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Detection of Antibodies Against the Open Reading Frame Three Protein of Porcine Circovirus 2 in Pigs

by

Aaron Hawkes

A THESIS

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Abstract

The swine pathogen porcine circovirus 2 (PCV2) is the primary etiological agent of a syndrome that is damaging to the swine industry. The PCV2 genome contains three major open reading frames (ORFs) and ORF3 appears to play a role in PCV2-related pathology and dissemination. No data have been published confirming that PCV2ORF3 protein is expressed in PCV2-infected swine. Here it is hypothesized that αPCV2ORF3 antibodies are present in the sera of most adult swine. Swine sera were screened for αPCV2ORF3 antibodies, the presence of which would imply PCV2ORF3 expression. About 90% of the tested sera from weaned swine appear to have at least some reactivity to rPCV2ORF3, implying that PCV2ORF3 is expressed *in vivo*. Serum reactivity to PCV2ORF3 did not correlate with PCV2 viral load or vaccination status. These results do not indicate or rule out any particular biological role for PCV2ORF3.

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Abbreviations, Symbols, Nomenclature

Abbreviation	Meaning
+ve	Positive
α	Alpha/Anti
AIDS	Acquired immunodeficiency virus
APC	Antigen-presenting cell
APC/C	Anaphase promoting complex/checkpoint
bp	Base-pair
BCR	B-cell receptor
BSA	Bovine serum albumin
BFDV	Psitticine *beak and feather disease virus*
CAV	*Chicken anemia virus*
CD	Cluster of differentiation
CSFV	*Classical swine fever virus*
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
dpi	Days post-infection
DuCV	*Duck circovirus*
DDR	DNA damage response
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
Fab	Fragment antigen-binding
Fc	Fragment crystallizable region
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
His	Histidine
HIV	Human immunodeficiency virus
HRP	Horse-radish peroxidase
IFA	Immunoflourescence assay
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl β-D-1 thiogalactopyranoside
ISH	*In situ hybridization*
KO	Knock-out
LB	Luria’s broth
LC-MS/MS	Liquid chromatography-mass spectroscopy
LPS	Lipopolysaccharide

MeOH	Methanol
MHC	Major histocompatibility complex
MDA	Maternally derived antibodies
MDC1	Mediator of checkpoint 1
N	Sample number
nAb	Neutralizing antibody
Nef	Negative factor
NIPC	Natural interferon producing cell
NLS	Nuclear localization signal
ORF	Open reading frame
p	Probability of occurring by chance
-P	Phosphorylated
p53	Protein 53 tumor suppressor protein
PiCV	*Pigeon circovirus*
Pirh2	p53-induced ubiquitin E3 protein ligase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PBST	PBS + Tween-20
PCR	Polymerase chain-reaction
PCV	*Porcine circovirus*
PCVAD	*Porcine circovirus* associated disease
PDNS	Porcine dermatitis and nephropathy syndrome
PK15	Porcine kidney -15 cell line
PMWS	Post-weaning multisystemic wasting syndrome
PRRSV	Porcine reproductive and respiratory virus
pORF	Plasmid construct bearing an ORF insert
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
RAP	Rabbit αpig
RCR	Rolling circle replication
Rep/Rep’	Replicase protein(s)
RGS	Regulator G-protein signaling
RT-PCR	Reverse-transcription PCR
SPF	Specific-pathogen free
SDS-PAGE	Sodium diodecyl sulfate-polyacrylamide gel electrophoresis
ssDNA	Single-stranded DNA
TCID	Tissue culture infectious dose
TNF	Tumor necrosis factor
Vif	Viral infectivity factor
VLP	Virus-like particle
Vpr	Viral protein R
Vpu	Viral protein unique
WT	Wild-type

The lame in the path outstrip the swift who wander from it.

-Francis Bacon

Chapter 1 Introduction

1.1 Porcine Circovirus-Associated Diseases

Porcine circoviruses (PCV) are non-enveloped icosahedral viruses of about 20 nm in diameter originally discovered as a contaminant of porcine tissue culture in the 1970s (2). PCV was not associated with any disease in pigs (3) until the late 1990s, when a strain of PCV antigenically distinct from the older PCV strain was isolated from pigs affected by a new disease (4). The two PCV strains were found to share 68% nucleotide identity (5). The non-pathogenic and pathogenic strains were labelled as PCV1 and PCV2 respectively. The new disease associated with PCV2 was called post-weaning multisystemic wasting syndrome (PMWS).

The swine industry has sustained massive costs from damage caused by PMWS and efforts to prevent it since its emergence in the 1990s. Upon weaning, affected piglets begin exhibiting jaundice, diarrhea, swollen lymph nodes, wasting, poor appetite, and a general 'failure to thrive' with lethality in some outbreaks as high as 80% (6). Swine lymph nodes in PMWS cases are populated with an abnormally high numbers of histiocytes and giant multinucleated cells, often containing cytoplasmic inclusion bodies (4, 7, 8). This is accompanied by granulomatous inflammation in the lymph nodes and immunosuppression resulting from massive lymphocyte depletion (8). Other lesions include non-collapsed lungs, interstitial pneumonia, interstitial nephritis of kidneys (8), liver atrophy, and hepatitis (6, 9). Changes to the cytokine response to antigens, particularly an increase in interleukin-10 (IL-10) secretion, are also observed (10). The inflammation and weight loss may be a consequence of increased tumor necrosis factor alpha (TNF-α) expression (7). A virus named porcine circovirus 2 (PCV2) has been identified as the primary etiological agent behind PMWS (4, 11). PCV2 has been linked to other swine pathologies such as enteritis (12) and porcine dermatitis and nephropathy syndrome

(PDNS) (13), although the role of PCV2 in these diseases is not clear. PMWS and other diseases that PCV2 has been linked to are grouped together as porcine circovirus associated diseases (PCVAD).

FIGURE 1. The pig on the left highlighted by the red arrow exhibits wasting and thinness typical of PMWS in contrast to an undiseased age-matched specimen on the right. Image taken from http://pcvd.net/background.php

1.2 Porcine Circoviruses and *Circoviridae*

PCV2 appears to be ubiquitous in the general swine population. The vast majority of pigs have antibodies to PCV2 (14) and antibodies to PCV2 are probably found in 100% of swine at least one point in their lives (15) but only some will develop clinical disease. Animals no longer showing clinical signs of PCV2 infection can still transmit the virus, and infection can persist for

125 days or longer (4). Transmission probably occurs by the oro-nasal route (16) and perhaps vertically in the womb (17). Cell tropism of PCV2 includes epithelial cells, hepatocytes, cells of the macrophage-monocyte lineage, and lymphocytes (4, 8, 18) Infected lymphocytes included cells bearing IgM+, CD4+, and CD8+ markers. PCV2 gene expression products were found in macrophages and IgM+ cells (18). PCV1 on the other hand infects and reproduces in cells of the macrophage-monocyte lineage and epithelial cells but it was not detected in lymphocytes in a study that looked for PCV1 infection of B and T lymphocytes (19).

PCVs have a circular ambisense ssDNA genome of 1.77 Kbp containing three major open reading frames (ORFs). ORF1 is differentially spliced to encode the Rep and Rep' proteins which together help carry out the replication of the viral genome by nicking it after conversion to dsDNA and unwinding it back into ssDNA. Rep and Rep' have MWs 35 KDa of and 28.5 KDa respectively. ORF2 encodes the 27.8 KDa capsid protein. This is the major immunogenic and only known structural component of PCV2 aside from the genome itself. The ORF3 sequence is embedded within ORF1 in the opposite orientation. Testing with bacterial two-hybrid assay and GST-pull down found that the Rep protein encoded by PCV2ORF1 is capable of binding the capsid protein encoded by PCV2ORF2, as well as c-*myc* and possibly syncoilin (20). C-*myc* is an important regulator of the transcription of many genes and syncoilin is an intermediate filament protein. The binding of Rep to the capsid protein and syncoilin could be important to localization of Rep, while binding of Rep to c-*myc* could cause interference with the expression of certain genes. The same study found that the capsid protein could bind to C1qB (20), which is a subunit of the C1 complex that activates the complement cascade upon binding to IgM or IgG antibody. The function of PCV2ORF3 is poorly understood and there is no published research on its promoter. Analysis by the online Neural Network Promoter Prediction tool from Berkeley found

regions of the PCV2 genome that might constitute the PCV2ORF3 promoter. The approximate location of the highest scoring region is highlighted in Fig. 2. The structure and genomic organization of PCVs is similar to most other members of the *Circoviridae* virus family.

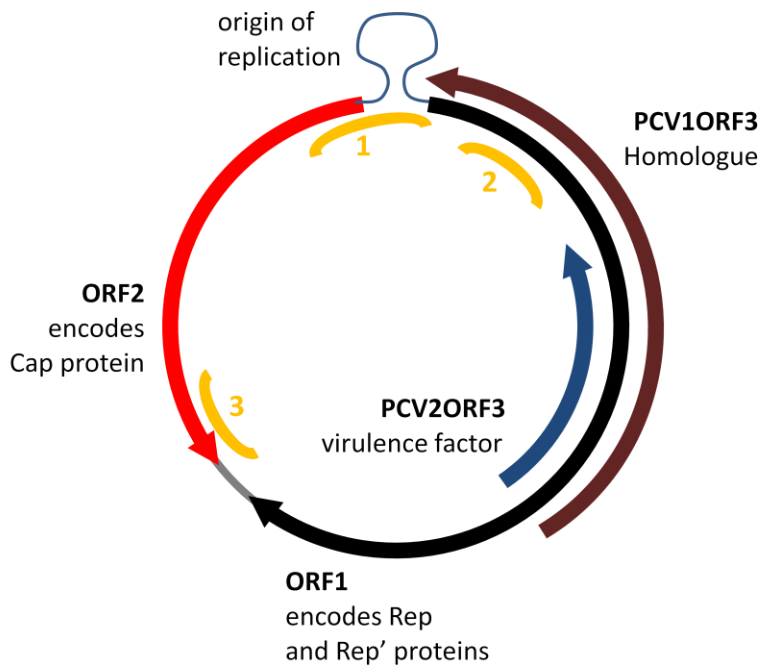

FIGURE 2. The PCV2 genome is 1.77Kbp long. The arrows in the diagram illustrate the location of each ORF and indicate its direction of transcription. Rolling circle replication is initiated from the stem loop structure in between the start of ORF1 and ORF2. A region predicted to contain the promoter for PCV2ORF3 and the known locations of the other two promoters are shaded in orange and labelled with the number of the ORF whose expression it regulates. The relative position and size of the PCV1 homologue of ORF3 is also shown alongside PCV2ORF3.

The *Circoviridae* taxon is a family of non-enveloped viruses with circular ssDNA genomes about 1-4 Kbp long that comprise at least two ORFs that are usually transcribed in opposite directions. They encode the protein(s) essential for replication (ORF1) and the

structural capsid protein (ORF2). The capsid is an icosahedral homopolymer (21). Members of this family also have a conserved nine bp secondary DNA structure called a hairpin loop. Hairpin loops are formed when two complementary regions of the same DNA or RNA strand in close proximity undergo base-pairing, forming a double-helix and an unpaired loop. This structure is the initiation site for rolling circle replication (RCR) of the *Circoviridae* genome. For RCR to take place the ssDNA genome must first be converted to dsDNA by DNA polymerase (DNAP). DNAP is most available in the cell nucleus when the cell genome is being replicated during the S-phase of the cell cycle and, at least in the case of PCV2, the viral genome cannot enter the nucleus on its own. For PCV2 to replicate, it must infect a cell; that cell must undergo mitosis resulting in incorporation of the PCV2 genome into the nuclei; and then the cell must go into S-phase (22). Other *Circoviridae* likely have similar requirements because of their similar genomes. Then the protein or proteins encoded by ORF1 nicks one strand of the *Circoviridae* genome in the hairpin loop and 'unzips' the whole viral dsDNA genome starting from the nick. The new ssDNA strand of the viral genome can be replicated by DNAP and the original strand of ssDNA can then either be packaged into a virion or replicated again. While most *Circoviridae* encode only one protein in ORF1 called Rep others such as the PCVs encode both Rep and an alternative splicing product called Rep', both of which are required for genome replication (23).

Circoviridae can be divided into three genera. Members of genus *Circovirus* infect vertebrates such as fish (24), swine (25), and birds (26). Members include porcine circoviruses types 1 and 2, Psitticine beak and feather disease virus (BFDV), duck circovirus (DuCV), and pigeon circovirus (PiCV). Genus *Cyclovirus* was only recently proposed and its members infect dragonflies and vertebrates and have been very tentatively associated with human neurological disorders in Vietnam (27). Chicken anemia virus (CAV) is the sole member of Genus *Gyrovirus*.

CAV and BFDV and possibly other *Circoviridae* are known to cause immunosuppression and often lead to death by secondary infection. Lymphocyte depletion is a recurrent histopathology of infection by members of *Circoviridae*.

The member of *Circoviridae* most different from the rest of this taxon is probably CAV. The genomic organization is different from the ambisense genomes of other *Circoviridae*. There is only one promoter that drives transcription of the entire genome in the same direction. Expression is regulated at the translational level by the leaky scanning mechanism. Leaky scanning relies on certain mRNA sequences just upstream of the start codon of the regulated ORF. These sequences normally bind to the second ribosomal subunit but in leaky scanning they are mutated from the optimal sequence for binding, which sometimes results in non-binding by the second ribosome subunit. The first ribosome subunit then continues to read through this ORF to the next start codon without translating it. Also, whereas infection by many other well-studied *Circoviridae* such as PCV2 is persistent and depletes both T and B lymphocytes, infection by CAV is transient and only depletes T-lymphocytes in addition to red blood cells (28, 29). Infection with CAV has a mortality rate of about 10% (30) with death usually a result of immunosuppression. The resultant secondary infections can have a synergistic relationship with the CAV infection, amplifying the pathogenicity of both (31). This is interesting because of similarities to PCV2, which induces some immunosuppression in subclinical infection (32, 33) while further stimulation of the immune system can result in far worse immunosuppression and clinical disease (34). Like CAV, PCV2 causes disease and immunosuppression in young animals. It is likely that the members of *Circoviridae* share common strategies for evading the immune response and causing pathology in the infected host.

1.3 Features of the Protective Immune Response to PCV2

Control of PCV2 viremia is highly dependent on an adaptive immune response. Treatment of pigs with cyclosporin before PCV2 inoculation inhibits T lymphocyte proliferation and the thymus-dependent humoral immune response, resulting in higher PCV2 viremia (35). The Th1 immune response produces inflammation and is directed against intracellular pathogens such as viruses and the Th2 immune response is directed against parasites and extracellular bacteria (37). The 'polarity' of the immune response depends in part on the cytokines produced by cells of the innate immune response (38). Evidence suggests that a Th1 immune response to PCV2 better protects against disease and controls viremia than a Th2 response does (36).

1.3.1 The Innate Immune Response to PCV2

The innate immunity is the first line of defense against infection. Cell types include dendritic cells (DCs) and macrophages. Some macrophages are predominantly involved in tissue repair while 'killer' macrophages act by phagocytosing pathogens, destroying them, and then presenting processed antigen peptides to lymphocytes to activate the adaptive immune response. Macrophages are activated by and help induce a Th1 response to infection. DCs endocytose antigens and are much stronger antigen presenters. These cell types both activate and mature other parts of the innate and adaptive immune system by secreting cytokines such as IL-8, which is important to inducing chemotaxis of cells such as neutrophils. An increase in serum levels of IL-8 in PCV2-infected pigs indicates that innate immunity may also be involved in the response to PCV2 (39). Interestingly, DCs derived from porcine bone marrow produce a very strong IL-12 response to both whole PCV2 and PCV2 virus like particles (VLPs) (40). Since this cytokine is important to T lymphocyte regulation and development of a Th1 response (38) its secretion by

Comment [MR1]: Clarification requested by Rebekah

DCs could be fundamental to the function of PCV2 vaccines based on VLPs or inactivated PCV2 virions. Certain DCs called natural interferon producing cells (NIPCs) secrete interferon alpha (IFN-α) in response to specific stimuli, which is important to the autocrine maturation of NIPCs and paracrine maturation of other DCs. These mature DCs can then more efficiently activate the adaptive immune response by antigen presentation. A weak IFN-α response to PCV2 infection may correlate with prolonged viremia (39). This indicates that NIPCs in particular are important to controlling PCV2 viremia by activating the rest of the innate immunity and activating the adaptive immunity.

1.3.2 The Cell-Mediated Response to PCV2

The adaptive cell-mediated immune (CMI) response in the context of viral infection involves the destruction of virus-infected cells by cytotoxic T lymphocytes (T_{cyto}) which express the CD8 but not the CD4 transmembrane glycoprotein. Naive T_{cyto} lymphocytes are activated by presentation of a part of the virus by an antigen presenting cell (APC), such as a macrophage or DC. Their maturation and activity is strongly influence by cytokines secreted by another kind of T lymphocyte called the helper T cell (T_h). These express the CD4 but not the CD8 transmembrane glycoprotein and are divided into Th0, Th1 and Th2 helper T cells. Cytokines secreted by macrophages, DCs, and other cells of the innate immune system differentiate Th0 cells into either Th1 or Th2 cells, which then drive Th1 or Th2 responses to infection respectively speaking (38). Memory T cells are antigen-specific T lymphocytes that persist after the antigen is cleared and this makes possible a faster recall response to repeated challenge. They express both the CD4 and CD8 markers in pigs. Adaptive CMI is involved in the porcine immune response to PCV2. In one study, extracted peripheral blood mononuclear cells (PBMCs)

from subclinical PCV2-infected swine were challenged with re-exposure to PCV2 antigen *in vitro*. PBMCs are blood cells with a round nucleus such as lymphocytes, macrophages, and monocytes. In response, these PBMCs secreted increased amounts of interferon gamma (IFN-γ) (41). IFN-γ is a critical cytokine for activating innate and Th1 adaptive immune responses, particularly in macrophages, and inhibiting viral reproduction. IFN-γ is produced by cells belonging to the innate wing of the immune system as well as antigen-specific CD4+ and CD8+ T lymphocytes. In the same study CD4+ and CD8+ T lymphocytes were selectively depleted to find out how much they contribute to the antigen-specific IFN-γ response. Comparing the response of CD4 and CD8-depleted animals to non-depleted control animals revealed that antigen-specific CD4+ and CD8+ T lymphocytes contributed to the IFN-γ response to PCV2. An IFN-γ response and a neutralizing antibody response in PCV2-infected swine seems to coincide with a decrease in viremia (39). The IFN-γ response both indicates and drives an adaptive CMI response to PCV2 infection. PCV2-challenged mice with more T_{cyto} cells in their spleen had better protection against disease and lower viremia (36), indicating the CMI response against PCV2 may be protective. CMI is definitely involved in the host response to PCV2 infection and it probably confers some protection against disease but it is unclear just how much CMI is responsible for controlling viremia or preventing disease in healthy PCV2-infected animals.

1.3.3 The Humoral Immune Response to PCV2

The humoral immune response consists of antibodies that target and bind to pathogens. Antibodies are produced by B lymphocytes, which carry the IgM cell surface protein. Naive B lymphocytes are also APCs. They are primed by direct interaction between the B-cell receptor (BCR) and the antigen. Primed B lymphocytes usually require activation by an activated T_h cell

before they start dividing and secreting IgM antibody. The B lymphocyte will initially secrete IgM antibodies but further interaction with an activated T_h cell can induce isotype switching (38). Interaction with T_h cells is not always required for isotype switching (42). Neutralizing antibodies (nAbs) in particular are strongly correlated with the control of PCV2 viremia (35) and avoidance of PCVAD (43). nAbs are antibodies that eliminate or reduce the infectivity of a virus. Mechanisms of neutralization include aggregating virus, blocking the virus-cell interaction, and others that are less well understood (44, 45). While αPCV2 antibodies can be detected 14 dpi (33, 35), nAbs are detected much later at 29 dpi using a standard nAb assay. In the standard nAb assay a serum is titred by serially diluting it and those dilutions are tested for neutralizing ability. The last dilution to achieve a certain level of neutralization of PCV2 is that serum's nAb titre (39). However, αPCV2 nAbs can be detected at 10-15 dpi using a more sensitive assay where the serum is added to PCV2 at a single dilution before it is applied to cells. The percentage reduction in the number of cells that are infected by PCV2 compared to a virus-only control is the serum's nAb titre (35, 43). αPCV2 nAbs are likely IgG_{2a} isotype antibodies (36), which are part of the Th1 immune response in pigs. IgM antibodies may also neutralize PCV2 (43). The immune response with clearest correlation to protection from disease according to previous research is the production of neutralizing antibodies, and for this reason this thesis focuses on measuring the neutralization titre of swine sera.

1.4 PCV2 Pathogenesis

Infection by PCV2 is necessary but not sufficient for PMWS development. A secondary stimulation of the immune system is also required to induce full-blown PMWS (34) which can come in the form of a co-infection (7) and even then only some swine infected by PCV2 will

develop PMWS. It is not known how PCV2 causes the massive lymphocyte depletion seen in PMWS. Vaccination against PCV2 largely prevents PCVAD, reduces mortality rates, and improves other outcomes such as weight gain but it does not prevent PCV2 infection (46). Subclinical PCV2 infection clearly modulates various aspects of the immune response with important consequences for swine health.

1.4.1 Immunomodulation

1.4.1.1 PCV2 Modulation of Innate Immunity

The innate immune system includes macrophages and DCs and is the first line of defense against pathogens. Some DCs called NIPCs activate themselves and other DCs by secreting IFN-α, and then in turn activate the adaptive immune response. Macrophages are sites of PCV2 replication (47) but DCs are not and PCV2 infection does not result in increased cell death in either DCs or macrophages (48, 49), but their cytokine secretion is strongly affected by PCV2 infection (50, 51). It appears that PCV2 can infect NIPCs and inhibit their ability to produce IFN-α and TNF-α which in turn inhibits the maturation of other DCs and themselves (51). TNF-α is an important pro-inflammatory cytokine. The underlying mechanism is unclear but it appears that the PCV2 genome affects a dominant negative signalling pattern that overrides a broad spectrum of cytokine responses to TLR-7 and TLR-9 agonists, resulting in lower NIPC secretion of IFN-α and TNF-α (50). PCV1 DNA does not appear to have the ability to inhibit TNF-α secretion in DCs (50). The PCV2 genome contains CpG oligodeoxynucleotides (ODNs), which are regions of DNA containing cytosine and guanine nucleotides linked by phosphodiester bonds. Unmethylated CpG ODNs tend to activate innate immune responses such as IFN-α

secretion but one of the CpG ODNs in the PCV2 genome actually has a strong inhibitory effect on IFN-α secretion (52). This inhibitory effect is dependent upon formation of a hairpin loop secondary structure in the inhibitory CpG-ODN (53). Similarly, infection of swine alveolar macrophages by PCV2 does not change their survival rate but does change their cytokine secretion profile. Much like in the NIPCs, IFN-α secretion is reduced but whereas PCV2 infection of DCs results in decreased TNF-α secretion, PCV2 infection of swine alveolar macrophages results in increased TNF-α secretion. There was also increased expression of IL-8 and other chemotactic factors (49). Increased levels of pro-inflammatory TNF-α were correlated with increased wasting in PMWS potentiated by PCV2 and porcine parvovirus (PPV) co-infection, strongly indicating a central role for TNF-α in the wasting and other pathologies related to PMWS (7).

The effects of PCV2 infection on alveolar macrophages goes beyond altered cytokine secretion. Swine alveolar macrophages infected with PCV2 are less able to phagocytose and create a microbiocidal response to an opportunistic swine pathogen called *Candida albicans* (49). Infection of macrophages by PCV1 does not inhibit phagocytosis and killing of a related yeast organism called *Candida krusei* (54). The microbiocidal response of the macrophages is its ability to release H_2O_2 and O_2^-, which are both used to destroy endocytosed pathogens. Down-regulation of the microbiocidal response and phagocytosis in macrophages is consistent with the increased susceptibility of PMWS-affected pigs to opportunistic pathogens. Also, given that phagocytosis is a necessary pre-requisite for antigen presentation, this could result in decreased activation of the adaptive immune response to PCV2 and secondary infections (49).

In summary, inhibition of the innate immune system by PCV2 does not involve destruction of infected cells of the innate immune system. Instead, PCV2 inhibits the ability of these cells to destroy pathogens and activate the adaptive immune response.

1.4.1.2 Other Cytokine Responses Modulated by PCV2

The process by which PCV2 depletes lymphocytes and otherwise modulates the immune system is not understood. Infection by PCV2 is linked to changes in many cytokine responses besides IFN-α and TNF-α, which could explain the systemic nature of the immunomodulation caused by PCV2. Another cytokine response altered by PCV2 infection is IL-10, which is an anti-inflammatory cytokine that down-regulates certain T lymphocyte cytokine responses and MHC-2 levels while increasing B lymphocyte proliferation and survival. Secretion of IL-10 is upregulated in both PMWS animals (55) and subclinical PCV2-infected animals (56). IL-10 upregulation can inhibit the clearance of different viruses in mice (57) and it may allow PCV2 to establish persistent infection in pigs (39). PBMCs from PMWS swine seem to secrete more IL-10 than those from undiseased swine in response to recall PCV2 stimulation (10) and this IL-10 recall response seems to correlate with PCV2 viremia (56). It appears that PCV2-infected monocytes in particular become strong producers of IL-10 and this in turn strongly reduces IL-12 and IFN-γ responses to PCV2 and recall responses to other pathogens. IL-2 was also down-regulated by PCV2 infection but not as a consequence of IL-10 up-regulation (58). IL-2 is necessary for T lymphocyte proliferation and inducing the Th1 response that is most protective against PCV2. Reducing IL-12, IL-2, and IFN-γ responses likely reduces the Th1 response strength. Infection by PCV1 did not modulate secretion of these cytokines nearly as much or at all (58). It seems that PCV2 changes many cytokine responses and increased levels of IL-10 in

particular are correlated with poor clinical outcome. Altogether, these cytokine response alterations can push the immune response away from the Th1 adaptive response that is most protective against PCV2 (36).

1.4.1.3 PCV2 Modulation of Adaptive Immunity

The B and T lymphocytes respectively comprise the humoral and cell-mediated wings of the adaptive immune response, which is necessary for controlling PCV2 infection (35). Lymphocytes are extremely important to the immune response and their depletion during PMWS destroys most of the adaptive immunity and is central to the severe immunosuppression associated with PMWS.

Development of PMWS is marked by the destruction of lymph node follicle structure (4, 6, 8) and a decrease in B and T lymphocyte counts (59, 60). Animals with PMWS have lower overall and neutralizing αPCV2 titres than animals with subclinical PCV2-infection (33). Subclinical PCV2 infection is also detrimental to the adaptive immune system, especially during recall response. Animals subclinically infected with PCV2 and vaccinated against porcine reproductive and respiratory virus (PRRSV) had worse pathological lesions after PRRSV challenge than PCV2-free animals vaccinated for PRRSV (61). Another study compared the protection conferred by CSFV vaccination in PCV2-free animals to animals subclinically infected with PCV2. The animals with subclinical PCV2 infection took longer to develop a neutralizing αCSFV antibody response and had lower counts of IgM+, CD4+, and CD8+ cells for at least one time point in the study. The impairment of vaccine protection associated with PCV2 infection means even subclinical infection is an important detriment to the health of swine. How PCV2 weakens the protection offered by vaccination is not understood. B

lymphocytes and possibly T lymphocytes can be productively infected by PCV2 (8, 18) therefore it is possible that PCV2 could destroy or modulate the lymphocytes after infection. The ability of B lymphocytes to switch isotypes independent in response to TLR9 signalling (42) suggests a role in humoral response suppression for the CpG-ODN element in the PCV2 genome that is capable of inhibiting TLR9 signalling (50), but this is speculative.

1.4.2 Genetic Determinants of Virulence

There are two main strains of PCV2 called PCV2a and PCV2b and both can instigate PCVAD (62). According to sequence analysis, they diverged from a common ancestor about 100 years ago, almost immediately after the divergence of PCV1 and PCV2 (62). The commercially available PCV2 vaccines all use the ORF2 sequence of the PCV2a strain, which is probably why an analysis of PCV2 strains in swine sera could only find PCV2a in unvaccinated swine (63). This is also probably why the last decade has seen an increase in the prevalence of PCV2b relative to PCV2a (62, 64) coincident with increased PCV2 vaccination.

Despite being present in swine for decades (65), PCV2 did not cause PMWS until the 1990s. A comparison of archived PCV2 strains extracted from pigs in 1970-1971 and contemporary PCV2 strains found a consistent nine basepair difference in ORF2 at base positions 1331-1339. This translated into a change in the capsid protein at amino acids 133-135 from threonine-glycine-asparagine in the archival PCV2 to alanine-threonine-alanine in contemporary PCV2 (47). The result is a change from a hydrophilic to a hydrophobic sequence in the second immunogenic epitope of the PCV2 capsid protein. No difference between the archival and contemporary PCV2 strains was seen in the ORF1 sequence or in the ORF3 sequence, which is embedded in ORF1 (47). Animals infected with the archival and

contemporary PCV2 strains without further immunostimulation had similar clinical signs and viral load in bronchial lymph nodes. Immunostimulation of pigs infected with contemporary PCV2 potentiated clinical signs bordering on low level PMWS and increased viral load in bronchial lymph nodes and sera. Neither clinical signs nor increased viral load in any tissue were observed in immunostimulated animals infected with the archival PCV2 (47). While infection of porcine kidney epithelial (PK15) cells by both archival and contemporary PCV2 yielded a similar amount of PCV2 DNA when normalized to cellular DNA, the infection of porcine alveolar macrophages by archival PCV2 yielded far less viral DNA than infection by contemporary PCV2. This could reflect either a lesser ability to enter and infect or less efficient replication kinetics within porcine macrophages on the part of archival PCV2 compared to contemporary PCV2 (47). This could be critical to their differing ability to cause disease because the lymph nodes of PMWS-animals typically contain an abnormally high number of histiocytes and giant multinucleated cells that stain positive for PCV2 (6, 8, 34) and express TNF-α (7). This expression of TNF-α may be a consequence of infection by PCV2 (49, 66) and it may drive PMWS-related pathogenesis (7). Lymphoid tissues of pigs infected with archival PCV2 were less heavily infected than lymphoid tissues of pigs infected with contemporary PCV2. Further, infection by archival PCV2 was restricted to lymphoid tissue while contemporary PCV2 was found in other tissues particularly in the liver (47). PCV2 antigen was found in hepatocytes and histiocytes in the liver in other studies and hepatitis or liver atrophy is often a feature of PMWS alongside hepatocyte apoptosis and necrosis (6, 8, 9). When PMWS was induced with mitogen in gnotobiotic pigs infected with PCV2, massive liver damage resulted (34). The ability of contemporary PCV2 to infect and damage the liver likely contributes to PMWS pathology. The difference in the capsid protein sequence observed between archival and contemporary PCV2

likely expands the range of tissues that PCV2 can infect and increases its ability to either infect and/or replicate in macrophages. This may enable the contemporary PCV2 to greatly increase its replication and cause pathogenicity upon immunostimulation of the infected host. While this helps explain the emergence of PMWS, it does not explain why only a minority of PCV2-infected pigs develop PMWS even if they are infected with the same strain.

Given that the PCV2 genome has the highest known rate of mutation for a ssDNA virus according to one analysis (62), it is possible that mutations in the PCV2 genome could give rise to strains with greater virulence. One such mutation bequeathed a PCV2b strain with an extra lysine at the C-terminal of the capsid protein (ORF2). Animals infected with this new PCV2b strain had more severe lesions and lymphocyte depletion and greater viral load in all tissues except the lungs when compared to animals infected by PCV2a/b strains lacking the extra lysine at the end of the capsid protein (67). In another recent study, two farms where PCV2 vaccine failure had occurred were investigated and the PCV2 strain with the extra lysine at the end of the capsid protein was again found, but with these additional mutations in the capsid protein: 59R/A → 59K, 68A → 68N, 134T → 134N, and 215V → 215I (68). It is clear that mutations in the PCV2 genome can create strains with enhanced virulence and that this is an ongoing process.

Aside from the cases involving the PCV2 strain with an extra lysine in the capsid protein, the differing clinical outcomes of pigs infected with PCV2 cannot be explained by differences in the genomes of different PCV2 strains. It is possible to induce PMWS using a PCV2 strain never previously associated with disease (55). A comparison of PCV2 genomes from healthy and PMWS animals could not find any consistent difference between strains associated with disease and strains that were not (69), although that study may be confounded by the presence of

multiple strains in a single pig. The clinical outcome of PCV2 infection may be determined by additional cofactors such as other infections rather than mutations in the PCV2 sequence.

1.5 PCV2ORF3

1.5.1 Significance of PCV2ORF3

Intriguingly, expression of a non-structural ORF protein has independently evolved at least three times in the *Circoviridae* family in CAV, and PCV2, and DuCV. CAV expresses a pro-apoptotic protein encoded by its third ORF called apoptin, whose cytotoxicity is regulated by localization to the nucleus (70). CAV induces apoptosis in thymocytes and when the neighboring cells absorb the resultant CAV-bearing apoptotic bodies they too become infected (71). This is similar to the manner in which PCV2 furthers its dissemination by apoptosis. In the case of PCV2 , the protein encoded by the third ORF induces apoptosis early in the infection cycle (1, 72) to convey the virus into macrophages and thereby aide in viral dissemination (66). It was recently found that DuCV also expresses a non-structural protein encoded by a third ORF, and there is some evidence suggesting it too is pro-apoptotic (73). The independent evolution of three functionally analogous ORF3 proteins indicates that the pro-apoptotic effect of these non-structural proteins confers a strong evolutionary advantage to members of *Circoviridae*. An analysis of 20 PCV2 strains found that the ORF3 region was highly conserved with greater than 94.5% identity at the amino acid level (72). Conservation implies that PCV2ORF3 has an important role in PCV2 infection.

1.5.1.1 PCV ORF3 Homologues

PCV1 is closely related to PCV2 (74) but does not cause any known disease (3). Of the three ORF homologues shared by PCV1 and PCV2, the ORF3 homologues are most different in terms of amino acid sequence. The PCV2 homologue of the ORF3 protein is 104 amino acids (315 bp) long, which is only half the length of the 206 amino acid (621 bp) long PCV1

Amino acid conservation between
the PCV ORF3 homologues

Unconserved 0 1 2 3 4 5 6 7 8 9 10 Conserved

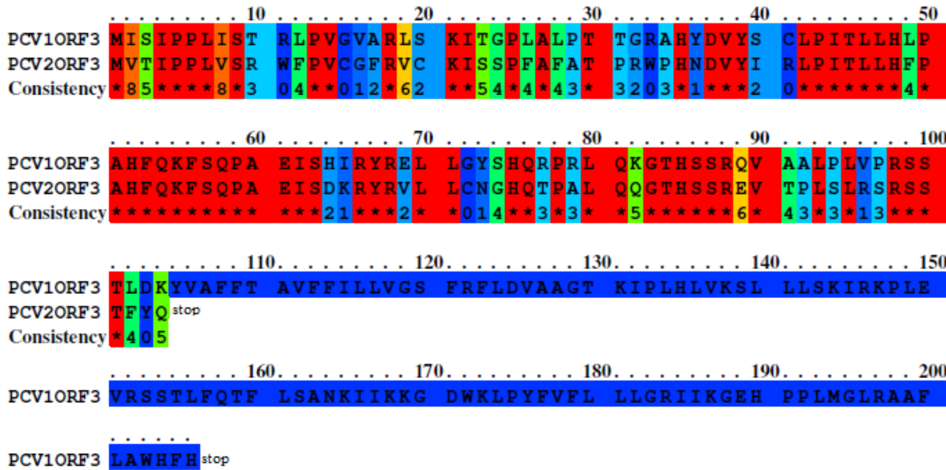

FIGURE 3. Comparison of the ORF3 homologues from PCV1 and PCV2 using the PRALINE multiple sequence alignment tool. Relative degree of conservation between the two proteins is determined according the BLOSUM62 matrix and color coded. Compared to PCV**1**ORF3, PCV**2**ORF3 is truncated by a stop codon. The terminus of both proteins is indicated by 'stop'. The 104 aa region translated in both homologues has only 61.5% amino acid homology (1).

homologue. The difference in length is due to a stop codon created by a difference of a single nucleotide. In the 104 amino acids translated in both homologues, there is only 61.5% amino acid identity between the two ORF3 homologues (1). This raises the possibility that the difference between the ORF3 homologues may account for the differing virulence of PCV1 and PCV2.

1.5.1.2 PCV2ORF3 and Apoptosis

Increased apoptosis and activation of caspases 3 and 8 but not 9 were seen in PK15 cells either infected with wild-type (WT) PCV2 or transfected with an ORF3-expressing plasmid compared to PK15 cells infected with an ORF3 knock-out (ORF3-KO) strain of PCV2 (72). Caspase 8 is an 'initiator' caspase that is activated extrinsically and then activates the 'executioner' class caspase 3 that directly instigates cellular apoptosis. In a study on PCV2ORF3's contribution to PCV2 dissemination, PK15 cell culture infected with WT PCV2 had a much higher number of viral genome copies in the cells and cell-free media than PK15 cell culture infected with the ORF3-KO PCV2. When the PK15 cells infected with WT PCV2 were also treated with a pan-caspase inhibitor called zVAD, the number of PCV2 genomes in the cells and in the cell-free media were reduced and not different from the cultures infected with ORF3-KO PCV2. zVAD affected no such decrease in genome copy numbers in PK15 cell cultures infected with ORF3-KO PCV2 (66). Similar results were found by comparing the serum viremia of mice infected with either WT or ORF3-KO PCV2 and treated or not treated with zVAD (66), indicating that caspase activation is essential to the mechanism exploited by PCV2ORF3 to enhance PCV2 replication and dissemination. Furthermore, when mice were infected with WT or ORF3-KO PCV2, the DNA of WT PCV2 was found in macrophages at much greater quantity

than the DNA of ORF3-KO PCV2. Treatment of the mice with zVAD ablated the apparent ability of PCV2ORF3 to enhance PCV2 entry into macrophages (66). Infection of mice by WT PCV2 also increased TNF-α transcription in macrophages, much as PCV2 infection of swine alveolar macrophages *in vitro* was observed to (49), but this increase was ablated by either knocking out ORF3 expression or treatment with zVAD. This implies that PCV2ORF3 and the apoptosis it initiates are responsible for the increase in TNF-α transcription. The possibility that PCV2ORF3 expression indirectly leads to increased TNF-α expression by macrophages is very important in light of the correlation between higher TNF-α levels and poor clinical outcome of PCV2 infection of swine (7). It is possible that PCV2ORF3 expression drives PCV2-related pathology by inducing increased TNF-α secretion. Altogether, it would appear that PCV2ORF3-instigated apoptosis enhances the dissemination of PCV2 by creating PCV2-bearing apoptotic bodies that are phagocytised by macrophages. These macrophages can then transport the virus throughout the infected host. Further, PCV2ORF3-induced apoptosis generates a TNF-α response that may lead to pathogenesis. This means PCV2ORF3 expression could be fundamental to the induction of PMWS.

The pro-apoptotic effect of PCV2ORF3 expression was also observed in porcine PBMCs transfected with a PCV2ORF3-expression construct. PBMCs include lymphocytes and macrophages in their lineage. These cells are integral to the immune system and the proposed ability of PCV2ORF3 to induce apoptosis in them could be vital to the immunosuppression seen in PCVAD and subclinical PCV2 infection. The PCV2ORF3 protein fused with green fluorescent protein (GFP) localized in the nucleus after 48 hours of expression and caspases 3, 8, and 9 were activated, leading to apoptosis (75). Similar observations were made when the C-terminal half of PCV2ORF3 fused with GFP was expressed transiently. Expression of the N-

terminal half of PCV2ORF3 fused with GFP did not result in increased apoptosis and the protein was localized to the cytoplasm (75). The difference in localization between the N-terminal and C-terminal halves of PCV2ORF3 may be due to the presence of two putative nuclear localization signals (NLSs) in the C-terminal half of PCV2ORF3. The N-terminal half of PCV2ORF3 has no NLS. These putative NLSs were tested by fusion with transiently expressed GFP in PBMCs. Whereas GFP itself localized in the cytoplasm, GFP attached to either of the two putative NLSs localized largely in nucleus (75), indicating that these NLSs are relevant to the localization and therefore function of PCV2ORF3. The pro-apoptotic function of PCV2ORF3 may be regulated by localization to the nucleus much like apoptin of CAV is, at least in PBMCs.

Interestingly, PCV1ORF3 appears to be more cytotoxic than PCV2ORF3 when transiently expressed in cells (76), indicating that either ORF3 is not the primary determinant of virulence or that PCV2ORF3 does not rely mainly on inducing apoptosis to augment PCV2 virulence. It is also possible that PCV2ORF3 is expressed *in vivo* and PCV1ORF3 is not. Another possibility is that PCV1ORF3 is expressed *in vivo* and causes apoptosis much earlier in the infection cycle than PCV2ORF3 does, which could shorten and impede PCV1 infection.

The mechanisms by which PCV2ORF3 induces apoptosis and otherwise enhances PCV2 virulence cannot be understood without identifying its interaction partners and those are still only just being elucidated. Screening by yeast-two-hybrid assay and GST-pull down experiments revealed a strong interaction between PCV2ORF3 and Pirh2 (77, 78). Pirh2 is an E3 ubiquitin ligase that regulates the ubiquitination of p53. Polyubiquitination of p53 protein leads to its destruction in the proteasome. p53 can be phosphorylated and phosphorylated p53 (p53-P) can initiate apoptosis. The constant basal expression and turnover of p53 maintains cellular homeostasis and disruption of this homeostasis can result in apoptosis. Infection of PK15 cells

with WT PCV2 was followed with a decrease in cellular levels of Pirh2. This was concomitant with increased levels of p53 and p53-P by 96 hpi. The ORF3-KO PCV2 did not change the expression of Pirh2 or increase the expression or phosphorylation of p53. Transient expression of PCV2ORF3 had a similar effect on Pirh2 and p53 expression as infection by WT PCV2 (77). A follow-up study revealed that PCV2ORF3 displaces p53 from binding to Pirh2 and transient PCV2ORF3 expression changes the Pirh2 localization from the nucleus to the cytoplasm and increases its turnover (78). Transfection of PK15 cells with pORF3 had similar results on Pirh2 and p53 levels as did infection with WT PCV2 but when amino acids 20-65 of PCV2ORF3 were deleted apoptosis was greatly reduced. Deletion of amino acids 30-60 abolished interaction with Pirh2 and no change in Pirh2 localization or increase in Pirh2 turnover was seen (78). These findings are roughly congruent with a study where transient expression of amino acids 53-104 of PCV2ORF3 was found to be sufficient to induce apoptosis (75) and the results of another study that tested the impact of various mutations in ORF3 on the ability of PCV2 to induce cell death by 72 hpi (79). These mutations in ORF3 were designed to produce truncated PCV2ORF3 protein or disrupt its predicted secondary structure. WT PCV2 induced about 80% cell death in PK15 cells by 72 hpi. Infection by PCV2 with truncated ORF3 induced far less cell death than WT PCV2 as did two of the mutants designed to disrupt PCV2ORF3 secondary structure. Two other mutations designed to disrupt secondary structure, at amino acids 52 and 85, did not change the amount of cell death induced by PCV2 infection. It is possible that these mutants did not disrupt secondary protein structure or that the cytotoxicity of PCV2ORF3 is not dependent on the secondary structure at amino acids 52 and 85. Amino acid 52 lies in the 30-60 amino acid section defined as essential for interaction with Pirh2 by Karuppannan *et al.*, 2010 (78), but it is outside of the 53-104 section found essential for apoptosis in PBMCs by Lin *et al.*, 2011 (75).

Amino acid 85 is well outside both regions. The mechanism by which PCV2ORF3 induces apoptosis is poorly understood although Pirh2 is likely involved. It appears that PCV2ORF3 both displaces p53 from binding to Pirh2 and mediates destruction of Pirh2. The resulting decrease in polyubiquitination of p53 enhances its stability and increases cellular levels of p53 and p53-P, leading to apoptosis.

Another likely interaction partner of PCV2ORF3 identified by two-hybrid and pull-down assays is regulator of G-protein signalling 16 (RGS16) (20). Intriguingly, neither PCV1ORF3 nor its first 104 aa were capable of pulling down recombinant RGS16. It is possible this is related to the differing ability of PCV1 and PCV2 to cause disease. Further, the PCV2ORF3 proteins cloned from two different PCV2**b** strains pulled down about twice as much recombinant RGS16 protein as did the PCV2ORF3 protein cloned from a PCV2**a** strain (80). Regulators of G-protein signalling accelerate the conversion of guanosine-5'-triphosphate (GTP) to GDP in certain proteins that are activated by binding to GTP. Conversion to GDP inactivates these proteins. The functions of RGS16 are largely unknown but it may be important to attenuation of signalling by chemokine receptors in T lymphocytes, which suggests a role in T lymphocyte trafficking (81). Interaction with RGS16 may be of importance to PCV2 infection because treatment with IL-10 and lipopolysaccharide (LPS) appears to induce an increase in RGS16 expression (82) and increased levels of IL-10 correlate with PCV2 viremia in infected pigs (56). PBMCs from PMWS pigs were also found to secrete more IL-10 than PBMCs from healthy pigs (10). The putative interaction between RGS16 and PCV2ORF3 would offer an intriguing basis for the correlation between viremia and IL-10 secretion.

The possible interaction between PCV2ORF3 and Pirh2 offers a mechanism underlying the cytotoxicity of the former and would explain why nuclear localization of PCV2ORF3 is

necessary for it to instigate apoptosis. The differing ability of the two PCV ORF3 homologues to interact with RGS16 offers a possible explanation for the differing virulence of PCV1 and PCV2; however, this is extremely tentative. There may be other binding partners of PCV2ORF3 and understanding the role of PCV2ORF3 in PCV2-infection entails finding them.

1.5.1.3 *In Vivo* Infection Experiments using PCV2ORF3 Knockouts

Two studies comparing infection of pigs WT and ORF3-KO strains of PCV2 both found that PCV2ORF3 is not essential for PCV2 replication *in vivo*. The quantity of PCV2 genome copies in the serum of pigs infected with the ORF3-KO was lower than in the serum of pigs infected with WT PCV2 in both studies; however, the study by Juhan *et al.* (2010) could not find a difference in lesion scoring between pigs infected with the WT and ORF3-KO PCV2 (83) while the study by Karuppannan *et al.* (2009) did (79). The study that did find a difference in lesion scoring also found that the lymph nodes of pigs infected with WT PCV2 had a higher viral load than lymph nodes of pigs infected with ORF3-KO PCV2. Lymph nodes of pigs infected with WT PCV2 in the study by Karuppannan *et al.* (2009) lost their architecture while the lymph nodes of pigs infected with the ORF3-KO PCV2 did not (79). The study that did not find a difference between infection by WT and ORF3-KO PCV2 in lesion scoring also did not find a difference in the amount PCV2-specific antigen in lymph nodes except in mesenteric lymph nodes (83). Neither of these studies induced PCVAD in the infected pigs, but the subclinical manifestation of PCV2 infection was more severe in the study that found a difference in lesion scoring between WT and ORF3-KO PCV2 than the one that did not. These two studies used different metrics for scoring pathology and also used very different methods to infect pigs. Karuppannan *et al.* inoculated pigs with PCV2 in the form of actual virus while Juhan *et al.*

injected PCV2 in the form of a DNA construct. The time required to establish viremia was longer and more variable between pigs in the study by Juhan *et al*. That study also had less stringent standards for what pigs were considered PCV2-free; at least one ostensibly PCV2-free pig tested positive for PCV2. Differences in methodology may account for the differing conclusions of these two studies. Nonetheless, these knockout studies indicate that PCV2ORF3 may be important to the virulence of PCV2 or at least development of higher viremia. The proposition that PCV2ORF3 expression is important to PCV2 virulence is backed by other knockout studies in mice.

Mice infected with WT PCV2 had a higher lesion score, greater viremia, more apoptosis in examined tissue, and more PCV2 DNA and antigenic load in the lymph nodes when compared to mice infected with ORF3-KO PCV2 (1). This same study also examined the immunological differences of mice infected by WT or ORF3-KO PCV2. Serum from mice infected with WT PCV2 had a weaker αPCV2 antibody titre than serum from mice infected with the ORF3-KO PCV2. Mice infected with WT PCV2 also had fewer CD4+, CD8+, and CD4+CD8+ cells during at least one time point in the experiment. These cell count results are similar to the earlier mentioned study of ORF3-KO and WT PCV2 infection in pigs by Karuppannan *et al*. (79) and could result in weaker CMI and T_h-directed activation of the humoral immune response. The study by Karuppannan *et al.* also counted IgM+ cells and found fewer of them in pigs infected with WT PCV2 compared to the pigs infected with ORF3-KO PCV2. The expression of PCV2ORF3 may have resulted in a weaker humoral immune response to PCV2 because the overall αPCV2 IgG titre was also lower in pigs infected with WT PCV2 than ORF3-KO PCV2. CD8+ cell counts did not significantly differ between pigs infected with ORF3-KO or WT PCV2 at any time point however (79). These findings may indicate that PCV2ORF3 down regulates or

kills different cell-types of the immune system and thereby contributes to PCV2-induced immunosuppression.

1.5.1.4 PCV2ORF3 DNA Vaccination

This contention is further backed by another study that compared mice vaccinated with a plasmid construct bearing PCV2ORF2 (pORF2) to mice vaccinated with both pORF2 and pORF3. Vaccination of mice with pORF2 had previously given mice high αPCV2 neutralization titres and a strong Th1 immune response to PCV2 challenge that resulted in protection in disease and lower viremia (36). Addition of the pORF3 to the DNA vaccine dramatically shortened the Th1 humoral response, which manifested as a decrease in the titre of αPCV2 IgG_{2a} antibodies and a relative increase in IgG_1 antibodies, and resulted in no development of a virus-neutralizing antibody response. The negative vaccine controls, which did not receive any DNA vaccination for PCV2, had stronger αPCV2 neutralization responses than mice vaccinated with both pORF2 and pORF3 (84). After challenge with PCV2, the mice vaccinated with both pORF2 and pORF3 had the same lesion score and greater viremia than mice that received no vaccination at all. In summary, addition of pORF3 to a DNA vaccine containing pORF2 abrogated the protection against PCV2 challenge conferred by pORF2 vaccination and altered the αPCV2 immune response in mice. This indicates PCV2ORF3 mRNA or protein is capable of causing immunosuppression. It is also possible that certain regions of DNA within PCV2ORF3 could be affecting this immunosuppression rather than a PCV2ORF3 expression product. The exact mechanism by which PCV2ORF3 affects immunosuppression is not known but apoptosis is likely involved given the many studies demonstrating that PCV2ORF3 is cytotoxic.

1.5.2 PCV2ORF3 Expression in Pigs

A previous study tested the role of PCV2ORF3 protein in PCV2-induced apoptosis with PCV2 strains with mutations in ORF3 that truncated or possibly altered the secondary structure of the PCV2ORF3 protein. Most of these mutants were less able to induce apoptosis in PK15 cells than WT PCV2, strongly indicating that the PCV2ORF3 protein expression product is important to apoptosis in PCV2-infected cells (79). Studies on PCV2ORF3 expression *in vivo* are limited. Expression of PCV2ORF3 was detected by IHC in the lymph tissues of PCV2-infected mice (1) but there has not yet been any published evidence of PCV2ORF3 expression in pig tissue. Members of the Czub lab have attempted to identify PCV2ORF3 using IHC but it was never detected in pig tissue in our experience. This thesis relies on detecting serum antibodies to PCV2ORF3 as a means of confirming its expression. This method is indirect but has the advantage that no matter where or when PCV2ORF3 is expressed, antibodies will be made against it and these are easy to detect with serological methods.

1.6 Thesis

Hypothesis: The ORF3 protein of PCV2 is expressed in PCV2-infected swine.

Objective 1: Test swine sera for antibodies to PCV2ORF3 to confirm that PCV2ORF3 was expressed in these swine.

Objective 2: Confirm that the apparent antibodies to PCV2ORF3 are not actually antibodies to PCV1ORF3.

Objective 3: Look for correlations between αPCV2ORF3 sera reactivity and other serological or clinical data that could suggest possible roles and functions for PCV2ORF3 and the biological importance of antibodies to it.

Chapter 2 Materials and methods

2.1 Serum Samples

The swine sera screened here come from three different Albertan farms that are summarized in Table 1. All sera sampling was performed under conditions adhering to Canadian guidelines for animal welfare in biomedical research. One cohort came from a farm with animals that were vaccinated at weaning and is labelled as ‘vaccinated’. These animals did not show any apparent signs of PCVAD. The vaccine used is a commercially available vaccine that is composed of PCV2ORF2 protein expressed in insect cells. Another cohort came from a farm with only unvaccinated animals without any apparent signs of PCVAD and is labelled as ‘unvaccinated apparently undiseased’. One cohort came from a farm with a history of PCVAD and only has unvaccinated animals several of which exhibited clinical signs of PMWS. This cohort is labelled as ‘unvaccinated diseased’. Animal ages ranged from sucklings to six month old adults. All sera were heat-inactivated at 56°C for 1 hour to inactivate complement. The PCV2 virus does not degrade or lose infectivity from this treatment (85).

TABLE 1. Summary of the swine cohorts sampled for sera.

Cohort	PCV2 Vaccination status	PCVAD status	Number of weaned animals	Number of suckling animals
Vaccinated for PCV2 & apparently undiseased	Yes (at weaning)	No apparent disease	30	31
Unvaccinated for PCV2 & diseased	No	Several animals with clinical signs; farm history of PCVAD	24	4
Unvaccinated for PCV2 & apparently undiseased	No	No apparent disease	43	5

2.2 Creation of the PCV1ORF3/pET28a+ and PCV2ORF3/pET28a+ Constructs

To express the ORF3 proteins, their DNA sequences had to be first synthesized and ligated into a plasmid appropriate for recombinant protein expression in bacteria. Our lab synthesized the PCV2ORF3 DNA by PCR because we did not have any available for use.

The PCV2ORF3 sequence was created by PCR-based gene synthesis using a PCV2 strain (GenBank accession number AY094619) as the template and cloned into the pET28a+ plasmid using *NdeI/XhoI* restriction enzyme cut sites. The resulting construct encoded a recombinant PCV2ORF3 protein with an N-terminal his-tag whose expression is induced by isopropyl β-D-1 thiogalactopyranoside (IPTG) and is driven by the upstream T7 promoter. The construct was then transformed into DH5α *E. coli* cells (Novagen, San Diego, CA) by heat shock at 42°C for 30 seconds, incubation on ice for 2 minutes, and then incubation in 800 µL of LB for 1 hour at 37°C with agitation. Transformants were spread-plated on LB-Kan plates and incubated overnight at 37°C. Colonies were picked, grown up, and their pDNA extracted for screening at the McGill University and Genome Quebec Innovation Centre in Montreal, QC for the correct sequence.

PCV1ORF3 was PCR-amplified from a synthetic PCV1 clone (GenBank accession number AY184287) and cloned into pET28a+ in a similar manner to PCV2ORF3. Transformants were also screened at the McGill University and Genome Quebec Innovation Centre in Montreal, QC for the correct sequence. The construction of the PCV2ORF3/pET28a+ and PCV1ORF3/pET28a+ constructs through to its transformation into DH5α cells was carried out for a previous M.Sc. thesis by Mark Chaiyakul (86).

2.3 Expression and Purification of rPCV2ORF3

The rPCV2ORF3 protein is not readily expressed in mammalian cells. To get a sufficient amount of rPCV2ORF3 to screen sera for αPCV2ORF3 antibodies it was expressed in bacteria. The rPCV2ORF3 was mostly insoluble and for that reason had to be extracted under denaturing conditions, which solubilises all of the protein. The rPCV2ORF3 includes a histidine tag, which binds nickel. This is why immobilized metal affinity chromatography (IMAC) was chosen as the purification method.

The PCV2ORF3/pET28a+ construct was extracted from the DH5α cells and transformed into BL21-DE3 Rosetta Blue cells (Novagen) for recombinant protein expression. The bacteria were induced at a large scale with 1 mM IPTG to express rPCV2ORF3 which was then extracted and purified by denaturing IMAC using Profinity IMAC Ni-Charged Resin (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. In accordance with that protocol, the culture was pelletted and suspended in a buffered solution of 8 M urea at a ratio of 1g pellet/10 mL lysis buffer and sonicated to solubilise all proteins. It was then centrifuged at 20,000xg for 30 minutes to separate the resolubilized protein from insoluble debris. The urea was required to keep all of the rPCV2ORF3 soluble. At this step, the manufacturer's protocol dictates that the supernatant be disposed of; however, this supernatant contained almost all of the rPCV2ORF3. For this reason, the supernatant was subjected to IMAC purification. The supernatant was clarified by filtration through a 0.8 μM filter to remove any debris. The clarified lysate was incubated with the Profinity IMAC Ni-Charged Resin, allowing the N-terminal histidine tag on the rPCV2ORF3 to bind the Ni^{+2} ions in the resin. After washing to remove impurities, the recombinant protein was eluted out of the resin with a buffered solution of 8 M urea and 500 mM imidazole. The imidazole is structurally similar to histidine and competitively binds to the Ni^{+2} ions, displacing

the histidine-tagged rPCV2ORF3. The IMAC-purified rPCV2ORF3 was subject to dialysis over 6 days to remove imidazole and urea using SpectraPor dialysis membrane type 6 with a 2 KDa MWCO and SpectraPor clamps (Spectrum, Rancho Dominguez, CA) in conditions summarized in Table 2. This caused the rPCV2ORF3 to precipitate, in effect concentrating it.

TABLE 2. Dialysis conditions of rPCV2ORF3 IMAC fractions over a 6 day time period. After IMAC purification, the rPCV2ORF3 fractions were dialyzed to remove imidazole immediately and remove urea in a stepwise fashion over 6 days. Solution at end of dialysis contains 300 mM NaCl and 50 mM NaH_2PO_4.

Dialysis Step	Time (hours)	Concentration of urea in the dialysate (M)	Temperature of the dialysate
1	24	6	Room temperature
2	24	4	Room temperature
3	24	2	Room temperature
4	48	1	Room temperature
5	24	0	4°C

The precipitated rPCV2ORF3 had to be resolubulized. Methanol (MeOH) is a protic solvent that can form hydrogen bonds and is at least as good as SDS at solubilising peptides (87). The resulting precipitate was resuspended in a 50% MeOH solution for 48 hours at room temperature to resolubilize the rPCV2ORF3. Extraction was repeated on each precipitate once to maximize the amount of protein extracted. A sample of the extracted protein was subject to SDS-PAGE, Coomassie-stained, and then the putative rPCV2ORF3 was excised with a clean razor blade in sterile conditions for LC-MS/MS by the Southern Alberta Mass Spectroscopy (SAMS) which confirmed its identity as PCV2ORF3 (see appendix).

2.4 Expression and Purification of rPCV1ORF3

The rPCV1ORF3 protein is also difficult to express in mammalian cells and therefore it was expressed in bacteria and purified for screening sera for αPCV1ORF3 antibodies. The PCV1ORF3/pET28a+ construct was transformed into *E. coli* BL21-DE3 Rosetta Blue cells (Novagen) and the sequence verified (Eurofins MWG Operon, Luxembourg). Expression protocol was similar to rPCV2ORF3 however rPCV1ORF3 expression was induced during the stationary growth phase, at an $O.D._{600}$ of just above 1.0. Expression during the stationary phase is a common strategy for expression of toxic proteins such as PCV1ORF3. IMAC purification was similar to rPCV2ORF3 and further purification was not necessary for these purposes. The identity of the putative rPCV1ORF3 was confirmed by immunoblotting with mouse αhis (H1029, St. Louis, MO, Sigma-Aldrich) and goat αmouse (A4416, Sigma-Aldrich) and by LC-MS/MS at the Southern Alberta Mass Spectroscopy lab. The first LC-MS/MS analysis failed to confirm the identity of rPCV1ORF3 because PCV1ORF3 is not annotated in the NCBI database, although it did match it to PCV2ORF3. The analysis was reiterated using the PCV1ORF3 sequence from the PCV2 strain used here (AY184287) and this confirmed the identity of rPCV1ORF3 (see appendix).

Comment [MR2]: Not 'PubMed'

2.5 SDS-PAGE and Immunoblot

SDS-PAGE, western blotting, and Coomassie staining were performed in accordance with established lab protocols. The Mini-PROTEAN Tetra gel apparatus from Bio-Rad was used for this study. All gels were 1 mm thick and either 12 or 15% acrylamide. Sample was combined with an equal volume of 2x SDS sample buffer prior to electrophoresis at 80V. Pyronin Y (Sigma-Aldrich) was added to the stacking gel because it runs ahead of the Coomassie dye front

at about 8 KDa and transfers with protein to polyvinylidene fluoride (PVDF) membrane during semi-dry transfer. This makes Pyronin Y useful as a transfer control and for marking the bottom of strips cut from PVDF after transfer. Electrophoresis was stopped when the Pyronin Y was less than 1 cm from the end of the gel. Gels were either stained with Coomassie dye or transferred to PVDF for immunoblotting.

All transfers were performed using the Semidry Transfer Apparatus (Bio-Rad) in accordance with the manufacturer's protocol. The gel and PVDF were first equilibrated in transfer buffer and then electro-transfer was carried out at 15V for 40 min. Blots were blocked for at least 1 hour in 5% skim milk solution. Incubation in primary antibody was carried out for at least 1.5 hours, followed by three washings in PBS-Tween 20 (PBST). The blots were then incubated in secondary antibody conjugated to HRP for 1 hour at room temperature, washed as before, and developed in either ECL (GE Healthcare, Baie d'Urfe, QC) or LuminaForte (Millipore, Billeric, MA). Blots were imaged in either the VersaDoc 5000 MP (Bio-Rad) or the Fluor-S MultiImager (Bio-Rad).

2.6 Use of rPCV2ORF3 in Immunoblot Detection of Sera Antibodies

For use in screening sera, the rPCV2ORF3 was quantified and transferred to PVDF membrane. To maximize the number of sera screened the rPCV2ORF3 blot was cut into strips and these strips were incubated with a dilution of the sera. The protein concentration of the 50% MeOH solubilization of rPCV2ORF3 was determined by Bradford assay (Bio-Rad) using a BSA curve in accordance with the Microtitre Assay procedure. The assay was optimized with varying amounts of rPCV2ORF3 and it was found that 21 μg of rPCV2ORF3 worked best. The rPCV2ORF3 in 50% MeOH was subjected to electrophoresis on a single-well 15% SDS-PAGE

gel with Pyronin Y (Sigma-Aldrich) in the stacking gel. Then the gel and PVDF (GE Healthcare) were equilibrated in semi-dry transfer buffer (20% MeOH, 25 mM Tris, 192 mM glycine, 0.2% SDS) for 15 minutes. The gel was subject to semi-dry transfer onto the PVDF membrane at 15 V for 40 minutes using a semidry transfer apparatus (Bio-Rad). Transfer was confirmed using Ponceau staining (Sigma-Aldrich) and blot was blocked overnight at 4°C in blocking buffer consisting of 5% skim milk in PBS containing 0.025% Tween (PBST) with 0.01% Thimerosal (Sigma-Aldrich). All blocking buffer has been filtered through a Grade 4 Whatman filter (GE Healthcare). The blot was then washed twice in PBST for about 15 min. The blot was cut into narrow strips and these strips were each incubated with a swine serum diluted 1/100 in blocking buffer. The dilution was made in a total volume of 400 µL and then spun down for at least 10 seconds at maximum speed to clear out insoluble complexes. The rPCV2ORF3 PVDF strip and 350 µL of the swine sera dilution were incubated together in a cut section of a 1 mL pipette sealed at both ends with Parafilm (Pechiney, Chicago, IL) overnight at 4°C with agitation. The next day these strips were washed three times in PBST about 5-10 minutes each time and then incubated in 1 mL diluted rabbit αpig (RAP) conjugated to horse-radish peroxidase (HRP) (A5670, Sigma-Aldrich) for one hour at room temperature with agitation. The strips were washed as before and developed in either ECL Plus (GE Healthcare) or ECL Prime (GE Healthcare) and visualized on the VersaDoc 5000 MP (Bio-Rad) on the chemiluminescent channel or on the Flour-S Multilmager (Bio-Rad) on the chemiluminescent ultrasensitive channel.

2.7 Controls and Quantification of αPCV2ORF3 Serum Reactivity Assay

The highly variable nature of chemiluminescence-based blotting necessitated that each 'test batch' of sera be standardized. Sera of the same 'test batch' were tested at the same time and the rPCV2ORF3 strips used to test them were cut from the same PVDF blot. There was a standard set of controls run with each test batch of sera. These controls were four newborn swine sera, a mouse αhis antibody (81663, Abcam, Cambridge, England), a highly reactive swine serum, and the 'null' control where no primary antibody was used. The four newborn sera were taken from swine immediately after birth before they could receive colostrum. Having never received colostrum, these newborns did not have any maternal antibodies to PCV2ORF3 in their sera and are therefore suitable as negative controls. The newborn sera were used to control for non-specific IgG binding and the 'null' to control for non-specific binding of the RAP conjugate.

The signals of every tested serum were quantified using the QuantityOne program (Bio-Rad) and background was subtracted. The background signal used to adjust a given serum signal was taken from within the same strip as the serum being adjusted. The signal readout of each tested serum was normalized by conversion to a sample/positive (S/P) ratio using the previously mentioned positive standard. The positive standard itself was given and S/P value of 1. Sera with background values greater than the sera signal were given final reactivity values of 0 because negative serum reactivity does not make sense.

$$\text{S/P value} = \frac{(\text{serum signal} - \text{background})}{(\text{positive standard signal} - \text{background})}$$

2.8 Serological Testing of Swine Sera for Presence of αPCV1ORF3 Antibodies

Only 2.4% of swine are positive for PCV1 by PCR according to one study (14), but it is still possible that many swine are infected by PCV1 at least once in their lives. Therefore, any

antibodies that react with rPCV2ORF3 could have actually been raised against PCV1ORF3 originally. No previous research regarding the cross-reactivity of antibodies to the ORF3 homologues has been published. To check for this possibility, several swine sera moderately to strongly reactive to PCV2ORF3 were screened for antibodies to PCV1ORF3. rPCV1ORF3 was electrophoresed in a 12% SDS-PAGE gel and transferred to PVDF in the same manner as for rPCV2ORF3. The amount of antigen was doubled to approximately 42 µg to make the amount of rPCV1ORF3 roughly equimolar to the rPCV2ORF3. For this serological assay, the ORF3 homologue proteins were only subject to IMAC and not further purified. This was sufficiently pure to check for the presence of antibodies to either ORF3 homologue. Membrane blocking and incubation in sera/control antibodies were the same except that the primary incubation was carried out at room temperature for 2 hours in an 800 µL volume instead of overnight at 4°C in a 350 µL volume. Also, the αmouse secondary was a sheep αmouse HRP (NA931, GE Life Sciences) and the mouse αhis was also different (H1029, Sigma-Aldrich). Only seven sera were tested for αPCV1ORF3 antibodies. These strips were developed in Luminata Forte (Millipore) and exposed on the VersaDoc (Bio-Rad) on the chemiluminescence channel.

Initially, there were problems with getting the sera to react as strongly with the rPCV2ORF3 in 8 M urea as they had reacted with rPCV2ORF3 in 50% MeOH. Most of the sera simply did not react with the IMAC-purified rPCV2ORF3 or even any contaminant proteins; however the rabbit αPCV2ORF3 and mouse αhis hyperimmune sera did. This was due to the presence of urea alongside both of the IMAC-purified ORF3 proteins. When protein is boiled in urea, as is typically done prior to gel electrophoresis, the high temperature promotes breakdown of urea into isocyanic acid and consequent carbamylation of lysine and arginine residues, which tend to be important to epitopes. Carbamylation must have rendered these epitopes non-reactive

with the swine sera αPCV2ORF3 antibodies. This problem was remedied by elimination of the boiling step prior to electrophoresis. The antigens were simply incubated for 30 minutes with the SDS sample buffer at room temperature prior to gel electrophoresis. All sera reacted moderately to very strongly with rPCV2ORF3 after this change was implemented.

2.9 DNA Extraction

To see if the αPCV2ORF3 reactivity of the sera had any association with change in PCV2 viral load, the number of PCV2 genome copies in the sera was measured. Before quantifying the serum viral load the PCV2 DNA had to be extracted. The QIAamp DNA Mini Kit was used to extract DNA from our sera samples in accordance with the manufacturer's instructions (QIAGEN, Germantown, MD) with some modifications. Only 100 µL of sera was used for extraction after being added to 100 µL sterile PBS and only 100 µL of elution solution was used to elute the DNA. In the few cases where elution was carried out with 200 µL of elution solution the DNA copy number was multiplied by a factor of 2 to compensate for the dilution. Initial treatment with proteinase K and then lysis buffer removes the nuclear material from the cell membrane and cytoplasm and disrupts the nuclear membrane. When applied to the silica matrix, the negatively charged DNA will adsorb to the silica at a pH below the pKa of the silica. It is thought that this reduces the electrostatic repulsion of the DNA and silica, while dehydration of both by salts causes the silica and DNA to bind. Salts and proteins are washed off with wash solutions containing ethanol, which keeps the DNA insoluble and adsorbed to the silica. Application of a solution with a pH of about 9 gives the silica a negative charge because it is above its pKa. This creates an electrostatic repulsion between the silica and the DNA, resulting in the elution of the latter.

2.10 Quantitative PCR

To quantify the number of PCV2 genome copies in the serum, qPCR was performed using the DNA extract of each serum and primers that amplified a section of PCV2ORF2. For the qPCR, the forward primer sequence was GTGACTGTGGTTCGCTTGAT and the reverse primer sequence was GTTACCGCTGGAGAAGGAAA both at a final concentration of 300 nM. The rest of the reaction included 12.5 µL of SYBR Green Master Mix low ROX (Quanta BioSciences, Gaithersburg, MD) and 2.5 µL of extracted DNA in a total volume of 25 µL. The qPCR cycle used was as follows: 1) 95°C for 5 min 2) 40 cycles of 95°C for 15 sec and 60°C for 1 min 3) 1 cycle of 95°C for 1 min, 55°C for 30 sec, 95°C for 30 sec. The qPCR reactions were carried out on the MX3005P (Agilent Technologies, Mississauga, Ontario, Canada). For each serum tested there were two internal duplicates within the sample plate and two duplicates of the plate ('duplicate of duplicates') except for the unvaccinated apparently undiseased cohort and all but seven of the vaccinated sucklings. These could only be subject to one qPCR run. The equations used to calculate copy numbers from cycle number were readjusted based on the assumption that all of the PCV2 genomes detected were in ssDNA form. For each set of qPCR a negative control made by extraction of PBS was run in duplicate. The background was subtracted and the result was then adjusted from per microlitre to per millilitre and subject to logarithmic transformation with a base of 10. Any result that was negative after subtracting the background was given a value of '0' after logarithmic transformation. After the logarithmic transformation the internal duplicates were averaged. Then the external duplicate values were averaged to get the final qPCR value in $\log_{10}$(genome copies/mL) for each serum. This work was performed partially by our lab technician and completed by myself.

2.11 Growing PCV2 Virus in PK15 Cells

The PCV2b strain 05-32650 (Accession # EF394779) was chosen for use in a virus neutralization assay and propagated in PK15 cells. The virus was cloned into the PJ201 plasmid twice to make a tandem construct. To grow up more of the tandem construct, it was transformed into *E. coli* for propagation and then extracted using the QIAGEN Plasmid Maxi Kit. The tandem DNA construct was transfected into PK15 cells using Lipofectamine 2000 (Invitrogen) and OptiMEM (Gibco) and the cells were incubated at 37°C and 5% CO_2. After three passages of the transfected cells, the supernatant was collected and used for superinfection. PK15 cells were infected with the transfection supernatant and one passage was performed after one week of incubation at 37°C and 5% CO_2. The supernatant was collected, freeze/thawed three times, sonicated, and clarified. The virus was titrated by diluting the virus from 10^{-1} to 10^{-10} in a 96-well plate and applying these dilutions to 60-80% confluent PK15 cells in each well. The virus titre was reported as tissue culture infective dose 50 per ml ($TCID_{50}$/ml). The virus dilutions were incubated and then the number of infected cells in each dilution was observed. The Reed-Muench equation (88) was applied to these results to determine the final virus titre in $TCID_{50}$/ml. $TCID_{50}$/ml is an endpoint dilution assay that quantifies the amount of virus required to infect 50% of an inoculated tissue culture. The virus produced had a titer of $4.6x10^4$ $TCID_{50}$/ml infecting 8-10% of the cells in the well. The creation of the tandem construct, propagation of the virus, and its titration were carried out by other members of the Czub lab.

2.12 Neutralizing Antibody Titration

To test whether serum αPCV2 neutralizing ability was correlated with its αPCV2ORF3 reactivity, all of the sera from vaccinated weaned animals and a random selection of the sera

from the other two cohorts had their αPCV2 neutralization titre quantified. These sera included all 30 of the weaned animals and 6 of the sucklings from the vaccinated cohort; 14 of the weaned animals and 4 of the sucklings from the unvaccinated diseased cohort; and 15 of the weaned animals and 5 of the sucklings from the unvaccinated apparently undiseased cohort for a total of 74 sera titred for αPCV2 nAbs. Previously heat-inactivated sera were diluted in three-fold steps in MEM (Gibco) + 10% fetal bovine serum, 2% penicillin-streptomycin solution, and 4% 200 mM L-glutamine and incubated with an equal volume of the previously described PCV2b virus stock for 1 hour at 37°C. This incubation period allowed for virus-antibody interaction and binding. This mixture was then applied to PK15 cells grown to about 50% confluence in a 96-well plate and incubated for 90 min at 37°C. Two of the wells of each plate were positive controls with only virus and no serum added; another two wells had PCV2 incubated with a 1/3 dilution of a newborn swine serum; and four wells had no PCV2 added to them whatsoever and controlled for background. After 90 min, the wells were washed with sterile 1X PBS and incubated with fresh media for 48 hours. The cells were then fixed with 4% paraformaldehyde for 30 min at room temperature. After washing three times with 1X PBS, cells were permeabilized at room temperature for 30 min in permeabilization buffer (1X PBS, 0.1% saponin, 0.1% BSA). Cells were then washed three times in PBS-0.1% Tween and stained by incubating with rabbit αPCV2ORF2 for 90 min at 37°C, washing three times with PBS-0.1% Tween, and then incubating in goat αrabbit Alexa Flour 568 (A11011, Invitrogen). Washed each well three times in PBS-0.1% Tween and stained with DAPI (Invitrogen). After 10 min the DAPI was washed away three times with 1X PBS. Plates were kept at -20°C in 100 µL/well PBS-buffered 90% glycerol until analysis with the InCell 2000 (GE Life Sciences). The wells with no virus added were used to adjust out the background of each plate. The percentage of cells

with positive nuclear staining and the percentage of cells with positive cytoplasmic staining were combined to get the total percent positive cells for each well. This was converted to a percent reduction of infectivity relative to the virus-only positive control wells using the formula below.

$$\% \text{ Reduction in infectivity} = \frac{\text{total \% positive in +ve control - total \% +ve in sample}}{\text{total \% positive in +ve control}} \times 100\%$$

The raw neutralizing titre of each serum is the last dilution that results in a 50% reduction in the number of infected cells relative to the positive control wells of the plate as determined by the InCell 2000. Neutralization was carried out in duplicate and the raw neutralizing titres were log transformed to base 10 and averaged to yield the final neutralization titre of each serum. This work was carried out collaboratively by all members of Czub lab. Although I did not process all of the sera from start to finish, I did carry out each step of the above process at least once on a large number of serum samples.

2.13 Statistical Analysis

For comparing the serum αPCV2ORF3 reactivity of two different groups of sera, the Mann-Whitney U test was used. The Mann-Whitney U test is a non-parametric method of testing whether two populations are significantly different or not. While parametric tests are more powerful, they require the data to be normally distributed and equally variant in both groups. The data compared here were not suitable for parametric testing because they were not normally distributed. The αPCV2ORF3 reactivity of the three different age categories was compared using the Kruskal-Wallis test, which is an extension of the Mann-Whitney U test to three or more groups. All tests for correlation were performed using the Spearman rank order correlation. The

Spearman rank order correlation can detect all monotonic relationships between two variables, not just linear relationships as is the case with the Pearson product correlation. The Spearman rank order correlation can be used on non-normally distributed data because it is a non-parametric test. A p value of less than 0.05 was considered significant. SigmaPlot 11.0 was used for all statistical analysis.

Chapter 3 Results

No data has yet been published demonstrating that PCV2ORF3 is expressed in PCV2-infected pigs. Our lab has attempted to directly detect PCV2ORF3 protein in the tissues of PCV2-infected pigs using IHC without success. Direct detection of PCV2ORF3 protein is difficult because it is not known what tissue it is expressed in or at what point during infection it is expressed at. It may be expressed only very briefly. The fact that antibodies to PCV2ORF3 will be made no matter where it is expressed or if it is expressed only briefly makes indirect detection by screening for αPCV2ORF3 serum antibodies a far more practical means of demonstrating PCV2ORF3 expression in PCV2-infected pigs. For this reason, my thesis relies on serological means of demonstrating PCV2ORF3 expression in PCV2-infected pigs.

There is also no data regarding the clinical relevance of antibodies to PCV2ORF3, such as a correlation between serum reactivity to PCV2ORF3 and viral load or nAb titre. In this thesis the αPCV2ORF3 reactivity of the sera is quantified using a standard and compared to the sera nAb titre and viral load and cohort age to see if antibodies to PCV2ORF3 are perhaps biologically or even clinically relevant.

3.1 Recombinant Expression and Purification of both ORF3 Homologues

To detect αPCV2ORF3 antibodies, PCV2ORF3 was recombinantly expressed in bacteria and purified for use in a serological assay. The results of rPCV2ORF3 extraction, IMAC purification, and resolubilization are shown in the Coomassie-stained SDS-PAGE in Fig. 4. There is a large band between the 10 KDa and 15 KDa ladder markers matching with the expected size of rPCV2ORF3 in all the lanes except the negative control. There are fewer bands in the E8 and E9 lanes than the lysate lanes and fewer still in the 50% MeOH resolubilized

rPCV2ORF3 lane. The IMAC elution fractions E8 and E9 were each subject to precipitating dialysis and two rounds of resolubilization in 50% MeOH. These are visualized by Coomassie stain and western blot in Fig. 5 using the same positive and negative controls as in Fig. 4. The Coomassie stained gel in Fig. 5 has a band between the 10 KDa and 15 KDa markers consistent with the expected size of rPCV2ORF3. This band was visible below 20 KDa in a western blot probed with the mouse αhis antibody (Abcam) to detect rPCV2ORF3.

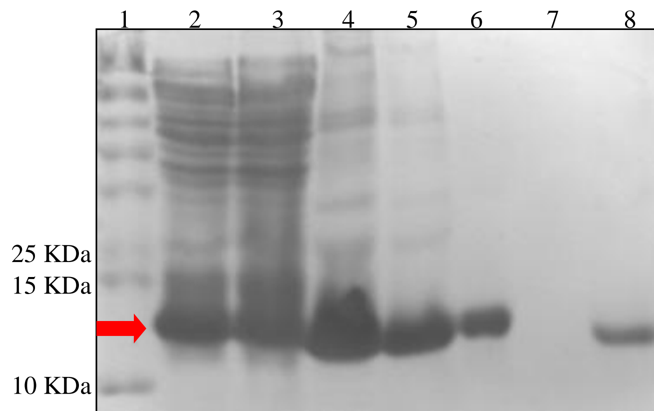

FIGURE 4. Coomassie stained SDS-PAGE of selected fractions from extraction and purification of rPCV2ORF3. The crude lysate (lane 2) was micro-filtered to produce cleared lysate (lane 3) which was subject to IMAC. Elution fractions E8 and E9 (lanes 4 and 5) were later precipitated by dialysis, resolubilized in 50% MeOH, and pooled (lane 6). The negative control was SDS sample buffer with no rPCV2ORF3 (lane 7) and the positive control was a previously extracted rPCV2ORF3 (lane 8). The red arrow indicates the position of rPCV2ORF3. Lane 1 is a pre-stained ladder from Bio-Rad with molecular weight labels in kilodaltons.

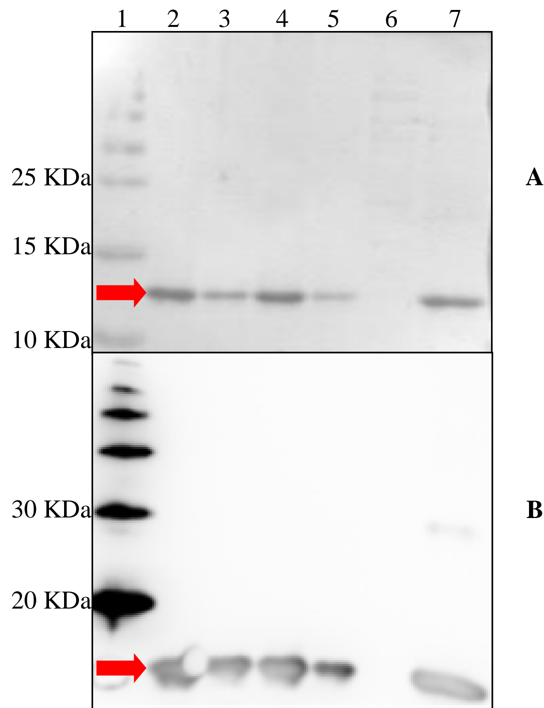

FIGURE 5. Coomassie-stained SDS-PAGE (A) and matching western blot (B) of the rPCV2ORF3 resolubilizations in 50% MeOH. The IMAC elution fractions E8 and E9 were each precipitated and then the precipitates were solubilised in 50% MeOH twice. Lanes 2 and 3 are the first and second solubilizations of fraction E8 respectively; lanes 4 and 5 are the same for E9. These were later pooled to use as antigen for probing sera. The same negative and positive controls as used previously were used in lanes 6 and 7 respectively. Mouse αhis (Abcam) was used as the primary antibody to identify the protein as rPCV2ORF3. The red arrow indicates the position of rPCV2ORF3. Lane 1 is a ladder with molecular weights labelled in kilodaltons. A pre-stained ladder (Bio-Rad) is used in the SDS-PAGE while MagicMark (Invitrogen) is used in the western blot.

The two PCV ORF3 homologues share 61.5% homology (1). There is no literature regarding cross-reactivity of antibodies to the ORF3 homologues. It is possible that any antibodies that react with rPCV2ORF3 could actually have been raised against PCV1ORF3. To control for this possibility and confirm that the antibodies reacting with rPCV2ORF3 do in fact confirm the expression of PCV2ORF3 *in vivo*, several swine sera were screened for antibodies to PCV1ORF3. To this end, PCV1ORF3 was recombinantly expressed and IMAC purified much as the rPCV2ORF3 was; however, induction of expression was attempted in both the stationary and logarithmic growth phases in separate cultures for rPCV1ORF3 whereas rPCV2ORF3 expression was only induced in the logarithmic phase. Induction during the stationary growth phase resulted in greatly increased amounts of overall and target protein compared to induction during the logarithmic growth phase as can be seen in the comparison displayed in Fig. 6. The novel elution fractions of the Coomassie-stained SDS-PAGE in Fig. 6 all contain an intense band between 15 and 25 KDa, at the expected rPCV1ORF3 size which LC-MS/MS confirmed as rPCV1ORF3. The E3 fraction from the stationary phase induction of expression was the source of rPCV1ORF3 used for testing sera for the presence of αPCV1ORF3 antibodies.

Indirect detection of PCV2ORF3 expression *in vivo* relies on using rPCV2ORF3 in serological assays to detect αPCV2ORF3 antibodies in swine serum. The rPCV2ORF3 in PVDF strips had to be tested with swine serum to make sure it could detect αPCV2ORF3 antibodies at a serum dilution that uses up a minimal amount of serum. The rPCV2ORF3 in 50% MeOH was transferred to PVDF which was cut into strips that were used for probing swine sera. The serum from an undiseased adult pig positive for PCV2 by qPCR was chosen for the pilot test because a healthy PCV2-infected immunocompetent pig will express antibodies to a viral antigen like PCV2ORF3 that is expressed *in vivo* if it is immunogenic.

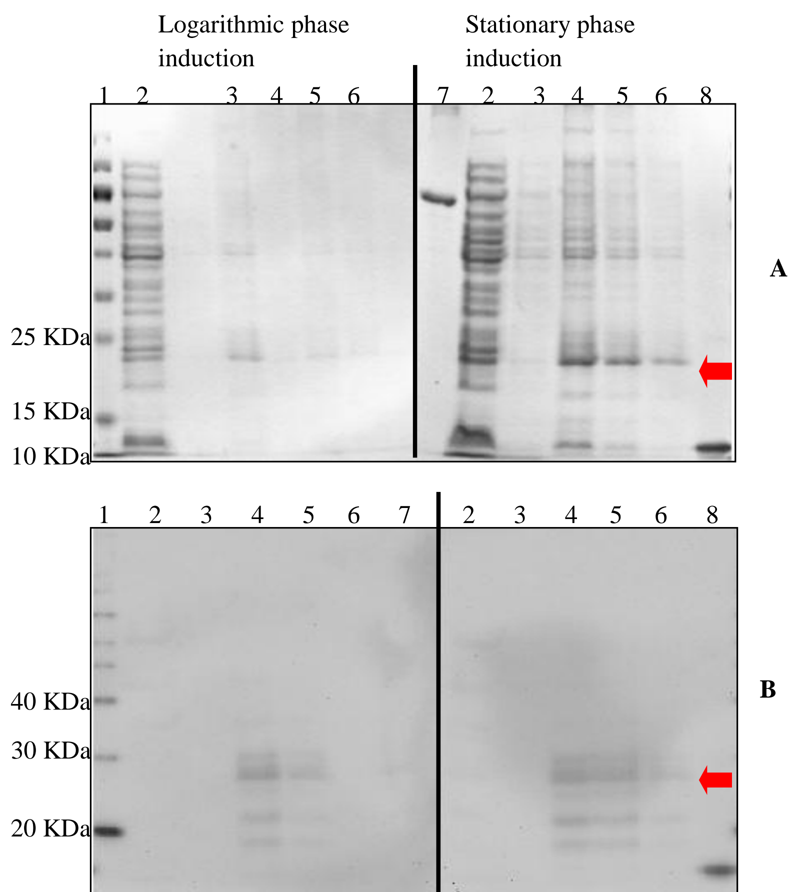

FIGURE 6. Coomassie-stained SDS-PAGE (A) and matching immunoblot (B) of select fractions from rPCV1ORF3 extraction and purification. The flow-through (lane 2), third wash fraction (lane 3), and three elution fractions (lanes 4-6) from induction at the stationary (right side) and logarithmic (left side) phases are displayed above. BSA was loaded into lane 7 and rPCV2ORF3 in 50% MeOH was loaded into lane 8. Induction during stationary phase resulted in more recombinant and overall protein expression than induction during the logarithmic phase. Pre-stained ladder from Bio-Rad was loaded into lane 1 of the SDS-PAGE and MagicMark from Invitrogen was loaded into lane 1 of the immunoblot. Molecular weights are labelled on the left in kilodaltons. Red arrow indicates position of rPCV1ORF3.

PVDF strips containing rPCV2ORF3 were used to probe diluted adult pig serum for αPCV2ORF3 antibodies. Serum taken from a newborn piglet before it could receive maternal antibody was used as a negative control. Incubation with the adult swine serum but not the newborn swine serum produced signal at the same MW as the mouse αhis control in Fig. 7.

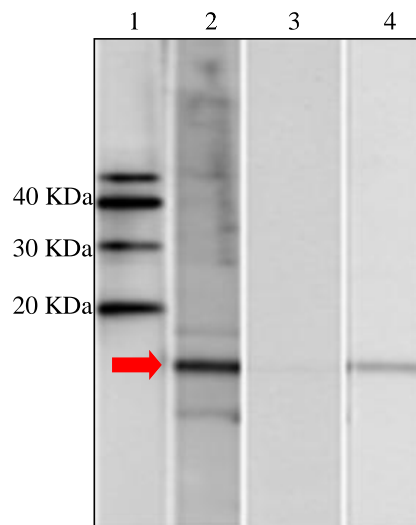

FIGURE 7. Antibodies in adult swine serum react with rPCV2ORF3 in a PVDF strip. The highly reactive adult swine serum is represented in lane 2. The newborn serum (lane 3) was taken before the piglet could receive colostrum and acts as a negative control. The mouse αhis (lane 4) confirms the identity of the antigen as rPCV2ORF3. The ladder is MagicMark (Invitrogen) and its molecular weights are labelled in kilodaltons. The red arrow highlights position of rPCV2ORF3.

3.2 The Vast Majority of Swine Sera React with rPCV2ORF3 but not with rPCV1ORF3

It is possible that antibodies to one PCV ORF3 homologue could cross-react with the other PCV ORF3 homologue. To confirm that the antibodies that reacted with rPCV2ORF3 in the previously tested adult swine serum were not in fact originally raised against PCV1ORF3,

several sera were screened for antibodies to both PCV ORF3 homologues to find at least one unambiguously positive for antibodies to PCV2ORF3. Mouse αhis incubation and development resulted in signal that was observed at approximately the expected MW for rPCV1ORF3 as well as at lower MWs to just under 20 KDa in the western blot on the left in Fig 8. None of the sera

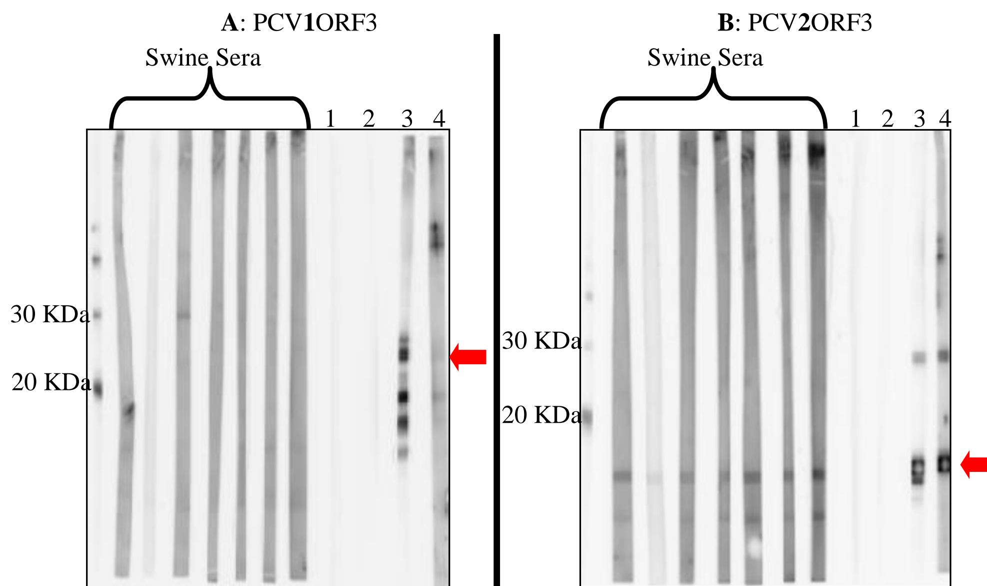

FIGURE 8. PVDF strips loaded with either rPCV1ORF3 (A) or rPCV2ORF3 (B) were used to probe swine sera for antibodies. Mouse αhis (lane 3) was used as a positive control to confirm the presence of the recombinant proteins at the expected MW. Rabbit αPCV2ORF3 serum (lane 4) was also used as a positive control. Negative controls consisted of newborn serum (lane 2) and no serum added (lane 1). Incubating the rPCV2ORF3 strips with the swine sera produced signal lining up with the positive control signals but incubating the rPCV1ORF3 strips with swine sera did not produce any signal lining up with the mouse αhis signal between 20-30 KDa. The position of the ORF3 homologue protein in each image is indicated by a red arrow. Antibodies to PCV1ORF3 were not detected. The molecular weight ladder used in each image is MagicMark (Invitrogen).

tested with rPCV1ORF3 produced signal that lined up with those produced by the mouse αhis. Incubation of rPCV2ORF3 with swine sera and development resulted in signal at the same position as the signal created by incubation with mouse αhis and rabbit αPCV2ORF3 sera. Rabbit αPCV2ORF3 serum did not react with rPCV1ORF3. Antibodies to PCV1ORF3 were not detected.

If PCV2ORF3 is a virulence factor, it must be expressed *in vivo* and the vast majority of PCV2-infected swine will make antibodies to it if it is immunogenic. To evaluate how many animals in the cohorts were positive for antibodies to PCV2ORF3, many sera were probed using PVDF strips loaded with rPCV2ORF3 as described previously. Sera from weaned animals were analyzed because sucklings still receive maternal antibodies by colostrum. The results are summarized in Fig. 9.

Of a total 97 sera from weaned swine about 89.8% (88) tested positive for antibodies to PCV2ORF3. 'Positive' is defined as producing any signal above background at the same molecular weight as rPCV2ORF3. All of the animals from the vaccinated cohort tested positive. The vaccine was subunit vaccine containing PCV2ORF2 protein expressed in insect cells and it did not contain PCV2ORF3. Antibodies to PCV2ORF3 were detected in 87.5% of the diseased unvaccinated cohort and in 86.0% of the apparently undiseased unvaccinated cohort.

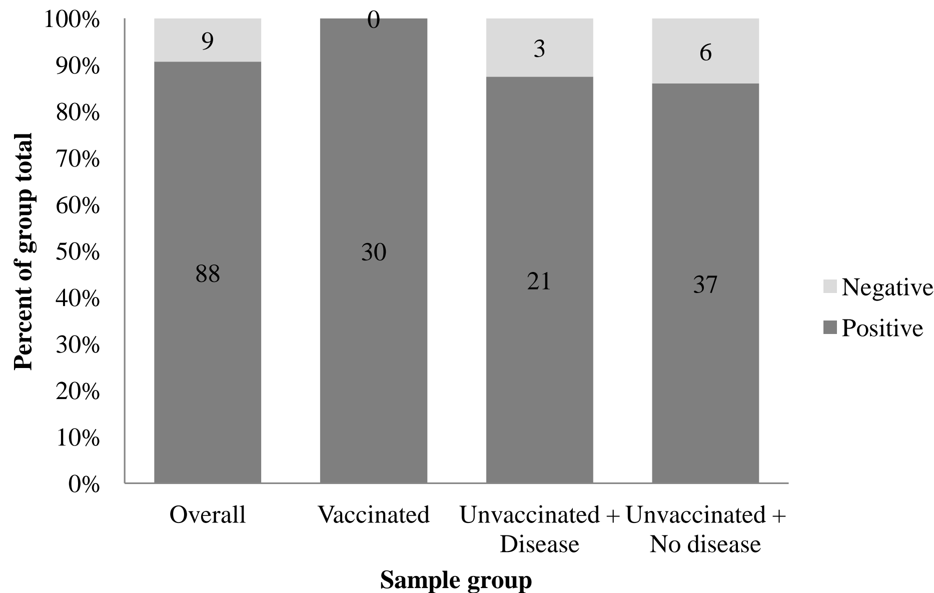

FIGURE 9. The number and percent of sera that are reactive and nonreactive with rPCV2ORF3 are displayed in a bar graph. The categories are the vaccinated cohort (N=30), the unvaccinated diseased cohort (N=24), and the unvaccinated undiseased cohort (N=43), and overall (N=97). This chart displays data obtained from the sera of weaned animals only. Positive is defined as 'any signal at the MW of rPCV2ORF3 above background'.

3.3 No Correlation Between Vaccination Status and αPCV2ORF3 Reactivity Found

No data has been published regarding the effect of PCV2 vaccination on αPCV2ORF3 serum antibodies. It is possible that the improvement in clinical outcomes associated with PCV2 vaccination are partly mediated by αPCV2ORF3 antibodies. To determine whether the protective effects of vaccination correlates with αPCV2ORF3 antibodies, the αPCV2ORF3 reactivities of the vaccinated and unvaccinated animals were compared. There is only one vaccinated cohort but there are two unvaccinated cohorts. This necessitated combining the αPCV2ORF3 reactivity

data from both of the unvaccinated cohorts and then comparing the pooled unvaccinated data to the vaccinated cohort using the Mann-Whitney U test. Only data obtained from sera from weaned animals (>26 days of age) were used for this comparison because animals are vaccinated at or close to weaning. No significant difference in αPCV2ORF3 reactivity was found between the vaccinated and unvaccinated groups.

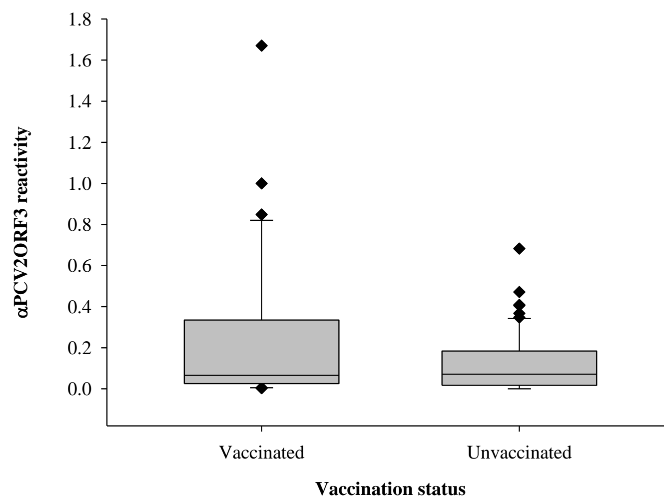

FIGURE 10. Box-plot chart comparing the αPCV2ORF3 reactivity of the vaccinated (N= 30) and unvaccinated animals (N=67). αPCV2ORF3 reactivity value is plotted on the y-axis. This is an S/P value is calculated by normalizing the background-adjusted generated by a sample signal to the background-adjust signal generated by a highly reactive standard. Only data from animals older than 26 days were included. Mann-Whitney U testing did not find a significant difference between the two groups of animals. The lines of each box-plot from the bottom to the top represent the 10th and 25th percentiles, the median, and the 75th and 90th percentiles.

3.4 No Correlation Between Viremia and αPCV2ORF3 Reactivity Found in Weaned Animals

Antibodies to non-structural viral proteins cannot neutralize a virus but they can be important in protection against disease. For example antibodies to the non-structural protein 1 (NS1) protein of dengue virus helps prevent disease in mice (89). On the other hand, antibodies to the Nef protein of HIV do not appear to protect against disease (90). To test whether the αPCV2ORF3 serum IgG antibody levels correlate with controlling viremia and therefore might have biological significance, they were compared to the serum viral load within each cohort of weaned animals and in the overall weaned animal dataset. The αPCV2ORF3 reactivity of each serum is an S/P value. The qPCR results displayed in Fig. 11 show that all the cohorts are overwhelmingly positive for PCV2 viremia where a final qPCR value of '0' $\log_{10}$(genome copies/mL) was considered negative and any value greater than 0 was considered positive. Statistical testing revealed that there was no correlation between αPCV2ORF3 sera reactivity and viral load overall or within any particular cohort.

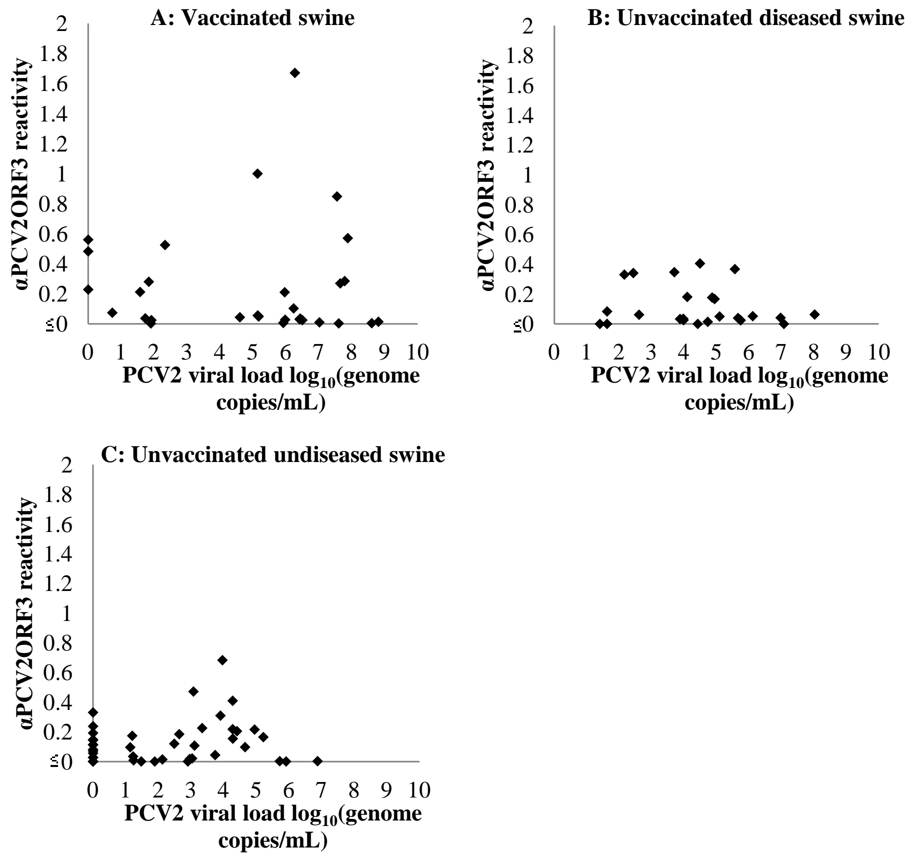

FIGURE 11. The αPCV2ORF3 reactivity of the sera is scatter-plotted against viral load using only data from weaned animals. The vaccinated animals had been vaccinated with a commercial vaccine.

3.5 Older Animals in One Cohort Have a Significantly Higher Serum αPCV2ORF3 Reactivity than Post-weaning Animals

Animals of different ages were sampled and their sera analyzed for αPCV2ORF3 reactivity to examine how αPCV2ORF3 reactivity changes with age. Only the unvaccinated undiseased cohort had discrete age data for every animal and this cohort was tested using the Spearman rank correlation coefficient, which did find a statistically significant correlation coefficient of 0.516 between age of an animal and its serum reactivity to rPCV2ORF3 in this cohort ($p<0.001$). The other two cohorts contained animals with both discrete age data and age data in the form of a broad range such as 40-47 days. It is not possible to run a statistical test for correlation with non-discrete data mixed with discrete data. The animals were grouped by age into three categories that coincide with the suckling, post-weaning, and grower/nursery phases of swine development. These age categories were 0-26 days, 27-84 days, and >84 days old respectively. The suckling category cut off of 26 days is also when the animals of the vaccinated cohort received vaccination. Then the αPCV2ORF3 reactivity of these age groups was compared using Kruskal-Wallis testing within each cohort and for the whole data set and graphically displayed in box-plots in Fig. 12. Statistically significant differences ($p<0.05$) were found between the 27-84 and >84 day old groups in the unvaccinated and undiseased cohort and in the overall data set.

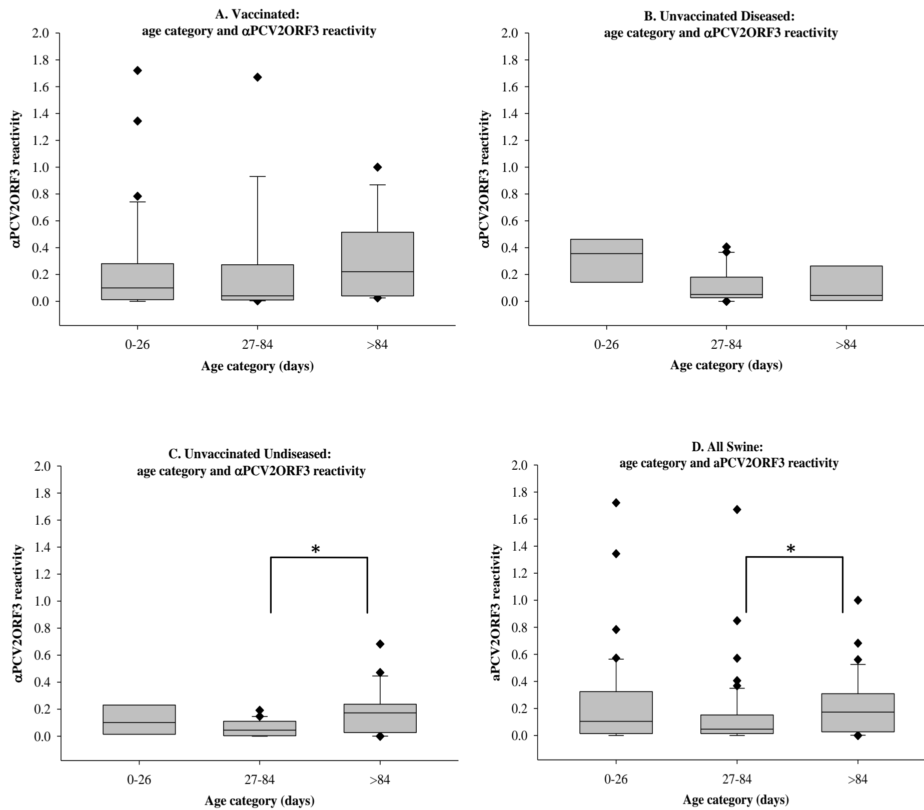

FIGURE 12. The animals were grouped according to age and cohort and the αPCV2ORF3 serum reactivity of each age group is displayed graphically in box-plots. The lines of each box-plot from the bottom to the top represent the 10th and 25th percentiles, the median, and the 75th and 90th percentiles. Age groups within each cohort were compared using Kruskal-Wallis testing and the statistically significant differences ($p<0.05$) are highlighted with an asterisk. The animals >84 days old have higher αPCV2ORF3 serum reactivity than the 27-84 day old animals in the unvaccinated undiseased cohort and the overall data set.

3.6 The αPCV2ORF3 Reactivity of the Diseased and Undiseased Unvaccinated Cohorts are not Significantly Different From Each Other

In some cases, antibodies to non-structural viral proteins can protect against disease (91). It is possible that the αPCV2ORF3 antibodies detected here might have a protective effect. To test for this possibility, the αPCV2ORF3 reactivity of weaned animals from the two unvaccinated cohorts was compared. One of these cohorts has only undiseased animals while the other cohort has a history of PCVAD. The vaccinated cohort was excluded from this comparison

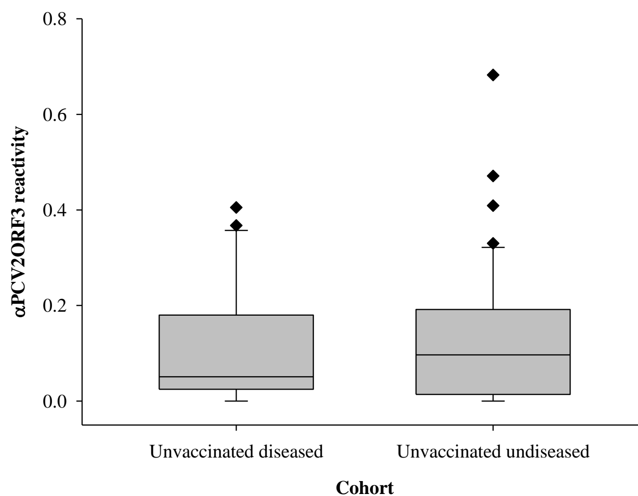

FIGURE 13. Box-plot chart comparing the αPCV2ORF3 reactivity of the unvaccinated disease cohort (N=24) and unvaccinated undiseased cohort (N=43). Only animals older than 26 days were included. The lines of each box-plot from the bottom to the top represent the 10th and 25th percentiles, the median, and the 75th and 90th percentiles. Mann-Whitney U testing did not find a significant difference between the two cohorts.

to eliminate the extra variable of vaccine status. Sucklings were excluded because these animals are rarely affected by PMWS. If the undiseased animals have greater serum reactivity towards rPCV2ORF3, it may indicate that αPCV2ORF3 antibodies are protective against disease. The αPCV2ORF3 reactivity of the two cohorts was compared using the Mann-Whitney U test. No significant difference between the αPCV2ORF3 reactivity of the two cohorts was detected.

3.7 Neutralizing Antibody Titre does not Correlate with Serum αPCV2ORF3 Reactivity

Neutralizing antibodies are highly important to controlling PCV2 viremia and avoiding PCVAD (33, 43). To test if there is an association between the serum reactivity to PCV2ORF3 and serum neutralization of PCV2, some of the sera tested for reactivity to PCV2ORF3 were also titred for virus neutralization activity and the nAb titre and αPCV2ORF3 reactivity were compared. The last dilution of a serum resulting in 50% reduction in infectivity relative to the positive controls was defined as the raw neutralizing titre of the serum. The duplicate raw neutralizing titres of each serum were averaged and subjected to logarithmic transformation base 10 for the final neutralizing titre. Spearman rank order testing did not reveal a correlation between nAb titre and αPCV2ORF3 reactivity. The lack of a relationship between nAb titre and αPCV2ORF3 sera reactivity is illustrated in Fig. 14.

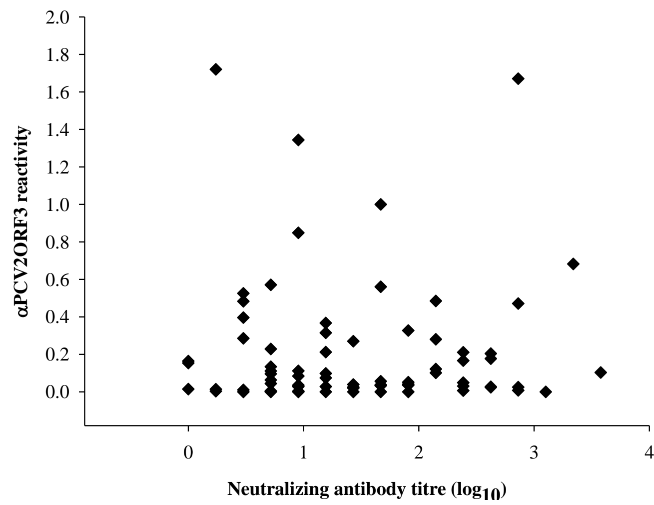

FIGURE 14. Scatter-plot of αPCV2ORF3 reactivity and nAb titre. There was no correlation found between nAb titre and either swine age category or αPCV2ORF3 reactivity.

Chapter 4 Discussion

While there is evidence indicating that PCV2ORF3 is involved in viral pathogenesis (1, 66, 79), the role of PCV2ORF3 is still poorly understood and its expression *in vivo* has not been demonstrated in published literature. Our lab has not previously observed PCV2ORF3 expression in pig tissues despite attempting to find it with IHC. Direct detection of PCV2ORF3 expression is difficult because it is not known where or when this protein is expressed during infection or for how long. Serum antibodies to PCV2ORF3 will however be produced regardless of these factors and they will persist in the serum. For these reasons this thesis aims to indirectly demonstrate PCV2ORF3 expression *in vivo* by screening swine sera for reactivity towards rPCV2ORF3. Antibodies to PCV2ORF3 were detected in many swine sera, strongly indicating that PCV2ORF3 is expressed during PCV2-infection of pigs.

Antibodies to non-structural viral proteins can protect against disease in certain cases (89, 91, 92). If the antibodies to PCV2ORF3 detected here are protective, then there should be correlations between serum reactivity to rPCV2ORF3 and other data such as viral load, nAb titre, or clinical outcomes. These correlations could also yield insights into possible PCV2ORF3 roles in PCV2 infection. No correlation between serum reactivity to PCV2ORF3 and viral load or nAb titre or clinical status could be found in the data presented here.

4.1 Detection and Prevalence of Antibodies to ORF3 Homologues

The ORF3 protein of PCV2 is not essential to viral replication but it has been implicated in promoting apoptosis of infected cells (72, 79). Phagocytosis of the resultant PCV2-bearing apoptotic bodies by macrophages leads to increased TNF-α secretion by those macrophages and enhances dissemination of PCV2 (66). A study that compared WT and ORF3-KO PCV2

infection in mice found that expression of PCV2ORF3 appears to be important to increased viremia, decreased numbers of T lymphocytes, and decreased levels of αPCV2 antibody (1). A similar knockout study in pigs produced similar results (79). A different knockout study in pigs found that the pigs infected with ORF3-KO PCV2 were less viremic than pigs infected with WT PCV2 but could not find a difference in virulence between the WT and ORF3-KO PCV2 (83). These studies seem to indicate that PCV2ORF3 is a virulence factor. If this is true, PCV2ORF3 protein must be expressed *in vivo*. The expression of PCV2ORF3 protein has been detected using IHC on sections of lymph node tissue from PCV2-infected mice (1) but no data confirming PCV2ORF3 expression in swine tissue has been published. PCV2ORF3 protein expression in tissue from PCV2-infected swine has not been detected using IHC in the experience of Czub lab (86). While the data from attempts at direct detection of PCV2ORF3 expression are inconclusive, it still appears to be an important virulence factor for PCV2 infection. Therefore, in this thesis I hypothesize that PCV2ORF3 is expressed and that this expression can be demonstrated by detection of αPCV2ORF3 antibodies in swine sera. A similar serology-based approach was used to demonstrate the expression of the ORF3 protein of DuCV (73).

The data obtained from testing sera for reactivity to rPCV2ORF3 indicate that the vast majority of animals in all three cohorts are reactive with rPCV2ORF3 and almost 90% of all the tested animals have at least some reactivity to rPCV2ORF3 (Fig. 9). The presence of antibodies to PCV2ORF3 protein strongly indicates that the pigs have been exposed to PCV2ORF3 protein. This implies that the pigs were exposed by vaccination, or during PCV2-infection, or by some other means without PCV2 infection. The vaccine used to vaccinate some of the tested animals is a subunit vaccine containing capsid protein expressed in insect cells and it therefore does not contain PCV2ORF3 protein. Also, only one cohort of swine was vaccinated. This eliminates the

possibility that the swine were exposed to PCV2ORF3 by vaccination. The PCV2ORF3 protein is a non-structural protein, making it unlikely that the swine were exposed to it without infection by PCV2. Therefore, it is highly likely that the presence of these antibodies in swine serum indicates that PCV2ORF3 was expressed in the vast majority of pigs that were sampled for analysis. Altogether this strongly implies that PCV2ORF3 is expressed *in vivo* during PCV2-infection of pigs. The presence of antibodies to PCV2ORF3 in apparently undiseased swine implies that PCV2ORF3 was also expressed in these animals during PCV2-infection. Therefore, PCV2ORF3 expression does not necessarily lead to disease.

There is no published data confirming that PCV1ORF3 is expressed. PCV1 is far less prevalent than PCV2 (14), but it is possible that many or even all pigs are infected by PCV1 at least once in their lives. These two homologues share 61.5% homology in the mutually translated region (1), making it possible that antibodies to one homologue could cross-react with the other. For these reasons it was prudent to confirm that the antibodies detected using rPCV2ORF3 were originally raised against PCV2ORF3 and not its PCV1 homologue. This required screening sera for antibodies to both ORF3 homologues to find sera that reacted only with rPCV2ORF3 and not rPCV1ORF3. Only sera positive for one ORF3 homologue would unambiguously imply expression of that ORF3 homologue. Seven sera positive for αPCV2ORF3 antibodies were tested for reactivity to rPCV1ORF3. Serum reactivity to PCV1ORF3 was not detected in any of these sera. Although this does not necessarily mean that αPCV1ORF3 antibodies were absent from the sera, it does mean that the αPCV2ORF3 antibodies detected in these sera were indeed originally raised against PCV2ORF3 protein. This lends further support to the hypothesis that PCV2ORF3 is widely expressed in PCV2-infected swine.

In light of this evidence in favor of the expression of PCV2ORF3 in pigs, it is not clear why our group and possibly others have not directly detected PCV2ORF3 in pig tissue using IHC. Other groups may have failed because of incompatibility between the antibody they used and the fixation process or a lack of epitope retrieval. Epitopes can be hidden by the fixation process and there are certain means of retrieving these epitopes without which the antibody used may not react with them, and the protein of interest will not be detected by IHC. The antigen could also have been altered more directly resulting in loss of conformational epitopes. The PCV2 virion and its epitopes are perhaps resistant to alteration during the fixation process due to the extreme stability of the PCV2 virion (85), but PCV2ORF3 probably lacks this unusual stability. Attempts by members of our lab to detect PCV2ORF3 expression by IHC included testing the primary antibody beforehand to make sure it was compatible with the fixation process used and included an optimized epitope retrieval step by heat-induction. Also, the αPCV2ORF3 antibody generated was a rabbit polyclonal that reacted with linear epitopes (86). The lack of conclusive results from our lab's previous IHC efforts may be due to PCV2ORF3 expression levels being beneath the detection threshold. Alternatively, PCV2ORF3 was expressed but only briefly and for a discrete time period before sampling. It is also possible that PCV2ORF3 was expressed in other tissues than those that were sampled. It should be noted that some other studies that examined the distribution of PCV2 antigen in PMWS and PCV2-infected pigs with IHC used rabbit polyclonal sera raised against supernatant of PCV2-infected cell culture or swine serum that reacted with PCV2 (8, 11). It is possible that PCV2ORF3 could have been expressed in either the infected swine or cell culture and therefore the rabbit and/or swine sera might have contained αPCV2ORF3 antibodies. These could have reacted with PCV2ORF3

protein in the swine tissues that were tested by IHC. Therefore it is possible that PCV2ORF3 expression has already been detected in published literature without anyone being aware of it.

4.2 Possible Roles for PCV2ORF3 as an Accessory Protein

Strains of PCV2 engineered to block expression of PCV2ORF3 can still replicate (66, 72, 79, 83) and therefore PCV2ORF3 does not appear to be essential for PCV2 replication. Based on this I suggest that PCV2ORF3 is an accessory protein. Also known as auxiliary proteins, these are viral proteins that are dispensable to viral replication but usually enhance virulence a great deal. They carry out multiple functions by interfering with and manipulating normal cellular processes. Accessory proteins often induce apoptosis, target intrinsic antiviral pathways, or otherwise subtly modify target cells and even virions themselves. Accessory proteins are not enzymes. A brief review of other accessory proteins may suggest potential roles for PCV2ORF3 in PCV2 infection.

A great example of an accessory protein is the Negative Factor (Nef) protein of HIV. Nef is thought to be important to HIV pathogenesis and maintenance of high viremia. Nef deletion from the closely related simian immunodeficiency virus (SIV) results in greatly reduced viral loads *in vivo* and severely delays AIDS onset in macaques (93). Nef rearranges signalling and trafficking pathways in the cell, acting as a kind of elaborate molecular ‘short circuit’. Its functions are highly varied. For example, it down-regulates MHC-1 surface expression by linking it to endocytic machinery, resulting in the endocytosis and destruction of MHC-1 and consequently reducing the chance of the infected cell being recognized and destroyed by CD8+ T_{cyto} cells (94). Other functions of Nef include modifying HIV-1 virions as they are produced (95) and inhibiting isotype switching (96). This is by no means a complete list of known Nef

functions but demonstrates that one accessory protein can act on many targets by forcing an association between cellular proteins.

A very different accessory protein is the apoptin protein of CAV. Apoptin can induce G_2/M arrest and p53-independent apoptosis in cancer cells. Its activity is regulated by localization. Relocation from the cytoplasm to the nucleus and subsequent apoptosis appear to be dependent on activation of the DNA Damage Response (DDR) pathway. The DDR may be of great importance to other *Circoviridae* members as well because they all have ssDNA genomes that replicate by RCR, both of which are likely recognized by the host cell as damaged DNA. The DDR can suppress the replication of many viruses and is manipulated by others to enhance replication. In the case of CAV, apoptin uses the DDR to target the anaphase promoting complex/checkpoint (APC/C) and mediator of checkpoint 1 (MDC-1) protein, leading to their degradation and in turn apoptosis (70), which probably contributes to viral dissemination (71). Apoptin appears to be an example of an accessory protein that operates by triggering apoptosis under specific conditions rather than subtly modifying multiple targets as is the case with Nef.

While they are not essential for viral replication, accessory proteins often boost viral replication and virulence. They can have many functions which can range from down-regulating cell surface proteins important to immune function to inducing apoptosis. The function of PCV2ORF3 is poorly understood. It is probably cytotoxic and appears to be important to increased viremia (79, 83), viral persistence (79), and PCV2 dissemination and pathogenicity (66). It is not essential to PCV2 replication (66, 72, 79, 83) but the evidence in this thesis suggests it is expressed and it therefore fits the definition of an accessory protein.

4.3 Vaccination Status and αPCV2ORF3 Sera Reactivity

The αPCV2ORF3 serum reactivity of the unvaccinated and vaccinated animals were compared to see if vaccination had any effect on production of antibodies to PCV2ORF3 or if those antibodies could mediate the protection against disease conferred by PCV2 vaccination (46). The animals analyzed in this thesis come from three cohorts, one of which is composed of animals vaccinated upon weaning at 26 days of age. The weaned animals from the two unvaccinated cohorts were pooled together into one unvaccinated set of data and compared to the vaccinated weaned animals in terms of αPCV2ORF3 serum reactivity using the Mann-Whitney U test.

No statistical difference between the vaccinated and unvaccinated animals was detected. This indicates that the beneficial effects of PCV2 vaccination are not mediated by antibodies to PCV2ORF3 and that PCV2 vaccination does not alter the production of αPCV2ORF3 antibodies.

4.4 Serum αPCV2ORF3 Reactivity does not Correlate to Viral Load or nAb Titre or Cohort Health Status

The role and significance of PCV2ORF3 in PCV2 infection is not well understood. Previous studies compared infection of pigs with WT PCV2 to infection with ORF3-KO PCV2 and found that the pigs infected with ORF3-KO PCV2 had lower viremia (79, 83), implying PCV2ORF3 expression is important to maintaining a high viral load possibly by inducing apoptosis and thereby leading to dissemination of the virus (66). Another study found that DNA vaccination with pORF3 alongside pORF2 resulted in immune disruption (84), suggesting a role for PCV2ORF3 in immunomodulation.

Some antibodies that are protective against disease are not neutralizing antibodies (97). Antibodies to non-structural viral proteins can be very important to protection against disease, as is the case with antibodies to NS1 protein of dengue fever virus and various other flaviviruses (89, 92, 98). NS1 is a transmembrane protein that is conserved across flaviviruses and it localizes at the surface of cell membranes, where it can be bound by αNS1 antibodies. It appears that the protective αNS1 antibodies are IgG_{2a} and IgG_{2b} antibodies capable of inducing the complement cascade upon binding to NS1, leading to cytolysis of the infected cell and a tenfold to hundredfold reduction in release of virus (99, 100). Other antibodies can have diagnostic and prognostic value, with their appearance heralding worse clinical outcomes. The appearance of IgA antibodies to the Epstein-Barr virus (EBV) capsid and antibodies that neutralize the EBV DNase appear to prognosticate nasopharyngeal carcinoma or recurrence of nasopharyngeal carcinoma after treatment (101-103). In other cases antibodies to non-structural proteins do not appear to have any clinical relevance or prognostic value. Antibodies to the Nef protein of HIV do not correlate with protection against AIDS progression (90).

In order to look for biological significance of the expression of PCV2ORF3 protein or antibodies to it, the αPCV2ORF3 reactivity of swine sera was compared to their viral load and to their nAb titre. If higher serum αPCV2ORF3 reactivity coincides with lower viral load, the antibodies to PCV2ORF3 detected here might be relevant to reducing viremia *in vivo* much like antibodies to the NS1 protein in Japanese Encephalitis Virus infection are (99). The serum αPCV2ORF3 reactivity was also compared to the nAb titre because nAb titre is correlated with lower PCV2 viremia (35) and superior clinical outcomes (43). The data was also analyzed to see if there was any correlation between the serum αPCV2ORF3 reactivity of the animals in a cohort and the presence of PCVAD in that cohort. The two unvaccinated swine cohorts were compared

to see if the cohort with a history of PCVAD had a statistically different overall αPCV2ORF3 reactivity compared to the other unvaccinated cohort which did not have a history of disease. The cohort of vaccinated animals was not included in this analysis because vaccination prevents disease (104, 105) and earlier results indicated that antibodies to PCV2ORF3 do not mediate this protection. Sucklings were also excluded because these animals are rarely affected by PMWS. If the antibodies to PCV2ORF3 detected in this thesis are protective against disease, perhaps the αPCV2ORF3 reactivity of sera from the unvaccinated cohort with a history of PCVAD will be lower than sera from the other unvaccinated cohort.

Analysis of serological data using the Spearman rank order test found no correlation between serum αPCV2ORF3 reactivity and viral load in the overall data or within any particular cohort. There was also no correlation between serum αPCV2ORF3 reactivity and nAb titre. Further, the two unvaccinated cohorts were not statistically different from each other in terms of serum αPCV2ORF3 reactivity; therefore no role in preventing disease could be given to αPCV2ORF3 antibodies. An apparent difference in the biological importance of antibodies to NS1 and PCV2ORF3 may be a consequence of different localization. The protection against disease conferred by antibodies to NS1 is dependent on NS1 localization to the cell membrane, where it can be bound by antibodies that initiate the complement cascade (99, 100). PCV2ORF3 does not appear to be a transmembrane protein like NS1 is and it localizes to the cytoplasm and nucleus (75) and its roles are carried out in an intracellular environment, like Nef of HIV. Therefore it is not surprising that antibodies to PCV2ORF3 do not appear to protect against disease like antibodies to NS1 do.

Previous research has found that total αPCV2 antibody levels do not necessarily correlate with serum nAb titre and animals can develop a very high overall αPCV2 titre without any nAb

titre (33, 43). It is possible that some pigs can mount a humoral response to PCV2 but cannot evolve nAbs either due to PCV2-mediated immunosuppression or perhaps genetics. Analysis of the data in this thesis finds a similar lack of relationship between nAb titre and αPCV2ORF3 reactivity. Some sera have a very strong nAb titre but little or no αPCV2ORF3 reactivity and some have moderately strong αPCV2ORF3 reactivity and little neutralization activity. Whatever suppresses nAb titre in some PCV2-infected animals does not appear to inhibit the production of antibodies to PCV2ORF3. The lack of correlation between αPCV2ORF3 antibodies and clinical outcomes, decreased viral load, or higher nAb titre seem to suggest that these antibodies to PCV2ORF3 are not biologically important. These negative conclusions cannot be drawn from the data because this is a retrospective study of cohorts in the field. Certain data, such as the exact time of infection of each animal, are unknowable.

The inability to find correlations in the data may be due to the fact that all of the sera were taken from animals in the field that were infected by PCV2 at different and unknown time points. There was no coordination between sampling time and infection time. This has several ramifications. Animals with little or no serum reactivity to PCV2ORF3 may have been sampled at a point in PCV2 infection where PCV2ORF3 had not yet been expressed or it was expressed but IgG antibodies to PCV2ORF3 had not yet been produced. Alternatively, these animals may have been sampled long after PCV2ORF3 had been expressed and the αPCV2ORF3 antibodies had decayed by then. It is also possible that antibodies to PCV2ORF3 are biologically important at a particular point during PCV2 infection, in which case αPCV2ORF3 serum reactivity at that time point might correlate inversely with viral load then or later on in infection. If the development of a higher PCV2 viral load at this time point is critical to progressing to PCVAD, and αPCV2ORF3 antibodies reduce viral load or dissemination at this time point, then the

clinical outcome of an infected animal might be associated with that animal's αPCV2ORF3 serum reactivity at this critical time point. Animals that go on to develop PCVAD might have had lower αPCV2ORF3 serum reactivity at that time point than those that do not. This is highly speculative, but if there is such a correlation the experimental design used in this thesis would not detect it.

These data do not support any particular role for PCV2ORF3. While correlations between αPCV2ORF3 serum reactivity and other serological data or clinical data can not be found, this could be due to a lack of data regarding the time point of infection of each animal.

4.5 There is Variance in Serum Reactivity to PCV2ORF3 Across Age-groups

Suckling pigs get IgG antibodies from sows through colostrum intake. After the pigs are weaned these maternal antibodies decay and the pigs must produce their own antibodies to replace the maternal antibodies. A study of αPCV2 antibody levels in non-PMWS animals of various ages in a farrow-to-finish farm found that suckling sera had high αPCV2 antibody levels, probably due to intake of maternal antibodies, but the αPCV2 antibody levels were much lower in animals that were 40 and 72 days of age, likely due to breakdown of maternal antibodies. Animals 107 days old and older had αPCV2 antibody levels nearly as high or as high as the sucklings, because they were expressing their own endogenous αPCV2 antibodies which coincided with a decrease in viremia, although infection was never eliminated (15). If PCV2ORF3 is expressed in infected adult pigs as part of subclinical persistent PCV2 infection then these older cohorts should be positive for antibodies to PCV2ORF3. There is no comparison of αPCV2ORF3 antibody levels in swine of different ages in the published literature.

Only the unvaccinated undiseased cohort was tested for a correlation between age and serum reactivity to rPCV2ORF3 because it was the only cohort with discrete age data for every member. There is a positive correlation between age and serum reactivity to rPCV2ORF3 in the cohort of unvaccinated and apparently undiseased animals according to the Spearman correlation coefficient. The other cohorts included swine with age data in the form of a range such as 40-47 days and this is not compatible with statistical testing for a correlation. The members of the other two cohorts were grouped together in three age categories that reflect different stages of swine development, those being suckling (0-26 days old), post-weaning (27-84 days old), and grower/adult (>84 days old). These age categories were compared within each cohort for differences in serum reactivity to rPCV2ORF3 using Kruskal-Wallis testing. The overall data set was treated in a similar way to see if there was an overall trend. The unvaccinated undiseased cohort was also grouped into these age categories and analyzed by Kruskal-Wallis testing to see what age group contributed most to the correlation between age and serum reactivity to rPCV2ORF3 in this cohort. There was no statistical difference in terms of serum reactivity to rPCV2ORF3 between the three age categories of the vaccinated and unvaccinated diseased cohorts, although the negative results of testing the unvaccinated diseased cohort should be interpreted cautiously because the test power was low. A low test power makes it difficult to detect differences and could result in false negatives. On the other hand the age category of >84 days old in both the unvaccinated apparently undiseased cohort and the overall data set were statistically different and more serologically reactive to rPCV2ORF3 than the post-weaning animals.

The positive correlation between αPCV2ORF3 serum reactivity and age in the unvaccinated undiseased cohort was apparently due to the contribution of older animals (>84

days old) with higher αPCV2ORF3 antibody levels. Adult swine in field conditions also have higher levels of αPCV2 antibodies than post-weaning animals (15) and this reflects the evolution of IgG antibody to endogenously expressed PCV2 proteins during persistent PCV2 infection. Pigs that are infected with PCV2 but do not develop PMWS tend to evolve stronger αPCV2 total antibody and neutralizing antibody titres with time (33). As such, stronger αPCV2ORF3 reactivity in the older animals of this cohort may reflect their having overcome PCV2-induced immunosuppression. Sucklings in the study of field animals (15) also had much higher levels of αPCV2 antibodies than post-weaning animals and were equal to the adults, likely reflecting the maternal αPCV2 IgG antibodies through intake of colostrum. This was not the case in the αPCV2ORF3 antibody data presented here. Suckling sera reactivity to rPCV2ORF3 was not statistically different from either the post-weaning or grower/adult swine in any cohort or the overall data. The reason for this difference is not clear but it is possible that maternal αPCV2ORF3 antibodies were present but react with conformational epitopes in PCV2ORF3. Either way, this indicates that the control of PCV2 replication in sucklings associated with maternally derived antibodies (106) is not dependent on antibodies that react with linear epitopes in PCV2ORF3.

While older animals in the unvaccinated apparently undiseased cohort seem to have evolved stronger αPCV2ORF3 reactivity than the younger animals, this does not appear to the case in the other cohorts. In the case of the unvaccinated diseased animals, it is possible that the low test power is producing a false negative but it is also possible that this is a manifestation of PCVAD-related immunosuppression. Immunosuppression in the form of B and T lymphocyte depletion is a hallmark of PMWS (8, 60, 107) and lower IgG αPCV2 antibody levels have been observed in PMWS cases compared to subclinical PCV2 infection (33, 43). This

immunosuppression could delay normal antibody evolution events such isotype switching and affinity maturation by somatic hypermutation. This would explain the lack of elevated αPCV2ORF3 IgG antibody levels in the older animals of the unvaccinated diseased cohort, but not in the vaccinated cohort. This cohort was not affected by PCVAD. It is possible that vaccination abbreviates or eliminates the immunosuppression caused by subclinical PCV2 infection and that the post-weaning animals (27-84 days old) are as able to produce IgG αPCV2ORF3 antibodies as older animals, but the lack of statistically significant difference between vaccinated and unvaccinated animals' αPCV2ORF3 serum reactivity does not support this explanation.

The evolution of antibodies to PCV2ORF3 in unvaccinated undiseased animals of different ages seems to roughly mirror the evolution of αPCV2 antibodies in undiseased field animals (15) with older animals expressing more antibody to PCV2ORF3 than post-weaning animals, but this pattern was not seen in the other two cohorts. This may be a consequence of immunosuppression in the cohort with a history of disease, but it is not easy to explain for the vaccinated cohort. The presence of αPCV2ORF3 antibodies in all of the age categories indicates that expression of PCV2ORF3 in persistently infected animals does not permanently stop at any time point.

4.6 Limitations of the αPCV2ORF3 Serum Reactivity Assay

To quantify the αPCV2ORF3 reactivity of the serum analyzed here, rPCV2ORF3 was expressed in bacteria, purified in denaturing conditions, and then transferred to PVDF membrane. The PVDF membrane was cut into strips and then incubated with the serum. After development, the signal at the position of rPCV2ORF3 on each PVDF strip was quantified,

adjusted for background, and standardized to the signal produced by a highly reactive serum run with each test. This assay allows for quantification of serum αPCV2ORF3 reactivity but it has several limitations and caveats that must be discussed.

The αPCV2ORF3 serum reactivity is not reflective of PCV2ORF3 expression levels because the antibodies persist after the infection is cleared and antibody levels are dependent on the immune state of the host. The αPCV2ORF3 reactivity of a serum also does not solely depend on the amount of antibody to PCV2ORF3 present in the serum. It may be the case that a serum with weak αPCV2ORF3 reactivity has as much αPCV2ORF3 antibody as a serum with strong αPCV2ORF3 reactivity but the antibodies of the two sera have very different binding affinities to the rPCV2ORF3. Also, only some sera were confirmed to be non-reactive to rPCV1ORF3. This assay may be detecting cross-reactivity from αPCV1ORF3 antibodies in some of the other sera, which would inflate their apparent αPCV2ORF3 reactivity. On the technical side, the rPCV2ORF3 is completely linearized by gel electrophoresis and the secondary antibody only binds to IgG-class swine antibodies. Therefore this PVDF-based assay only detects IgG-class antibodies that react with linear epitopes in rPCV2ORF3. Consequently, any antibodies that bind conformational epitopes or are not IgG antibodies would not have contributed to measured αPCV2ORF3 serum reactivity. Given that IgM is an early-response isotype it is possible that some of the swine sera with no or low reactivity actually have IgM αPCV2ORF3 antibodies but these animals had not yet undergone isotype switching.

4.7 Summary

These data strongly support the hypothesis that PCV2ORF3 is expressed in pigs infected with PCV2. PCV2ORF3 expression does not necessarily lead to disease. These data do not

support or contraindicate any particular role for PCV2ORF3 protein in PCV2 infection. The ability to draw correlations from these data is made difficult by the fact that the animals were all infected at different and unknown time points. Production of antibodies to PCV2ORF3 does not appear to be influenced by PCV2 vaccination or important to its beneficial effects.

4.8 Future Directions

This paper shows that PCV2ORF3 protein is likely expressed *in vivo*. The attempts to find possible roles for PCV2ORF3 protein and αPCV2ORF3 antibodies in PCV2 infection by correlating αPCV2ORF3 serum reactivity to serological and clinical data was stymied by the fact that the animals were infected at different and unknown time points. These experiments can now be repeated under more controlled conditions and samples can be taken over a time course.

It is not currently known whether or not the antibodies to PCV2ORF3 are biologically relevant or not. They may be important to viral clearance, but the attempt to find a correlation between viral load and serum reactivity to rPCV2ORF3 in this thesis was made extremely difficult by the lack of control over when the animals were infected. This hypothesis could be re-tested by infecting pigs with PCV2 and measuring their serum viral load and serum reactivity to rPCV2ORF3 from the time of infection and onward. If serum reactivity to rPCV2ORF3 correlates inversely to viral load, it would indicate that antibodies to PCV2ORF3 are biologically important. This experimental design also allows for finding correlations at specific time points. Given that PCV2ORF3 is likely involved in PCV2 dissemination early in in PCV2 infection (66), it is possible that antibodies to it at an early time point will correlate to viral load later on. Any study of antibodies to PCV2ORF3 should consider producing rPCV2ORF3 in non-denaturing conditions and using it in an assay such as ELISA that can detect antibodies that bind

to conformational epitopes. Further, the use of secondary antibodies that bind to IgA and IgM isotypes would make it possible to analyze the whole antibody response to PCV2ORF3.

Previous research strongly indicates that subclinical PCV2 infection suppresses nAb titres (32, 43) and that DNA vaccination with expression constructs bearing PCV2ORF3 suppresses development of nAbs in mice (84). Is PCV2ORF3 protein responsible for suppression of nAb production? Is PCV2ORF3-mediated suppression of nAbs specific to αPCV2 nAbs or nAbs in general? These questions could be answered by an experiment that compares the evolution of neutralizing antibodies in pigs infected with WT PCV2 to pigs infected with an ORF3-KO PCV2 strain that does not express PCV2ORF3. To see if PCV2ORF3 expression suppresses nAb titres to PCV2 and other pathogens, the animals would be given a vaccine for classical swine fever virus (CSFV) after being inoculated with PCV2. Sera samples would be taken on a time course starting from the time of inoculation and assayed for the ability to neutralize both PCV2 and CSFV. Only non-PMWS animals could be used to screen out PMWS-related immunosuppression. If PCV2ORF3 protein reduces the titres of nAbs in general, then the sera from animals infected with WT PCV2 should be less able to neutralize both PCV2 and CSFV than sera from animals infected with ORF3-KO PCV2. If the effect is specific only to αPCV2 nAbs then the αCSFV nAb titres will not be different. The only problem with this experiment is the fact that viremia in animals infected by ORF3-KO PCV2 would likely be lower than in animals infected by WT PCV2 based on previous research (79, 83). For that reason, even if a difference in nAb titre were found it would not be clear if it was a direct consequence of knocking out PCV2ORF3 or of the lower viremia. A solution for this is not readily apparent.

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Appendix

1) IMAC Profinity Protocol (Bio-Rad)

A) Preparation of cleared lysate under denaturing conditions (summarized from p. 19)

Changes: the protocol calls for discarding the first lysate after centrifugation, but this lyate contained almost all of the recombinant protein. Instead, the lysate obtained during the centrifugation after sonication was subject to IMAC.

Materials

Reagents

• Denaturing lysis buffer, pH 8.0 (urea-based)

– 50 mM sodium phosphate (NaH_2PO_4)

– 300 mM NaCl

– 5 mM imidazole

–8 M urea

Equipment

• Filter apparatus (0.45 μm)

• Sonicator

• Centrifuge

Method

1. Harvest *E. coli* from an appropriate volume of bacterial culture by centrifugation at 4,000 x *g* for 20 min at 4°C.

2. Discard supernatant and freeze pellet at -20°C.

3. Determine weight of pellet.

4. Resuspend pellet in 1:10 ratio (w/v) in lysis buffer. Thoroughly resuspend the pellet by vortexing.

5. Sonicate the cell suspension/lysate 4 times at 1 min intervals each. Sonicate on ice at all times. Decrease interval time if the sample becomes warm. Keep samples cold at all times. Check for clarity and increase sonication if needed.

6. Centrifuge the homogenized sample at 12,000 g for 20 min at 4°C.

7. Retain lysate and filter through a 0.45 µM filter. Store at 4°C until ready to proceed with IMAC purification.

B) IMAC purification of histidine-tagged proteins (Summarized from p. 37)

Changes: no significant changes.

Reagents

• Binding/wash buffer

– 50 mM sodium phosphate (NaH_2PO_4)

– 300 mM NaCl

– 5 mM imidazole

–8 M urea

Adjust to pH 8.0.

• Elution buffer

– 50 mM sodium phosphate (NaH_2PO_4)

– 300 mM NaCl

– 500 mM imidazole

–8 M urea

Adjust to pH 8.0.

Biological Sample

• Clarified lysate (as prepared above)

Method

1. Prepare the resin. Ensure that all ethanol has been thoroughly washed away before proceeding to step 2.

Resin Preparation: Profinity IMAC and Profinity IMAC Ni-charged resins come supplied in a 20% ethanol solution for resin storage. Before purification, the resin storage solution provided must first be replaced with distilled water.

    1. Transfer an appropriate amount of resin slurry to a 5 mL polypropylene tube.
    2. Apply vacuum to the column for rapid removal of the storage solution. Exert caution to not allow the resin to dry out.
    3. Wash the column with 3 column volumes of distilled water.
    4. Add enough distilled water to make a 50% slurry.
    5. The resin is now ready to be packed.

2. Add an appropriate amount of the prepared resin slurry (see Resin Preparation above) to an appropriate amount of clarified lysate.

3. Swirl mixture gently in an appropriate container. Incubate the resin-lysate mixture at 4°C for up to 30 min.

4. Load the resin-lysate mixture into an appropriate-sized column. Cap the bottom outlet of the column.

5. Collect column flow-through.

6. Wash column with at least 5 column volumes of binding/washing buffer. Collect wash fractions. Pool with fractions collected in step 5.

7. Elute the protein with at least 5 column volumes of elution buffer.

8. Repeat elution step if necessary.

2) Bio-Rad Bradford Protein Assay (Bio-Rad instruction manual)

2.4 Microtiter Plate Protocols

Changes: no major changes.

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 µl of each standard and sample solution into separate microtiter plate wells.
4. Add 200 µl 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 hour.
6. Measure absorbance at 595 nm on NanoDrop 2000.

3) Qiagen DNA Mini Kit (DNA purification from blood or body fluids spin protocol summarized from p. 26)

Changes: 1) to conserve sera, only used 100 µL of serum for extraction instead of 200 µL, to which 100 µL of sterile 1X PBS was added. 2) eluted with only 100 µL of elution buffer instead of 200 µL.

Procedure

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.

2. Add 100 µL sample to the microcentrifuge tube and 100 100 µL of sterile 1X PBS.

3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s. To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

4. Incubate at 56°C for 10 min. DNA yield reaches a maximum after lysis for 10 min at 56°C. Longer incubation times have no effect on yield or quality of the purified DNA.

5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. Close each spin column to avoid aerosol formation during centrifugation.

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate..

9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.

10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 100 µl Buffer AE. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

LC-MS/MS Results

MASCOT Search Results

User : Kelvin
E-mail :
Search title : S006508
MS data file : S006508.mgf
Database : NCBInr 20120829 (20,093,899 sequences; 6,882,348,701 residues)
Taxonomy : Viruses (1,007,273 sequences)
Timestamp : 6 Dec 2012 at 22:02:09 GMT

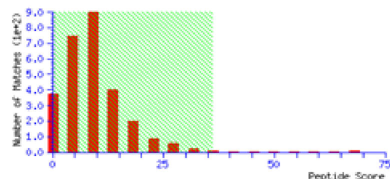
Re-search ◉ All ○ Non-significant ○ Unassigned [help] Export As XML

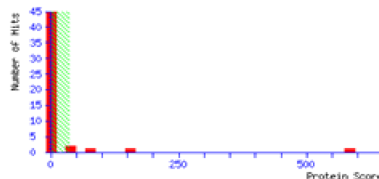
Not what you expected? Try the select summary.

Search parameters

Type of search	: MS/MS Ion Search
Enzyme	: Trypsin
Variable modifications	: Carbamidomethyl (C), Oxidation (M)
Mass values	: Monoisotopic
Protein mass	: Unrestricted
Peptide mass tolerance	: ± 10 ppm
Fragment mass tolerance	: ± 0.6 Da
Max missed cleavages	: 1
Instrument type	: Default
Number of queries	: 3,984

Score distribution





Peptide score distribution. Ions score is −10log(P), where P is the probability that the observed match is a random event. Individual ions scores **> 36** 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

Filter | Significance threshold p< 0.05 | Max. number of families AUTO | [help]
Ions score or expect cut-off 36 | Dendrograms cut at 0
Preferred taxonomy All entries

Proteins (5) | Report Builder | Unassigned (3957)

Protein families 1–5 (out of 5)

10 per page 1 Expand all Collapse all

Accession contains Find

1	**gi|9507572**	585	chloramphenicol acetyltransferase [Plasmid R100]
2	**gi|168747377**	138	NAD(P)H:quinone oxidoreductase, type IV [Escherichia coli O157:H7 str. EC4113]
3	**gi|3668368**	90	P12.1 [Porcine circovirus 2]
4	**gi|390635653**	41	polymerase, partial [Paramyxovirus bat/GH47/2008]
5	**gi|212286052**	40	E2 regulatory protein [Caretta caretta papillomavirus 1]

FIGURE 15. LC-MS/MS analysis of the IMAC-purified rPCV1ORF3 using only sequences from the NCBI database, which do not annotate the PCV1ORF3 sequence. The results show a match to various bacterial proteins as well as PCV2ORF3, the NCBI ID of which is P12.1.

MASCOT Search Results

User : menglin
E-mail :
MS data file : S006508.mgf
Database : Kelvin 20120924 (3 sequences; 507 residues)
Timestamp : 12 Dec 2012 at 18:06:47 GMT

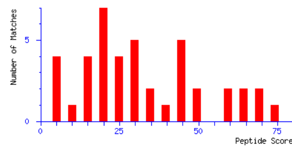
Re-search All Non-significant Unassigned [help] Export As XML

Not what you expected? Try the select summary.

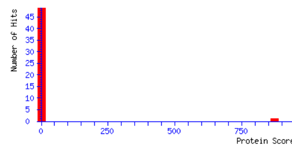
Search parameters

Type of search	: MS/MS Ion Search
Enzyme	: Trypsin
Variable modifications	: Carbamidomethyl (C), Oxidation (M)
Mass values	: Monoisotopic
Protein mass	: Unrestricted
Peptide mass tolerance	: ± 10 ppm
Fragment mass tolerance	: ± 0.6 Da
Max missed cleavages	: 1
Instrument type	: ESI-TRAP
Number of queries	: 3,984

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 **> 0** 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

Filter Significance threshold p< 0.05 Max. number of families AUTO [help]
Ions score or expect cut-off 0 Dendrograms cut at 0

Proteins (1) Report Builder Unassigned (3942) permalink

Protein family 1 (out of 1)

10 per page 1 Expand all Collapse all

Accession contains Find

		Score	Mass	Matches	Sequences	emPAI	
1	**PCV1**	872					ORF3
1.1	PCV1	872	23213	42 (37)	10 (10)	5.75	ORF3

FIGURE 16. LC-MS/MS analysis of the IMAC-purified rPCV1ORF3 using the PCV1ORF3 sequence from GenBank accession number AY094619 supplied by our lab. The results show a match to the PCV1ORF3 sequence from GenBank accession number AY094619.

MASCOT Search Results

User : MK
E-mail :
Search title : 6341
MS data file : ORF3.mgf
Database : NCBInr 20120421 (17,893,860 sequences; 6,141,683,785 residues)
Taxonomy : Viruses (4,710,071 sequences)
Timestamp : 29 Jun 2012 at 16:19:51 GMT

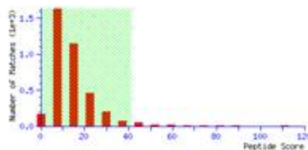
Re-search ◉ All ○ Non-significant ○ Unassigned [help] Export As XML

Not what you expected? Try the select summary.

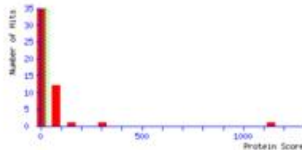
Search parameters

Type of search : MS/MS Ion Search
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein mass : Unrestricted
Peptide mass tolerance : ± 10 ppm
Fragment mass tolerance : ± 0.6 Da
Max missed cleavages : 1
Instrument type : ESI-TRAP
Number of queries : 3,968

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 **> 41** 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

Filter Significance threshold p< 0.05 Max. number of families AUTO [help]
Ions score or expect cut-off 41 Dendrograms cut at 0
Preferred taxonomy All entries

Proteins (15) | Report Builder | Unassigned (3865)

Protein hits (16 proteins)

Columns: Standard (12 out of 12)

Filters: (none)

Export as CSV

Family	M	DB	Accession	Score	Mass	Matches	Pep(sig)	Sequences	Seq(sig)	emPAI	Description
1	1	NCBInr	gi|4106899	1134	12008	71	71	6	6	4.83	ORF-3 [porcine circovirus type 2-E]
1	2	NCBInr	gi|330688655	485	11974	36	36	6	6	4.83	11.9 kDa apoptosis protein [Porcine circovirus 2]
2	1	NCBInr	gi|299818255	279	62422	5	5	1	1	0.11	heat shock protein cognate 5, partial [Proctophyllodes polyxenus]
3	1	NCBInr	gi|4106889	147	6830	4	4	2	2	1.30	ORF-4 [porcine circovirus type 2-C]
4	1	NCBInr	gi|15804792	110	15759	2	2	1	1	0.22	rplI gene product [Escherichia coli O157:H7 str. EDL933]
5	1	NCBInr	gi|333987742	64	44195	2	2	1	1	0.07	aspartate transaminase [Methanobacterium sp. SWAN-1]
6	1	NCBInr	gi|15640393	61	14240	2	2	1	1	0.24	30S ribosomal protein S6 [Vibrio cholerae O1 biovar El Tor str. N16961]
7	1	NCBInr	gi|7245665	54	26046	2	2	1	1	0.27	Chain A, Crystal Structure Of The Complete Transactivation Domain Of E2 Pro
8	1	NCBInr	gi|15599453	53	25822	2	2	1	1	0.13	rpsC gene product [Pseudomonas aeruginosa PAO1]
9	1	NCBInr	gi|378754723	46	58585	1	1	1	1	0.06	hypothetical protein NERG_02155 [Nematocida sp. 1 ERTm2]
10	1	NCBInr	gi|342806187	46	31700	2	2	1	1	0.10	MSHA biogenesis protein MshM [Vibrio splendidus ATCC 33789]
11	1	NCBInr	gi|343499645	43	34525	3	3	1	1	0.10	HTH-type transcriptional regulator [Vibrio tubiashii ATCC 19109]
12	1	NCBInr	gi|325521381	43	18233	3	3	1	1	0.19	dihydroneopterin triphosphate pyrophosphatase [Burkholderia sp. TJI49]
13	1	NCBInr	gi|197305007	42	19811	1	1	1	1	0.17	Chain A, Structure Of Vaccinia Virus Protein B14
14	1	NCBInr	gi|15804734	42	10381	1	1	1	1	0.33	groES gene product [Escherichia coli O157:H7 str. EDL933]
15	1	NCBInr	gi|15603277	42	30090	1	1	1	1	0.11	50S ribosomal protein L2 [Pasteurella multocida subsp. multocida str. Pm70]

FIGURE 17. LC-MS/MS analysis of the rPCV2ORF3 in 50% MeOH. The results show a match to the PCV2ORF3 sequence with GenBank accession number AY094619.