in different regions of the protein, but close together in three-dimensional space. These amino acids are brought together by folding the protein and form a native structure (Owen et al., 2013). The linear and conformational epitopes are recognized by different immune cells. T-cells usually recognize linear epitopes, which are presented by MHC molecules. B-cells recognize complete, small compounds or part of large molecules; thereby, they can recognize both linear and conformational epitopes (Owen et al., 2013).

Defining the type of antigenic epitopes and specifically the neutralizing epitope helps researchers to improve the vaccine efficacy. Detailed information on epitope dominance and neutralizing epitope of PCV2-Cap, as documented in the published literature, are summarized in the next section.

### **1.5.1 Linear Epitopes of PCV2-Cap Protein**

Several antigenic regions have been identified in PCV2-Cap protein using swine polyclonal antibodies (Mahe et al., 2000; Truong et al., 2001; Tribble et al., 2011) or mouse monoclonal antibodies (mAbs) (Lekcharoensuk et al., 2004; Shang et al., 2009). Although

multiple regions on PCV2-Cap are identified as the immunoreactive regions in the literature, none of the studies have considered testing of these regions as neutralization epitopes.

The pepscan analysis of overlapping fragments of the entire Cap protein showed that there are five linear immunoreactive areas on Cap: residues 25-43, 69-83, 117-131, 169-183, and 193-207 (Mahe et al., 2000). In another study, a subset of sera which were taken from experimentally-infected pigs over a period of 11 weeks was used to assess the antibody response towards the epitope 117-131. All sera had detectable level of antibodies at the end of the experiments (Truong et al., 2001). In addition, 26% of swine sera collected from PMWS-free herds and 45% of the sera from PMWS-affected herds reacted with the epitope 117-131. Based on these results, it has been suggested that the epitope 117-131 can be used as a serological marker for PCV2 infection (Truong et al., 2001). Reactivity of swine sera, collected from SPF pigs or field pigs, with different recombinant Cap (rCap) subunits demonstrates that subunit 101-150 has the highest specificity and sensitivity in an indirect ELISA (Wu et al., 2008). More recently, residues 169-180 in the Cap protein have been shown to be highly immunogenic in experimentally-infected animals, and weakly immunogenic in vaccinated animals (Trible & Rowland, 2012).

Four linear B-cell epitopes (residues 156-162, 175-192, 195-202, and 231-233) have been identified using mAbs (Shang et al., 2009). Furthermore, the alanine scanning mutagenesis has helped identifying the 173-tyrosine, 174-phenylalanine, 175-glutamine, 179-lysine (Trible et al., 2011), as well as 156-tyrosine and 233-proline (Shang et al., 2009) as the individual amino acids that contribute to antibody recognition.

Figure 1–3 summarizes the location of immunoreactive linear epitopes in PCV2-Cap from previous studies.

```

1  mtyprrrryrr rhrprshlg qilrrrpwlv hprhryrwr r kngifntrls rtfgytikrt
61  tvrtpswavd mmrfnindfl ppgggsnprs vpfeyyrirk vkvefwpcsp itqgdrqvgvs
121 savilddnfv tkataltydp yvnyssrhti tqpfsyhsry ftpkpvldst idyfqpnkr
181 nqlwlrqlta gnyvchvgigt ahensiydqe ynirvtmyvq frefnlkdp lnp

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**Figure 1-3. Location of immunoreactive linear epitopes in PCV2-Cap** (strain 05-32650, GenBank accession no. EF394779).

Underlined regions show the location of the immunoreactive regions described by Mahe et al. (2000) using pepscan analysis. The red box shows the location of immunodominant peptides described by Truong et al. (2001). The blue box shows the synthetic peptide specific for PCV2 (Ha et al., 2005). The bold region has the highest sensitivity and specificity in indirect ELISA (Wu et al., 2008). The green boxes identify the receptor binding regions of mAbs described by Shang et al. (2009).

### 1.5.2 Conformational Epitopes of PCV2-Cap Protein

Several conformational epitopes within PCV2-Cap have been identified using mAbs (Lekcharoensuk et al., 2004; Shang et al., 2009) or swine polyclonal antibodies (Trible et al., 2011; Trible et al., 2012).

To map the conformational epitopes of PCV2-Cap protein, a set of PCV1/PCV2 chimeras and 7 mAbs against the PCV2-Cap protein were generated. The immunofluorescence assay (IFA) was used to detect the reactivity of chimeras by the mouse mAbs in transfected PK-15 cells. Five overlapping conformational epitopes have been identified within the amino acid residues 47-63, 165-200, and 230-233 (Lekcharoensuk et al., 2004). Moreover, mAbs with neutralizing activity to PCV2 react with amino acid residues 47-57 and 165-200, which means that these residues are likely involved in forming the conformational neutralizing epitope (Lekcharoensuk et al., 2004). Similarly, PK-15 cells transfected with the truncated Cap gene fragments and stained with mAbs demonstrated that the residues 231-233 and 1-60 participate in the formation of neutralizing conformational epitope (Shang et al., 2009).

Single mutations in the PCV2-Cap have also been shown to change the neutralizing phenotype of the virus (Huang et al., 2011; Saha et al., 2012). One amino acid that determines a conformational neutralizing epitope was recognized at the position 59 in the Cap protein of PCV2a using mAb. It was also shown that a substitution of alanine by arginine at position A59R inhibits the immunoreactivity of PCV2a with mAb (Huang et al., 2011). In addition, a double mutation at positions E191R and T131P results in the loss of neutralization; however, amino acids mutation at positions A190T and P151T lead to the gain of neutralization (Saha et al., 2012). The amino acids at positions R59A and A60T in the Cap protein of PCV2 participate in the formation of conformational neutralizing epitopes (Liu et al., 2013).

Previous studies have used mAbs to determine the neutralizing conformational epitope of PCV2-Cap; however, polyclonal antibodies present in swine sera may recognize a different conformational epitope. Only two studies have indirectly investigated the presence of a neutralizing conformational epitope in Cap protein, under experimental conditions (Trible et al., 2011; Tribble et al., 2012). In the first study, the authors proposed that the neutralizing antibodies, present in the serum of vaccinated animals, react towards the truncated Cap (amino acids 43-233) (Trible et al., 2011). The second study demonstrated that animals immunized with a commercial vaccine have higher NA titers than animals immunized with monomeric Cap (Trible et al., 2012). These studies were based on the assumption that Cap residues 43-233 and PCV2 vaccines represent the conformational epitope. However, the precise location of a neutralizing conformational epitope on Cap residues 43-233 or PCV2 vaccine was not demonstrated in these studies.

## 1.6 Hypothesis and Objectives

Characterization of PCV2 neutralizing epitopes is important for PCV2 vaccines improvement. Although the mouse mAbs recognize a neutralizing conformational epitope of PCV2-Cap (Lekcharoensuk et al., 2004), it is still not clear whether the same neutralizing epitope is recognized by serum antibodies of naturally-infected pigs. Thus, more research is needed to determine whether the neutralizing epitope(s) is a conformational or linear epitope.

**Hypothesis:** Under field conditions, protective NAs recognize conformational epitope(s) that exist only on the surface of PCV2 particles.

The main goal of this thesis is to determine whether NAs against PCV2 recognize conformational epitope(s) on the surface of virus particles using sera collected from farmed pigs. In this thesis, several questions will be answered in order to reach the goal:

**Question 1:** Do antibodies in swine sera collected from farmed pigs react with PCV2-Cap linear and/or conformational epitopes?

**Question 2:** Do antibodies that react with PCV2-Cap linear epitopes have virus neutralizing activity?

**Question 3:** Do antibodies that react with PCV2-Cap conformational epitope(s) have virus neutralizing activity?

## **Chapter Two: Materials and Methods**

### **2.1 Serum Samples**

One hundred sixty serum samples were collected from naturally-infected pigs, which were randomly selected from 13 different farms in Alberta. These animals were categorized into two groups according to their vaccination status, vaccinated (n=80) and non-vaccinated (n=80). No information is available about the clinical status of these animals. Negative control sera were obtained by bleeding four newborn piglets immediately after birth. The piglets did not consume colostrum and consequently did not have maternal antibodies against PCV2. All sera were heat-inactivated at 56°C for 1h and kept at -20°C until used.

### **2.2 Cell Culture**

PK-15 cell line was kindly provided by Dr. Carl A. Gagnon (Faculty of Veterinary Medicine, University of Montreal). PK-15, free of PCV, was propagated in Eagle's Minimum Essential Medium (MEM) (Sigma- Aldrich ) supplemented with 1% penicillin-streptomycin solution (Sigma-Aldrich), 5% heat-inactivated fetal bovine serum (FBS) (Gibco), 1% essential amino acids (Sigma- Aldrich), and 1% sodium pyruvate (Gibco). PK-15 cells were maintained at 37°C in the presence of 5% CO<sub>2</sub> to obtain a confluent monolayer cells. To subculture PK-15 cells, the monolayer of cells was incubated with trypsin-EDTA (0.25%) (Invitrogen) for 5 min at 37°C to promote cell detachment. Five mL of complete growth medium were added to the detached cells, and cells were aspirated by a gentle pipetting. Centrifugation was performed at 400×g for 5 min and the pellet was re-suspended in appropriate volume of MEM media with a sub-cultivation ratio of 1/2 to 1/4.

To assess the cell viability, 10  $\mu$ l of cell suspension were diluted 1/10 in trypan blue dye and loaded into the hemacytometer (Propper). Unstained (viable) cells were counted under a light microscope in the four corner squares, and total cells/mL was determined using the following formula:

$$\text{Total cells/mL} = \text{Total cells counted} \times (\text{dilution factor} / \text{number of squares}) \times 10^4$$

## **2.3 Production and Purification of PCV2 Particles**

To test the reactivity of swine sera to the PCV2 epitopes, PCV2 infectious particles were produced, concentrated with polyethylene glycol (PEG) (Promega) and purified through sucrose gradient (Sigma-Aldrich). The procedure is explained in more detail in the following sections.

### **2.3.1 Production and Propagation of PCV2 Particles**

PCV2 infectious particles were produced using a reverse genetic, which was introduced for the first time by Fenaux et al. (2002). This approach eliminates the problems associated with the presence of other pig agents in tissue homogenate in animal studies.

Similar approach was adopted by our group with some modifications. Briefly, two copies of the full-length PCV2b genome (Strain 05-32650, GenBank accession # EF39477) were cloned into the pJ201 vector to make a tandem construct. Competent *E.coli* cells were transformed with a PCV2 tandem construct. The DNA was extracted from the bacterial culture using the plasmid Maxi Kit (Qiagen) and used to transfect PK-15 cells using Lipofectamine 2000 (Invitrogen). The transfected PK-15 cells were collected after the fourth passage and subjected to freeze/thaw three times. The cell lysate was sonicated in an ice bath at 80% power for 1 min, and then centrifuged at 500 $\times$ g for 5 min. To propagate PCV2, PK15 cells were subjected to four rounds of infection.

The supernatant was collected, titrated, aliquoted, and kept at -80°C for further use. The 50% tissue culture infectious dose (TCID<sub>50</sub>) per mL was calculated as  $6.3 \times 10^4$ . The virus propagation and titration was done by other members of Dr. Markus Czub's lab.

### **2.3.2 Purification of PCV2 Particles**

The crude virus was precipitated using 10% PEG 8000 and 0.5 M sodium chloride (NaCl), and subjected to a constant gentle agitation at 4°C overnight. The precipitated virus was collected by centrifugation at 15000×g for 30 min, then the pellet was dissolved in TNE buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA). The pellet was layered on top of 30% sucrose (dissolved in TNE buffer) and centrifuged at 185000×g for 2 h, 50 min. The pellet containing the PCV2 virus was resuspended in TNE buffer. For further purification, the virus suspension was loaded onto the discontinuous sucrose gradient (30% and 65% dissolved in TNE buffer) and centrifuged at 185000×g for 2 h, 50 min. A milky white band in the interface of 30% and 65% sucrose was collected gently. As a negative control, non-infected PK-15 cells were prepared using exactly the same procedure as the PCV2 particle purification.

### **2.3.3 Dialysis of PCV2 Particles**

The purified PCV2 was subjected to dialysis to remove any sucrose residual that may interfere with the downstream experiment. Spectra/Por dialysis membrane (50 kDa molecular weight cut-off) was cut into 10 cm tubes, where 1 mL of purified virus was placed. The tubes were kept in 1000-fold volume of sterile phosphate-buffered saline (PBS) 1X at pH 7.4 over night at 4°C under slow stirring (Fisher Scientific Isotemp ceramic stirrer). The PBS was

changed twice. The purified virus was collected after dialysis and analyzed for the presence of PCV2 particles. The same procedure was used for non-infected PK-15 cell lysates.

## **2.4 Analysis of the Purified PCV2 Particles**

Multiple assays were used to test the presence of PCV2 particles including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, and transmission electron microscopy (TEM). The Reed Muench equation was used to measure the titer of PCV2 (Reed & Muench, 1938).

### **2.4.1 SDS-PAGE and Western Blot**

In order to load the same amount of antigen in the gel, protein concentrations of both purified PCV2 and PK-15 cell lysates were determined using Bio-Rad's protein assay before proceeding with SDS-PAGE (Appendix 4). To perform the SDS-PAGE (Appendix 5), the purified PCV2 and PK-15 lysates were mixed with an equal volume of 2X SDS sample buffer, which contains the SDS (Sigma-Aldrich) and 2-Mercaptoethanol (BME) (Sigma-Aldrich). The mixture was heated for 10 min at 95°C and was subjected to electrophoresis in a 12% denaturing polyacrylamide gel for 2 h at 100 V. Gels were either stained with Coomassie blue (Appendix 6) or transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare) via a Bio-Rad semi-dry transfer apparatus for 50 min at 20 V (Appendix 7). The PVDF membrane was stained with Ponceau (Sigma-Aldrich) to confirm the success of protein transfer. After washing the membrane with distilled water, 5% skim milk diluted in PBS with 0.25% Tween-20 (PBST-0.25%) (blocking buffer) was used for blocking step in order to prevent any non-specific binding of the antibodies to the membrane. The membrane was incubated with the rabbit anti-Cap protein

(diluted 1/2000 in blocking buffer) overnight at 4°C or for 2 h at room temperature, then washed with PBST-0.25% for 10 min three times. After washing step, the membrane was incubated with the goat anti-rabbit conjugated with horseradish peroxidase (HRP) (Sigma-Aldrich) (diluted 1/10000 in blocking buffer) for 1 h at room temperature. The membrane was washed with PBST three times, and incubated with chemiluminescence (GE Healthcare) (Appendix 8). Images were viewed and analyzed using Bio-Rad VersaDoc 5000 MP imaging system and Quantity-one software (Bio-Rad).

#### **2.4.2 Transmission Electron Microscopy (TEM) of the Purified PCV2 Particles**

The PCV2 particles morphology and size were confirmed by TEM. TEM images were produced by the microscopy and imaging facility at the University of Calgary. Briefly, purified PCV2 was absorbed to the carbon-coated TEM copper grid for 5 min. The grid was collected by forceps and placed on filter paper. The dried grid was placed on the drop of uranyl acetate solution for 30 seconds, and dried by filter paper to absorb the excess solution. Samples were examined using a Hitachi H7650 TEM at 80 kV.

#### **2.4.3 Titration of the Purified PCV2 Particles**

In a 96-well tissue culture plate,  $10^4$  cells/well were seeded and incubated at 37°C with 5% CO<sub>2</sub> to get 70% confluent cells. The PCV2 was added at different dilutions ( $10^{-1}$  to  $10^{-7}$ ) in five replications to 70% confluent PK-15 cells. Cells were incubated with PCV2 for 90 min at 37°C. PCV2 was removed and fresh media was added, followed by incubating the plate for 48 h at 37°C. The infected PK-15 cells were fixed for 30 min with 4% paraformaldehyde (PFA) (Sigma-Aldrich) at room temperature. The PFA was removed and cells were incubated with permeabilization buffer (0.1% bovine serum albumin (BSA) (Sigma-Aldrich) and 1% saponin

(Sigma-Aldrich) diluted in PBS), for 30 min at room temperature. Plate was incubated with a rabbit polyclonal anti-Cap antibody (diluted 1/500 in the permeabilization buffer) for 90 min at 37°C. After washing the plate three times with PBST-0.1%, a secondary antibody goat anti-rabbit Alexa Fluor 568 (Invitrogen) (diluted 1/400 in the permeabilization buffer), was added to each well and the plate was incubated for 1 h at 37°C. The plate was washed three times with PBST-0.1% and 300nM 4', 6-diamidino-2-phenylindol (DAPI) (Invitrogen) in PBS was added to the plate. The cells were incubated for 5 min in the dark at room temperature to stain the nucleus of the cells. After three washes with PBS, the number of infected cells in each dilution were counted using Olympus 1X51 inverted fluorescence microscope. The TCID<sub>50</sub> of the purified PCV2 was calculated using Reed Muench equation (Reed & Muench, 1938).

## **2.5 Detection of Antibodies Reacting with Denatured PCV2 Particles**

To assess the reactivity of 160 sera with PCV2 linear epitopes, SDS-PAGE and Western blot were used. The reactivity of sera with PCV2 linear epitopes was further quantified with densitometry assay.

### **2.5.1 SDS-PAGE and Western Blot**

Before running SDS-PAGE and Western blots, the protein concentration of PCV2 particles was quantified by Bradford assay (Bio-Rad).

PCV2 particles and PK-15 lysates were mixed with 2X SDS and BME, and heated at 95°C for 10 min to denature the particles. Denatured particles were loaded into 12% SDS-PAGE and subjected to electrophoresis for 2 h at 100 V. Pre-stained ladder (Bio-Rad) was mixed 1:1 with MajicMark (Invitrogen) and used as a marker in each SDS-PAGE gel. The gel was transferred

onto a PVDF membrane for 50 min at 20 V. The membrane was blocked with 5% skim milk diluted in PBST-0.25%, and then cut longitudinally into several strips. The strips were individually placed into a tube made from a 5 cm cut section of a 1mL pipette to minimize the use of a large volume of swine serum. The strips were incubated with swine sera diluted 1/100 in the blocking buffer. The 5 cm cut section of a 1 mL pipette was sealed at both ends with parafilm. Incubation of the strips with swine sera was performed overnight at 4°C with a constant agitation. After incubation, the strips were washed three times with PBST-0.25% for 10 min each. After washing, the strips were incubated with goat anti-rabbit HRP whole IgG diluted 1/150000 for 1 h at room temperature. Following the washing step, the membrane strips were incubated with chemiluminescence, and images were captured by VersaDoc 5000 MP imaging system (Bio-Rad). Serum from a newborn piglet and a serum with a medium reactivity against denatured particles served as negative and positive controls, respectively. Controls were run side by side with swine sera in all batches.

### **2.5.2 Densitometric Analysis**

Densitometric analysis of Western blot assay enabled quantification of the antibody responses against the linear epitopes of PCV2-Cap protein, which was represented by a band at 28 kDa. The images were captured by Versadoc 5000 MP imaging system, analyzed by Quantity one software, and quantified by densitometric assay.

Densitometric analysis was performed by placing the strips beside each other using pre-stained molecular weight marker. Two individual tight boxes were drawn, one for the band and another one for the background on each strip. The intensity of each band was measured by subtracting the band intensity from the background. Normalization was done against internal

controls to increase accuracy; thereby positive and negative controls were run with each batch of sera. The density for each serum was calculated from the ratio of the densities produced by tested serum and by positive and negative controls. The band intensity was displayed in Excel format to be used for more statistical analysis. To have reliable results, all the strips from an individual PVDF membrane were developed at the same time.

## **2.6 Optimization of the Enzyme-linked Immunosorbent Assay (ELISA)**

Particle-based indirect ELISA was optimized to detect IgG/IgM antibodies towards the surface-exposed conformational epitope(s) of PCV2 particles.

### **2.6.1 Detection of IgG by PCV2 Particle-based ELISA**

PCV2 particles and the PK-15 cell lysate were serially diluted in 50 mM bicarbonate buffer (pH 9.6) and added to the 96-well ELISA plate (Corning) as antigens. The plate was incubated overnight at 4°C, then washed three times with PBST-0.05%. Skim milk (5%) diluted in PBST-0.05% was added to the wells to block the non-specific sites and the plate was incubated at 37°C for 1 h. After two washes with PBST-0.05%, 50 µL of two-fold serially diluted sera in blocking buffer were added to the plate and incubated for 90 min at 37°C. After five washes with PBST-0.05%, HRP-conjugated rabbit anti-pig whole IgG (Sigma-Aldrich) was diluted 1/2000 in the blocking buffer, added to the plate, and incubated at 37°C for 1 h. After the incubation period, the plate was washed five times with PBST-0.05%. The colorimetric reaction was developed by adding 50 µL 2, 2'-Azino-bis (3-Ethylbenzothiazoline-6-Sulphonic Acid) (ABTS) (Invitrogen) and incubating at 37°C in the dark for 25 min. The color reaction was stopped by adding 50 µL

of sulphuric acid solution (0.18 M) (Acros Organics). The plate was read at 415 nm using a microtiter plate reader (Bio-Rad).

### **2.6.2 Detection of IgM by PCV2 Particle-based ELISA**

All the optimizing steps of detecting IgM antibodies towards PCV2 particles were similar to the IgG capture particle-based ELISA with few modifications. Three swine sera were collected from PCV2 experimentally-infected pigs before infection (BI) and 10 and 17 DPI. Sera obtained from the pigs prior to the infection have been used as a negative control in the assay. Two-fold serial dilution of sera starting from 1/10 to 1/80 was used. In addition, goat anti-pig HRP recognizing porcine IgM (Abcam), diluted 1/10000 in blocking buffer, was used as a secondary antibody.

### **2.6.3 Determination of the Cut-off for Particle-based ELISA (IgG/IgM)**

To set a cut-off value, sera from four newborn piglets were tested in particle-based ELISA (IgG/IgM). The cut-off was performed as mean of the reactivity of sera to the PCV2 particles (IgG or IgM) plus three standard deviations (SD).

## **2.7 PCV2 Neutralization Assay**

Serial three-fold dilutions of swine sera were prepared in a final volume of 50  $\mu$ L and mixed with 50  $\mu$ L of non-purified PCV2 that contains  $3.1 \times 10^3$  TCID<sub>50</sub>. The mixture was incubated for 1 h at 37°C, and then added to 50% confluent PK-15 cells. The cells were incubated with the mixture for 90 min at 37°C and then washed with PBS. Cells were incubated with fresh media for 48 h at 37°C. After the incubation time, cells were fixed with 4% PFA for 30 min at room temperature and washed three times with 1X PBS. Fixed cells were incubated

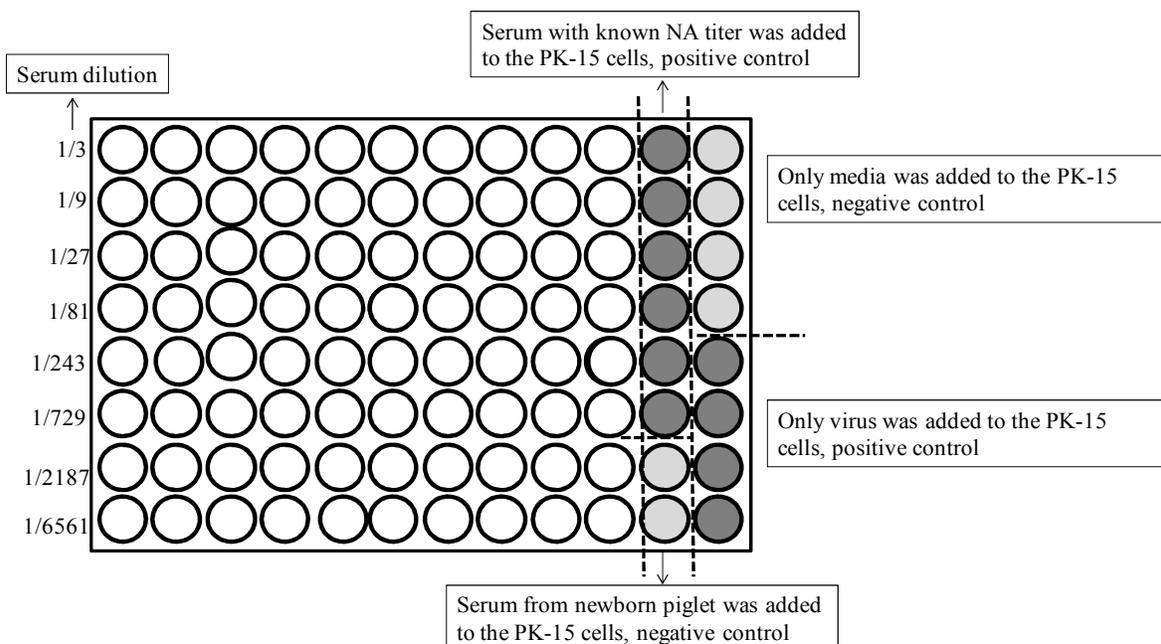
with permeabilization buffer for 30 min at room temperature. The 96-well plates were filled out with 100  $\mu$ L PBS. Fixed cells were incubated with the rabbit polyclonal anti-Cap diluted 1/500 in the permeabilization buffer for 90 min at 37°C. Cells were washed three times with PBST-0.1% and incubated with 1/400 diluted goat anti-rabbit Alexa Fluor 568 for 1 h at 37°C. Cells were washed three times with PBST-0.1%, and cells were stained with 300nM DAPI for 5 min at room temperature in the dark. Cells were washed with PBS three times and wells were kept with 100  $\mu$ L PBS for further image analysis.

In each tissue culture plate, proper negative and positive controls were used. Figure 2–1 shows the position of the positive and negative controls in a 96-well plate. All the neutralization tests were performed in duplicate at two different days (Appendix 9).

Plates were first visualized with an Olympus 1X51 inverted fluorescence microscope and then analyzed with the In Cell Analyzer 2000 automated microscope (GE Healthcare). The percent reduction of infectivity was performed with following formula:

$$\text{Percent reduction infectivity} = \frac{(\% \text{ total infected cells in positive control} - \% \text{ total of infected cells in sample})}{\% \text{ total infected cells in positive control}}$$

Virus neutralization assay was optimized by Cristina Solis Worsfold and the level of NA titer for these sera was determined with the help of other members of Dr. Czub's laboratory and myself.



**Figure 2-1. Position of negative and positive controls for virus neutralization assay in a 96-well plate.**

Negative controls in light gray colors: four wells only contained PK-15 cells without PCV2 and serum; two wells contained PK-15 cells with PCV2 and serum, serum was taken from newborn piglet. Positive controls in dark gray colors: four wells only contained PCV2 and PK-15 cells; a column contained PK-15 cells with PCV2, which incubated with a specific serum with known level of NA titer. Serum samples with unknown level of NA titer were diluted from 1/3 to 1/6561 in each individual column. The NA titer is a dilution of the serum where it is able to neutralize 50% of the infected cells as compared to positive controls.

## 2.8 Depletion of Antibodies Reacting to PCV2-Cap Linear Epitopes

To test whether purified PCV2 particles expose only conformational epitope(s) on their surface, antibodies that target the PCV2-Cap linear epitopes were depleted in three swine sera. Sera before and after depletion of antibodies were tested in particle-based ELISA. In addition, to test whether NAs target linear and/or conformational epitope(s), sera before and after depletion of antibodies were tested in PCV2 neutralization assay. To deplete antibodies towards linear epitopes, a recombinant Cap (rCap), which was expressed and purified by immobilized metal affinity chromatography (IMAC), was used as a source of antigen. As a negative control for

antibodies depletion, a recombinant green fluorescent protein (rGFP) was generated and purified the same way as rCap.

### **2.8.1 Recombinant Cap Protein Expression**

To produce rCap, the full length ORF2 gene that encodes Cap protein was amplified and cloned in a bacterial expression vector. Briefly, ORF2 was amplified from strain EF394779 using appropriate primers. The 700-bp PCR product was digested with restriction enzymes *XhoI* and *NdeI* and then cloned into a bacterial expression vector pET-28a(+). PCV2ORF2/pET28a(+) constructs through to its transformation into Top-10 cells (Invitrogen) were carried out with other members of Dr. Czub's lab. The recombinant PCV2-Cap protein was expressed as a fusion protein with N-terminal peptide of six histidines (His-tag). His-tag allows the purification of rCap protein using IMAC.

#### **2.8.1.1 Bacterial Transformation**

The transformed Top-10 cells with pET-PCV2-Cap DNA were thawed and seeded on a plate that contain 50 mg/mL kanamycin, and incubated at 37°C overnight. On the following day, a single colony was collected and transferred in 5 mL Luria Broth (LB) (Invitrogen) medium containing 50 mg/mL kanamycin (5 µL) and incubated at 37°C overnight. The next day, 3 mL of bacterial culture was used to extract the plasmid DNA with the aid of GeneJET plasmid mini-prep (Fermentas) as instructed by the manufacturer. The concentration of the purified plasmid DNA was determined by Nano-drop, which was 68 ng/µL. The purified plasmid DNA was sent to University Core DNA services at University of Calgary for sequencing.

The recombinant expression plasmids were transformed into *E.coli* BL21 (DE3) Rosetta blue competent cells (Novagen). Fifty µL aliquot of cells was thawed on ice, incubated with 50

ng PCV2-DNA for 30 min following heat shock at 42°C for 30 seconds, and was incubated on ice for 2 min. Pre-warmed LB (500 µL) was added to the transformed competent cells and incubated in a shaker at 225 rpm at 37°C for 1 h. After incubation time, 100-200 µL of transformants were spread on to a LB-agar plate containing kanamycin 30 µg/mL and incubated overnight at 37°C. The plasmid contains a kanamycin resistant gene; therefore, these bacteria that were transformed effectively with the plasmid were able to grow on the plate containing the antibiotic.

#### ***2.8.1.2 Amplification of rCap Protein***

One single colony was selected and inoculated in 1 mL LB with 30 mg/mL kanamycin at 37°C while shaking for 8-12 h. Five hundred microliters of bacterial culture was used to inoculate 10 mL of LB containing the kanamycin while shaking at 37°C for 24 h until reaching an optical density 600 (OD600) of 0.4 to 0.8. The OD of bacterial culture was determined with a biophotometer (Eppendorf). The expression of Cap was induced in the logarithmic phase by adding 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) to the 500 mL of culture and the bacterial culture was incubated 3 h at 37°C. The bacteria were harvested by centrifugation at 3000×g at 4°C for 10 min and the pellet was stored at -20°C until protein purification.

#### ***2.8.1.3 Purification of rCap Protein***

The N-terminal His-tag helps to purify rCap protein by Profinity IMAC nickel (Ni)-charged resin (Qiagen) under denaturing conditions. According to the protocol (Appendix 10), the bacterial culture was pelleted and treated with a lysis buffer containing 8 M urea. One to 2 g of the pellet was resuspended in 5 to 10 mL of lysis buffer and were kept overnight at 4°C while

shaking. To solubilize all proteins, sonication was done on ice (80% power, 20 seconds on and 20 seconds pulse). The clear solution was centrifuged at 3200×g for 20 min to separate resolubilized proteins from insoluble ones. The supernatant that contains the protein of interest was incubated with pre-treated Ni beads at room temperature for 1 h while shaking. This step allowed the His-tag on the rCap to bind to the Ni in the resin. After incubation time, the mixture was spun down and the supernatant was collected as flow-through. The lysis buffer was used to wash and remove the impurities. After washing, the rCap protein was eluted out of with the elution buffer containing 8 M urea and 500 mM imidazole (Sigma-Aldrich), which competed for the binding to Ni and His-tag rCap was released. Urea was required to solubilize the polypeptide. The same approach was performed to produce and purify rGFP.

## **2.8.2 Analysis of the Recombinant Proteins**

Samples were collected from all the washing and elutions steps and tested with Coomassie blue dye and Western blot. Expression of rCap with 6X His-tag made the visible band 1 kDa higher than Cap alone; therefore the band was observed at 29 kDa. The band was excised with a clean razor blade and sent to Southern Alberta Mass Spectroscopy (SAMS) at University of Calgary to confirm the presence of rCap (Appendix 11).

### **2.8.2.1 Bio-Rad protein Assay**

To determine the concentration of total protein of rCap and rGFP, the Bio-Rad protein assay was used. Before protein estimation, the recombinant proteins were diluted two times in PBS as the Bio-Rad protein assay is not compatible with the presence of 8 M urea in solutions.

### **2.8.2.2 SDS-PAGE and Western blot**

To analyze the rCap/rGFP proteins, SDS-PAGE and Western blot were carried out with the methods previously discussed. Eluted rCap/rGFP (2.5  $\mu$ g) was mixed with equal volume of 2X SDS sample buffer, BME, and kept at room temperature for 30 min before electrophoresis. There was no need to heat the mixture of rCap/rGFP and sample buffer due to the breakdown of urea into isocyanic acid and carbamylation of proteins/peptides. The carbamylation has effects on these epitopes and make them non-reactive with the swine sera.

After electrophoresing, the gel was stained with Coomassie blue and transferred to PVDF membrane. The blot was probed with rabbit anti-Cap (diluted 1/2000), following incubation with a secondary antibody, goat anti-rabbit (diluted 1:10000) conjugated to HRP. PVDF membrane containing rGFP was probed with the rabbit anti-GFP (diluted 1/2000) (Sigma-Aldrich) following incubation with the goat anti-rabbit secondary antibody (diluted 1:10000).

### **2.8.3 Depletion of Antibodies Reacting to PCV2-Cap Linear Epitopes**

To deplete antibodies towards linear epitopes, three swine sera were selected. These sera were positive in particle-based ELISA (IgG) and Western blot. The level of NA titer in two sera was 1/200, and in one serum was 1/375. Recombinant Cap/GFP (50  $\mu$ g) was subjected to electrophoresis on a single well. The gel was subjected to semi-dry, transfer to the PVDF membrane, and was blocked with 5% skim milk. Since rCap/rGFP are 29 kDa, PVDF membrane was cut horizontally between 25 and 35 kDa. Sera with 1/200 NA titer were diluted 10 times in PBST-0.25%; the other serum diluted 20 times in PBST-0.25%. Diluted sera were incubated for 2 h at room temperature with an individual narrow strip of rCap in 15 mL conical centrifuge tube while shaking. As a negative control for the depletion of antibodies, another aliquot of each

individual serum was incubated with rGFP side by side with the tube containing rCap strip. In total, an aliquot of each serum was incubated with five rCap blots (250 µg rCap, positive control) and another aliquot was incubated with five rGFP strips (250 µg rGFP, negative control).

#### **2.8.4 Swine Sera Status after Depletion of Antibodies Reacting to Linear Epitopes**

To ascertain whether antibodies that bind to PCV2-Cap linear epitopes were depleted, the PVDF membrane containing 11 µg denatured PCV2 particles was cut longitudinally into 30 strips. Therefore, each strip contains 0.3 µg PCV2-Cap. The strips were probed with sera before and after depletion of antibodies. As positive controls, one of the strips was probed with rabbit anti-Cap to confirm the efficient transfer of Cap protein from the gel to PVDF membrane. Another positive control was the incubation of a strip with PCV2-positive swine serum. Negative controls comprised a strip probed with newborn serum and a strip probed only with rabbit anti-pig HRP to detect any non-specific binding of secondary antibody to the membrane. The strips were analyzed by VersaDoc 5000 MP imaging system and quantity-one software. To compare the intensity of bands on each strip, densitometric analysis was performed before and after depletion of antibodies (antibodies that bind to PCV2-Cap linear epitopes).

Swine sera before and after depletion of antibodies (antibodies that target linear epitopes) were subjected to the PCV2 particle-based ELISA to assess whether linear epitopes are present on the purified PCV2 particles. In addition, sera before and after depletion of antibodies were tested in virus neutralization assay to determine whether depletion of antibodies reacting to PCV2-Cap linear epitopes has any impact on the level of NA titer.

## 2.9 Statistical Analyses

To normalize the NA data for statistical comparisons, the NA titer obtained from the virus neutralization assay was log<sub>2</sub>-transformed. The Kolmogorov-Smirnov and the Shapiro-Wilk statistics were computed for normality test. The non-normally distributed data sets (particle-based ELISA and Western blot) were computed with non-parametric tests such as Mann-Whitney U test or Kruskal Wallis test. To compare serum reactivity to native and denatured forms of PCV2 in the vaccinated and non-vaccinated groups, the Mann-Whitney U test was used. The Mann-Whitney U test is a nonparametric test alternative to the independent sample t-test that allows two groups to be compared without being normally distributed. Finally, Spearman's rank order correlation was employed to study the relationship between IgG antibodies bind to the PCV2 particles and the level of NA titer. Spearman's rank order correlation is a statistical measure of monotonic relationship strength between two variables. Differences were considered significant for all test procedures when  $P < 0.05$ . SPSS 17.0 software was used for all statistical analysis.

## **Chapter Three: Results**

The type of epitope (linear vs. conformational) targeted by NAs in naturally-infected swine sera has not been studied. Previously, I hypothesize that NAs target conformational epitope(s) on the surface of PCV2 particles using sera collected from farmed pigs. Particle-based ELISA was used to test the reactivity of 160 swine sera towards surface-exposed conformational epitope(s) of PCV2 particles. In addition, the reactivity of sera with linear epitopes of PCV2-Cap was assessed by Western blot. Finally, the reactivity of the swine antibodies towards conformational and linear epitopes of PCV2-Cap was correlated with the level of NA titer to determine the type of epitope (linear vs. conformational) that NAs bind.

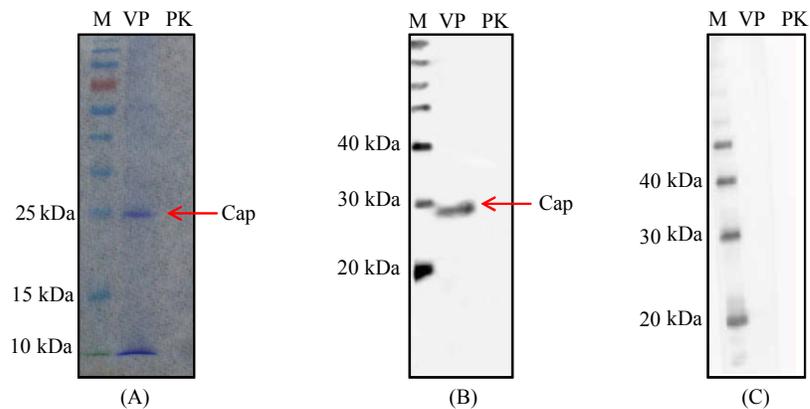
### **3.1 Analysis of the Purified PCV2 Particles**

PCV2 particles were generated, purified, and tested with several assays to determine the presence of PCV2 particles in the purified fractions. These tests comprised of SDS-PAGE with Coomassie blue staining, Western blot, and TEM. The reactivity of 160 swine sera towards PCV2-Cap linear epitopes was assessed by Western blot and quantified by densitometric analysis.

#### **3.1.1 SDS-PAGE and Western Blot**

Coomassie blue staining and Western blot were used to show the presence of the PCV2-Cap protein in the purified PCV2 particles. Proteins from non-infected PK-15 cells and purified PCV2 particles were subjected to SDS-PAGE and staining with Coomassie blue. A band at the expected size for PCV2-Cap was indicated by Coomassie blue staining (Fig 3-1 A).

In addition, the denatured PCV2 particles and non-infected PK-15 cells were transferred to the PVDF membrane after electrophoresis. The PVDF membrane was incubated with rabbit anti-Cap serum, following incubation with goat anti-rabbit conjugated with HRP. A Cap protein of approximately 28 kDa was detected as a structural protein of the virus with Western blot (Figure 3-1B). As shown in Figure 3-1C, no signal was detected when serum from a newborn pig was used as a primary antibody. This result was expected because no contact with the maternal antibodies has been reported for this animal. Since PK-15 cells were not infected with PCV2, a band at PCV2-Cap position (28 kDa) was not detected.



**Figure 3-1. Immunoblot and Coomassie stained SDS-PAGE of PCV2 particles and mock PK-15 cells.**

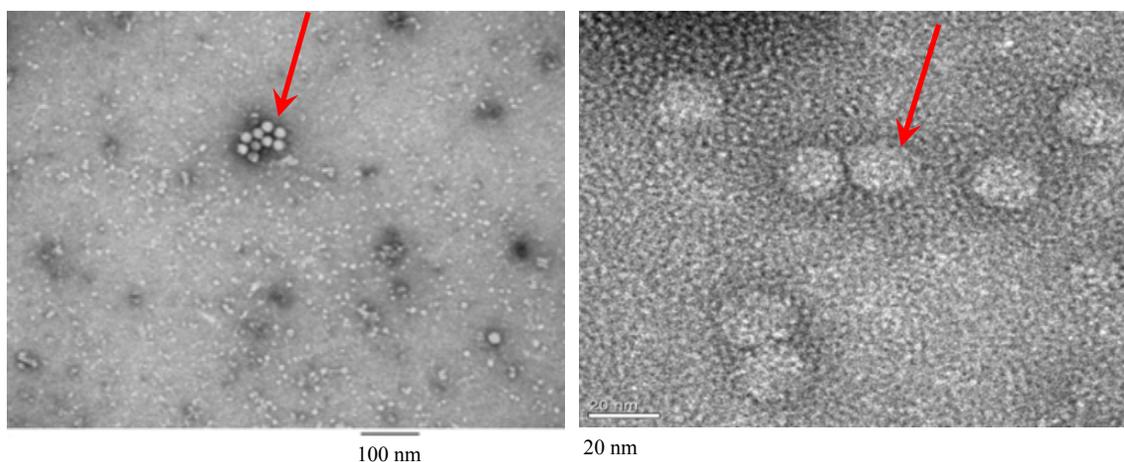
Coomassie staining shows a band at 28 kDa for PCV2 particles matching with immunoblot, the non-infected PK-15 cells did not display a band at the PCV2-Cap position (28 kDa) (A). Immunoblot analysis of PCV2 and mock PK-15 cells were probed with rabbit anti-Cap hyperimmune serum, which acts as a positive control to confirm the presence of Cap in the blot. (B). PVDF membranes loaded with PCV2 particles and mock PK-15 cells were probed with newborn serum, which acts as a negative control (C). Prestained ladder (Fermentas) mixed with MagicMark (Invitrogen) was loaded into immunoblots and SDS-PAGE gel. Red arrow indicates position of PCV2-Cap.

Abbreviations: M, molecular weight marker (M); kDa, Kilodaltons; VP, purified PCV2 particles; PK-15, porcine kidney cell line.

In conclusion, both SDS-PAGE and Western blot assays were able to confirm the presence of PCV2-Cap in the purified PCV2 particles. PK-15 cells, not infected with PCV2, act as a negative control.

### 3.1.2 TEM of the Purified PCV2 Particles

TEM was used to detect PCV2 particles based on the morphology and size of the particles. PCV2 particles are approximately 17 nm in diameter (Mankertz, 2008). Figure 3–2 shows representative images, which were obtained from the purified PCV2 by TEM. These images confirm the presence of virus particles at the expected size (17 nm) in the purified virus fraction.



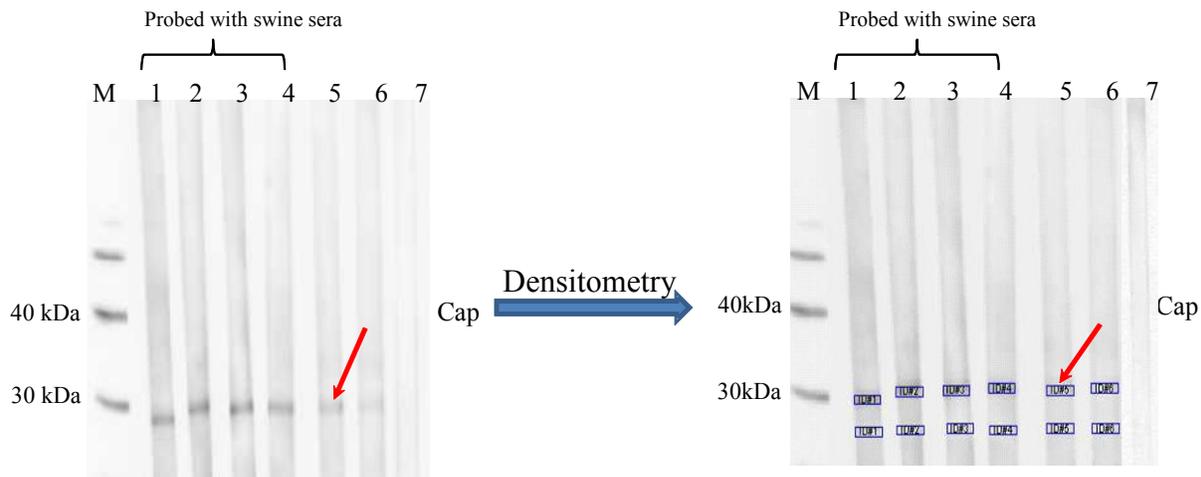
**Figure 3-2. TEM of purified PCV2 particles (red arrows).**

PCV2 particles were produced in cell culture and purified by PEG and sucrose gradients. Scale bar: left image 100 nm, right image 20 nm. Magnification: left image 40,000, and right image 250,000. The diameter of PCV2 particles was about 17 nm, corresponding to the size reported in the literature (Mankertz, 2008).

## 3.2 Do Antibodies in Swine Sera Collected from Farmed Pigs React with PCV2-Cap Linear Epitopes?

PCV2 particles were electrophoresed in SDS-PAGE and transferred to PVDF membrane. The PVDF membrane was cut into strips and incubated with swine sera. After development, the

signal at the position PCV2-Cap (28 kDa) was quantified by densitometric analysis. Densitometric analysis was performed by drawing a tight boundary around the PCV2-Cap, as well as same size box for the background for each individual serum. Background intensity was subtracted from the blot image (Figure 3–3). In each test proper positive and negative controls were run and the S/P value was calculated.

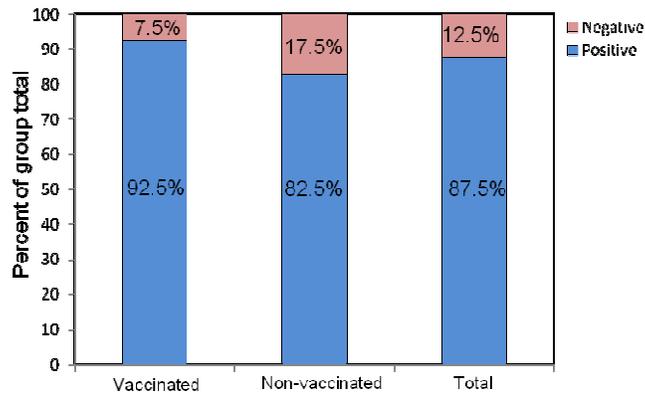


**Figure 3-3. Reactivity of swine sera with PCV2 linear epitopes in PVDF strips.**

Denatured PCV2 particles were loaded on the SDS-PAGE gel and transferred to PVDF membrane. Membrane was cut into several strips and used to probe with the cohort sera. Left picture only shows reactivity of different sera with denatured particles. Right picture shows same strips as left one, except the densitometry assay was also developed by drawing boxes around the band and background of each strip. Strips 1,2,3,4 show reactivity of different individual sera with denatured particles. There was a standard set of controls run with each test batch of sera, including positive and negative controls. Rabbit anti-Cap hyperimmune serum acts as a positive control to confirm the presence of Cap at the expected molecular weight in a blot (strip 5). Another positive control was the reactive adult swine serum with denatured particles, shown in strip 6. One strip was used to probe with newborn serum, which was collected before the piglet could receive colostrums and acts as a negative control (lane 7). The ladder is MagicMark (Invitrogen) and its molecular weights are labelled in kilodaltons. The red arrow highlights position of PCV2-Cap.

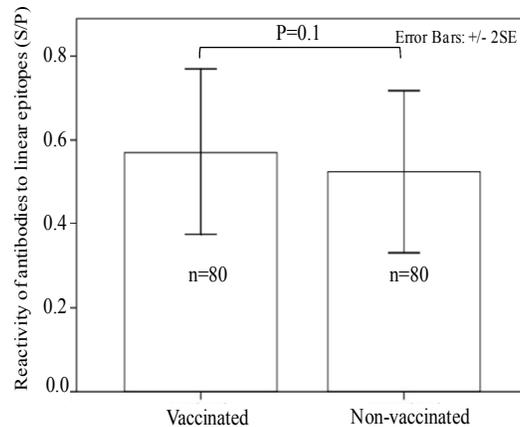
The vast majority of the cohort (87.5%) reacted with PCV2-Cap linear epitopes regardless of their vaccination status. The prevalence of reactivity of swine sera to linear epitopes of PCV2-Cap in vaccinated (n=80) and non-vaccinated (n=80) groups were 92.5% and 82.5%,

respectively (Figure 3–4). Moreover, there was no significant difference between reactivity of swine sera towards PCV2-Cap linear epitopes in vaccinated and non-vaccinated groups ( $p=0.1$ ) (Figure 3–5).



**Figure 3-4. Percentage of reactive (positive) and non-reactive (negative) sera with denatured PCV2 particles using Western blot according to vaccination status.**

The categories are vaccinated cohort ( $n=80$ ) with  $n=74$  positive and  $n=6$  negative sera, the non-vaccinated cohort ( $n=80$ ) with  $n=66$  positive and  $n=14$  negative sera, and overall ( $n=160$ ).



**Figure 3-5. Bar graph comparing the mean reactivity of swine antibodies towards PCV2-Cap linear epitopes between vaccinated ( $n=80$ ) and non-vaccinated pigs ( $n=80$ ).**

Reactivity of swine sera towards PCV2-Cap linear epitopes is plotted on the y-axis. This is an S/P value which is calculated by normalizing the data. Mann-Whitney U test did not find a significant difference between the two groups.

Taken together, these results indicate that the majority of swine sera reacted with PCV2-Cap linear epitopes and no significant difference was observed in the mean values of densitometry between vaccinated and non-vaccinated animals.

### **3.3 Do Antibodies in Swine Sera Collected from Farmed Pigs React with PCV2-Cap Conformational Epitope(s)?**

Assuming that PCV2 particles contain surface-exposed conformational epitope(s), PCV2 particle-based ELISA was optimized to assess the reactivity of swine sera to the conformational epitope(s) of PCV2 particles.

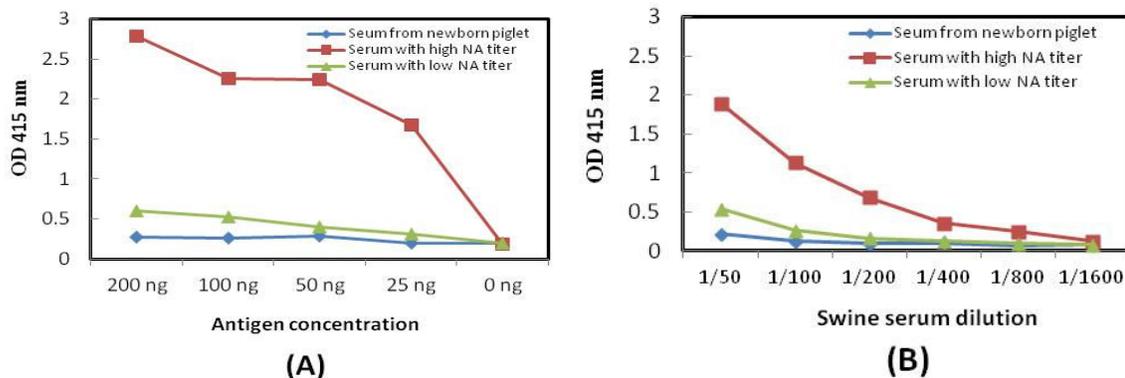
#### **3.3.1 Optimization of the IgG Capture Particle-based ELISA**

To optimize the particle-based ELISA, purified PCV2 particles (TCID<sub>50</sub>= 2×10<sup>6</sup>) and non-infected PK-15 cells (negative control) were used as a source of antigens. The protein concentration of purified particles was 168 µg/mL and of PK-15 cells was 346 µg/mL. The optimal working antigen concentrations were determined by adding varying concentrations of antigens to the 96-well ELISA plate (Figure 3–6A). Two sera with high and low NA titers were used as positive controls and newborn serum was selected as a negative control. A conjugate control was also included in each plate, which is represented by a well containing only secondary antibody and substrate-chromogen mixture. The conjugate control was used to detect the non-specific binding of the secondary antibody to the antigens.

Figure 3–6A shows that serum with high NA titer has a signal that amplifies gradually with the increase in the PCV2 protein concentration, reaching a plateau at 100 ng, where the entire antigen is saturated with antibody. After the plateau, the signal increases again because of over-saturation and background. The value of the signal is dependent on the amount of the antigen that was attached to the plate and the amount of antibody. The low NA serum had weaker reactivity towards PCV2 particles, and the negative control (serum from newborn piglet) had no reactivity with PCV2 particles.

To determine the optimal dilution of swine sera for particle-based ELISA optimization, the sera were two-fold serially diluted from 1/50 to 1/1600 (Figure 3–6B). Strong signal at 1/50 was observed for the serum with high NA titer, which decreased with serial dilution. The last dilution gave low OD values that were equivalent to the background. The serum with low NA titer and the newborn serum showed very low OD values at 1/50, and no change in these values was observed after dilution. The signal of the conjugate control was close to zero meaning there was not non-specific reaction between secondary antibody, rabbit anti-Pig HRP, and coated antigens.

The same procedure was performed with 8 more serum samples, and similar results were found (Appendix 12). As a result, the optimal working protein concentration and serum dilution that were chosen to run the particle-based ELISA were 100 ng and 1/50 dilution, respectively.



**Figure 3-6. Optimization of particle-based ELISA to detect the reactivity of IgG antibodies towards PCV2 particles.**

Three sera were used to optimize the particle-based ELISA assay, one serum with high NA titer, one with low NA titer, and a newborn serum. A two-fold dilution reduced the concentration of PCV2 particles from 200 ng to 0 ng, and a plateau at 100 ng represents the region where the entire antigen is saturated with antibody and works as an optimum protein concentration for the assay (A). Graph shows the titration curves of two-fold serum sample dilutions from 1/50 to 1/1600 towards PCV2 particles. There was an increase with decreasing the dilution of sera; 1/50 gave the highest value, which represents the optimum working dilution for the particle-based ELISA (B). Newborn serum acts as a negative control and did not react with PCV2 particles.

### **3.3.2 Validation of the IgG Capture Particle-based ELISA**

Particle-based ELISA was validated to provide a test to quantify the IgG antibodies against PCV2 particles in swine sera. The validation parameters comprised performing data normalization, determining the cut-off, and testing for the assay reproducibility.

#### ***3.3.2.1 Data Normalization***

To compare the data from one plate to another plate, positive and negative controls were included in each plate. Data normalization was achieved by expressing the sample to positive ratio (S/P) of individual samples, based on the OD values of positive and negative controls using following formula:

$$\text{S/P value} = \frac{(\text{OD of the sample} - \text{OD of the negative control})}{(\text{OD of the positive control} - \text{OD of the negative control})}$$

#### ***3.3.2.2 Cut-Off Determination***

The threshold or cut-off is defined as the level of antibody that discriminates positive results from background reading. The most appropriate method for determining the cut-off is to test the known negative samples. Therefore, four sera from newborn animals (negative controls) were selected, and the threshold was calculated by the mean OD values of negative controls plus three SD. Samples were considered negative when the S/P ratio was less than 0.2 and positive when the S/P ratio was greater or equal to 0.2.

#### ***3.3.2.3 Reproducibility of the Assay***

The reproducibility of the assay means the agreement between replicates within and between runs of the assay, and usually reported as the coefficient of variability (CV). The CV is the ratio of the standard deviation to the mean and usually present as percentage [CV%=

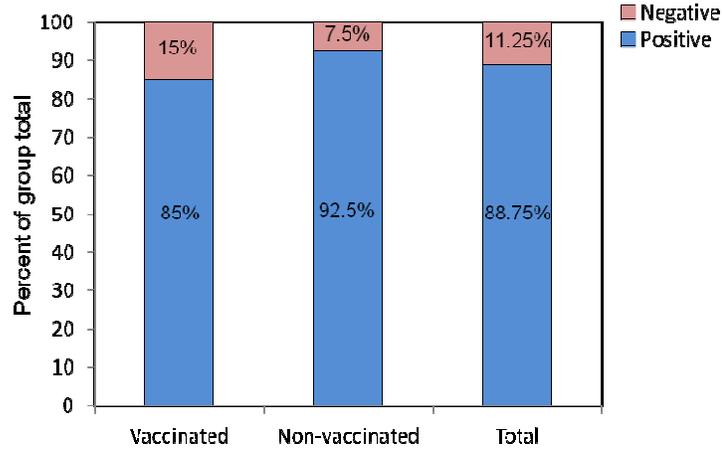
SD/mean) $\times$ 100]. The inter- and intra-assay CV% less than 10% is acceptable (Selman et al., 2012).

The inter-assay CV% represents the consistency of the results from one plate to another plate. To calculate the inter-assay CV%, 5 PCV2-positive sera were selected and run in duplicate on 5 different plates and occasions. The plate means for each serum were calculated and used to measure the overall SD, mean, and CV%. The inter-assay CV% for particle-based ELISA was 6.5% (Appendix 13).

To measure intra-assay CV%, 20 different swine sera were tested in duplicate in one plate. The intra-assay CV% was calculated by determining the average of CV% for an individual serum in duplicate. The intra-assay CV% for particle-based ELISA was 2.4% (Appendix 13), which shows the degree of difference between duplicates of an individual sample.

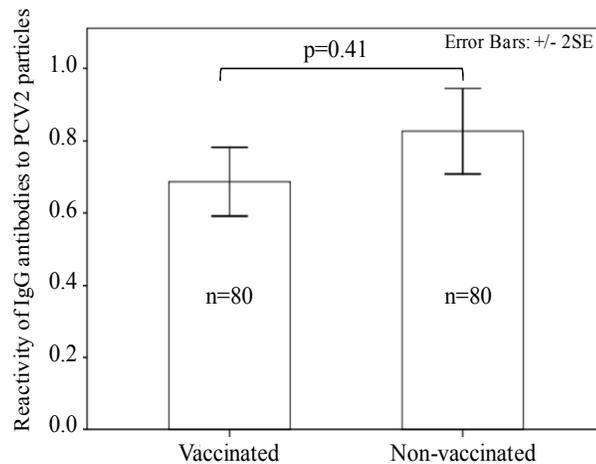
### **3.3.3 Reactivity of IgG Antibodies towards PCV2-Cap Conformational Epitope(s)**

To test whether swine sera reacted with the surface-exposed conformational epitope(s) of PCV2 particles, 160 swine sera were tested in particle-based ELISA. The vast majority of sera in the cohort (88.75%) reacted with the surface-exposed conformational epitope(s) of PCV2 particles. With regard to their vaccination status, the data analysis showed that 85% of the vaccinated pigs, and 92.5% of the non-vaccinated animals were positive (Figure 3–7). A serum was recognized as positive in particle-based ELISA when S/P values were equal to or greater than cut-off (OD=0.2). The Mann-Whitney U test showed no significant difference between vaccinated and non-vaccinated pigs ( $p=0.41$ ) (Figure 3–8).



**Figure 3-7. Percentage of reactive (positive) and non-reactive (negative) sera with PCV2 in particle-based ELISA according to vaccination status.**

The categories are the vaccinated cohort (n=80) with n=68 positive and n=12 negative sera, the non-vaccinated cohort (n=80) with n=74 positive and n=6 negative sera, and overall (n=160). Positive is defined as any signal above the cut-off.



**Figure 3-8. Antibody reactivity towards PCV2 particles according to vaccination status.**

No significant difference was observed in the reactivity of swine IgG antibodies towards PCV2 particles between two groups of animals using Mann-Whitney U test (p=0.41).

In conclusion, most of sera in the cohort reacted with the surface-exposed conformational epitope(s) of PCV2 particles regardless of their vaccination status.

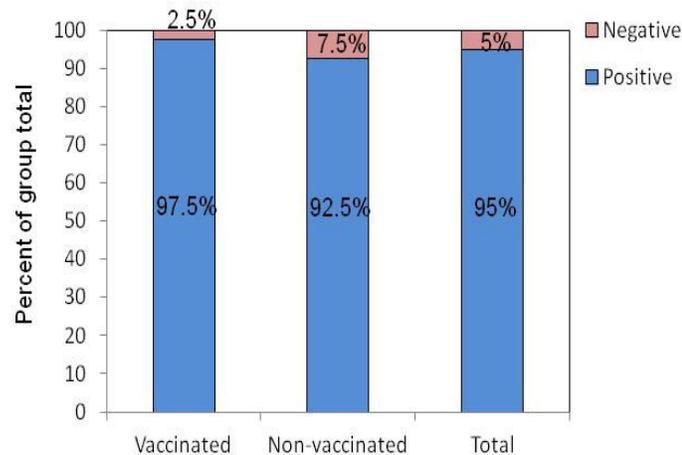
### **3.4 Do Antibodies that React with PCV2-Cap Linear Epitopes have Virus Neutralizing Activity?**

To ascertain whether NAs bind to the linear epitopes of PCV2-Cap, the virus neutralizing activity was determined in 160 swine sera and the results were correlated with the reactivity of swine sera towards PCV2-Cap linear epitopes.

#### **3.4.1 Virus Neutralizing Titer in Swine Sera**

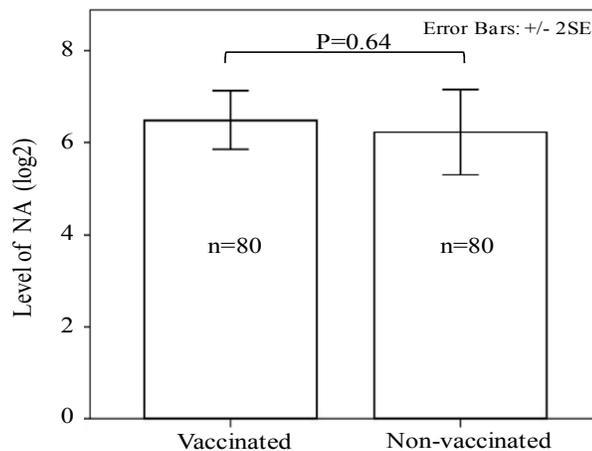
The vast majority of the cohort (95%) was positive in PCV2 neutralization assay (Figure 3–9). A serum was recognized as positive in virus neutralization assay when the level of NA titer was greater than zero. Different levels of NA titer were observed in the cohort with minimum and maximum values of 1/3 (serum diluted 3 times) and 1/5135 (serum diluted 5135 times), respectively. This is a dilution required to cause a 50% reduction in the number of PCV2-infected cells as compared to the positive control.

Although the level of NA titer was higher in vaccinated pigs than non-vaccinated ones, no significant difference was observed between the two groups using independent-sample t-test ( $p=0.64$ ) (Figure 3–10). Therefore, both groups of animals could induce NAs regardless of their vaccination status.



**Figure 3-9. Percentage of reactive (positive) and non-reactive (negative) sera in virus neutralization assay according to vaccination status.**

The categories are the vaccinated cohort (n=80) with n=78 positive and n=2 negative sera, the non-vaccinated cohort (n=80) with n=74 positive and n=6 negative sera, and overall (n=160). Data for the PCV2 neutralizing antibody titer of these sera was taken from Solis Worsfold et al., 2015.



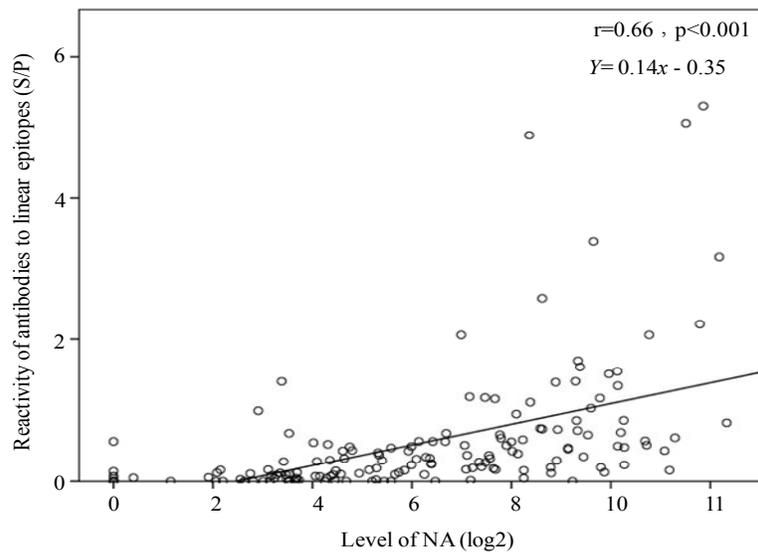
**Figure 3-10. The level of NA titer (log<sub>2</sub>-transformed) in the swine sera according to vaccination status.**

No significant difference was observed in the level of NA titer between sera collected from vaccinated and non-vaccinated animals using independent-sample t-test (p=0.64). Data for the PCV2 neutralizing antibody titer of these sera was taken from Solis Worsfold et al., 2015.

As a conclusion, 95% of the cohort had different levels of NA titer due to the vaccination or exposure of animals to the PCV2. In addition, NAs were induced in both vaccinated and non-vaccinated animals, and no significant difference was observed between the two groups.

### 3.4.2 Antibodies towards PCV2-Cap Linear Epitopes have Virus Neutralizing Activity

To assess whether antibodies that bind to PCV2-Cap linear epitopes has virus neutralizing activity, correlation between the level of NA titer and antibody reactivity towards PCV2-Cap linear epitopes was performed. A significant positive correlation was observed between IgG antibodies reacting to PCV2-Cap linear epitopes and NA titer using Spearman's rank order correlation ( $r=0.66$ ,  $p<0.001$ ) (Figure 3–11). These results suggest that NAs target the PCV2-Cap linear epitopes.



**Figure 3-11. Positive correlation between the level of NA titer (log-2 transformed) and antibody reactivity towards PCV2-Cap linear epitopes in Western blot.**

Individual samples are depicted as open symbols. Correlation between the level of NA titer (log2 transformed) and antibody reactivity towards PCV2-Cap linear epitopes ( $r=0.66$ ,  $p<0.001$ ) is shown in scatter plot. Linear regression was calculated and is represented as a line.

### 3.5 Do Antibodies that React with PCV2-Cap Conformational Epitope(s) have Virus Neutralizing Activity?

To ascertain whether NAs bind to the surface-exposed conformational epitope(s) of PCV2 particles, the correlation between the level of NA titer and antibody reactivity to the PCV2

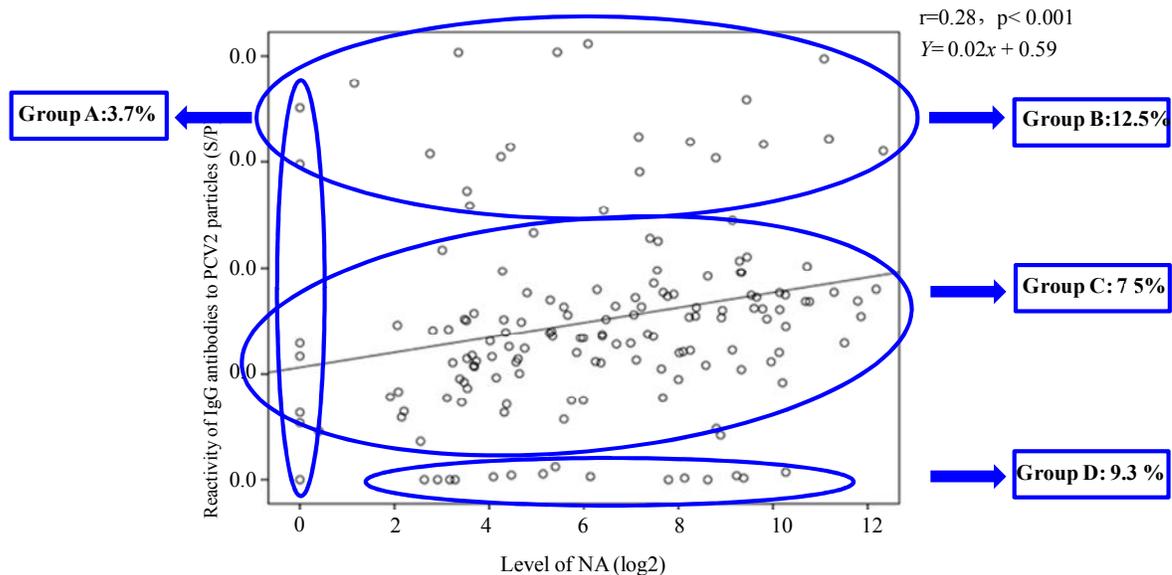
particles was assessed by Spearman's rank order correlation. Since the reactivity of swine sera towards PCV2 particles and the level of NA titer did not show significant difference between vaccinated and non-vaccinated animals, the data from all swine samples regardless of their vaccination status were used to perform the correlation test.

### **3.5.1 IgG Antibodies towards PCV2-Cap Conformational Epitope(s) have Virus Neutralizing Activity**

A significant positive correlation was observed between reactivity of IgG antibodies towards surface-exposed conformational epitope(s) of PCV2 particles and NA titer ( $r=0.28$ ,  $p<0.001$ ) (Figure 3–12), suggesting that NAs bind to the conformational epitope(s) on the surface of PCV2 particles.

The results of the correlation test (Figure 3–12) were divided into four groups (A, B, C, and D) based on their agreement with regression line. The vast majority of swine sera (group C: 77.5%) showed a significant positive correlation between the level of NA titer and reactivity of the sera with PCV2 particles ( $r=0.46$ ,  $p<0.001$ ), which indicates the presence of neutralizing conformational epitope(s) on the surface of PCV2 particles.

The strength of correlation was low or almost non-existent for 21.8 % of the sera (group A, B, and D). Group D of the sera (9.3%) had a detectable level of NA titer, but did not have IgG antibodies towards PCV2 particles (Figure 3–12). The presence of NAs in these sera might be due to the existence of other antibody isotypes such as IgM antibody. Group A and B will be discussed in more detail later.



**Figure 3-12. Positive correlation between the level of NA titer (log 2-transformed) and IgG antibodies reacting with PCV2 particles.**

Individual samples are depicted as open symbols. Correlation between the level of NA titer (log<sub>2</sub> transformed) and IgG antibody reactivity towards PCV2-Cap linear epitopes ( $r=0.28$ ,  $p < 0.001$ ) is shown in scatter plot. Linear regression was calculated and is represented as a line. The blue circle shows different populations of data sets.

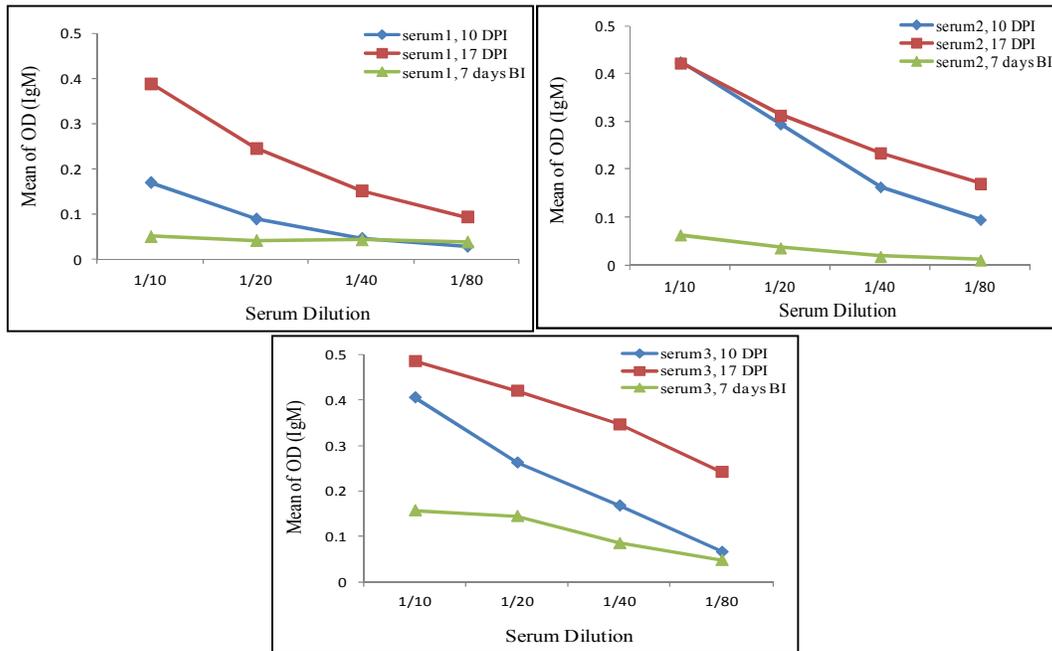
### 3.5.2 Optimization of IgM Capture Particle-based ELISA

To test whether swine sera from group D (9.3%) (Figure 3–12) had IgM antibodies towards surface-exposed conformational epitope(s) of PCV2 particles, IgM capture particle-based ELISA was developed and used to test this group of samples.

To optimize the IgM capture particle-based ELISA, three sera were obtained from experimentally PCV2 infected pigs. These sera were collected from animals before infection and 10 and 17 days after experimental PCV2 infection. Figure 3–13 shows the mean OD values of three different serum samples that were two-fold serially diluted from 1/10 to 1/80, using a constant antigen concentration (100 ng). The OD values were subtracted from the OD values of the negative control (non-infected PK-15 cells). The results demonstrate that less diluted sera

(1/10) had higher reactivity towards PCV2 particles. Since the concentration of IgM antibodies in the serum is almost 10 times lower than the IgG antibodies concentration (Bourne, 1973), a less diluted serum (1/10) was used in IgM capture ELISA as compared to serum dilution in IgG capture ELISA (1/50). Sera that were collected before PCV2 infection had no reactivity in IgM capture particle-based ELISA; however, sera had detectable OD values at 10 DPI, which increased over time (17 DPI).

Therefore, the IgM capture particle-based ELISA was optimized and could be used to test the group D sera. This group (Figure 3–12, Group D) had different levels of NA titer without detectable levels of IgG antibodies towards PCV2 particles. A cut-off value of 0.01 was set using the same procedure as described before.



**Figure 3-13. Optimization of particle-based ELISA to detect IgM antibodies.**

Graphs show the titration curves of three swine sera that were obtained, before and after experimental PCV2 infection. All three sera showed no reactivity in IgM capture particle-based ELISA before PCV2 infection. Sera collected from animals after experimental PCV2 infection have reactivity against PCV2 particles that increased over time.

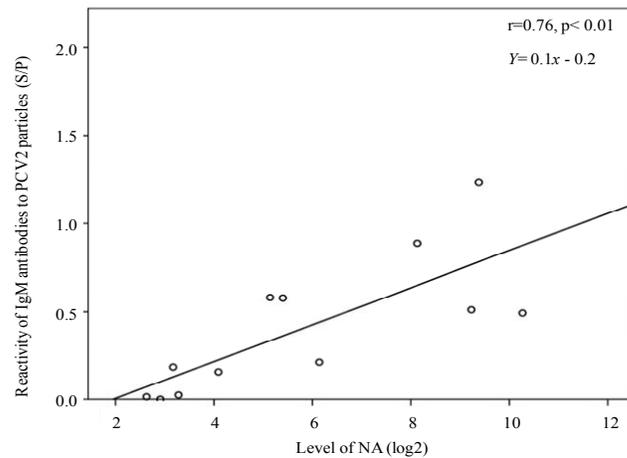
Abbreviations: BI, before experimentally PCV2 infection; DPI, after experimental PCV2 infection

### **3.5.3 IgM Antibodies towards PCV2-Cap Conformational Epitope(s) have Virus Neutralizing Activity**

To test whether animals in group D (n=15) (Figure 3–12) had IgM-NA, a correlation between the level of NA titer and reactivity of IgM antibodies towards PCV2 particles was assessed in these samples.

IgM antibodies were tested in 13 out of 15 swine sera by IgM capture particle-based ELISA. Two of the sera were not tested by IgM capture ELISA because of limited sample volume. A significant positive correlation was observed between IgM antibodies towards PCV2 particles and NA titer in the group D of cohort using Spearman's rank order correlation ( $r=0.76$ ,  $p < 0.01$ ) (Figure 3–14). The positive correlation between the results of NA titer and particle-based ELISA suggests that IgM-NA but not IgG-NA bind to surface-exposed conformational epitope(s) of PCV2 particles in these sera.

Thus, there was a significant positive correlation between reactivity of sera towards surface-exposed conformational epitope(s) of PCV2 particles and NA titer, suggesting that IgM-NA target conformational epitope(s) on the surface of PCV2 particles.



**Figure 3-14. Strong positive correlation between the level of NA titer (log 2-transformed) and IgM antibodies reacting to PCV2 particles.**

Individual samples are depicted as open symbols. Correlation between the level of NA titer (log2 transformed) and IgM antibody reactivity towards PCV2-Cap linear epitopes ( $r=0.76$ ,  $p < 0.01$ ) is shown in scatter plot. Linear regression was calculated and is represented as a line.

### **3.6 Do Neutralizing Antibodies Target both PCV2-Cap Linear and Conformational Epitopes?**

The positive correlation between the NA titer and antibody reactivity towards PCV2-Cap linear epitopes (in Western blot) and surface-exposed conformational epitope(s) of PCV2 particles (in particles-based ELISA), raises a question whether NAs target both linear and conformational epitopes of PCV2.

To ascertain whether NAs target both surface-exposed conformational epitope(s) of PCV2 particles and linear epitopes, two experimental studies were conducted. First, rabbit serum raised against PCV2-Cap linear epitopes was tested in particle-based ELISA and PCV2 neutralization assay. In the second study, antibodies towards linear epitopes were depleted in three swine sera. These sera had medium NA titer and medium reactivity in Western blot and particles-based ELISA. Sera before and after depletion of antibodies (antibodies that bind to PCV2-Cap linear epitopes) were tested in Western blot, particle-based ELISA, and PCV2 neutralization assay.

### 3.6.1 Testing Rabbit Serum Raised against PCV2-Cap Linear Epitopes

Rabbit serum raised against PCV2-Cap linear epitopes was available in Czub's lab. This serum was tested in Western blot to make sure that the serum reacts with PCV2-Cap linear epitopes (Figure 3–1B). Rabbit anti-Cap serum was tested in particle-based ELISA and virus neutralization assay. No reactivity was observed between rabbit serum raised against PCV2-Cap linear epitopes and PCV2 particles in particle-based ELISA. Rabbit anti-Cap was tested in virus neutralization assay and the results were summarized in table 3-1. PCV2 virus neutralization assay was reported as a percentage of reduction in infectivity, and the NA titer is a dilution of the serum where it is able to neutralize 50% of the viral infectivity as compared to positive controls. Since the percentage of reduction in PCV2 infectivity in virus neutralization assay is less than 50% in all dilutions, rabbit serum do not neutralize the virus.

**Table 3-1. PCV2 neutralizing activity of rabbit serum, which raised against PCV2-Cap linear epitopes.**

Serum dilution	% Reduction of infectivity
1/3	39.5
1/9	20.7
1/27	0
1/81	0
1/243	0
1/729	0
1/2187	0
1/6561	0

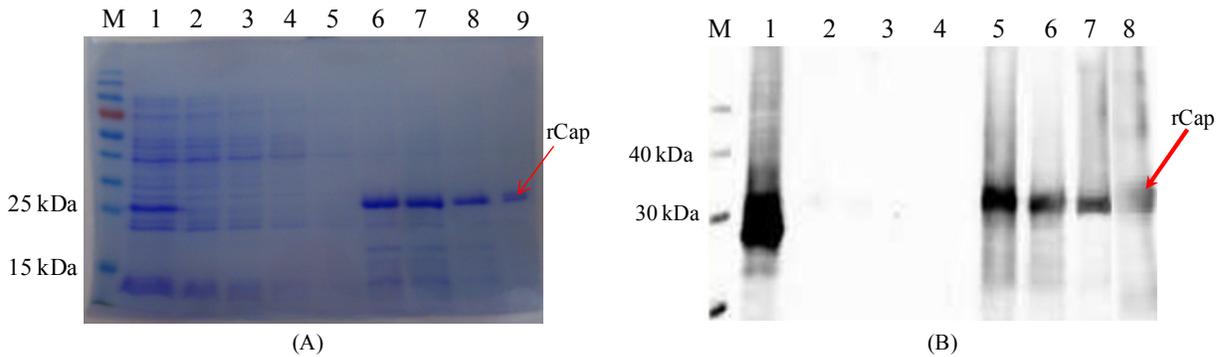
### **3.6.2 Depletion of Serum Antibodies towards PCV2-Cap Linear Epitopes**

To deplete the antibodies, which bind to the linear epitopes, rCap/rGFP proteins were expressed in a bacterial expression vector and purified using IMAC. The use of recombinant proteins has advantages over the purified virus for the depletion of antibodies, including high yield of the product, less expensive, and short generation time.

#### ***3.6.2.1 Expression and Purification of Recombinant Proteins***

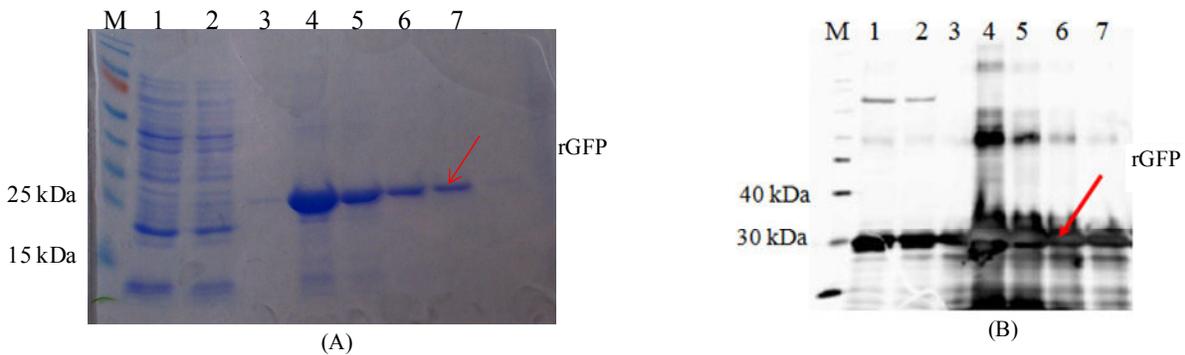
Recombinant Cap/GFP proteins were expressed in bacteria and were subjected to IMAC purification. The protein concentrations of rCap and rGFP were 550  $\mu\text{g/mL}$  and 1146  $\mu\text{g/mL}$ , respectively. The expected size for recombinant Cap/GFP proteins was approximately 29 kDa. The presence of recombinant proteins (rCap and rGFP) in the purified fractions was tested by SDS-PAGE and Western blot.

Recombinant Cap was detected by Coomassie blue staining following SDS-PAGE (Figure 3–15A). For blotting, the PVDF membrane was probed with rabbit anti-Cap; a band corresponding to the same size was also detected by immunoblot (Figure 3–15B). A large band was also detected by Coomassie blue (Figure 3–16A), and blotting (Figure 3–16B) for rGFP fractions at expected size (approximately 29 kDa).



**Figure 3-15. Coomassie-stained SDS-PAGE and Western blot analysis of PCV2 rCap protein.**

Coomassie stained SDS-PAGE (A): the crude lysate (lane 1) was subject to IMAC. Flow through (lane 2), wash 1 to 3 (lane 3-4-5), elution fractions (lanes 6-7-8), positive control (lane 9). The red arrow indicates the position of PCV2 recombinant Cap. Western blot analysis of PCV2 rCap (B): Flow through (lane 1), washes 1-4 (lane 2-4), elutions 1-3 (lane 5-7), positive control (lane 8).



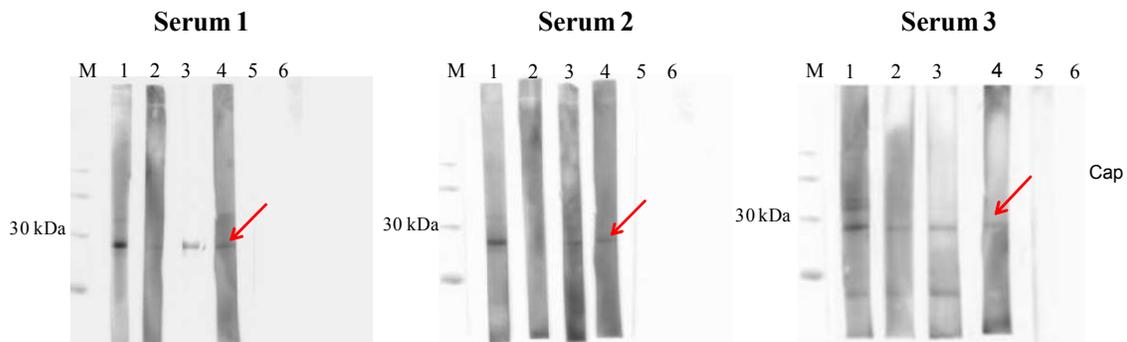
**Figure 3-16. Coomassie stained SDS-PAGE and Western blot analysis of rGFP protein.**

Coomassie-stained SDS-PAGE (A), and Western blot analysis of PCV2 rGFP protein expression (B). Molecular weight markers (M) are in kDa, Flow through (lane 1), washes 1-2 (lane 2-3), elutions 1-3 (lane 4-6), positive control (lane 7).

### ***3.6.2.2 Swine Sera Status in Western blot after Depletion of Antibodies Reacting to PCV2-Cap Linear Epitopes***

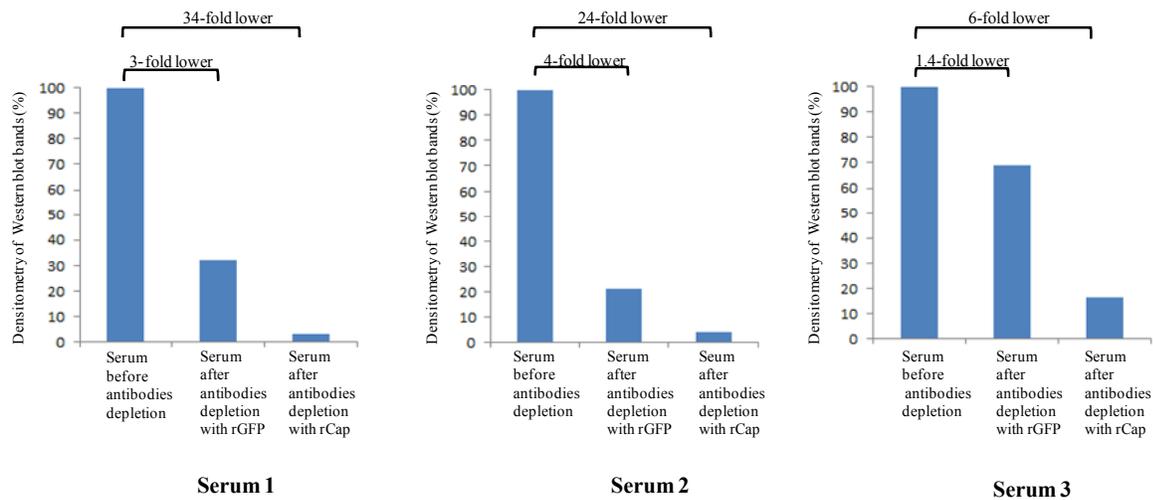
To test the success of antibodies depletion (those antibodies that target PCV2-Cap linear epitopes), denatured PCV2 was transferred to the PVDF membrane and probed with sera before and after depletion of antibodies.

As depicted in Figure 3–17, strips probed with swine sera, which antibodies were depleted with rCap (positive control), had lower reactivity towards PCV2-Cap than strips probed with non-depleted or depleted antibodies with rGFP (negative control). Figure 3–18 shows the quantification of data using densitometric analysis.



**Figure 3-17. Reactivity of swine sera towards denatured PCV2 before and after depletion of antibodies reacting to PCV2-Cap linear epitopes.**

Denatured PCV2 particles have been transferred to a PVDF membrane. The PVDF membrane was cut into strips. Strips were used to probe the serum before depletion of antibodies (lane 1). Strip was used to probe the serum after depletion of antibodies using rCap (lane 2). Strip was used to probe the serum after depletion of antibodies using rGFP, act as a negative control for the assay (lane 3). Polyclonal rabbit anti-Cap confirms the presence of PCV2-Cap in the blot (positive control) (lane 4). The newborn serum collected before receiving colostrum acts as a negative control (lane 5). Strip was used to probe only the secondary antibody to check for non-specific bindings (negative control) (lane 6).



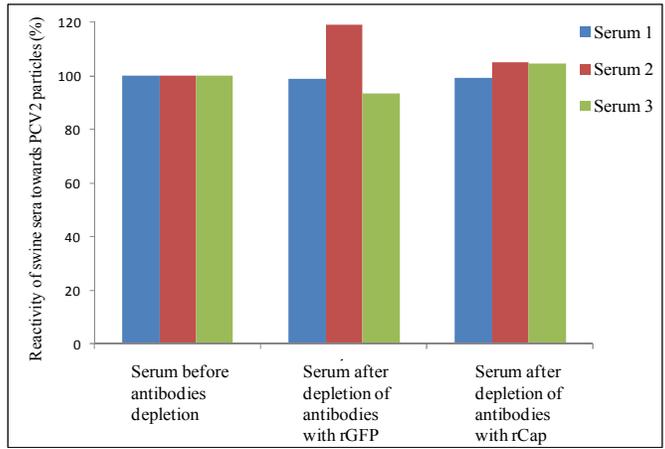
**Figure 3-18. Densitometric analysis of swine sera reactivity towards denatured PCV2 particles before and after depletion of antibodies (antibodies that target PCV2-Cap linear epitopes).**

Three sera were selected to deplete antibodies towards linear epitopes using rCap and rGFP (negative control). The reactivity of serum antibodies towards PCV2-Cap linear epitopes before and after depletion of antibodies, which target linear epitopes, was quantified by densitometric analysis and the results are shown as a percentage of the signal.

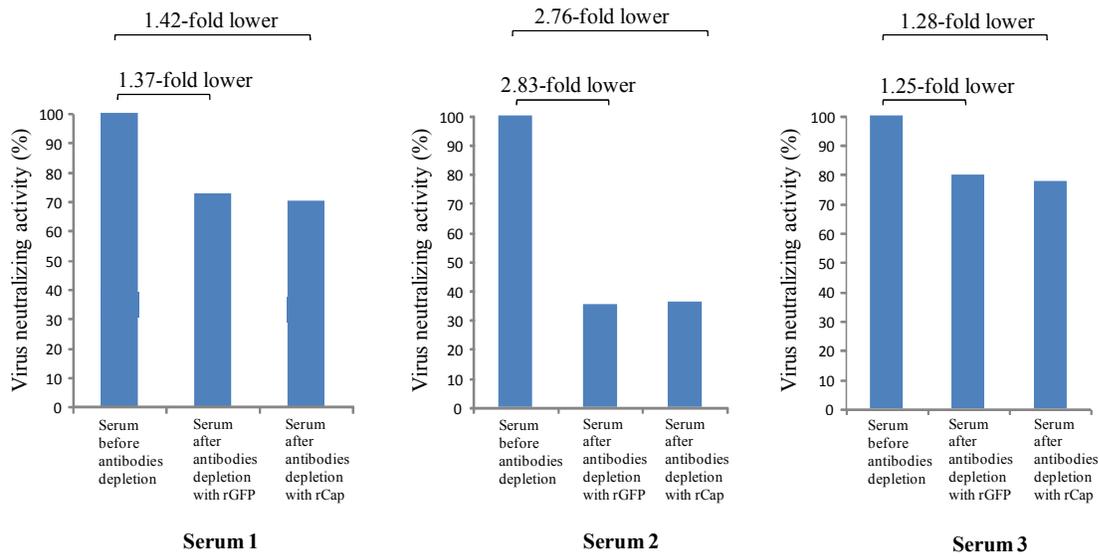
### ***3.6.2.3 Swine Sera Status in Particles-based ELISA and Virus Neutralization Assay after Depletion of Antibodies Reacting to PCV2-Cap Linear Epitopes***

To ascertain whether PCV2 particles only contain surface-exposed conformational epitope(s), sera before and after depletion of antibodies (antibodies that bind to linear epitopes) were tested in duplicates in particle-based ELISA (Figure 3–19). Depletion of antibodies with either rCap or rGFP did not have any impact on the reactivity of sera towards PCV2 particles.

To test whether NAs bind only to surface-exposed conformational epitope(s) of PCV2, sera before and after depletion of antibodies were tested in virus neutralization assay. The results are summarized in Figure 3–20 as percentage PCV2 neutralizing activity. Depletion of antibodies with either rCap or rGFP reduced the level of NA titer; the percentage reduction of NA titer in serum antibodies depleted with rCap (positive control) was the same as the level of NA titer in serum antibodies depleted with rGFP (negative control).



**Figure 3-19. Reactivity of swine sera towards PCV2 particles in particle-based ELISA before and after antibodies depletion using rCap and rGFP (antibodies that target PCV2-Cap linear epitopes).** Three sera were selected to deplete antibodies towards linear epitopes using rCap and rGFP (negative control). These three sera were subjected to the PCV2 particle-based ELISA before and after depletion of antibodies which target linear epitopes.



**Figure 3-20. Virus neutralizing activity of swine sera before and after depletion of antibodies (antibodies that target PCV2-Cap linear epitopes).**

Three sera were selected to deplete antibodies towards linear epitopes using rCap and rGFP (negative control). These three sera, before and after depletion of antibodies, were subjected to the PCV2 neutralization assay.

## Chapter Four: Discussion

Several experimental (Fort et al., 2008; Opriessnig et al., 2008a) and field studies (Horlen et al., 2008; Kixmoller et al., 2008) have demonstrated that PCV2 vaccines induce NAs, decrease viral load levels, and reduce PCV2-associated microscopic lesions. However, another study demonstrated the co-existence of NAs with viremia (Gerber et al., 2012). Along the same lines, a recent study conducted in our laboratory showed that vaccination has a positive impact on the induction of NAs in the nursery/weaner pigs but has no effect on the level of PCV2 viral load in farmed pigs (Solis Worsfold et al., 2015).

The identification of the antigenic sites of Cap protein, targeted by the NAs response, has been the focus of several studies. Initially, chimeric PCV1/PCV2-ORF2 and seven mAbs against PCV2-Cap were used to map the antigenic sites of PCV2-Cap protein (Lekcharoensuk et al., 2004). Lekcharoensuk et al. (2004) suggested that three antigenic sites of the Cap are likely involved in the formation of one neutralizing conformational epitope on PCV2 virions. In this study, mouse mAbs helped identify a PCV2 neutralizing epitope; however, in viruses such as lactate dehydrogenase-elevating virus the neutralizing epitopes recognized by polyclonal anti-sera have been shown to differ from those of neutralizing mAbs (Harty & Plagemann, 1988). Altogether, these data raise the question whether NAs in naturally-PCV2 infected pigs target the conformational epitope as mAbs.

In this study, I attempted to determine whether NAs recognize conformational epitope(s) on the surface of PCV2 particles using sera from farmed pigs.

In the context of other viral infections, it has been suggested that ELISA based on VLP or virus particles could be used to assess the antibody reactivity against conformational epitope(s)

(DOS SANTOS et al., 2004), whereas Western blot has been widely used to assess the antibody responses against linear epitopes (Kerr et al., 1999; Huang et al., 2011; Lekcharoensuk et al., 2004). Linear epitopes may exist either on the surface of the virion or buried in virus particles. Linear epitopes can be exposed under denaturing conditions that break down the three dimensional structure of the entire protein. For instance, detection of IgM antibodies against conformational epitopes of parvovirus VP1 and VP2 structural proteins were detected by ELISA using un-denatured VP1 and VP2 antigens, whereas the reactivity of antibodies towards linear epitopes was evaluated by Western blot (Kerr et al., 1999; Manaresi et al., 2001).

#### **4.1 Do Antibodies in Swine Sera Collected from Farmed Pigs React with PCV2-Cap Linear and/or Conformational Epitopes?**

Our results showed that the majority of swine sera were reactive to PCV2 particles in ELISA (88.75%). These data are consistent with previous studies that reported a high prevalence of antibodies against PCV2 in swine populations using ELISA. Indeed, more than 82% of swine serum samples (n= 6046) collected from a non-vaccinated finisher population of 185 farms throughout the USA were positive for anti-Cap antibodies (Puvanendiran et al., 2011). In a similar study, 365 out of 517 swine sera, which were collected from vaccinated and naturally-infected pigs, were positive in a commercial ELISA (Shin et al., 2014). In addition, 83.14% (n=1080) of swine sera collected from naturally-infected pigs were positive in an ELISA based on rCap (Shang et al., 2009). All these ELISA were based either on the bacterial expression of Cap (Yin et al., 2010), synthetic peptide (Truong et al., 2001), or live PCV2 produced in cell culture (Nawagitgul et al., 2002; Shang et al., 2008).

Although, our assay has not been compared to the gold standard methods such as IFA or immunoperoxidase monolayer assay (IPMA), several studies have shown that ELISA has a high sensitivity and specificity in detecting PCV2 antibodies, and also increases the accuracy of results interpretation over the IFA and IPMA (Nawagitgul et al., 2002; Blanchard et al., 2003). Our data show a variation in the reactivity of swine sera in particle-based ELISA. The weak reaction of some sera may have been a reflection of the time of infection, where IgG antibodies may have not been high enough to be manifested, or individual animals may have been infected at different and un-known time points. The agreement between this study and previous studies demonstrates that our established PCV2-based ELISA is a reliable tool to assess PCV2 infection.

I have assessed the level of antibodies towards PCV2 linear epitopes by Western blot and a similar frequency of positive samples was observed (87.5%) as compared to PCV2-based ELISA. Moreover, 95% of all tested animals had at least some reactivity to PCV2 in ELISA and/or Western blot, which indicates that pigs have been exposed to different epitopes (conformational and/or linear epitopes) of PCV2.

The high prevalence of antibodies observed in swine sera is due to the ubiquitous nature of PCV2; most of animals are exposed to the virus several times in their life (Zhou et al., 2006). Moreover, PCV2 antibodies are prevalent in swine herds regardless of their clinical (Rodriguez-Arrijo et al., 2002) or vaccination status (Gerber et al., 2012). As I have demonstrated in this study, there was no significant difference between vaccinated and non-vaccinated cohorts in terms of reactivity to PCV2 particles.

#### **4.2 Do Antibodies that React with PCV2-Cap Linear Epitopes have Virus Neutralizing Activity?**

The significant positive correlation between the level of NA titer and reactivity of antibodies towards linear epitopes of PCV2 in Western blot ( $r=0.664$ ,  $p< 0.001$ ), raises the question whether NAs were produced against PCV2-Cap linear epitopes and if particles expose linear epitopes.

Three swine sera were selected and subjected to the depletion of antibodies towards linear epitopes; sera before and after depletion of antibodies were tested in particle-based ELISA and virus neutralization assay. For the depletion experiment, rCap (positive control) and rGFP (negative control) were expressed and purified successfully as confirmed by SDS-PAGE and Western blot. Purified recombinant proteins were blotted to PVDF membranes, and the membranes were incubated with swine sera multiple times to deplete antibodies that target PCV2-Cap linear epitopes. The depleted and non-depleted serum antibodies were tested by particle-based ELISA. The results showed no difference in the reactivity of swine sera to the PCV2 particles before and after depletion of antibodies, which suggests that PCV2 particles do not expose linear epitopes on their surface. In addition, sera before and after depletion of antibodies were tested in virus neutralization assay. The NA titer was at the same level for both positive (serum antibodies depleted with rCap) and negative (serum antibodies depleted with rGFP) controls. Therefore, depletion of antibodies towards linear epitopes did not have an impact on the level of NA titer, suggesting that NAs most likely do not target linear epitopes.

Since a Western blot assay was used to keep epitopes in a linear form, one may ask whether all epitopes are in a linear form in Western blot. It has been shown that epitopes might renature during or after the transfer of protein to the PVDF membrane (Zhou et al., 2007). Thus,

the current depletion method might lead to the depletion of some antibodies towards conformational epitopes as well.

Another set of experiments was conducted to confirm the presence of only conformational epitope(s) on the PCV2 particles. The rabbit was immunized with purified full-length PCV2-Cap eluted in 8 M urea. Purification of PCV2-Cap with the presence of urea denatured the conformational epitopes; therefore, serum that was collected from the rabbit only detected the PCV2-Cap linear epitopes. Rabbit serum did not neutralize the PCV2. In addition, the serum was tested in the PCV2 particle-based ELISA, and no reactivity was observed between PCV2 particles and rabbit serum. As a result, rabbit serum raised against linear epitopes of PCV2-Cap neither revealed neutralization nor reacted with PCV2 particles. These results indicate that PCV2 particles do not expose linear epitopes on their surface.

Under field conditions, the continuous exposure of animals to PCV2 may affect the antibody response in these animals. The correlation that exists between NA titer and the reactivity of swine sera towards PCV2-Cap linear epitopes ( $r=0.66$ ,  $p< 0.001$ ) might result from the interaction between PCV2 and the immune system in these animals. Cap monomer or Cap fragments could be recognized by the immune system during virus replication (Trible et al., 2011). The immune cells detect and kill PCV2-infected cells, leading to the exposure of PCV2 linear epitopes to the immune system. B-cells detect the PCV2 linear epitopes directly by the help of  $T_H$  (Owen et al., 2013) and produce antibodies against linear epitopes of PCV2. As a conclusion, the interaction between PCV2 and immune system leads to not only the induction of NAs, but also production of antibodies against linear epitopes.

### **4.3 Do Antibodies that React with PCV2-Cap Conformational Epitope(s) have Virus Neutralizing Activity?**

Previous studies indicate that NAs contribute to the protection of pigs from PMWS (Fort et al., 2008; Opriessnig et al., 2008a). Therefore, identification of the NAs and its target epitope is important for PCV2 vaccine development. The significant positive correlation between the level of NA titer and antibody reactivity towards conformational epitope(s) in particle-based ELISA ( $r=0.46$ ,  $p<0.001$ ), raises the question whether NAs were produced against PCV2 conformational epitope(s) and if particles expose only neutralizing conformational epitope(s).

There are only two experimental pig studies that have indirectly investigated the type of epitopes (linear vs. conformational) targeted by NAs using swine sera (Trible et al., 2011; Tribble et al., 2012). Tribble et al. (2011) demonstrated that sera collected from experimentally vaccinated pigs have higher antibody responses to the nearly full-length rCap (residues 43-233) than small size rCaps. The authors suggested that rCap (43-233) is large enough to fold and make a conformational epitope, which can be targeted by NAs in vaccinated animals. In a follow-up study, PCV2 negative pigs were vaccinated with either a baculovirus-expressed Cap vaccine or a truncated Cap (43-233) fused with ubiquitin (Ub) (Tribble et al., 2012). Fusion of Cap with Ub is believed to keep the Cap protein in monomeric form. Baculovirus-expressed Cap vaccine and Cap-Ub are representative of conformational and monomeric Cap, respectively. The immunization of pigs with vaccine or Cap-Ub was followed by a double challenge with PCV2/PRRSV viruses. The results demonstrated that pigs immunized with Cap-Ub fusion protein and challenged with PCV2/PRRSV have low NA titer, whereas animals immunized with PCV2 vaccine have high NA titer.

In this study, the majority of swine sera showed a significant positive correlation between the level of NA titer and antibody reactivity towards particle-based ELISA ( $r=0.46$ ,  $p<0.001$ ). Our results aligned with previous studies suggesting that NAs recognize conformational epitope(s) on the surface of PCV2 particles if we take into account that 60 Cap molecules would fold into tridimensional structure and form conformational epitope(s) (Crowther et al., 2003). In addition, the PCV2 particles used in the current study may decrease the risk of false-negative results, because these particles were produced by reverse genetics from the full-length genome; the Cap-NLS was deleted in other studies (Trible et al., 2011; Tribble et al., 2012). Importantly, the NLS region of Cap includes immune-reactive regions (Mahe et al., 2000) and NLS deletion may change the folding of Cap protein. Thus, PCV2 particle-based ELISA might have an advantage over other rCap-based ELISA in assessing antibody reactivity towards conformational epitope(s). In addition, the use of PCV2 particles as an antigen overcomes some issues related to the cross-reactivity of pig antibodies with other contaminants when bacterial rCap protein is used as a source of an antigen.

#### **4.3.1 IgM-NAs Target Conformational Epitope(s) on the Surface of PCV2 Particles**

NAs are mainly restricted to IgGs, as shown by the positive correlation between ELISA specific to isotypes and NA titer (Fort et al., 2007). In mouse models, NAs against PCV2 are mainly IgG2a (Shen et al., 2008). In our study, 15 sera (group D: 9.3%) (Figure 3–12) from both vaccinated and non-vaccinated cohorts and with different levels of NA titer, showed no detectable level of IgG antibodies towards PCV2 particles. This raised the question whether other antibodies isotypes were involved in virus neutralizing activity in pigs.

The IgM antibody is the first class of antibody produced during infection (Owen et al., 2013) and can neutralize PCV2 (Meerts et al., 2006). Here, we showed a strong positive correlation between NA titer and IgM reactivity towards PCV2 particles ( $r=0.76$ ,  $p < 0.01$ ) in group D of sera, which explains the lack of reactivity in particle-based ELISA (IgG). Our study does not support a previous study showing no correlation between NA titer and IgM reactivity towards PCV2-rCap using a commercial kit (Fort et al., 2007). The negative correlation between NA titer and IgM-capture ELISA might be due to the absence of proper folding of rCap in the ELISA plate, which prevents exposure of the neutralizing epitope. Notably, in the current study presence of PCV2-IgM was confirmed in both vaccinated and non-vaccinated cohorts, while vaccination against the influenza A virus suppresses IgM and accelerates IgG responses (Panyasing et al., 2013).

The maximum OD values of IgM antibodies towards PCV2 in these sera was 0.23, which was lower than the maximum OD values of anti-PCV2 IgG antibodies (maximum OD= 2.16). The low OD values of anti-PCV2 IgM antibodies might due to the affinity maturation and class-switching process. Typically, higher affinity was observed for IgG antibodies than IgM. Although IgM antibodies have low affinity, they have high avidity towards the antigen due to the pentameric structure and presence of 10 antigen-binding sites, which enable IgM to bind to the antigen at different sites (Owen et al., 2013). In addition, the concentration of IgM antibodies (2.9 mg/mL) in pig serum is lower than IgG antibodies (24.3 mg/mL) (Bourne, 1973).

IgM antibodies against PCV2 are observed between 7 and 14 DPI, reaching a peak at 21 DPI, and decreasing until 49 DPI using experimentally-infected pigs (Fort et al., 2007). The presence of IgM in the sera indicates that pigs are in the beginning of the infection or they are re-infected with PCV2 and have not yet undergone isotype switching. However, detection of IgM

antibodies does not always result after recent virus infection, for example, IgM antibodies against chikungunya virus (Borgherini et al., 2008; Grivard et al., 2007) and West Nile virus (Carson et al., 2006) can persist for 18 months. However, the reason for this prolonged presence of IgM antibodies has not been defined.

In conclusion, neutralizing capacity of PCV2 antibodies is due to both IgG and IgM antibodies.

#### **4.3.2 Swine Antibodies Target Non-neutralizing Conformational Epitope on the Surface of PCV2 Particles**

Despite the positive correlation between NA titer and reactivity of sera towards PCV2 particles, there are few swine sera (group A: 3.7%) (Figure 3–12), that react with PCV2 particles and do not show detectable level of NAs. This raises the question whether neutralizing conformational epitope(s) is the only epitope exposed on the surface of PCV2 particles.

There were four non-neutralizing sera in group A of the cohort, without reactivity towards linear epitopes in a Western blot, but with positive reactivity towards PCV2 particles. Therefore, these sera neither have neutralizing antibodies nor have antibodies directed to linear epitopes. Reactivity of these sera with PCV2 particles was explained by the presence of non-neutralizing conformational epitope(s) on the PCV2 particles. Altogether, these data suggest that PCV2 particles expose both neutralizing and non-neutralizing conformational epitopes. This result may not be unexpected, since the presence of both neutralizing and non-neutralizing epitopes is observed in the structural protein of other viruses such as PRRSV (Ostrowski et al., 2002) and foot and mouth disease virus (Ouldridge et al., 1984).

Few sera in the cohort (group B: 12.5%) (Figure 3–12) had higher reactivity towards PCV2 particles compared to other samples. I hypothesize that these sera react with both neutralizing and non-neutralizing conformational epitopes on the surface of PCV2 particles. However, uncovering the reason for reactivity of only few swine sera in the cohort with both conformational neutralizing and non-neutralizing epitopes requires further research.

#### **4.4 Conclusions**

Our data showed that NAs mainly target surface-exposed conformational epitope(s) on the surface of PCV2 particles, regardless of the antibody isotype (IgG or IgM). Furthermore, the combinational approach of different assays, i.e. virus neutralization assay, Western blot, and particles-based ELISA, has helped identify different profiles of antibody response in pigs and the existence of non-neutralizing conformational epitope(s) on the surface of PCV2 particles.

#### **4.5 Future Directions**

PCV2 particles that contain surface-exposed neutralizing conformational epitope(s) can be used as a target to identify the exact sequence of neutralizing epitope. Different methods have been used to identify the amino acid sequence of the epitope such as X-ray crystallography and phage display library. X-ray crystallography is considered a gold standard method for the identification of epitopes, although it is very expensive and requires mAbs in a crystal form. The phage display would be an alternative way to define the precise location of a PCV2 neutralizing epitope. The approach involves inserting into the phage the DNA fragments encoding the protein of interest, and then screening the phages using swine sera that have high levels of NA titer. The selected phages would be analyzed by sequencing to determine the sequence of peptides targeted

by the NAs. This method offers sufficient depth of coverage to characterize the PCV2 neutralizing epitope, which will help us to develop a new vaccine. A neutralizing epitope-based vaccine will restrict the immune response to the production of high level of NAs and provide protection not only against the disease but also against the infection.

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## Appendix

### Appendix 1. Chemicals and Consumables

Product Name	Provider	Code
ABTS(500ml)	Invitrogen	00-2024
Acetic Acid Glacial	Sigma-Aldrich	695084
Acrylamide (30% solution)	Sigma-Aldrich	A3699
Amersham ECL Prime	GE-Healthcare	RPN2232
Ammonium Persulfate (APS)	Sigma-Aldrich	A9164
BL21 (DE3) Rosetta blue competent cells	Novagen	71059-4
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A7906
2-Mercaptoethanol (BME)	Sigma-Aldrich	M3148
Bradford Assay Reagent	Bio-Rad	500-0006
Brilliant Blue R	Sigma-Aldrich	B0149
Bromophenol Blue	Sigma-Aldrich	B0126
Cell Culture Flasks (T25)	BD Falcon	353108
Cell Culture Flasks (T75)	BD Falcon	353109
Conical Centrifuge Tube (25ml)	VWR	525-0400
Conical Centrifuge Tube (50ml)	VWR	525-0403
4',6-Diamidino-2-Phenylindole (DAPI)	Invitrogen	D3571
DNA Miniprep Kit	Fermentas	K0503
Eagle's Minimum Essential Medium(500ml)	Gibco	11095-080
ECL Plus Western Blotting Detection	GE Healthcare	RPN2132
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich	E0399
ELISA Plates	Corning	9018
Fetal Bovine Serum (FBS)	Gibco	12483-020
GeneJet Plasmid Miniprep Kit	Fermentas	K0502
Glacial Acetic Acid	Sigma-Aldrich	695092
Glycerol	Invitrogen	15514-011
Glycin	Sigma-Aldrich	G8790
Goat anti Pig HRP (IgM)	Abcam	ab112745
Goat anti Rabbit Alexa Fluor 568	Invitrogen	A11011
Goat anti Rabbit HRP (whole IgG)	Sigma-Aldrich	A0545
Hydrochloride Acid (HCl) (37%)	Sigma-Aldrich	258148
Imidazole	Sigma Aldrich	15513
Isopropyl $\beta$ -D-1-thiogalactopyranoside (IPTG)	Sigma Aldrich	15502
Kanamycin Disulfate Salt	Sigma Aldrich	K1876
Lipofectamine 2000 Transfection Reagent	Invitrogen	P/N 52887
Luria Broth (LB)	Invitrogen	12795-084
Magic Mark	Invitrogen	LC5602
Essential Amino Acid Solution	Sigma- Aldrich	M7145
Methanol	Sigma-Aldrich	179337
N,N,N',N'-Tetramethylethylenediamine(TEMED)	Sigma-Aldrich	T9281
Ni-NTA Agarose (25ml)	Qiagen	30210

Page Ruler Prestained Ladder Protein	Fermentas	SM0671
Parafilm	Pechiney, Chicago	
Paraformaldehyde Powder(PFA)	Sigma-Aldrich	158127
Penicillin/Streptomycin	Sigma-Aldrich	P4458
pET28a (+)	Novagen	69864-3
Plasmid Maxi Kit	Qiagen	12163
Polyethylene Glycol (PEG) 8000 Powder	Promega	V3011
Polyvinylidene Fluoride (PVDF)	GE Healthcare	RPN303F
Ponceau	Sigma-Aldrich	P3504
Potassium Chloride (KCl)	Sigma-Aldrich	P5405
Potassium Phosphate Monobasic(KH <sub>2</sub> PO <sub>4</sub> )	Sigma	P9791
Rabbit anti Pig HRP (whole IgG)	Sigma Aldrich	A5670
Rabbit anti GFP	Sigma Aldrich	G1544-100
Saponin	Sigma-Aldrich	47036
Serological Pipets (1mL)	Falcon	357521
Serological Pipets (5mL)	VWR	89130-896
Serological Pipets (10mL)	VWR	89130-898
Serological Pipets (25mL)	VWR	89130-900
Skim Milk Powder	Safeway	
Sodium Azide	Sigma-Aldrich	71289
Sodium Bicarbonate (NaHCO <sub>3</sub> )	Sigma-Aldrich	144-55-8
Sodium Chloride (NaCl)	Sigma-Aldrich	S3014
Sodium Phosphate Monobasic (NaH <sub>2</sub> PO <sub>4</sub> )	Sigma-Aldrich	S8282
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich	L4390
Sodium phosphate dibasic (Na <sub>2</sub> HPO <sub>4</sub> )	Sigma-Aldrich	S9763
Sodium Pyruvate	Gibco	11360-070
Spectra/pro Dialysis Membrane	Spectrum Labs	132128
Sucrose	Sigma-Aldrich	S0389
Sulfuric acid (H <sub>2</sub> SO <sub>4</sub> )	Acros Organics	13361-0010
Surgical Blade Stainless Steel	Feather	11086410
Top 10	Invitrogen	C4040-10
Trizma base	Sigma-Aldrich	93362
Trypan Blue Stain (0.4%)	Gibco	15250-061
Trypsin-EDTA (0.25%) (1X)	Invitrogen	25200
Tween-20	Sigma-Aldrich	P7949
Urea	Sigma-Aldrich	U1250

## Appendix 2. Buffers/Solutions

<ul style="list-style-type: none"> <li>• <u>lysis buffer, pH 8.0</u>  NaH<sub>2</sub>PO<sub>4</sub> 100mM  NaCl 300mM  Imidazole 20mM  Tween 20 0.05%  Urea 8M</li> </ul>	<ul style="list-style-type: none"> <li>• <u>Elution Buffer, pH 8.0</u>  NaH<sub>2</sub>PO<sub>4</sub> 100mM  NaCl 300mM  Imidazole 500mM  Tween 20 0.05%  Urea 8M</li> </ul>
<ul style="list-style-type: none"> <li>• <u>PBST 0.25% (Western blot)</u>  PBS 1000mL  Tween 20 2.5mL</li> </ul>	<ul style="list-style-type: none"> <li>• <u>Ponceau Staining (500 mL)</u>  Ponceau 5 g  Acetic Acid 5mL  H<sub>2</sub>O 475mL</li> </ul>
<ul style="list-style-type: none"> <li>• <u>Blocking buffer (Western blot)</u>  Skim milk 5%  PBST 0.25%</li> </ul>	<ul style="list-style-type: none"> <li>• <u>Blocking buffer (ELISA)</u>  Skim milk 5%  PBST 0.05%</li> </ul>
<ul style="list-style-type: none"> <li>• <u>Coomassie staining solution(200mL)</u>  Brilliant blue 0.5g  dH<sub>2</sub>O 91mL  Methanol 91mL  Glacial acetic acid 18mL</li> </ul>	<ul style="list-style-type: none"> <li>• <u>Coomassie destaining solution (1L)</u>  dH<sub>2</sub>O 455mL  Methanol 455mL  Glacial acetic acid 90mL</li> </ul>
<ul style="list-style-type: none"> <li>• <u>Resolving SDS PAGE gel 12%</u>  dH<sub>2</sub>O 1.6mL  Acrylamide 30% 2mL  Tris (pH 8.8), 1.5M 1.3m  SDS 10% 50μL  APS 10% 50μL  TEMED 2μL</li> </ul>	<ul style="list-style-type: none"> <li>• <u>Stacking SDS PAGE gel</u>  dH<sub>2</sub>O 1.05mL  Acrylamide 30% 250μL  Tris (pH 6.8), 0.5M 190μL  SDS 10% 15μL  APS 10% 15μL  TEMED 1.5μL</li> </ul>
<ul style="list-style-type: none"> <li>• <u>10% APS (10mL)</u>  APS 1 g  dH<sub>2</sub>O 10 mL</li> </ul>	<ul style="list-style-type: none"> <li>• <u>10% SDS</u>  SDS 50 g  dH<sub>2</sub>O 500 mL</li> </ul>
<ul style="list-style-type: none"> <li>• <u>2X SDS PAGE sample buffer</u>  Bromophenol blue 27.5μM  BME 15%  Glycerol 1 30%  SDS 0.12%  Tris (pH 6.8) 0.15M</li> </ul>	<ul style="list-style-type: none"> <li>• <u>10X PBS (1 L)</u>  NaCl 1.4M  KCl 27mM  Na<sub>2</sub>HPO<sub>4</sub> 65mM  KH<sub>2</sub>PO<sub>4</sub> 15mM  pH 7</li> </ul>
<ul style="list-style-type: none"> <li>• <u>10X SDS PAGE running buffer (1 L)</u>  Tris 0.25M  Glycin 1.92M  SDS 10g</li> </ul>	<ul style="list-style-type: none"> <li>• <u>Western blot transfer buffer (1 L)</u>  Tris 25mM  Glycin 192mM  Methanol 20%  SDS 0.2%</li> </ul>
<ul style="list-style-type: none"> <li>• <u>Permeabilization buffer (IFA)</u>  PBS 1X  Saponin 0.1%  BSA 0.1%</li> </ul>	<ul style="list-style-type: none"> <li>• <u>TNE buffer</u>  Tris 10mM  EDTA 2mM  NaCl 150mM</li> </ul>

<ul style="list-style-type: none"> <li>• <u>LB Medium</u> 25g/L LB base in dH<sub>2</sub>O</li> </ul>	
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### **Appendix 3. Equipment and Software**

- SDS PAGE and Western blot
  - Bio-Rad Mini-protean Tetra Cell system for 1.0 mm gels
  - Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell
  - Bio-Rad VersaDoc Imaging System
  
- Centrifuges
  - Beckman Avanti J-26 XPI high speed centrifuge; rotors: JLA-16.250
  - Beckman coulter-optima ultracentrifuge, MLS50
  - Eppendorf Centrifuge 5810R; rotors: A-4-62, F-34-6-38
  - Eppendorf Centrifuge 5415R
  
- Scales
  - Mettler Toledo PB153-S
  - Satorius TE212
  
- Microscopes
  - Olympus CKX41 upright microscope
  - Olympus 1X51 inverted microscope
  - Incell analyzer 2000 automated microscope
  
- Softwares
  - Quantity-One imaging software
  - SPSS Statistical software 17.0

- Others

Thermo Scientific NanoDrop 1000 Spectrophotometer

Eppendorf Biophotometer

Eppendorf Thermomixer

New Brunswick Scientific Innova 40 Incubator Shaker

Fischer Isotemp 220 series water bath

Bio-Rad iMark plate reader

Stovall Life Science the Belly Dancer rotating platform

VWR Symphony pH meter

Fischer Scientific Vortex Mixer

Fischer Scientific Isotemp ceramic stirrer

#### **Appendix 4. Bio-Rad Bradford Protein Assay (Bio-Rad instruction manual)**

##### **Microtiter Plate Protocols**

The Bio-Rad Protein Assay can also be used with a microplate reader. The linear range of the Standard and Microassay procedures when used in the microtiter plate format is slightly changed, since the ratio of sample to dye is modified.

##### **Standard Procedure for Microtiter Plates**

1. Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts DDI water. Filter through a Whatman #1 filter (or equivalent) to remove particulates. This diluted reagent may be used for about 2 weeks when kept at room temperature.
2. Prepare three to five dilutions of a BSA protein standard. The linear range of this microtiter plate assay is 0.05 mg/ml to approximately 0.5 mg/ml. Protein solutions are normally assayed in duplicate or triplicate.
3. Pipet 10  $\mu$ l of each standard and sample solution into separate microtiter plate wells.

4. Add 200  $\mu$ l of diluted dye reagent to each well. Mix the sample and reagent thoroughly using a microplate mixer. Depress the plunger repeatedly to mix the sample and reagent in the well. Replace with clean tips and add reagent to the next set of wells.
5. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 h.
6. Measure absorbance at 595 nm.

## **Appendix 5. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

### Objective:

To separate proteins according to their size for analysis via coomassie staining or western blot.

### Buffers and Reagents:

#### 1. 1.5 M Tris/HCl, pH 8.8

90.855 g Tris or Trizma base  
500 mL distilled water

#### 2. 0.5 M Tris/HCl, pH 6.8

30.285 g Tris or Trizma base  
500 mL distilled water

#### 3. 10% SDS

50 g SDS  
500 mL distilled water

#### 4. 10% APS

0.2 g APS  
2 mL distilled water

#### 5. TEMED

#### 6. 30% acrylamide-bis/acrylamide solution

### 7. 10X SDS Running Buffer

30.3 g Tris or Trizma base

144.1 g Glycine

10 g SDS powder

1L distilled water

### 8. 2X SDS Sample Loading Buffer

10 mL 1.5 M Tris, pH 6.8

6 mL 20% SDS

30 mL Glycerol

15 mL 2-mercaptoethanol

1.8 mg Bromophenol blue

100 mL distilled water

### Protocol:

#### Phase I – Casting SDS-PAGE gel

1. Wash the glass plates and spacers well with detergent before use and wipe them dry.  
Assemble the glass plates and spacers as shown in the figure below. Fill the space between glass plate and spacer with water to make sure there is a good seal. If there is a leak, readjust the plates.

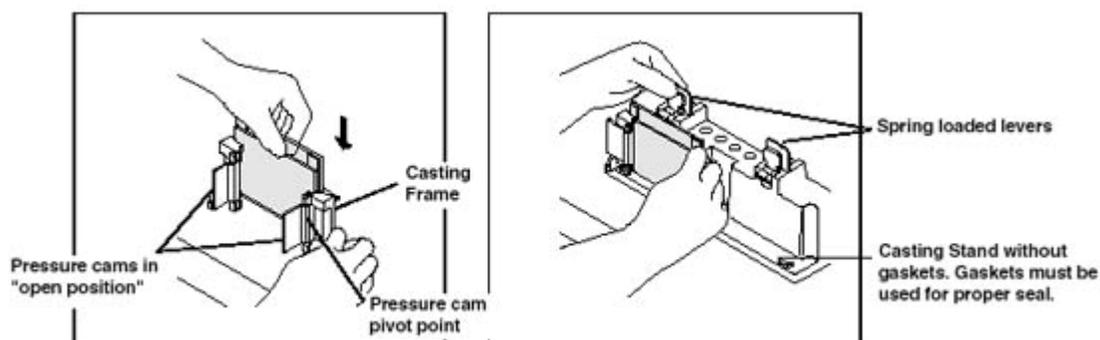


Fig. 3. Assembling the Mini-PROTEAN 3 Casting Frame and Casting Stand.

(c) Bio-Rad

2. Prepare resolving and stacking gels in 50 mL and 15 mL Falcon tube, respectively. The recipe of each gel is shown in table 1.

Table 1. Ingredients for SDS-PAGE gels commonly used in this laboratory. Other percentage gels can be made. Please refer to Bio-Rad manual/protocol for other percentage gels.

\* refers to ‘add immediately before use’. 10 mL of resolving gel is for 2 gels while 10 mL of stacking gel is for more than 2 gels.

<b>Ingredients</b>	<b>12% Resolving Gel</b>	<b>4% Stacking Gel</b>
Distilled water	3.4 mL	6.1 mL
30% acrylamide/bis-acrylamide solution	4.0 mL	1.3 mL
1.5 M Tris/HCl, pH 8.8	2.5 mL	-
0.5 M Tris/HCl, pH 6.8	-	2.5 mL
10% SDS	100 $\mu$ L	100 $\mu$ L
10% APS*	50 $\mu$ L	50 $\mu$ L
TEMED*	5 $\mu$ L	10 $\mu$ L
Total Volume	10 mL	10 mL

3. When the gel is ready to be cast, pour out the water used in step #1. Add APS and TEMED to the resolving gel solution and mix thoroughly.
4. Pour the resolving gel solution into the space between glass plates and spacer up until the green line on the gel casting apparatus. Make sure to leave enough space for the stacking gel solution and the length of the comb.
5. Fill to the top with isopropanol. This will help make a sharp line on the resolving gel solution. Incubate at room temperature until the gel polymerizes.
6. When the resolving gel polymerizes, discard the isopropanol by pouring and by soaking with filter paper.
7. Add APS and TEMED to the stacking gel solution and mix thoroughly. Fill the remaining space between glass plates and spacer with this stacking gel solution to the

rim. Immediately add the comb into the slot. If a 1mm spacer plate is used, the corresponding 1mm comb is to be used, and so on. Incubate at room temperature until the gel polymerizes.

8. Once the gel completely polymerizes, proceed to running SDS-PAGE. If SDS-PAGE was not ran on the same day, gel cassettes were removed from the casting frame and stored wrapped in moist paper towel and sera wrap at 4°C.

#### Phase II – Running SDS-PAGE

9. Assemble two gel cassettes onto the electrode assembly with short, spacer plates facing inward. If only one gel cassette was made, use a mock gel cassette.
10. Slide the electrode assembly into the clamping frame. Press down on the electrode assembly while closing the cam levers of the clamping frame.
11. Lower the clamping frame into the mini-tank. Fill the inner chamber (formed from the two gel cassettes in the clamping frame) with 1X SDS Running Buffer up to the rim. Fill further until the  $\frac{1}{4}$  of the tank is filled with 1X SDS Running Buffer.
12. Prepare and load protein samples:
13. Cell lysates, resuspended in 2X sample buffer, are boiled at 99°C for 5-10 minutes and vortexed. Samples can be spun down briefly to remove the condensation droplets from the top of the tube. Load protein samples (5-10  $\mu$ L depending on the size of the well) into the well. Usually, a reference pre-stained ladder and a ladder that can show up when exposed to chemiluminescence are loaded along with the protein samples. Loading of protein samples was often done in the fume hood because 2X sample buffer contains 2-mercaptoethanol. Be careful not to overload the wells as cross-contamination can occur.

14. Close the lid of the SDS-PAGE apparatus. Make sure the colors of the electrodes match those on the apparatus. Run SDS-PAGE at 100V for 1 h and 45 minutes.

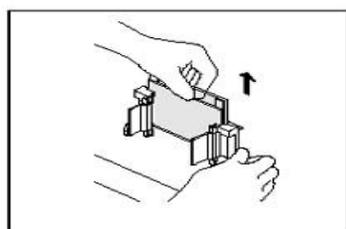
Notes:

**Caution:** Read the MSDS before using 30% acrylamide-bis/acrylamide solution as this chemical is a strong nerve-toxic reagent.

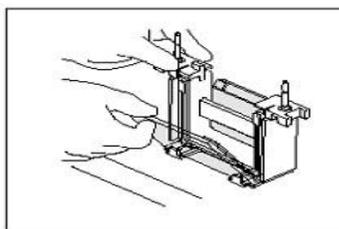
**Caution:** Treat SDS powder with extreme caution. SDS powder should be put into solution in the fume hood.

For more information, refer to the Bio-Rad manual/protocol for running SDS-PAGE with the mini-protean system.

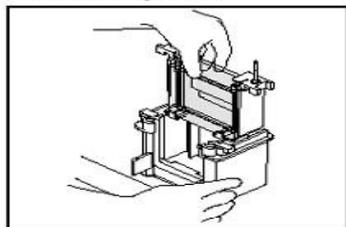
**Caution:** 1X SDS Running Buffer can be reused once



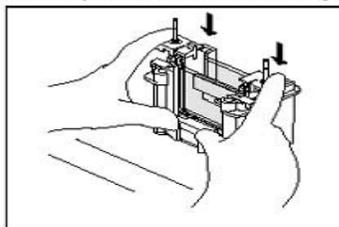
5a. Remove the Gel Cassette Sandwich from the Casting Frame.



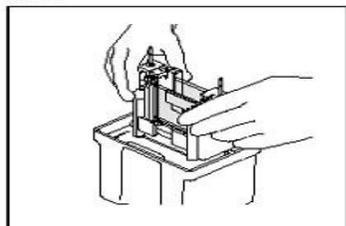
5b. Place Gel Cassette Sandwich into the Electrode Assembly with the Short Plate facing inward.



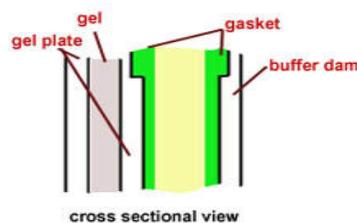
5c. Slide Gel Cassette Sandwiches and Electrode Assembly into the clamping frame.



5d. Press down on the Electrode Assembly while closing the two cam levers of the Clamping Frame.



5e. Lower the Inner Chamber into the Mini Tank.  
Fig. 5. Mini-PROTEAN 3 assembly.



Modified from Bio-Rad's source

## **Appendix 6. Coomassie Staining**

*Objective:* To stain SDS-PAGE gels for proteins

*Buffers:*

### 1. Coomassie Staining Solution

0.5 g Coomassie Brilliant Blue

91 mL sterile water

91 mL methanol

18 mL glacial acetic acid

### 2. Coomassie Destaining Solution

455 mL sterile water

455 mL methanol

90 mL glacial acetic acid

*Protocol:*

1. Remove protein gel from SDS-PAGE running machine and incubate the gel in coomassie staining solution for 30 minutes with rocking.
2. Remove the coomassie staining solution. Do not discard as the solution can be reused. When the solution becomes 'gunky' and quite different from the first time the solution was made, discard the solution.
3. Incubate the stained gel in the coomassie destaining solution until protein bands can be seen. Change the solution periodically.
4. The coomassie destaining solution can be reused by filtering the solution onto a filter paper coated with charcoal.

*Notes:*

Apparently, coomassie-stained gels can also be destained using sterile water with heating in a microwave. Do NOT microwave coomassie destaining solution because it contains methanol.

## Appendix 7. Semi-dry Transfer (Bio-Rad)

### Objective:

To transfer resolved proteins from protein (*i.e.* SDS-PAGE) gels onto membrane (nitrocellulose or polyvinylidene fluoride [PVDF]) for analysis of protein bands by western blot.

### Reagents:

#### 1. Semi-dry transfer buffer, pH 8.3

- 3.029 g Tris or Trizma Base
- 14.4 g Glycine
- 200 mL Methanol
- 2 g SDS
- 1L distilled water

### Protocol:

1. Equilibrate the protein (*i.e.* SDS-PAGE) gel in semi-dry transfer buffer for 15-30 minutes with rocking. This will remove some salts and SDS from the SDS-PAGE running buffer and prepare gels for semi-dry transfer. Gels are not recommended to be in equilibration buffer for too long as some proteins may be lost to diffusion.
2. Equilibrate semi-dry transfer filter papers (2) and membrane (1) for each gel in semi-dry transfer buffer in the same manner as gel-equilibration. **IMPORTANT:** PVDF membrane needs to be activated in methanol for 5-10 seconds before equilibration.
3. Pre-damp (NOT soak) the semi-dry tray with semi-dry transfer buffer before preparing the sandwich in step 4.
4. Prepare a semi-dry transfer ‘sandwich’ as shown in figure 1. At each layer of the sandwich, a roller or a serological pipet is used to firmly roll bubbles from each layer. Pre-damp (NOT soak) the semi-dry cover with semi-dry transfer buffer.

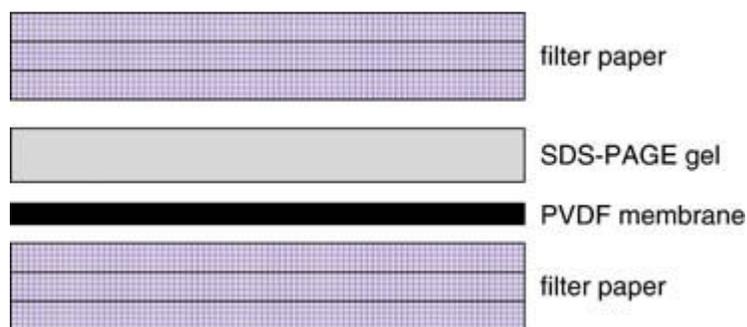


Figure 1. Semi-dry transfer ‘sandwich’. Note that a PVDF membrane can be replaced with a nitrocellulose membrane.

5. Two methods can be used to run the semi-dry transfer: 1) constant amps and 2) constant volts. This protocol describes a constant amp method: run *each blot* at 60mA or 0.06A for 50 minutes. The maximum number of blots for each transfer is four (240mA or 0.24A). This protocol is preferred to reduce the amount of heat generated. Transfer of proteins with larger molecular weight may require a longer run-time (i.e. up to 90 minutes). Do NOT exceed 25V.
6. Recommended optional step: After the transfer is finished, stain the SDS-PAGE gel with coomassie and stain the blot with Ponceau Red to determine the efficiency of transfer. This is recommended for first-time user. Usually, the efficiency of transfer can also be determined with a pre-stained ladder that was initially loaded onto a lane in the SDS-PAGE gel and subsequently transferred.
7. Block the membrane in 5% skim milk/PBST overnight at 4°C or for 1 h at room temperature. **NOTE:** This is step #1 of Western blotting protocol.

Notes:

1. Membrane selection for western blotting (one-sided focus on the advantage of PVDF over nitrocellulose membrane):

<http://www.millipore.com/immunodetection/id3/membraneselection>.

2. Use a forceps, NOT gloves, to touch the membrane.
3. Please have a look at the semi-dry transfer manual from Bio-Rad for more information.

## **Appendix 8. Western Blotting**

Objective: To detect specific proteins using antibodies.

Reagents:

### 10X Phosphate buffered saline (PBS) pH 7.4

- 81.816 g 1.4M Sodium chloride
- 2.013 g 27mM Potassium chloride
- 9.2274 g 65mM Sodium phosphate dibasic
- 2.04135 g 15mM Potassium phosphate monobasic
- 1L distilled water
- Adjust pH to 7.4, autoclave

### 2.1X PBS with 0.5% Tween 20 (PBST)

- 200mL 10X PBS
- 10mL Tween 20
- 2L distilled water

### 3. 5% skim milk

- 5 g skim milk powder
- 100 mL 1X PBST

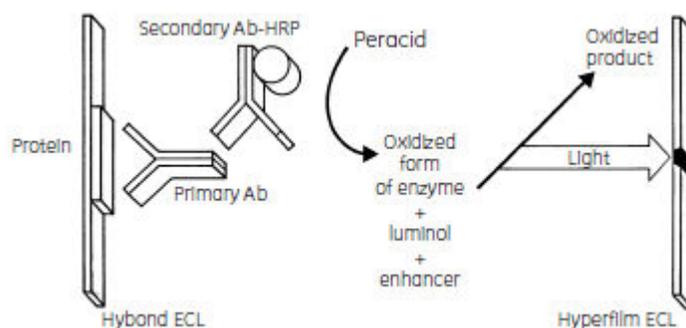
### 4. Enhanced chemiluminescent substrate reagent mix

- 2 mL Solution A
- 50  $\mu$ L Solution B

5. Primary and secondary antibodies (Vary depending on applications and assays)

Protocol:

1. Block membrane in 5% skim milk dissolved in PBST for 1 h at room temperature or at 4°C overnight. Purpose: Using milk or bovine serum albumin (BSA) will block nonspecific protein binding sites.
2. Optional: Rinse or wash the membrane three times in PBST for 5 minutes at room temperature with rocking.
3. Incubate the membrane in primary antibody for 1 h at room temperature or overnight at 4°C. Optimized concentration of primary antibody is diluted in 5% skim milk/PBST.
4. Wash the membrane three times in PBST for 5 minutes at room temperature with rocking.
5. Incubate the membrane in secondary antibody for 1 h at room temperature. Optimized concentration of secondary antibody is diluted in 5% skim milk/PBST. Incubation in secondary antibody for 50 minutes is fine, but longer incubation beyond 1 h is not recommended.
6. Wash the membrane three times in PBST for 5 minutes at room temperature with rocking.
7. Incubate the membrane in enhanced chemiluminescent substrate reagent mix at room temperature for 5 minutes.



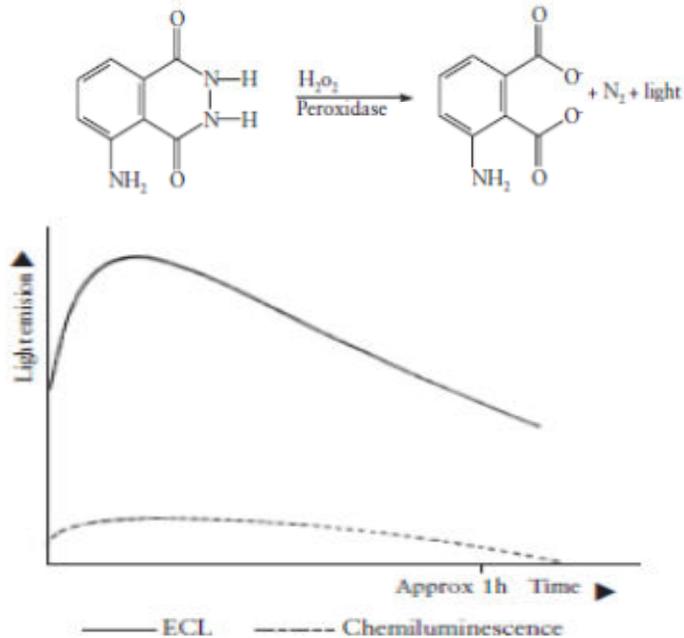


Figure 1. Principle of ECL detection (taken from GE Healthcare reference sheet, RPN2132). Important points: 1) Chemiluminescent reaction peaks after 5-20 minutes and decays slowly thereafter. Half-life is 60 minutes.

8. Membranes were analyzed with a VersaDoc 5000 Imaging System and a Quantity One software.

Notes:

1. The rocking speed can be increased for the 5 minute-washing step. Longer washing time is fine.
2. Incubation of membrane in primary antibody at 4°C overnight is often better than incubation at room temperature for 1 h.

**Appendix 9. PCV2 Neutralization Assay (Optimized by Cristina Solis Worsfold)**

Objective:

Determine the titer of neutralizing antibodies against PCV2 in pig serum samples.

*Materials and reagents:*

<b>For neutralization assay:</b>	<b>1 plate</b>
96-well plate	1
96-well PCR skirted plate	2
MEM media (add 5% FBS, antibiotics, amino-acids and sodium pyruvate)	15ml
PCV2b stock	5ml
200ul tips non-barrier	1 box
100ul tips barrier	2.5 boxes
PK-15	1x10 <sup>6</sup> cells
Serum sample (Positive control)	25µl
Reagent container	2
<b>For staining:</b>	
4% Paraformaldehyde	4ml
Permeabilization buffer (1X PBS, 0.1% Saponin, 0.1% BSA)	12ml
Rabbit anti-CAP (1:200)	20 µl
Goat anti- rabbit (1:400)	10 µl
DAPI (300nM) in PBS	4ml
PBS-tween 0.1%	60ml
200ul tips non-barrier	1 box
Non-sterile PBS	70ml

*Protocol Virus Neutralization Assay:*

1. Place 50µl of MEM media in the wells of a 96-well PCR skirted plate.
2. Place 25µl of the serum sample in the first well and make three fold dilutions going down the column for a final volume of 50µl<sup>1</sup>. Barrier tips should be used in this step, changing them with every dilution. Discard 25µl of the last dilution.
3. Add 50µl of the PCV2 stock into each well. Final volume: 100µl. Place 2 wells of positive infection control with the virus only (no serum).
4. Place a plastic adhesive film to avoid evaporation of the sample. Shortly vortex and spin down the plate.
5. Incubate the virus and the samples for 1 h at 37°C incubator.
6. Take the 96-well plate containing the PK-15 cells out from the incubator. Take all media out using the extractor.

7. Place the samples in the plate following the same order as the skirted plate using barrier tips.
8. Incubate for 90 minutes at 37°C.
9. After the incubation, take all media out, wash 1X with sterile PBS and place 100µl of fresh media.
10. Incubate 48 h at 37°C. After incubation, take all media out with the extractor.

*Protocol for indirect-immune fluorescence staining:*

1. Place 50µl of 4% paraformaldehyde to fix the cells and incubate at room temperature for 30 minutes. Afterwards, wash with 100µl of non-sterile PBS three times. Discard the PBS-PFM in the correct container.
2. Place 50µl of permeabilization buffer into each well and incubate at room temperature for 30 minutes. Take out with extractor after incubation.
3. Place 40µl of the primary antibody (rabbit anti-CAP) 1:200 dilution in permeabilization buffer on each well. Incubate 90 minutes at 37°C.
4. Wash 3X with 100µl PBS-Tween.
5. Place 40 µl of the secondary antibody (goat anti-rabbit Alexa Fluor) 1:400 dilution in permeabilization buffer on each well. Incubate 60 minutes at 37°C.
6. Wash 3X with 100µl PBS-Tween.
7. Place 40ul of DAPI 300nM on each well and incubate 10 minutes room temperature. Afterwards, wash 3X with 100µl of non-sterile 1X PBS. Discard liquid in correct container.
8. Place 100µl of 1X PBS on each well. Cover in aluminium foil and refrigerate until analysis.

- Analyze the plate in InCell Analyzer (Dr. Yates Lab).

## **Appendix 10. Recombinant Cap Purification (Optimized by Narges Nourozieh)**

### Reagents:

pET vector with HIS-tagged CAP  
BL-21 *E. coli*  
Luria Broth  
Kanamycin  
Ni-NTA charged beads  
Amicon Ultra 30K tubes  
1x PBS

### Buffers/Solutions:

#### 1. Imidazole Lysis/Wash Buffer, pH 8.0

100mM NaH<sub>2</sub>PO<sub>4</sub>  
300mM NaCl  
20mM imidazole  
0.05% Tween 20  
8M Urea

#### 2. Imidazole Elution Buffer, pH 8.0

100mM NaH<sub>2</sub>PO<sub>4</sub>  
300mM NaCl  
500mM imidazole  
0.05% Tween 20  
8M Urea

#### 3. pH Lysis Buffer, pH 8.0

0.1M NaH<sub>2</sub>PO<sub>4</sub>  
0.01M Tris  
8M Urea

Protocol:

BL-21 bacteria are transformed with the pET CAP plasmid and grow up to the appropriate OD. IPTG is then added to induce protein expression. Bacteria is then collected and lysed and CAP protein is purified via Ni-NTA beads that interact with the HIS tag.

Amplification of Bacterial CAP protein

1. Transform BL-21 *E. coli* with the CAP pET plasmid following the Czub lab protocol.
2. Select a single colony and grow up in 1 ml of LB broth with Kanamycin added. Grow up 8 to 12 h at 37°C while shaking.
3. Inoculate 10ml of LB broth with Kanamycin with 500 µL of the 1ml culture. Grow overnight at 37°C while shaking.
4. The next day inoculate 500ml of LB broth and Kanamycin with the whole 10ml culture. Grow for appropriately 4 h at 37°C while shaking to an OD600 of appropriately 0.5. The cells should be in mid-log phase.
5. Add 1mM IPTG to induce expression.
6. Grow at 37°C with shaking for 4 h or at 30°C with shaking overnight. Harvest the cells by centrifugation at 3000 x g for 10 minutes at +4°C.
7. Proceed to purification or store the cells at -80°C for future use.

Purification of Bacterial CAP by Imizadole

1. Resuspend bacterial cell pellet in the imizadole lysis/wash buffer; 5ml to 10ml of buffer for every 1-2 ml of cell pellet. Leave shaking at 4°C overnight. Sonicate at low setting for three 1 minute repeats on ice. The solution should be clear.
2. Centrifuge at 4000 rpm for 20 minutes.

3. While centrifuging, prepare beads by pre-clearing them with the lysis/wash buffer. Use 0.5ml of beads for every 5 to 10ml of lysate. Add 1ml of buffer to 0.5ml of beads and gently mix. Spin down at minimum spin for 10 seconds. Remove supernatant. Repeat two more times.
4. Collect supernatant from lysate and discard the cell debris. Add supernatant to beads and incubate with shaking at room temperature for 1 h.
5. Spin down at minimum spin for 10 seconds and collect the supernatant. This is the flow through and should be run on a SDS-PAGE gel to check if any CAP protein is present.
6. Add 1ml of lysis/wash buffer to the beads, mix then spin down at minimum spin for 10 seconds and collect the supernatant. This is the wash and should be run on a SDS-PAGE gel to check if any CAP protein is present.
7. Repeat two more times.
8. Add 1ml of the imidazole elution buffer to the beads and incubate at room temperature for 20 minutes while shaking.
9. Spin down at minimum spin for 10 seconds and collect the supernatant. This is the first elution and there should be CAP protein present.
10. Add 1ml of the imizadole elution buffer to the beads and incubate at room temperature for another 20 minutes while shaking.
11. Spin down at minimum spin for 10 seconds and collect the supernatant. This is the second elution and there should be CAP protein present.
12. Add 1ml of the imizadole elution buffer to the beads and incubate at room temperature for another 20 minutes while shaking.

13. Spin down at minimum spin for 10 seconds and collect the supernatant. This is the third elution and there maybe CAP protein present.
14. Take each elution and concentrate them (if there is more than 2ml of each elute this is recommended) by using the 30K Amicon Ultra Centrifugal filter units. The concentrated protein will remain in the filter and should be resuspended in 1.5ml of 1x PBS.
15. Take aliquots of each elute and the saved flow through and wash and run on two SDS-PAGE gel, one for a Coomassie stain and one for Western Blotting. The western blot with either the Abcam anti-HIS antibody (use 1:1000 in 5% milk in PBS-Tween) or the anti-CAP rabbit polyclonal (1:3000 in 5% milk in PBS-Tween) to determine if the protein is present in the elute. The Coomassie will show how pure the elutions are. A second round of purification is likely needed to get rid of any contaminating proteins.

## Appendix 11. Mascot Results

1/12/2015

./data/20121105/F003428.dat (Mascot Search Results)

### MASCOT Search Results

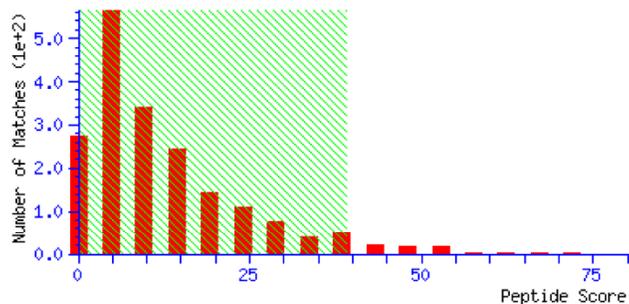
**User** : Kelvin  
**E-mail** :  
**MS data file** : Cap.mgf  
**Database** : NCBI nr 20120829 (20,093,899 sequences; 6,882,348,701 residues)  
**Taxonomy** : Viruses (1,007,273 sequences)  
**Timestamp** : 5 Nov 2012 at 17:05:01 GMT

Not what you expected? Try [the select summary](#).

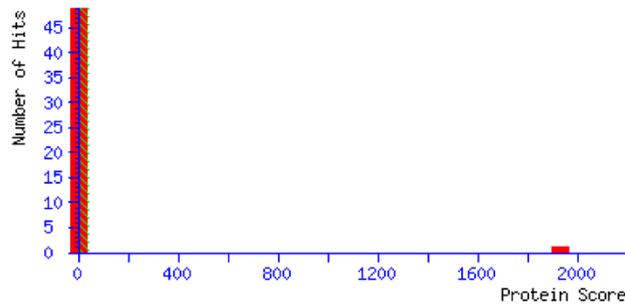
#### ▼ Search parameters

**Type of search** : MS/MS Ion Search  
**Enzyme** : Trypsin  
**Variable modifications** : **Oxidation (M)**, **Carbamidomethyl (C)**  
**Mass values** : Monoisotopic  
**Protein mass** : Unrestricted  
**Peptide mass tolerance** :  $\pm 20$  ppm  
**Fragment mass tolerance** :  $\pm 0.2$  Da  
**Max missed cleavages** : 1  
**Instrument type** : ESI-QUAD-TOF  
**Number of queries** : 3,462

#### ▼ Score distribution



Peptide score distribution. Ions score is  $-10 \log(P)$ , where  $P$  is the probability that the observed match is a random event. Individual ions scores  $> 39$  indicate **identity** or **extensive homology** ( $p < 0.05$ ).



**[Deprecated]** Score distribution for family members in the first 50 proteins. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein families.

### ► Legend

### Protein Family Summary

Significance threshold  $p <$   Max. number of families   
 Ions score or expect cut-off  Dendrograms cut at   
 Preferred taxonomy

### Protein families 1–2 (out of 2)

per page 1

►1	<b>gi 11907589</b>	1933	ORF2 [Porcine circovirus 2]
►2	<b>gi 1125703</b>	47	hemagglutinin [Influenza A virus (A/chicken/Mexico/31381-..

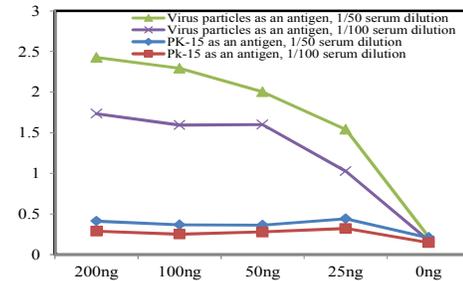
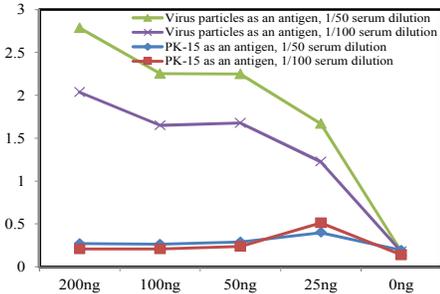
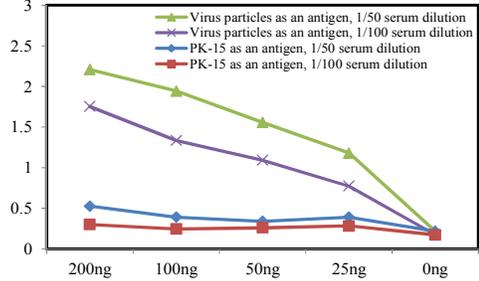
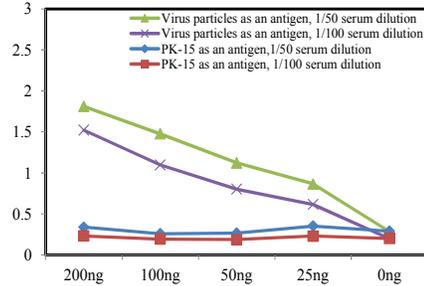
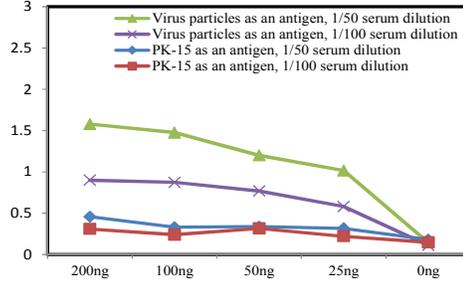
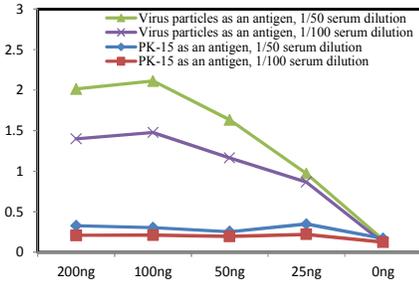
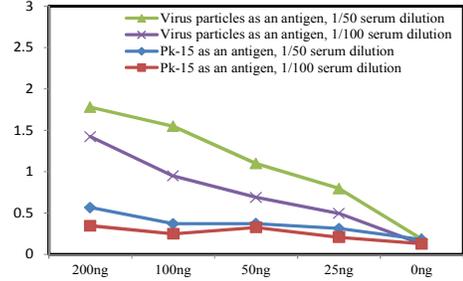
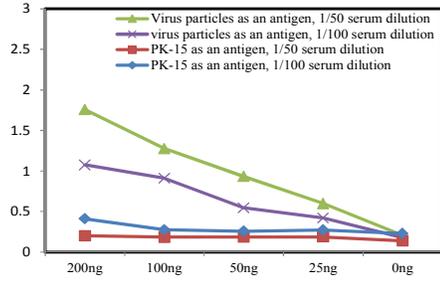
per page 1

Not what you expected? Try [the select summary](#).

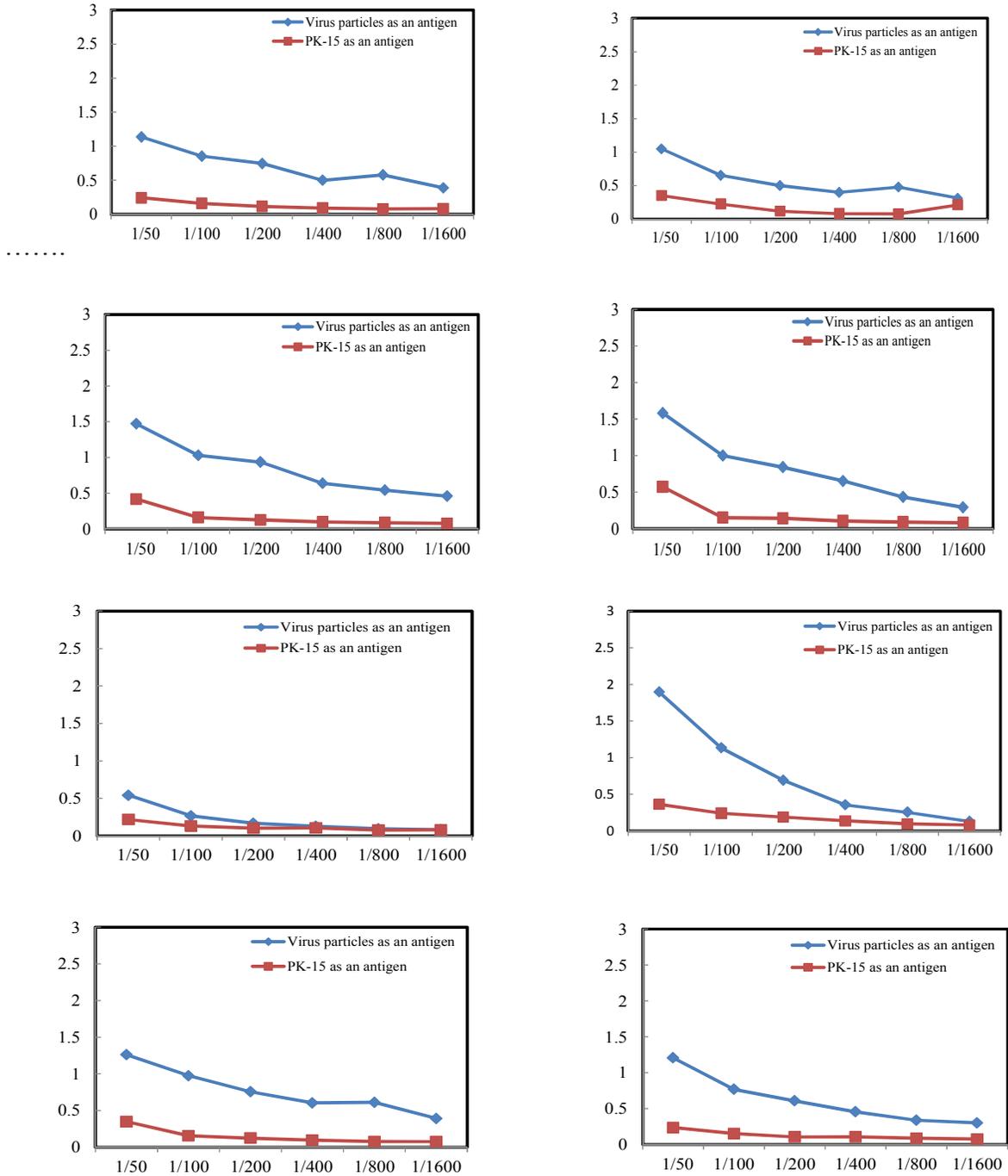
Mascot: <http://www.matrixscience.com/>

## Appendix 12. Optimization of Particles-based ELISA

### 12.1. Optimal Working Protein Concentration



## 12.2. Optimal Working Dilution



## Appendix 13. Coefficient of variability (CV)

### 13.1. Intra-assay CV%

Sample	Run 1	Run 2	Mean	Standard deviation	CV%
1	2.859	2.51	2.68	0.24	9.19
2	2.55	2.56	2.56	0.002	0.11
3	2.71	2.66	2.68	0.03	1.34
4	2.7	2.91	2.8	0.14	5.26
5	2.9	2.78	2.84	0.082	2.88
6	2.82	2.66	2.74	0.11	4.12
7	2.56	2.44	2.5	0.08	3.27
8	1.82	1.88	1.85	0.04	2.2
9	2.11	2.1	2.1	0.007	0.36
10	2.61	2.65	2.63	0.02	1.15
11	2.81	2.78	2.8	0.023	0.83
12	1.11	1.17	1.14	0.038	3.34
13	1.43	1.47	1.45	0.029	2.04
14	1.29	1.31	1.3	0.01	1.46
15	1.87	1.88	1.87	0.007	0.41
16	2.03	2.04	2.04	0.007	0.34
17	1.59	1.61	1.6	0.014	0.88
18	0.94	0.92	0.93	0.012	1.36
19	1.43	1.41	1.42	0.012	0.89
20	1.72	1.9	1.81	0.12	6.9

Intra-assay CV (n=20)= average CV%= 2.4

13.2. Inter-assay CV%

<b>Plate</b>	<b>Run 1</b>	<b>Run 2</b>	<b>Mean</b>
<b>Plate 1</b>	1.04	1.05	1.049
	0.9	0.93	0.91
	0.56	0.57	0.57
	0.74	0.73	0.74
	1.9	1.8	1.85
<b>Plate 2</b>	1.1	1.2	1.15
	0.99	0.94	0.96
	0.5	0.54	0.52
	0.81	0.82	0.815
	1.8	1.7	1.75
<b>Plate 3</b>	1.2	1.3	1.25
	1	1.1	1.05
	0.7	0.6	0.65
	0.8	0.81	0.805
	1.7	1.9	1.8
<b>Plate 4</b>	1.3	1.4	1.35
	0.9	0.85	0.87
	0.4	0.48	0.44
	0.73	0.91	0.82
	1.9	1.7	1.8
<b>Plate 5</b>	1.26	1.34	1.3
	1.21	1.02	1.11
	0.43	0.56	0.49
	0.74	0.89	0.81
	1.8	1.96	1.88

<b>Mean plate1</b>	<b>Mean plate 2</b>	<b>Mean plate 3</b>	<b>Mean plate 4</b>	<b>Mean plate 5</b>	<b>Mean of Means</b>	<b>Standard deviation of means</b>	<b>CV% of means</b>
1.049	1.15	1.25	1.35	1.3	1.21	1.1	8.84
0.91	0.96	1.05	0.87	1.11	0.95	0.05	6.08
0.57	0.52	0.65	0.44	0.49	0.54	0.06	12.53
0.74	0.815	0.805	0.82	0.81	0.79	0.02	3.68
1.85	1.75	1.8	1.8	1.88	1.8	0.03	1.75

Inter-assay CV (n=5)= average CV%= 6.5