VIROLOGIC, EPIDEMIOLOGIC, AND PROPHYLACTIC INVESTIGATION ON PORCINE CIRCOVIRUS TYPE 2 INFECTION IN PIGS

A THESIS

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL

OF THE UNIVERSITY OF MINNESOTA

BY

KWANG SOO LYOO

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTORE OF PHILOSOPHY

JULY, 2009

ACKNOWLEDGMENTS

I deeply appreciate Dr. HanSoo Joo, my advisor, for giving me the opportunity to study and to achieve PhD degree in University of Minnesota. He always gave me a lot of inspirations for my research, encouragement not to lose my goal, and counsel as a mentor. I would like to recognize the value of the education by Dr. Joo and I could look over my life during the time with him.

I sincerely thank my committee members, Dr. Sagar M. Goyal, Dr. Peter Davies, and Dr. Jerry Torrison, for their participation in my thesis research. They taught me a lot of scientific knowledge, gave me idea for my project, and provide how to complete great results. I also appreciate their excellent personality and I was happy to have the interesting discussion developing research skills.

I thank Dr. Brian Caldwell who provided the field study for the main chapters in my thesis. He ingeniously designed the study model to demonstrate the efficacy of vaccines and devoted his effort to practical farm work and communication.

Dr. HyeunBum Kim, a previous member in my laboratory, supported to visit and collect samples in farms. Dr. SeungWook Kang and Dr. NamHo So helped me laboratory techniques and statistical analysis for my research. I learned a lot of knowledge from them that I had not recognized before. It was interesting and educable to have conservation with them in school or personal places.

I also thank Dr. KeumHwa Choi, Dr. JungJoo Hong, Dr. ByungWhi Kong, Dr. RaMi Cha, Dr. BitNa Kang, and MinHo Song. Their professional and personal advices encouraged me to pursue my degree successfully.

I really thank Bill Marks, CEO of MJ biologics, for supporting of my PhD degree. I could complete the entire courses in University of Minnesota by his incredible confidence and patience.

I appreciate all faculties and students of swine center and the staff of the department who gave me scientific advice and assisted my progress was gone favorably.

Finally, to my wife MinJin An and my daughter GaIn, I have not enough words in the world to thank for their assistance and trust. I can not imagine how to finish my study in United States without my family. They are a part and all of my life.

DEDICATION

This thesis is dedicated to my parents, Lyoo JoongDon and Shin HeaSook, and parents-in-law, An InKi and Lee SahIm, who sacrificed to me and supported my education in United States.

ABSTRACT

Porcine circovirus 2 (PCV2) is associated with several diseases in pigs including postweaning multisystemic wasting syndrome (PMWS). The virus has been classified into two genetic types of PCV2a and 2b by phylogenetic analysis. Although the PCV2b was commonly associated with PMWS, the genetic groups were not reflected or explained the difference of viral virulence. In an examination of viral characteristics, PCV2 isolates showed unusual resistance in high temperature and low pH.

In order to differentiate two PCV2 genotypes, a nested polymerase chain reaction (nPCR) assay was developed. The nPCR was found to be highly specific and sensitive for detecting and differentiating between the two genotypes.

For quantification of the viral load, a real-time PCR assay based on SYBR green was developed for PCV2b and we found that the real-time PCR assay was more sensitive than nPCR and can be a useful diagnostic method for the detection and quantification of PCV2b in swine serum samples. Additionally, a serum neutralization (SN) assay was modified and standardized based on previously developed method.

PCV2 viremia or SN antibody levels could be a marker for the protection against PMWS. Therefore, these were compared with pigs in the farms during and after the outbreak of clinical PMWS. The results indicated that the farms with clinical PMWS showed high percentages of PCV2 viremia, especially PCV2b, and low SN titers.

In the study to compare the efficacy of three PCV2 vaccines using 80 piglets, overall there were differences in the vaccine efficacy among commercial PCV2 vaccines, and the differences were based on the comparison of daily weigh gain, PCV2 antibody titers, percentage of the viremic pig, and PCV2b load in sera.

Lastly, PCV2 viremia and antibody levels were compared between heavy and light weight pigs at marketing age in conventional farms with routine PCV2 vaccination. Of the 7 farms, mean IFA titer, number of viremic pig, and mean the viral loads in 3 or 4 farms were significantly different between the heavy and light weight pigs. These results suggest that PCV2 infection can be one of the causes for the light weight pigs at marketing age.

TABLE OF CONTENTS

List of Tables

List of Figures

GENERAL INTRODUCTION

Porcine circovirus (PCV) was first discovered as a contaminant of the porcine kidney cell line, and the virus is classified into one of the family *Circoviridae*. There had been no attention with this virus in the past because there was no pathogenic evidence in pigs. Then in 1996, a novel PCV was first isolated from pigs in association with clinical signs of wasting in Western Canada, and the disease was later referred to as postweaning multisystemic wasting syndrome (PMWS). The new virus was shown to be less than 80% of nucleotide sequence identity as compared to the cell contaminant virus. Subsequently, the old and new viruses were named PCV1 and PCV2, respectively.

The PCV2 is one of the smallest autonomously replicating viruses, and the virion is approximately 17 nm in diameter with a genomic size of 1.7 kb. Two major open reading frames (ORF) 1 and 2 have been identified within the genome, and two functional proteins have been expressed by the two ORFs coding the replication enzyme proteins and the structural capsid protein, respectively.

The PCV2 is now accepted as an essential agent causing PMWS, although other factors may be involved for the development of clinical PMWS. During the last decade, significant economic losses due to PMWS have been observed with mortality of the growing pigs ranging between 5 and 50% in swine industry throughout Europe and Asia. The clinical signs and mortality were observed in pigs mainly between 8-16 weeks of age, and the affected pigs showed progressive weight loss, lymphoid depletion and others.

In North America, clinical PMWS has been observed from early 2000 in eastern Canada and from the late 2005 in the United States. Fortunately, effective PCV2 vaccine has been available commercially since the middle of 2006, and now clinical PMWS in

swine farms has been significantly reduced because of the routine use of the PCV2 vaccine.

When this thesis research was initiated during the late 2005, typical clinical signs of PMWS were reported from a few farms in Minnesota. The etiology of PMWS in association with PCV2 infection was still debating during that time. Before 2005, isolation of PCV2 was predominantly PCV2a genotype in the US farms, and the clinical signs with PCV2a genotype were not clinically apparent. However in mid 2005, a severe PMWS case with mortality of 14.1% (48 of 340 pigs) was first reported in a small finishing site in Minnesota. Later, PCV2 isolated from this farm was identified to be PCV2b genotype.

This thesis research was therefore designed to investigate the etiology and epidemiology of PCV2 infection in pigs. In addition, it was necessary to develop different diagnostic methods to investigate on-farm clinical PMWS cases. During the middle of these investigations, commercial PCV2 vaccines were available, and thus a study on the evaluation of different PCV2 vaccines was necessary. The overall objectives of this thesis were; 1) to isolate and characterize PCV2 isolates from pigs in farms with or without clinical PMWS, 2) to evaluate different diagnostic methods for PCV2 infection including nested-PCR assay to differentiate between the 2 genotypes, a SYBR green real-time PCR for the quantification of PCV2, and serologic methods for the detection of PCV2 antibody, 3) to perform epidemiologic investigation of PCV2 infection in swine farms during and after clinical PMWS, and finally 4) to compare efficacy of three commercial PC2 vaccines in conventional pigs. In addition, PCV2 viremia and antibody levels were compared between heavy and light weight pigs at marketing age.

CHAPTER 1

LITTERATRE REVIEW

1. 1. Porcine circovirus

Porcine circovirus (PCV) belongs to a member of the genus *Circovirus*, family *Circoviridae*. The virus is an icosahedral, non-enveloped, very small circular single-stranded DNA virus containing the genome size of 1.76kb, and has a diameter of 17nm (Tischer et al., 1982; Tischer and Buhk, 1988). Other members of this family group identified are beak and feather disease virus, goose circovirus, pigeon circovirus, and chicken anemia virus (Mankertz et al., 2004). The PCV was originally isolated from a porcine cell line (PK-15: ATCC-CCL 33), and it has been contaminated in the PK-15 cell line as a nonpathogenic agent in pigs (Tischer et al., 1987; Tischer and Buhk, 1988).

1. 1. 1 Genetic characteristics of PCV2

A novel PCV-like virus was first detected and isolated from wasting pigs in the US, Canada, and Europe in 1998. The initial PCV isolation was associated with post-weaning multi-systemic wasting syndrome (PMWS), and the virus was found to have 68% genetic homology with a previously published non-pathogenic PCV strain by genome sequence analysis (Ellis et al., 1998; Hamel et al., 1998; Allan and McNeilly, 2006). The pathogenic novel virus was proposed as PCV2, as compared to the non-pathogenic virus as PCV1 based on genetic and antigenic characterizations (Meehan et al., 1998). The viral genome of PCV2 has 1767-1768 nucleotides, and six open reading frames (ORFs) encode putative proteins in the genome (Hamel et al., 1998; Meehan et al., 1998). Among them, ORF1 and ORF2 are main functional genes, and they encode *Rep* protein and *Cap* protein, respectively (Mankertz et al., 2004). The ORF1 is a very conserve region in the circovirus genus, and it encodes enzyme proteins *Rep* and *Rep'*

which are essential for DNA replication in the host cell. However, the ORF2 gene encodes Cap protein of 39kDa which is the major structure protein and most likely associated with the host immune reaction (Mankertz et al., 2004; Cheung et al., 2007; Grau-Roma et al., 2007). The third protein that was encoded by ORF3 was recently described, and the protein was non-essential for virus replication but can induce cell apoptosis and affect in viral pathogenicity *in vivo* and *in vitro* systems (Liu et al., 2006).

1. 1. 2. Cultivation of PCV2

The PK-15 (ATCC-CCL 33) cell has been chronically contaminated with PCV1. PCV2 has been cultured using a PCV-free PK-15 cell line. The viral capsid protein was detected in the cytoplasm and nucleus of the infected cells after 18 hours post-inoculation (pi), and the cell-free progeny virus appeared 30 hours pi. Although viral antigens were first detected in the nucleus, they became more perinuclear and cytoplasmic as the infection progressed (Ellis et al., 1998; Cheung et al., 2007). In the cellular and viral mechanism, capsid protein was never found in the nucleus of infected cells in the absence of Rep protein. This indicates that the capsid has an interaction with Rep protein to cross the nuclear membrane. The Rep protein were mainly detected in the cytoplasm, and the infected cells showed irregular form of nuclei with genomic condensation (Cheung et al., 2007). Although porcine alveolar macrophages and fetal cardiomyocytes showed some replication, the kinetics of viral growth in those cells were much lower than in PK-15 cell line (Meerts et al., 2005). The virus replication was increased up to 50 times when the cell monolayers were treated with 300mM glucosamine and fresh growth medium (Tischer et al., 1987).

1. 1. 3. Evolution of PCV2 genotypes

Characterization of PCV2 genotypes was first organized and published by Olvera, et al 2007. Although there were attempts to find genetic differences between PCV2 isolates from farms with and without clinical PMWS, no obvious differences were found following analysis of PCV2 genome sequences in GenBank database until 2005 (Larochelle et al., 2002; de Boisseson et al., 2004; Delay et al., 2005). A few years later, PCV2 designated genotypes 1 and 2 were reported by a phylogenetic study (Grau-Roma et al., 2007). In a study of the phylogenetic tree, PCV2 genotype 1 was composed with PCV2 strains from European countries and China, while most US and Canadian strains along with a few European and Asian strains were included in genotype 2. It was suggested that the ORF2 gene of PCV2 could be used as a phylogenetic and epidemiologic marker, while none of the genetic clusters were apparently associated with clinical disease status (Grau-Roma et al., 2007).

From the late 2004 in Canada, PMWS/PCVAD cases of high mortality were observed and suddenly increased. The Canadian researchers isolated PCV2 from PMWS affected pigs and confirmed that the genetic type of the virus from the emerging case was a novel strain which had not been reported before in North America (Delay et al., 2005; Gagnon et al., 2007). The genotypes were designated initially as 4-2-2 (group 2 PCV2) and 3-2-1 (group 1 PCV2) type by restriction fragment length polymorphism (RFLP) technique. The PMWS cases by 3-2-1 genotype were significantly increases in 2005, whereas the percentage of cases by the 4-2-2 genotype showed no difference during the years (Delay et al., 2005).

The virus genotypes of PCV2a and PCV2b correspond to 4-2-2 (group 2) and 3-2-1 (group 1) genotype, respectively, that were reported in Canada (Horlen et al., 2007), and the terminology of PCV2a or 2b has now been widely accepted in swine industry. The PCV2b genotype, which had high genetic identity of approximately 99.5% with European isolates in 2005, was isolated in the US pig farms, and the detection of the genotype has been significantly increased in US swine industry after 2005 (Cheung et al., 2007).

There have been diversities in the opinion that pathogenic severity of PCV2 can depend on the specific genotype 2a or 2b in PMWS cases, because the two genotypes have been recovered from both clinical and subclinical PMWS cases (Allan and McNeilly, 2006; Grau-Roma et al., 2007; Hesse et al., 2008; Pesch and Ohlinger, 2007; Wiederkehr et al., 2007). However, a molecular epidemiology study suggested that PCV2b genotype was more closely related with PMWS development in swine herds, because PCV2a genotype was more commonly isolated from pigs in healthy swine herds. Submission of the PCV2 strains in GenBank database has also been shifted from PCV2a (European group 2) to 2b (European group 1) since 2003 (Dupont et al., 2007). Therefore, a hypothesis was proposed that there is a difference in the pathogenicity between the two PCV2 genotypes, although experimental evidence has not been well demonstrated for the pathogenic difference between the two genotypes.

1. 2. Postweaning multisystemic wasting syndrome (PMWS)

In 1991 a new disease, PMWS, was first reported in the retrospective samples of healthy pig herds in western Canada, but this disease was not recognized as a wide spread

disease until 1996 (Harding, 1997). The use of the term “postweaning multisystemic wasting syndrome - PMWS’ was first suggested to describe the clinical signs (Clark, 1997; Harding, 1997). Different terminologies, including porcine circovirus disease (PCVD) in Europe or porcine circovirus associated disease (PCVAD) in the US, were proposed for PMWS because the studies to reproduce PMWS under natural and experimental conditions did not demonstrate strong evidence that PCV2 is the causative agent for the disease (Lopez-Soria et al., 2004). Under field conditions, PMWS has been associated with a number of swine diseases; mycoplasma pneumonia, actinobacillary pneumonia, atrophic rhinitis, salmonellosis, swine dysentery, transmissible gastroenteritis, and pseudorabies (Allan and McNeilly, 2006). Porcine reproductive and respiratory syndrome (PRRS) was frequently observed in field conditions, and the co-infection with PRRS virus (PRRSV) and PCV2 was commonly detected in pigs with PMWS outbreaks. Although PRRSV is not an essential agent for PMWS, it may partially contribute to worsen the clinical severity (Lyoo et al., 2001; Allan and McNeilly, 2006). In addition, PCV2 infection showed an interaction or synergism in porcine respiratory disease complex (PRDC), which is associated with *Mycoplasma hyopneumoniae*, swine influenza virus, and PRRSV (Kim et al., 2003; Dorr et al., 2007).

1. 2. 1. Clinical signs and lesions in the pigs with PMWS

Pigs are most commonly affected by PMWS between 5 and 12 weeks of age (Allan and McNeilly, 2006). PMWS has been demonstrated in various pig farm types regardless of the herd sizes and the production systems (Grau-Roma et al., 2007). The predominant clinical signs include weight loss by emaciation, jaundice, and respiratory

problems with tachypnea and dyspnea. Other common clinical signs include diarrhea, coughing, and central nerve system problems (Harding, 1997). Morbidity and mortality in PMWS affected farms have been variable because they may depend on the farm's hygienic condition or management practice and co-infectious agents in the farm (Grau-Roma et al., 2007).

Pigs that die with PMWS showed poor body condition and moderate pale skin. All lymph nodes including inguinal, mesentery, bronchial, and peripheral lymph nodes are typically enlarged three to four times than the normal size. Lungs are heavily edematous, firm, and non-collapsed, and yellow to pink lobules, or dark red lobules are present on the lung surface. The spleen is moderately enlarged and the liver shows interlobular lesions and a mottled appearance. Kidneys are enlarged sometimes, and white spots and edema lesions are occasionally evident (Allan and McNeilly, 2006; Grau-Roma et al., 2007).

PCV2 has been detected from abortion and stillbirth cases, and linked with not only reproductive failure but also vertical transmission (West et al., 1999; Bogdan et al., 2001; Lyoo et al., 2001). Diffuse myocarditis lesions have been shown in aborted fetuses infected with PCV2 *in utero*, and the viral antigen has been detected from heart and other organs (West et al., 1999). It has been suggested that PCV2 is one of the causes of swine reproductive failure with other viral pathogens such as PRRSV, Aujeszky's disease virus (ADV), and porcine parvovirus (PPV) (Maldonado et al., 2005).

1. 2. 2. Microscopic lesions of PMWS affected pigs

Typical lung lesions are characterized by various degree of diffuse granulomatous

interstitial pneumonia with infiltration of alveolar macrophages and lymphohistocytic cells. The viral antigen has been found in airway epithelial cells with intranuclear inclusion bodies. Airways are affected by epithelial necrosis and bronchiolitis with infiltration of monocytes and neutrophils (Clark, 1997; Allan and McNeilly, 2006). Microscopic lesions of all lymphoid organs (lymph nodes, spleen, Peyer's patch of the ileum, and tonsil) are unique in PMWS affected pigs. In the lymph node, B cell follicles are lost in the early stage, and T cell area is expanded by large histocytic cells and multinuclear giant cells. This depletion of the lymphocytes has been described as a typical or hallmark lesion in lymphoid organs of the PMWS cases. The depletion is mainly observed within lymphoid follicles and paracortical zones. The syncytial cells, eosinophils, and dendritic cells are prominent (Clark, 1997; Rosell et al., 1999; Allan and McNeilly, 2006).

Kidneys developed lymphocytic and histocytic infiltration in peripelvic tissues, and localized vasculitis in cortical and medullar regions. Although inflammatory infiltration are scattered in widespread regions, clinical signs associated with those lesions have not been observed (Clark, 1997; Grau-Roma et al., 2007). In the gastrointestinal tract, atrophy of the villous is mild to severe. Often lymphohistiocytic infiltration and regeneration of glandular and cryptal epithelial cells are observed in the epithelial tissues. Viral antigen is detected within epithelial cells, mucosa, and sub-mucosa of the small intestine (Allan and McNeilly, 2006).

1. 2. 3. Viremia of PCV2 in PMWS affected pigs

Quantification of virus in the serum of PCV2 infected pigs has become a common

approach to measure the progression of the infection, because the viral load can be useful to distinguish between subclinical and clinical disease (Rovira et al., 2002; Brunborg et al., 2004). Quantification of the viral load in serum sample was first described using competitive polymerase chain reaction (PCR) method. It was reported that the amount of the circulating virus in serum could be used to differentiate between pig groups of different clinical conditions, because development of clinical PMWS is associated with a certain viremic level of PCV2 in the blood (Liu et al., 2000).

Nested PCR (nPCR) assay has also been used to detect PCV2 DNA in serum samples. Although the nPCR is more sensitive than one-step PCR, the assay cannot measure virus quantity (Larochelle et al., 2000; McIntosh et al., 2006a). Virus quantification has been achieved with a real-time PCR assay which is the most specific and sensitive method (Rovira et al., 2002; Chung et al., 2005; Carasova et al., 2007; Grau-Roma et al., 2007). In these studies, serum has been recommended as a suitable sample for distinguishing between subclinical and clinical PMWS pigs. Serum, as compared to other tissues, can be collected from live animals and tested for the viral quantity with less labor (Brunborg et al., 2004; Grau-Roma et al., 2007). Although PCV2 quantity in the serum may predict the severity of PMWS in pigs, the viremic levels could be variable within pig groups that have different disease conditions (Grau-Roma et al., 2007).

1. 2. 4. Pathogenesis of PCV2 infection

Pathogenesis of PMWS/PCVAD by PCV2 infection is poorly understood, although viral genetic, immunologic, and pathologic observations were reported by

various research groups (Grau-Roma et al., 2007). According to the previous reports, PCV2 along with various co-factors including the strain of PCV2, the presence of co-infection with other pathogens, immunostimulation, and host susceptibility were involved in the PMWS affected pigs, and then those factors down regulated the immune system (Fig. 1.1) (Opriessnig et al., 2007). Lymphoid depletion and high level of viremia are the most prominent markers in the affected pigs, and development of clinical PMWS/PCVAD is linked with high mortality in pig herds.

It has been speculated that infection with a certain PCV2 genotype was likely associated with pathogenic virulence based on comparison of nucleotide sequences of PCV2 isolates from clinical and subclinical PMWS farms. Larochelle et al., 2002 reported that there were differences in the sequences of the isolates and some associations between the virus isolates and the virulence in pigs. In Canada, the severe form of PMWS markedly increased since 2005. Canadian researchers suggested that the increasing cases were associated with the infection of PCV2b genotype (Delay et al., 2005; Gagnon et al., 2007). However, both PCV2a and PCV2b strains could be detected from pigs in PMWS affected farms (Pogranichniy et al., 2002; de Boisseson et al., 2004).

A number of reports have been published on the experimental reproduction of the disease using PCV2 alone or co-infection of PCV2 with other pathogens such as porcine parvovirus (PPV), PRRSV, and *M. hyopneumoniae* (Kennedy et al., 2000; Harms et al., 2001; Rovira et al., 2002; Ostanello et al., 2005; Cheung et al., 2007). These experiments were performed in conventional pigs, specific pathogen free (SPF) pigs or cesarean-derived colostrum-deprived (CDCD) pigs. The results showed mild clinical signs, gross and microscopic lesions of PMWS in the target organs, and high antibody response

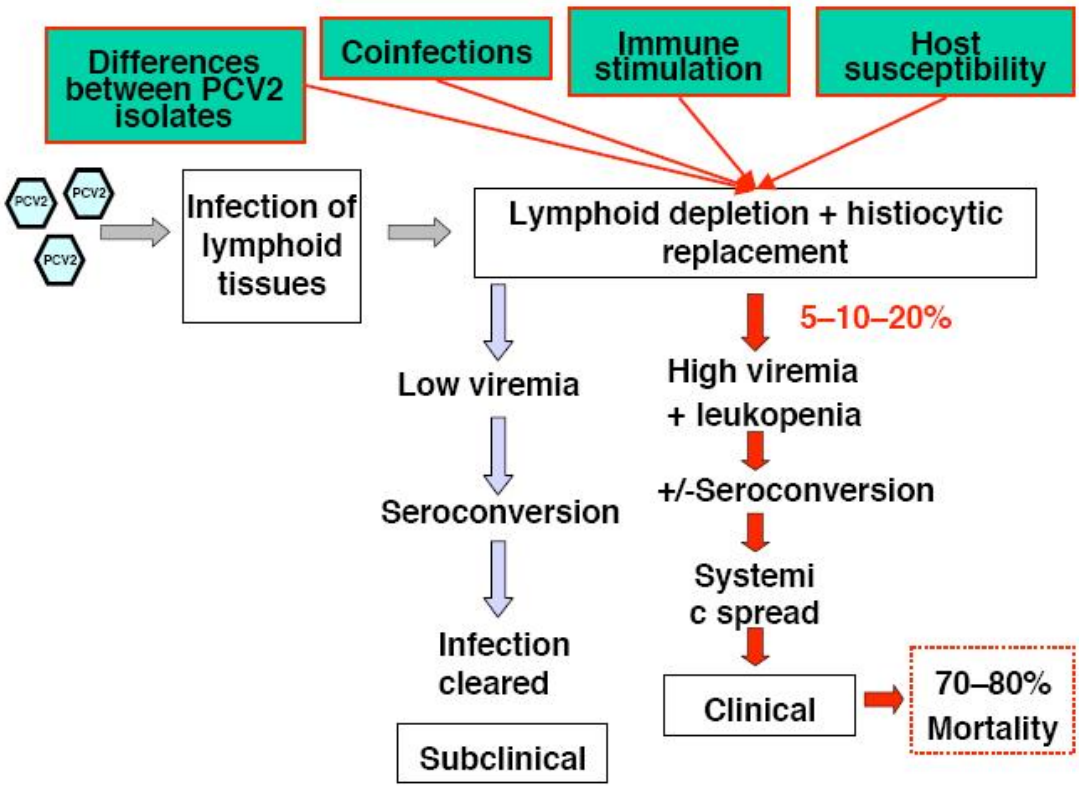


Fig. 1.1. A suggested pathogenesis of clinical and subclinical PMWS/PCVAD in pigs (Opriessnig et al., 2007)

against the virus, but severe clinical PMWS signs could not be consistently demonstrated in the experimental studies.

Immuostimulation was observed in the pigs when PCV2 infection and injection of a vaccine containing certain adjuvant were carried out at the same time (Krakowka et al., 2001; Kyriakis et al., 2002; Allan and McNeilly, 2006). In these cases, the amount of PCV2 antigen in the blood and the lymphoid depletion in the tissues were markedly increased. It was also confirmed in the experimental studies that clinical signs of PCV2 infected pigs with inoculation of an adjuvant were significantly different when compared to the non-adjuvant group (Cheung et al., 2007). In contrast**,** other studies demonstrated that there was no difference in the mortality and symptoms between experimental groups with or without an adjuvant (Ladekjaer-Mikkelsen et al., 2002; Resendes et al., 2004).

Some studies suggested that specific breeds of pig have low susceptibility to PMWS (Madec et al., 2000; Grau-Roma et al., 2007). The Landrace pigs showed significantly more development of PCV2 infection and the lesions as compared to other genetics (Cheung et al., 2007). However, it may not be concluded that the observed results were originated from a particular genetic line or boar, and the data were analyzed using a limited number of animals in the study (Grau-Roma et al., 2007).

1.2.5. Reproduction of clinical PMWS following experimental challenge

In order to reproduce the disease, different experimental challenge studies have been carried out. However, there has not been a consistently successful result for severe PMWS. Nevertheless, some studies have reported that experimental co-infection of PCV2 with other porcine pathogenic agents or immunostimulaton reagent could increase

the severity of lesions and clinical signs as compared with the infection of PCV2 alone. The experimental reproduction of PMWS was first reported by Ellis et al (1999) with PCV2 isolate and the tissue homogenates in gnotobiotic pigs. All challenged pigs showed typical gross and microscopic lesions of PMWS in the target organs and high antibody response against the virus. However, clinical signs were not observed in all of the experimental pigs (Ellis et al., 1999) Other studies of PMWS have failed to demonstrate severe and typical clinical signs in the challenged pigs, although moderate clinical signs, specific viral antigen, high titer of antibody, and microscopic lesions were detected in the pigs (Allan et al., 1999; Kennedy et al., 2000; Magar et al., 2000). The various experimental models, therefore, have suggested involvement of disease co-factors, other microorganisms, and pigs of different conditions.

A combination of PPV and PCV2 was injected into colostrum-deprived (CD) pigs. The lesions of the co-infected pigs were similar to the group that was infected with only PCV2 but clinical signs were noticed better in the co-infected group (Kennedy et al., 2000; Allan and McNeilly, 2006). Because PMWS was commonly reported in pigs during post-weaning age under field conditions, conventional weaned pigs were used for experimental reproduction of PMWS. Following intranasal inoculation with PCV2 isolate, microscopic lesions were detected but there were no major clinical signs (Magar et al., 2000).

PRRSV has been commonly detected with PCV2 in PMWS affected pigs as a co-infectious pathogen in field surveys (Lyoo et al., 2001; Allan and McNeilly, 2006). Therefore, PCV2 and PRRSV were intranasally inoculated single or in combination to CD or CDCD weaning pigs. The dual or single infection caused a mild clinical

respiratory disease or the clinical disease was not evident in all groups (Harms et al., 2001; Allan and McNeilly, 2006). The PCV2 and PRRSV dual infection was tried in conventional weaned pigs, and the results were similar to those of the previous co-infection studies, but one of the pigs in the dual infection group died with severe respiratory and wasting signs (Rovira et al., 2002). When immune cells of the pigs were infected with PPV, there was a transient immunosuppression, and thus it was suggested that the immune condition could be associated with PMWS development (Krakowka et al., 2001).

A hypothesis was made that a non-infectious immunogen could act as a strong immunostimulant, and it was later confirmed that an adjuvant could increased the severity of PMWS, even though pigs were individually challenged only with PCV2 without other pathogenic microorganisms. Gnotobiotic piglets were injected with keyhole limpet hemocyanin (KLH) emulsified in incomplete Freund's adjuvant (ICFA) in intramuscular route, and then with PCV2 oronasally. All piglets immunized with KLH/ICFA showed moderate to severe clinical PMWS, whereas the piglets infected with PCV2 alone did not develop PMWS. This study, therefore, led to specialization that immune-stimulation could be one of the trigger components in the pathogenesis of PMWS in swine (Krakowka et al., 2001).

M. hyopneumoniae is the causative agent for porcine enzootic pneumonia and is highly associated with porcine respiratory disease complex (PRDC). PCV2 has also been one of the common agents in the pigs with PRDC. In one study, weaned pigs that had been injected with *M. hyopneumoniae* via intra-tracheal route 2 weeks before were inoculated intranasally with PCV2. Moderate clinical signs, higher amount of PCV2

genome copies and microscopic lesions associated with PMWS were observed in the dually infected pigs (Cheung et al., 2007). PCV2 DNA clone without a live virus was also used in the experiment for reproduction of PMWS. The cloned DNA was challenged with or without KLH/ICFA used as an immunostimulation promoter in SPF piglets. The inoculum was directly injected into the liver and lymph nodes or injected intramuscularly. Mild-to-moderate clinical signs were observed, and microscopic lesions and PCV2 antigen were detected in numerous tissues including blood (Fenaux et al., 2002; Grasland et al., 2005).

Experimental reproduction of reproductive abnormalities has been demonstrated because the reproductive failures associated with PCV2 have been described in field cases. The PCV2 isolate was directly inoculated into late-term fetuses by laparotomies or trans-abdominally injected by an ultrasound guided-needle. Some fetuses showed stillbirth, mummification, or death. These studies supported the results that PCV2 could cause reproductive failures in pregnant sows (Johnson et al., 2002; Yoon et al., 2004).

1. 2. 6. Diagnosis of PMWS in pigs

For the diagnosis of PMWS, three criteria have been established, and it is suggested to define the disease in single or groups of animals: (1) the presence of clinical signs of wasting and weight loss, with or without dyspnea, jaundice, enlarged lymph nodes, and icterus, (2) observation of the histopathological lesions including depletion of lymphocytes, granulomatous inflammation, and inclusion bodies in lymphoid tissues, lung, and less often liver and kidney, and (3) detection of PCV2 antigen within the tissue lesions (Grau-Roma et al., 2007; Sorden, 2000). The definitions for PMWS diagnosis

implied that only clinical signs or gross lesions were not sufficient, and PCV2 infection did not mean PMWS without both the clinical signs and the lesions in suspected PMWS cases (Sorden, 2000).

The PCV2 antigen has been detected by several methods in a laboratory. *In situ* hybridization (ISH) and immunohistochemistry were found to be highly sensitive and specific, and have been widely used to detect PCV2 antigen or nucleic acid in all of the infected tissues (Rosell et al., 1999; Allan and McNeilly, 2006). The level of lymphoid depletion or the amount of viral antigen has been suggested as a criterion for making a diagnosis of PMWS, but PCV2 antigen has been detected in the lymphoid organs in pigs with or without clinical PMWS. The severe microscopic lesions of lymphocyte depletion were commonly observed in fatal cases of PMWS (Rosell et al., 1999; Quintana et al., 2001).

The polymerase chain reaction (PCR) assay has been used as a specific and sensitive diagnostic method for the detection of PCV2 in field samples (Larochelle et al., 1999a; Lyoo et al., 2001; Calsamiglia et al., 2002). Using the PCR assay, PCV2 can readily be differentiated from PCV type 1 (PCV1). One-step PCR is known to be sensitive enough to detect PCV2 in tissue samples but it may not be sensitive for the serum or semen samples due to insufficient quantity of the viral DNA in the samples. Therefore, a nested PCR assay was developed and applied to detect PCV2 in serum or semen samples (McIntosh et al., 2006b).

The quantitative real-time PCR assays were developed to quantify PCV2 antigen in the tissues of clinical or subclinical PMWS pigs (Chung et al., 2005; Grau-Roma et al., 2007). The viral load determined by real-time PCR was differently distributed in the

organs of the naturally infected pigs. Higher viral load was detected in the tissues of spleen, lung, and lymphoid organ, while the virus load was relatively low in serum, kidney, heart, and intestines (Chung et al., 2005). In the similar manner by immunohistochemistry, significantly higher PCV2 viral load was detected in the lymphoid tissues and serum samples of PMWS pigs as compared to those in healthy pigs (Brunborg et al., 2004). These results indicate that the amount PCV2 in infected tissues can be an indicator to determine clinical severity of PMWS (Grau-Roma et al., 2007).

For the serologic methods for PCV2 infection, indirect immunofluorescence assay (IFA), immunoperoxidase monolayer assay (IPMA), and enzyme linked immunosorbent assay (ELISA) have been developed (Rosell et al., 2000; Nawagitgul et al., 2002; Allan, 2007). Because PCV2 is an ubiquitous antigen, PCV2 specific antibody could be detected in most pigs under natural conditions regardless of the presence of clinical PMWS (Larochelle et al., 2003). The antibody kinetics, therefore, have not been significantly accepted as a diagnostic method for clinical PMWS (Sorden, 2000) .

1. 2. 7. Prevention of PMWS

The control of PMWS or PCVAD was difficult under field conditions, because PCV2 is so ubiquitous and resistant to different environmental degradation. A physico-chemical characteristic study of PCV demonstrated that the virus was highly resistant to low pH, high temperature, and treatment with chloroform (Allan and McNeilly, 2006). PCV2, moreover, survived against most commercial disinfectants which are currently used in swine farms (Martin et al., 2007). Improvement of farm management skills could reduce the impact by PCVAD. Some management measures, therefore, have been

suggested and designed. Application of good management procedures such as improving hygiene, reducing stress and preventing other pathogens could reduce the percentage of mortality by PCVAD (Madec et al., 2000; Allan and McNeilly, 2006). The cause of PCVAD has been recognized by several factors including PCV2. For the control of the disease, therefore, understanding and elimination of the triggering should be achieved (Grau-Roma et al., 2007).

1. 2. 8. Vaccination

Since the late 2006, commercial PCV2 vaccines including Circumvent ™ PCV (Intervet Inc), Suvaxyn® PCV2 (Fort Dodge), Ingelvac® CircoFLEX ™ (Boehringer Ingelheim), and Circovac® (Merial Inc.) have been available in the swine industry, and the former three products have been licensed by the USDA.

The Circumvent ™ PCV vaccine was developed based on the baculovirus vector system for ORF2 gene transfection. The protective efficacy of the expressed ORF protein was examined by the challenge trial in SPF pigs following inoculation of PCV2 intratracheally and intramuscularly. The ORF2 vaccine group was compared with a non-vaccine group, ORF1 vaccine group, ORF1 and ORF2 combined vaccine group and DNA vaccine group. The results showed a significant improvement in the growth performance in all of the vaccinated groups as compared to the non-vaccine group (Madec et al., 2000).

The Ingelvac® CircoFLEX was also produced using the baculovirus vector system, and PCV2 ORF2 gene protein was expressed by the system in insect cells. The ORF2 protein was purified and substantially concentrated by the technical process of the

Boehringer Ingelheim company. This vaccine was examined under commercial farm conditions, and the results showed that clinical signs, mortality, and lesions of the vaccinated group were significantly reduced as compared to the non-vaccinated group (Boehringer Ingelheim technical bulletin).

The Suvaxyn® PCV2 was developed from the constructed chimeric virus of PCV1 and PCV2 that ORF2 gene of PCV2 was inserted into the backbone of PCV1. The efficacy of the chimeric virus vaccine was evaluated with SPF pigs, and gross and microscopic lesions of the non-vaccinated group were significantly severer than those of the vaccinated group (Fenaux et al., 2003).

The Circovac® was developed following inactivation of the whole virus and mixing with an oil adjuvant. The vaccine caused improvement in the performances by an experimental challenge in the piglets and sows, and has been suggested to use in pregnant sows (Charreyre et al., 2005).

CHAPTER 2

ISOLATION AND CHARATERIZATION OF PORCINE CIRCOVIRUS TYPE 2 ISOLATES FROM PIGS IN FARMS WITH OR WITHOUT CLINICAL POST-WEANING MULTI-SYSTEMIC WASTING SYNDROME

2.1. Summary

The purpose of this study was to isolate and characterize porcine circovirus type 2 (PCV2) from swine farms with or without clinical post-weaning multi-systemic wasting syndrome (PMWS) in Minnesota. A total of 76 samples that were collected from 6 farms with PMWS and 4 farms without PMWS, and that were positive for PCV2 by a polymerase chain reaction assay were used in this study. Of the 76 samples, 8 PCV2 were isolated and passed continuously on PK-15 cell line. Whole genome and phylogenetic characteristics of ORF2 gene sequences were compared between the isolates from PMWS affected and non-affected farms to find genetic difference. There was 95% genetic homology in whole genome sequence between the 4 PCV2 isolates. In the phylogenetic analysis, 20 PCV2 isolates were classified into two genetic subgroups. The PCV2b was composed with 12 isolates from 6 PMWS farms and 2 isolates from 1 none-PMWS farm, and PCV2a with 6 isolates form 3 none-PMWS farms. Although the PCV2b was commonly associated with PMWS, the genetic groups were not reflected or explained the difference of viral virulence. In addition, some physicochemical properties on the survivability of PCV2 were examined. PCV2 showed resistance in high temperature (56℃) and low pH (pH 2.0). The present results confirmed the previous results on the virus genetic, disease characteristic and the spread of PCV2b in association with clinical PMWS in Minnesota.

2.2. Introduction

Post-weaning multi-systemic wasting syndrome (PMWS) was first reported in western Canada but now the syndrome has been observed in many different countries (Allan et al., 1998; Allan and Ellis, 2000). Clinically PMWS is characterized by severe weight loss, respiratory signs, and high mortality, causing significant economic loss to the swine industry (Allan et al., 1998; Allan and Ellis, 2000). Porcine circovirus type 2 (PCV2) is considered the major causative agent for PMWS, although the virus has also been isolated from pigs without clinical history of PMWS (Allan *et al.*, 1998; Fenaux *et al.*, 2000; de Boisseson *et al.*, 2004). In Minnesota, severe PMWS with high mortality was not observed in swine farms until the mid 2005, although PCV2 was isolated before 2005 from pigs including stillborn fetuses (Farnham et al., 2003).

PCV2 is very small and non-enveloped DNA virus containing a 1.76 kilo base pair (bp) genome. Two main viral genes, ORF1 and ORF2, have been described. The ORF1 encoded the *Rep* protein participating in viral replication, and the ORF2 encoded *Cap* protein (Nawagitgul *et al.*, 2000; Cheung, 2003). Viral genomes showed more than 95% homology among PCV2 strains, however, the nucleotide variations of ORF1 and ORF2 were 97-100% and 91-100%, respectively (Fenaux *et al.*, 2000; Meehan *et al.*, 2001; de Boisseson *et al.*, 2004).

PCV2 isolates from different geographic areas showed some genetic variation (Fenaux *et al*., 2000). The US and Canadian PCV2 strains reported before 2000 were shown to be closely related to each other by phylogenetic analysis, while a few Canadian isolates had genetic sequence similar to those of the European isolates (Mankertz *et al*., 2000). Attempts have been made to find genetic difference between the PCV2 isolates

from farms with and without clinical PMWS but no obvious difference was found (de Boisseson *et al.*, 2004; Sibila *et al.*, 2004; Segales *et al.*, 2005). Olvera *et al* (2007) analyzed 148 PCV2 sequences and divided the virus strains into 2 different subgroups and 8 genetic clusters (Olvera *et al*., 2007). However, none of the genetic clusters were identified to be associated with clinical severity, while the analysis of ORF2 gene was suggested to use as phylogenetic and epidemiologic marker (Olvera *et al.*, 2007). The objectives of this study were to isolate PCV2 from swine farms with or without clinical history of PMWS in Minnesota and to examine some physio-chemical and genetic characteristics of the PCV2 isolates.

2.3. Materials and methods

2.3.1. Sample collection and virus isolation

A total of 76 samples (29 sera and 47 tissues of lung or lymph node) that were positive for PCV2 by a polymerase chain reaction (PCR) assay were used in this study. The samples were collected from nursery and grower pigs of 10 farms in Minnesota in 2003, 2005 and 2006, and stored in -20℃. Six of the 10 farms had clinical PMWS with history of having marked increases in number of wasting pigs and mortality. The grow-finish mortality in the 6 PMWS farms was more than 8% lasting for at least 8 weeks, and the mortality were increased more than twice as compared to those before the PMWS outbreak. In addition, PCV2 was detected by PCR assay and/or immunohistochemistry, and microscopic lesions including lymphoid depletion were confirmed following submission of the samples to a veterinary diagnostic laboratory by attending

veterinarians. The 4 farms without clinical PMWS, however, did not have increased mortality with wasting pigs even though PCV2 antigen and antibody were detected.

For PCV2 isolation, each tissue sample was homogenized, centrifuged at 3,000g for 20 min, and the supernatants were inoculated in PCV-free one-day old PK-15 cell monolayer. Three blind passages of the inoculated PK-15 cell monolayer were made every 4-5 days. On the second day of each passage, the monolayer was treated with 300mM *D*-glucosamine as previously described (Tischer et al., 1987). The presence of PCV2 was demonstrated by indirect immunofluorescence antibody (IFA) test using a reference PCV2 antibody positive swine serum (Johnson et al., 2002). Viral infectivity (50% tissue culture infective dose, $TCID_{50}$) was examined by a routine method, and the titers were read by IFA method. Ten fold serial dilutions of the isolated virus were inoculated into PK-15 cell in suspension. The cells were placed in a 96-well microtiter plate and incubated for 72 hours at 37℃ in a humidified 5% CO_2 atmosphere. The plate was fixed by acetone and ethanol mixture (1:1) for 20 min. The PCV2 positive reference serum was incubated in the IFA plate at 37℃ for 45 min and washed 3 times with phosphate buffered saline (PBS, pH 7.2). Then an optimal dilution of rabbit anti-swine IgG FITC (ICN Biomedicals, Inc. Aurora, OH) was incubated at 37℃ for 45 min and washed again. Viral infectivity was examined under a fluorescent microscope.

2.3.2. Stability of PCV2by heating and different pH

Stability of PCV2 was investigated following incubation at 56℃ and 70℃. Aliquots of PCV2a (EF452353) and 2b (EF452350) were maintained in a water bath at

56℃ or 70℃ for 1, 6, and 24 hours. After the incubation, each virus was tested for infectivity. These procedures were repeated twice, and mean infectivity titers were calculated. For the stability of PCV2 in different pH, the standard buffer solutions of pH 2, 3, 4, 10, 11, and 12 were used (Ricca Chemical Company, Arinton, TX). One ml of PCV2a (EF452353, $10^{5.5}$ $TCID_{50}$/0.1ml) and 9 ml of each pH buffer were mixed to minimize an effect that the medium component of viral fluid can modify pH of the test buffer. The mixtures were incubated at room temperature for 30 min and neutralized immediately to prevent cell toxicity by each buffer solution. All test mixtures and positive control virus samples were adjusted with PBS, pH 7.2 to make the same final volume. All reaction and positive viruses were diluted by 5-fold and inoculated into PK-15 cells. The infectivity of each virus was measured by IFA method. These procedures were repeated 2 times, and mean titers were calculated.

2.3.3. Replication kinetics of PCV2 in different cell types and chicken embryo

PCV2a (EF452353) and 2b (EF452350) strains were used to examine growth characteristics. Vero cells, MARC-145 cells, MDCK cells, and swine testicle (ST) cells were inoculated with $10^{4.0}$ $TCID_{50}$/0.1ml of each strain of the PCV2 and cultured for 4-5 days. The cell cultures were passed blindly 3 times. The cultivation of PCV2 was also attempted in embryonated chicken eggs. Nine days old SPF chicken embryonated eggs (Hy-Vac, Adel, IO) were inoculated via chorioallantoic cavity with each of the PCV2 strains and incubated for 6 days. Virus isolation was attempted five blind passages. For the viral infectivity, all cultivated samples, cell homogenates, allantoic fluid and embryo

tissue homogenates at 5^{th} passage, and reference virus strains were tested by the same titration method using PK-15 cells.

2.3.4. Growth characteristics of PCV2 isolate

To compare virus yields of the cell-free and cell-associated virus, one-step growth curves were determined with a PCV2 isolate. One-day old PK-15 cells monolayer was prepared in a 24-well cell culture plate, and PCV2 (EF452350) was inoculated onto the wells and absorbed for 1 hour at 37℃. Each well was washed twice to remove unabsorbed virus and incubated with fresh medium at 37℃. Cultured supernatants and cells were separately collected 12, 24, 36, 48, 60, and 72 hours post-inoculation (pi), and viral infectivity was tested. This procedure was repeated twice, and the mean titers of the viral infectivity were calculated.

2.3.5. Comparison of antibody response between PCV1 and PCV2

The PK-15 cells infected with either PCV1 or PCV2 in 96-well microtiter plates were prepared. The each virus specific plate was incubated for 72 hours at 37℃ in a humidified 5% CO_2 atmosphere. The plate was fixed by acetone and ethanol mixture (1:1) for 20 min and stored at -70℃ until use. Sera to be tested were serially diluted 4-fold up to 4^{-10} with PBS, and 100μl of each serum dilution was transferred to the PK-15 cell plate for PCV1 and PCV2 and incubated at 37℃ for 45 min, respectively. The IFA antibody titers were measured as previously described.

2.3.6. Viral DNA amplification and sequencing

Viral DNA was extracted using a DNeasy Tissue kit (Qiagen, Valencia, CA) according to the manufacturer‘s instruction. Primer pairs were separately designed for sequencings of the whole genome and ORF2 gene by published PCV2 sequences in GenBank. The primer sequences designed and used for PCR assays are shown in Table 2.1. For the full sequencing, FS1-f and -r primer pair were designed to amplify whole genome of 1767 bps or 1768 bps, and FS2 and FS3 primer pairs was designed to generate overlapping PCV2 gene fragments. The PCR reaction mixtures were prepared by adding 2 μl of extracted viral DNA, 2.0 mM $MgCl_2$, 1x PCR buffer, 0.2mM dNTPs, 1 μM each primer, and 1U of *Taq* polymerase (GoTaq™ DNA Polymerase; Promega, Madison, WI), and adjusted with distilled water to make 25 μl of the final volume. The amplification was performed with 30 cycles of 95℃ for 1 min, 52℃ for 1 min, and 72℃ for 1 min in case of FS1 primer pair. The optimal annealing temperatures of all primer pairs are given in Table 2.1.

2.3.7. Sequencing and phylogenic analysis

For sequencing of the whole genome and the ORF2 gene, PCR products were purified using a commercial gel purification kit (QIAEXII gel extraction kit; Quiagen) according to the manufacture’s instruction. Purified PCR products were sequenced by the electrophoretic DNA analyzers, ABI 3730xl (BioMedical Genomic Center, University of Minnesota). The nucleotide sequence alignment and phylogenic analysis were performed by Neighbor-Joining (NJ) tree using the MEGA version 3.1 alignment program. Bootstrap values were evaluated on 500 repeats of the alignment. The percentage of sequence identity among PCV2 isolates and different PCV2 strains was determined with

Table 2.1. The sequence and position of primer pairs and product sizes for PCV2 used in this study

	Name	Sequence(5'-3')	Position in genome	Amplification size (bp)	Anneal temperature (℃)
Whole genome	FS1-f	TAT TGT AGT CCT GGT CGT AT	1098-1117	1768	50
	FS1-r	TCC GTG TAA CCA TGT A	1082-1097		
	FS2-f	CAA CTG CTG TCC CAG CTG TA	844-863	915	55
	FS2-r	AGC GCA CTT CTT TCG TTT TC	1739-1758		
	FS3-f	GGT AAC GGT GGC GGG GGT	1626-1643	620	57
	FS3-r	TTT CTG ACA AAC GTT A	472-487		
ORF2	ORF2-f	TTA AGG GTT AAG TGG GGG GT	1033-1052	699	53
	ORF2-r	ACG TAT CCA AGG AGG CGT TA	1712-1731		

MegAlign program in the DNAstar version 5.0 package. The ORF2 sequences of PCV2 isolates were analyzed in comparison with 31 ORF2 gene sequences of PCV2 strains that were originated from the farms with or without PMWS and published in GenBank from different countries in North America, Europe, and Asia.

2.4. Results

2.4.1. PCV2 isolation

Isolation and identification of PCV2 in clinical samples from 10 different farms was summarized in Table 2.2. Eight PCV2 were isolated and passed continuously on PK-15 cells. PCV2 specific fluorescence was confirmed for each isolate in infected PK-15 cell monolayer using a reference PCV2 antiserum. Of 8 PCV2 isolates, 2 (MN-NP160 and MN-NP162) were isolated from pigs in a none-PMWS farm, and 6 (MN-R4, MN-R13, MN-B2, MN-B9, MN-PG34, and MN-H12) were made from PMWS pigs in 4 different farms. The MN-R4 and MN-R13 were isolated from tissue homogenate, while others were made from serum samples. Infectivity titers of MN-R4 and MN-B2 isolates were recorded up to $10^{6.0}$ $TCID_{50}$ /ml but those for other isolates were lower.

2.4.2. Evaluation of heat stability of virus isolates

The infectivity of heated PCV2 was compared with that of an untreated control and expressed as log10 $TCID_{50}$/0.1ml. The results of infectivity reduction of PCV2 before and after heating at 56°C and 70°C for 1, 6 and 24 hours are shown in Table 2.3. The infectivity titers of PCV2a and 2b ($10^{4.5}$ and $10^{4.0}$) remained after incubation at 56°C

Table 2.2. Isolation and identification of PCV2 genotypes and clinical history of PMWS in the originated swine farms (*: PCV2 was cultured using PK-15, **: ORF2 amplified)

Farm	Clinical PMWS	Year sampled	PCV2 Identification	PCV2 subgroup	PCV2 strain	Genbank No.
B	+	2005	Cultured*	2b	MN-B2	EF452354
	+	2005	PCR**	2b	MN-B7	EF452357
	+	2005	PCR	2b	MN-B8	EF452355
	+	2005	Cultured	2b	MN-B9	EF452356
BE	+	2006	PCR	2b	MN-BE1	EF452362
	+	2006	PCR	2b	MN-BE2	EF452363
H	+	2006	Cultured	2b	MN-H12	EF452361
NL	-	2006	PCR	2b	MN-NL2	EF619970
	-	2006	PCR	2b	MN-NL4	EF619971
PG	+	2006	Cultured	2b	MN-PG34	EF452358
R	+	2005	Cultured	2b	MN-R4	EF452350
	+	2005	Cultured	2b	MN-R13	EF452351
VB	+	2006	PCR	2b	MN-VB1	EF452360
	+	2006	PCR	2b	MN-VB2	EF452359
NP	-	2003	PCR	2a	MN-NP61	EF452364
	-	2003	Cultured	2a	MN-NP160	EF452352
	-	2003	Cultured	2a	MN-NP162	EF452353
PA	-	2003	PCR	2a	MN-PA20	EF452365
PC	-	2003	PCR	2a	MN-PC48	EF452366
	-	2003	PCR	2a	MN-PC60	EF452367

Table 2.3. Infectivity of PCV2 by heating at different temperatures

Incubation hours	Infectivity titer after exposure at different temperature (log10)			
	70℃		56℃	
	[a]PCV2a	[b]PCV2b	[a]PCV2a	[b]PCV2b
0	4.5	4.0	4.5	4.0
1	1.5	1.0	4.5	4.0
6	0	0	2.5	2.5
24	0	0	2.5	2.0

[a] PCV2a: Genbank access number EF452353

[b] PCV2b: Genbank access number EF452350

for 1 hour but were reduced from the original titer of $10^{4.5}$ and $10^{4.0}$ $TCID_{50}$/0.1 ml to $10^{1.0}$ and $10^{1..5}$ $TCID_{50}$/0.1ml, respectively, after exposure to 70°C for 1 hour. When PCV2 isolates were incubated at 70°C for 6 or 24 hours, the infectivity was not detected. The infectivity titers of PCV2a and PCV2b after incubation at 56°C for 6 or 24 hours were reduced to $10^{2.5}$ or $10^{2.0}$ $TCID_{50}$/0.1 ml, respectively.

2.4.3. Evaluation of pH stability of virus isolates

Infectivity titers of PCV2 at different pH are summarized in Table 2.4. Although the viral infectivity was somewhat decreased in acid buffers, PCV2 maintained the infectivity even under a strong acid of pH 2. The infectivity was significantly decreased at pH 11 and almost disappeared at pH 12.

2.4.4. Replication kinetics of PCV2 in different cell types

PCV2 was replicated in ST and PK-15 line cells but there was no evidence of the replication of the virus in Vero cells, MARC-145 cells, and MDCK cells, and chicken embryonated eggs. The infectivity titer of the virus in ST cells was $10^{3.5}$ $TCID_{50}$/0.1 ml, and decreased somewhat when compared to the original viral infectivity. Negative results on the cultures in other cell types and embryonated chicken eggs were confirmed by PCV2 PCR assay.

2.4.5. Growth characteristics of PCV2 isolate

In a comparison of one-step growth curves, the cell-free and the cell-associated progeny viruses showed a difference in growth characteristics (Fig. 2.1). The viral growth

Table 2.4. Infectivity of PCV2 after treatment at different pH for 30 minutes

	Acid			Neutral	Alkali		
pH	2	3	4	7	10	11	12
Viral titer (log5)	2.7	3.2	3.3	4.2	2.8	1.3	0.3

.

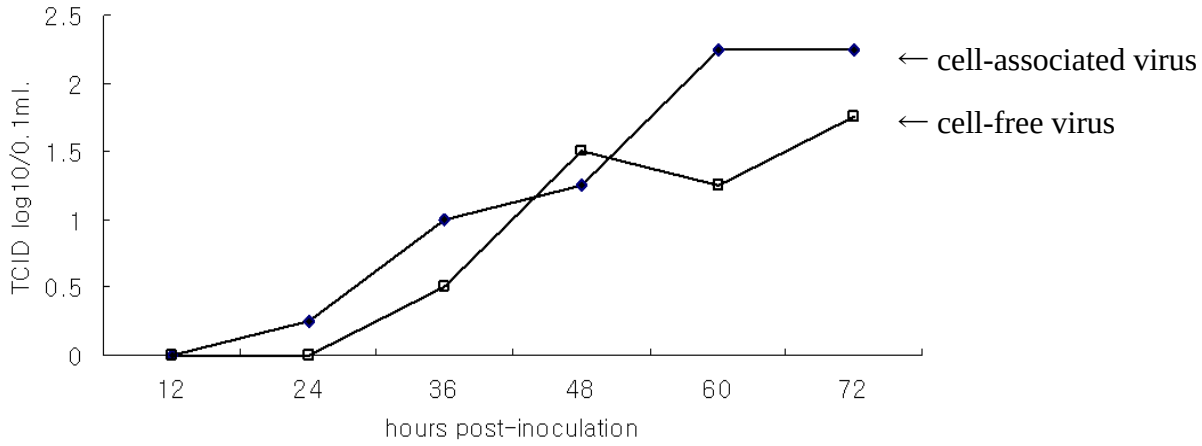


Figure 2.1. One-step growth curves for the cell-free and cell-associated progeny of PCV2.

of the cell-associated progeny virus was initially detected by IFA from approximately 24 hours pi, while the cell-free virus was initially detected from 36 hours pi. The infectivity titers of both cell-free and cell-associated progeny virus were gradually increased by 72 hours pi. Although the pattern of the growth curves were similar, infectivity titers of cell-associated viruses were higher than those of the cell-free virus

2.4.6. Sequence analysis of PCV2 whole genome

Whole genomic sequences of MN-R4 (EF452350) and MN-R13 (EF452351) isolates from PMWS affected pigs showed 1767 bp and were the highest genetic homology with a Dutch strain (AY484411 - Hamel *et al.*, 1998; Grierson *et al.*, 2004). The MN-NP160 (EF452352) and MN-NP162 (EF452353) isolates from pigs in a none-PMWS farm showed 1768 bp and were ≥99% genetic homology with one of the Canadian (AF027217) and the U.S. strains (AF055391) reported in 1998. The heterogeneity of whole genome between PCV2 isolates from samples collected in 2003 and 2005 was 5% (full sequence data are not shown).

2.4.7. Phylogenetic analysis and gene alignment of PCV2 ORF2 gene

The phylogenetic analysis was performed with ORF 2 gene of the 8 MN PCV2 isolates and 6 uncultured PCR positive samples from different farms (Table 2.2.). Two genetic clusters were identified in comparison with 22 published PCV2 strains from GenBank (Fig. 2.2.). Six cultured isolates (MN-R4, -R13, -B2, -B9, -PG34, and -H12) and 3 PCR positive samples (MN-BE2, -VB1, and -VB2) were classified into the lower cluster (PCV2b) along with PCV2 strains of Netherlands, France and one US strain in

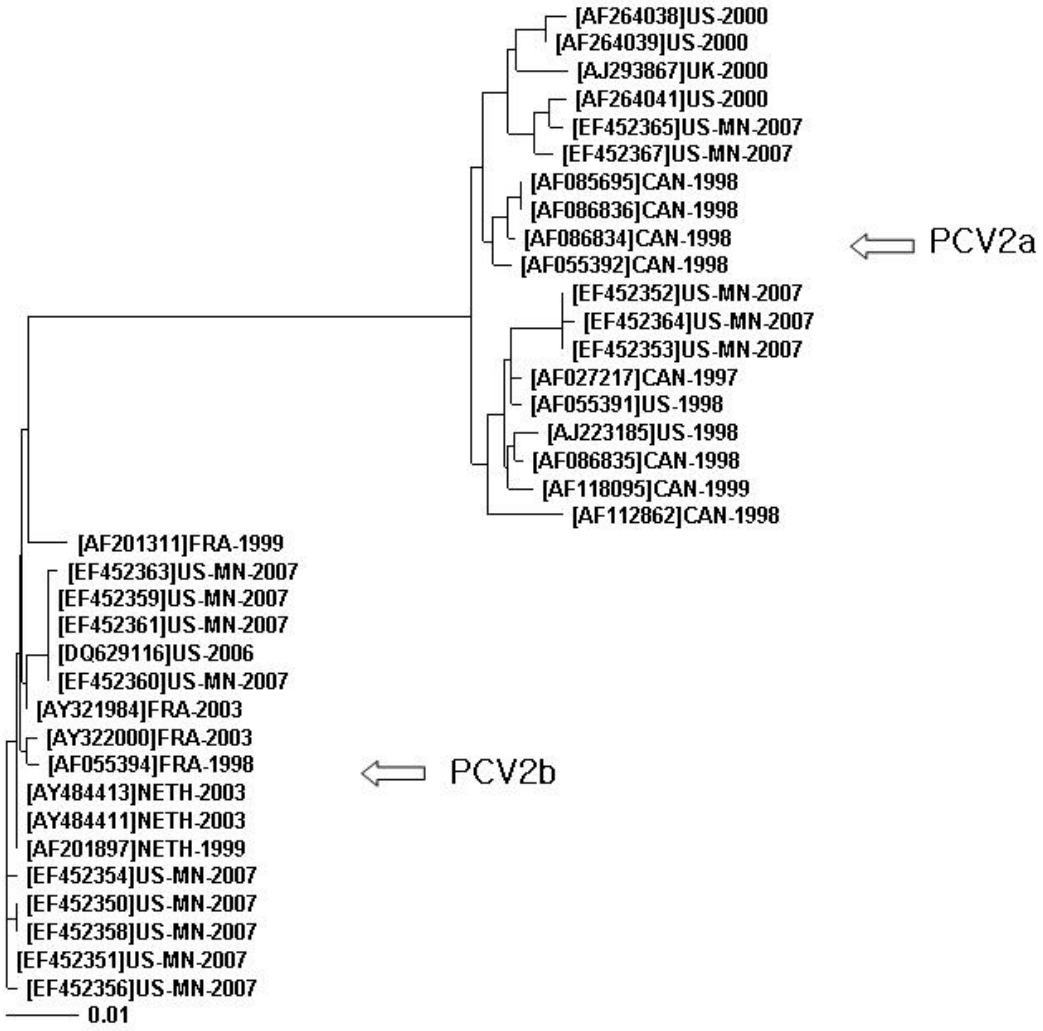


Fig. 2.2. Phylogenic tree based on the ORF2 gene of PCV2 MN isolates. Eight PCV2 isolates and 6 PCR positive samples from Minnesota and 22 PCV2 strains sequences from Genbank were compared. The geographic locations of accession number are indicated with abbreviation: CAN – Canada; FRA – France; UK - United Kingdom; NETH- Netherlands; US – United States.

2006. The upper cluster (PCV2a) was composed with two MN-NP160 and -NP162 isolates and 3 PCR positive products (MN-NP61, -PA20, and -PC60) along with PCV2 strains from the US, UK and Canada. All of the North American strains in the upper cluster were reported before 2001. PCV2 positive samples in lower cluster were collected in 2005 and 2006, while those in upper cluster were collected in 2003. The GenBank numbers assigned to the sequences determined in this study are listed in Table. 2.2.

Nucleotide percent identity of the ORF2 gene between 8 PCV2 isolates from pig farms with or without PMWS was compared to those isolates from other countries (Table 2.5). The 6 PCV2 isolates from PMWS pigs displayed nucleotide identity from 99.3 to 100% with the European and the Chinese strains. The 6 isolates showed percent identity less than 92.7% with the US, Canadian and the 2 isolates (MN-NP160 and -NP162) from farms without clinical PMWS.

The sequence alignment of the ORF2 gene was compared between 4 PCV2 isolates from PMWS pigs and the MN-NP160 from pig without PMWS (Fig. 2.3). There was one nucleotide deletion at the 703rd position for all 4 PCV2 isolates from PMWS pigs. The MN-NP160 isolate showed heterogeneity in 53 nucleotides of the 703 nucleotides when compared to those from clinical PMWS farms. Marked sequence heterogeneity in the ORF2 gene was shown in a fragment between the 255th and the 273rd position (Fig. 2.3.).

2.5. Discussion

PMWS is now considered a global disease causing significant economic losses in swine industry. The first case of typical PMWS in Minnesota was in a small finishing site

Table 2.5. Comparison of nucleotide sequence identities among the ORF2 genes of 8 PCV2 isolates from Minnesota and other countries. The values above the diagonal are corrected the percent identity and the values below the diagonal are the percent difference uncorrected for multiple base changes by the cluster method.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
1		98.1	99.4	99.4	99.7	96.1	98.1	92.9	93.1	93.0	92.9	92.9	92.7	92.6	92.9	93.0	99.1	99.1	**1**	AF055391US
2	1.9		98.1	98.1	98.1	95.1	98.6	92.3	92.4	92.3	92.1	92.1	92.0	92.1	92.1	92.3	97.9	97.9	**2**	AF264038US
3	0.6	1.9		99.1	99.4	96.1	98.1	92.6	92.9	92.7	92.7	92.6	92.4	92.4	92.6	92.7	98.9	98.9	**3**	AJ223185US-IO
4	0.6	1.9	0.9		99.4	96.1	98.4	92.6	92.9	92.7	92.6	92.6	92.4	92.4	92.6	92.7	98.9	98.9	**4**	AB072303 JAP
5	0.3	1.9	0.6	0.6		96.1	98.1	92.9	93.1	93.0	92.9	92.9	92.7	92.6	92.9	93.0	99.1	99.1	**5**	AF027217 CAN
6	4.0	5.0	4.0	4.0	4.0		95.1	92.0	92.3	92.1	92.0	92.0	91.8	91.9	92.0	92.1	95.6	95.6	**6**	AF201305GER
7	1.9	1.4	1.9	1.6	1.9	5.0		92.3	92.4	92.3	92.1	92.1	92.0	92.1	92.1	92.3	97.6	97.6	**7**	AJ293867 UK
8	7.5	8.1	7.8	7.8	7.5	8.5	8.1		99.7	99.9	99.4	99.4	99.6	99.4	99.4	99.6	92.3	92.3	**8**	AY322000 FRA
9	7.2	8.0	7.5	7.5	7.2	8.1	8.0	0.3		99.9	99.7	99.7	99.6	99.7	99.7	99.9	92.6	92.6	**9**	AY484413NETH
10	7.4	8.1	7.7	7.7	7.4	8.3	8.1	0.1	0.1		99.6	99.6	99.7	99.6	99.6	99.7	92.4	92.4	**10**	DQ180392CHA
11	7.5	8.3	7.7	7.8	7.5	8.5	8.3	0.6	0.3	0.4		99.7	99.3	99.7	99.7	99.9	92.3	92.3	**11**	MN-B2
12	7.5	8.3	7.8	7.8	7.5	8.5	8.3	0.6	0.3	0.4	0.3		99.3	99.7	99.7	99.9	92.3	92.3	**12**	MN-B9
13	7.7	8.5	8.0	8.0	7.7	8.6	8.5	0.4	0.4	0.3	0.7	0.7		99.3	99.3	99.4	92.1	92.1	**13**	MN-H12
14	7.7	8.4	8.1	8.1	7.7	8.6	8.4	0.6	0.3	0.4	0.3	0.3	0.7		100.0	99.9	92.1	92.1	**14**	MN-PG34
15	7.5	8.3	7.8	7.8	7.5	8.5	8.3	0.6	0.3	0.4	0.3	0.3	0.7	0.0		99.9	92.3	92.3	**15**	MN-R4
16	7.4	8.1	7.7	7.7	7.4	8.3	8.1	0.4	0.1	0.3	0.1	0.1	0.6	0.1	0.1		92.4	92.4	**16**	MN-R13
17	0.9	2.2	1.2	1.2	0.9	4.6	2.5	8.1	7.8	8.0	8.1	8.1	8.3	8.4	8.1	8.0		100.0	**17**	MN-NP160
18	0.9	2.2	1.2	1.2	0.9	4.6	2.5	8.1	7.8	8.0	8.1	8.1	8.3	8.4	8.1	8.0	0.0		**18**	MN-NP162
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**		

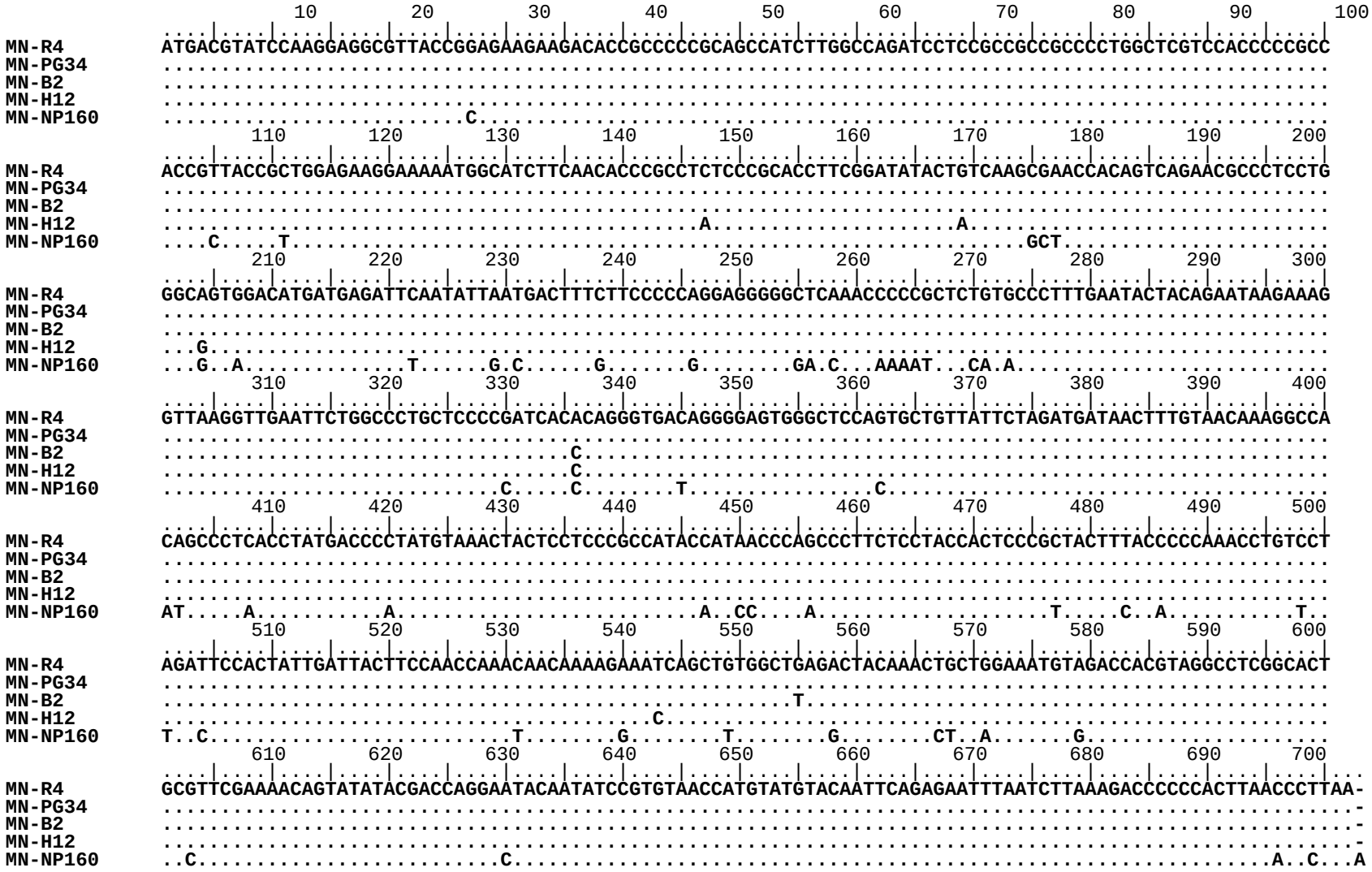


Fig 2.3. Nucleotide sequence alignment of the ORF2 gene among Minnesota PCV2 isolates.

in mid 2005 (Cano, unpublished data). In that farm, 48 (14.1%) out of 340 pigs died or were culled due to severe clinical signs of PMWS. The MN R-4 and R-13 of PCV2 were isolated from the pigs in that farm. Initially we experienced difficulty in isolation and cultivation of PCV2 from the samples. Following the passages of PCV2 infected PK-15 cells, cytopathic effect was not observed but low numbers of the infected cells were detected when the monolayer of the cells were examined by IFA method. Viral infectivity of the isolates was low and poorly increased even after several passages. It appears that *D*-glucosamine treatment and continues passages are necessary to isolate the virus and increase the infectivity titer. It is not known why only 2 isolates (MN-R4 and MN-B2) reached the infectivity titers of $10^{6.0}$ $TCID_{50}$/0.1 ml, although other isolates were propagated under the same condition.

The virus is ubiquitous and is extremely resilient in many environments (Segales et al., 2005). A related virus, PCV1, is resistant to inactivation at pH 3 and in lipid solvents, and is stable at 70°C for 15 minutes (Allan et al., 1994), but little is known about the stability at different pH and temperature of PCV2. Stability of PCV2 at different pH and high temperature was examined using similar methods in the previous studies (Deboer and Barber, 1964; Allan et al., 1994). Although physio-chemical characteristics of PCV1 were reported, there is no information for PCV2. The results in this study were obtained to provide expanded information on the physiochemical properties and could be compared to PCV1 characteristics in earlier study (Allan et al., 1994). PCV2 similar to PCV1 showed resistance in high temperature and low pH. The infectivity of both PCV2 genotypes was maintained for one hour at 56℃, but the virus was inactivated at 70℃. The chemical reagents of strong acid or alkali have been widely

used as a disinfectant but it is necessary to reconsider with certain disinfectants for circovirus family. In this study, PCV2 infectivity was also somewhat decreased even at strong acid, but the virus was inactivated at pH 11 or pH 12.

Antibody cross reaction has been commonly observed between PCV1 and PCV2 because their high genetic homology is directly related with antigenic structure. PCV1 and PCV2 have genetic homology of 68%, and it can induce a cross reaction between the type specific antibody. The PCV2 antibody produced from PCV1 free animals recognized PCV1 antigen structure in IFA test. The difference of IgG titer showed from 2^6 to 2^{12} in an animal following post infection days. Although, the difference of neutralizing activity between both genotypes IgG has not been demonstrated, there could be some immune-cross reaction.

Genetic similarity between the Minnesota isolates and various European strains was confirmed by gene aliment and phylogenic analysis. Five Minnesota isolates (MN-R4, -R13, -B2,-B9 and -PG34) and in the lower cluster (Fig. 2.2) were genetically very close to the Dutch and France strains (Grierson et al., 2004; Olvera et al., 2007). Another isolate (MN-H12) and 3 PCR positive samples (MN-BE2, -VB1and -VB2) in the lower cluster showed almost 100% genetic identity (Table 2.3) with a US strain (DQ629116) that was recently denominated as a European-like PCV2 (Cheung et al., 2007). These Minnesota isolates and the European-like strain could be classified into PCV2 group 1A (PCV2b) by the method used in PCV2 genome evolution study (Olvera et al., 2007). In the upper cluster (Fig. 2.2), two isolates (MN-NP160 and -NP162) and 3 PCR positive samples (MN-NP61, -PA20, and -PC60) originated from none-PMWS farms in 2003 were grouped with the Canadian and the US strains reported between 1998 and 2000, and

these were classified into the PCV2 group 2E (PCV2a) by the genome classify study (Olvera et al., 2007).

In the whole genomes comparison between the 2 PCV2 isolates each sampled in 2003 and 2005 showed 95% genetic homology, while the genetic homology of the ORF2 genes among the 4 isolates was less than 92.7%. The sequencing data of the ORF2 genes in this study were similar to those of the previous reports (de Boisseson et al., 2004), and thus we focused on ORF2 gene analysis to approach phylogenic discrimination between PCV2 isolates. In the present study, PCV2 positive samples that were classified into PCV2b were from both PMWS farms and none-PMWS farm. Therefore, the genetic subgroup of PCV2 did not reflect or explain the difference in viral virulence, although PCV2b was more associated with PMWS, as previously reported (Fenaux *et al.*, 2000; de Boisseson *et al.*, 2004).

Cheung et al. (2007) recently observed that sudden appearance of PCV2b was associated with increase in PMWS farms in North Carolina, Iowa, and Kansas states, and suggested that the outbreaks of PCV2b is a concern for the swine industry (Cheung et al., 2007). This is coincided with field observations of dramatic increase of wasting pigs with high mortality in Minnesota starting from late 2005 and beginning 2006. With the present results, it may be concluded that PCV2 isolates in Minnesota could also be classified into two phylogenetic subgroups, and PCV2b has been spread and caused PMWS in Minnesota swine farms. The present results confirmed the previous results by others regarding to the relation with virus genetic and disease characteristic and the spread of PCV2b at least after 2003 in Midwestern states. It would be interesting to examine the

pathogenic difference in pigs following experimental inoculation with PCV2 in two different phylogenetic clusters.

CHAPER 3

DEVELOPMENT AND EVALUATION OF DIFFERENT DIAGNOSTIC METHODS FOR PORCINE CIRCOVIRS TYPE 2 INFECTION IN PIGS

This chapter is divided into three different sections of 3A, 3B and 3C. Each section contains the works on development and evaluation of 3 different diagnostic methods that have been used in this thesis research.

CHAPTER 3

SECTION 3A.

Evaluation of a nested polymerase chain reaction assay to differentiate between two genotypes of porcine circovirus type 2

3A.1. Summary

Porcine circovirus 2 (PCV2) is associated with several diseases in pigs including postweaning multisystemic wasting syndrome (PMWS). A new genotype of PCV2 has been isolated from swine farms with or without clinical PMWS in North America. The new genotype was differentiated in a separate cluster by phylogenetic analyses and is now named PCV2b as compared to PCV2a for the previously known genotype. The purpose of this study was to develop and evaluate a nested polymerase chain reaction (nPCR) assay to detect and differentiate between PCV2a and 2b. Genotype-specific primer sets were designed using sequence data published for different PCV2 strains. Specificity and sensitivity of the nPCR were examined using PCV2 isolates with known genotype. The nPCR was found to be highly specific and sensitive for detecting and differentiating between the PCV2 genotypes compared to the conventional one-step PCR assay. Nested PCR was applied to detect PCV2 and identify the genotype in serum samples from swine farms with and without a clinical history of PMWS. Of 60 serum samples collected from 4 farms during clinical PMWS outbreaks, PCV2a and 2b were detected in 6 and 49 samples, respectively. Six of the 10 samples from 1 of the 4 farms had both PCV2a and 2b. Of 20 serum samples from 2 farms without PMWS, 11 were positive for PCV2a only. These results suggest that the nPCR can be used to detect PCV2 and differentiate between the 2 genotypes from field samples.

3A.2. Introduction

Porcine circovirus 2 (PCV2) is a very small, circular, single-stranded DNA virus that has been classified in the family *Circoviridae* and genus *Circovirus*. Two open reading frames (ORFs) of PCV2, ORF 1 and ORF 2, each encode a replication protein (*Rep*) and a structural protein (*Cap*) (Meehan *et al*., 1998). The virus has been associated with several disease syndromes in pigs, including postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome, reproductive failure, and porcine respiratory disease complex (Allan *et al.*, 1999; Rosell *et al.*, 2000; Ellis *et al.*, 2004). These syndromes together are considered porcine circovirus–associated disease (PCVAD) (Allan and Ellis, 2000; Horlen et al., 2007).

Isolation of a new genotype of PCV2 was reported from pig farms with high mortality due to PMWS in Canada (Carman et al., 2006; Gagnon et al., 2007). The new genotype was also reported in the U.S. farms both with and without clinical PMWS (Cheung et al., 2007; Horlen et al., 2007). In North America, the new PCV2 genotype is now commonly named PCV2b to distinguish it from PCV2a, the previously known genotype (Gagnon et al., 2007; Horlen et al., 2007). In a molecular evolution study, PCV2 strains from different geographical areas were also shown to be differentiated into genetic groups 1 and 2 (Olvera *et al*., 2007). Genetically, PCV2 groups 1 and 2 were found to contain 1,768 and 1,767 nucleotides, respectively (Cheung et al., 2007; Olvera et al., 2007). In phylogenetic trees, group 1 viruses were classified in the same cluster of PCV2b and mostly reported from European countries and China, while group 2 viruses were from countries throughout the world. All of the PCV2 reported from North America before 2004 was categorized in the PCV2a group (Cheung et al., 2007; Gagnon et al.,

2007). This coincided with the initial experience with high mortality due to PMWS in swine farms in Canada and the United States (Cheung et al., 2007; Gagnon et al., 2007).

The polymerase chain reaction (PCR) assay has been used as a specific and sensitive diagnostic method for the detection of PCV2 in field samples (Larochelle et al., 1999; Lyoo et al., 2001; Calsamiglia et al., 2002). Using the PCR assay, PCV2 can readily be differentiated from porcine circovirus 1 (PCV1). Conventional 1-step PCR is sensitive enough to detect PCV2 in tissue samples; however, it is not sensitive enough for use with serum and semen samples due to an insufficient quantity of the viral DNA. Therefore, nested PCR (nPCR) assays have been developed and applied to detect PCV2 for those samples (Larochelle et al., 2000; McIntosh et al., 2006).

Distinguishing between PCV2a and 2b is difficult and time consuming. PCV2 genotypes have been classified by several methods, including viral genomic sequencing, restriction fragment length polymorphism (RFLP) analysis, real-time PCR assay, and genotype-specific PCR (Carman et al., 2006; Gagnon et al., 2007; Horlen et al., 2007). Each of these methods has advantages and disadvantages in obtaining the results. It is generally accepted that nPCR is less expensive than real-time PCR, and less time and labor consuming than RFLP and sequencing methods.

It has been understood that the genotype-specific primers for differential PCR with high sensitivity are not easy to design, because the genetic identity between the 2 genotypes is very high (>95%) (Olvera et al., 2007). The differentiation between PCV2a and 2b using a nPCR assay has not been reported, and the nPCR reported previously was used solely to detect PCV2 without genotype differentiation from samples with low quantities of virus such as semen (McIntosh et al., 2006). Therefore, the purpose of the

current study was to develop and evaluate a nPCR assay to detect and differentiate between the 2 genotypes PCV2a and PCV2b. The nPCR with specific primer sets for each group was evaluated for specificity and sensitivity using PCV2 of known genotype. The nPCR was also applied on field serum samples for the detection and genotyping of PCV2.

3A.3. Materials and methods

3A.3.1. Primer design for nested polymerase chain reaction assay

Primer sets that could detect the 2 genotypes of PCV2 were designed. Nucleotide sequence data for PCV2 strains from GenBank and the 14 Minnesota PCV2 isolates were aligned using ClustalX 1.83 to identify regions that differed between the 2 genotypes. The most heterogeneous nucleotide portion of the 2 genotypes was detected from 1462–1480, and reverse primers of 2 types were designed (Fig. 3A.1). To amplify the PCR products of different sizes, the forward primer for PCV2a genotype was designed in both ORF1 and ORF2 regions, and the primer of PCV2b was in the ORF2 region. The primer sequences for PCV2a were 2a-F (forward): 5’-AACAATCCACGGAGGAAGG-3’ and 2a-R (reverse): 5’-GGGACCAACAAAATCTCY-3’, and for PCV2b were 2b-F (forward): 5’-CTGTTTTCGAACGCAGTG-3’ and 2b-R (reverse): 5’-CTCAAACCCCCGCTCTG-3’. The predicted product sizes of the PCV2a– and PCV2b–specific PCR were 360 bp and 568 bp, respectively. For the first round PCR, the reverse nested primer was designed from the highly conserved region outside 2a-R primer sites in ORF2. The forward nested primer, which can differentiate PCV-1 and PCV2, was

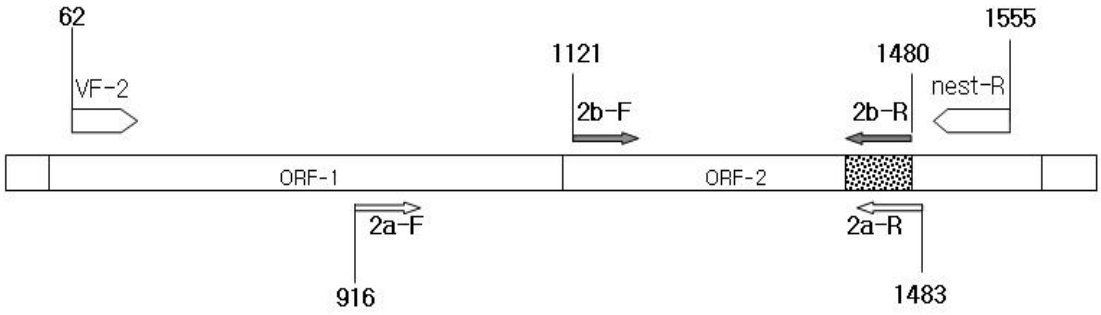


Fig. 3A.1. A diagram of primer design. The reverse primers 2a-R and 2b-R commonly recognize the most distinct portion between porcine circovirus 2 (PCV2) genotypes 2a and 2b. The forward primers 2a-F and 2b-F were designed to make different polymerase chain reaction product sizes in open reading frames of PCV2, ORF 1 and ORF 2, respectively. The forward primer of the first round, VF-2, can differentiate Porcine circovirus type 1 (PCV1) and PCV2 in which a few nucleotides are deleted in PCV1.

selected according to the results from a previously study (Lyoo *et al*., 2001). The primer sequences were VF-2 (forward): 5'-GAAGAATGGAAGAAGCGG-3' and nest-R (reverse): 5'-ACAGTCAGAACGCCCTCCT-3'. The predicted PCR product size of the first round primer was 1494 bp. The oligonucleotide primer sets were designed using the Primer3 (v. 0.3.0) program (Source Forge, Inc., Mountain View, CA/http://frodo.wi.mit.edu/primer3/input.htm).

3A.3.2. Polymerase chain reaction and nested polymerase chain reaction assays

Genomic viral DNA from PCV2 isolate and serum sample was extracted and purified using DNeasy blood & tissue kit (Qiagen, Inc., Valencia, CA) as described in the manufacturer's manual. The first round PCR was performed with the VF-2 and nest-R primer set. A final volume of 25 μl contained 2 μl of extracted viral DNA, 2.5 μl 5X reaction buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 2.0 mM of $MgCl_2$, 1 μl of 1 pM each primer, 0.25 mM of dNTPs (deoxyribonucleotide triphosphates), and 1U of Taq polymerase (Promega Corp., Madison, WI), and adjusted with DNase-free distilled water to make the final volume. The amplification was undertaken using a thermal cycler (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MS) and the following cycling profile: 5 min denaturation at 94°C, 30 repetitive cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 54°C, and 40 sec of elongation at 72°C. Elongation was extended for 7 additional min in the last cycle.

For differential nPCR, the amplified product of the first round PCR was reacted with each group specific primer set in separate tubes. The primary amplification product of 1 μl was added to a new PCR mixture of 5X reaction buffer (200 mM Tris–HCl, pH

8.4, 500 mM KCl), 2.0 mM of $MgCl_2$, 1µl of 3 pM each primer, 0.25 mM of dNTPs, and 1U of Taq polymerase, and adjusted with DNase-free distilled water to make 25 µl of final volume. The nPCR steps for both group-specific reactions were performed as described above for PCR except for 30 sec of annealing at 57°C, and 30 sec of elongation at 72°C. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis and examined under UV light.

3A.3.3. Specificity and sensitivity of the nested polymerase chain reaction

Initially, nPCR was used to evaluate specificity using 2 reference PCV2 isolates of known genotype, EF452353 (PCV2a) and EF452350 (PCV2b). With the reference strains, the viral DNA was reacted with first round primer set and then the PCR products were performed with 2 differential primer sets. In a tube, the first round PCR product of EF452353 (PCV2a) was reacted with PCV2a genotype-specific primer set. At the same time, the PCR product was reacted with PCV2b genotype-specific primer set in another tube. Similarly EF452350 (PCV2b) was also tested with PCV2a– and PCV2b– specific primer sets. PCV-1 and porcine parvovirus (PPV) were also tested with the same nPCR procedure for its specificity. Subsequently, all 14 Minnesota PCV2 isolates (PCV2a: EF452352, EF452353, EF452364, EF452365, and EF452367; PCV2b: EF452350, EF452351, EF452354, EF452356, EF452358, EF452359, EF452360, EF452361, and EF452363) were tested by nPCR. Viral DNA from each virus was tested with both differential primer sets and examined for the presence of nonspecific primer reaction. The PCR products of 360 and 568 bp obtained in the second round PCR during development stage were sequenced to confirm specificity.

The sensitivity of the nPCR was examined in comparison with conventional 1-step PCR. The EF452353 (PCV2a) and EF452350 (PCV2b) strains were pre-adjusted to be the same infectivity titer ($10^{4.0}$ $TCID_{50}$/ml) and were serially diluted 10 fold up to 10^{-10}. Viral DNA of each dilution was tested by both nPCR and one-step PCR without the first round step. The nPCR was further performed with 12 serum samples from 2 different farms in which each genotype of PCV2 had been detected. These samples were examined by conventional one-step and nPCR assays, and the results were compared.

3A.3.4. Application of nested polymerase chain reaction assay on serum samples

A total of 80 serum samples from pigs between 8 and 14 weeks old from 6 different swine farms were randomly selected from a serum bank. Farms A, B, C, and D had a history of typical clinical signs of PMWS during the time of blood collection in 2006. Sera collected in 2004 from farms E and F were also used. Farms E and F had no history of clinical PMWS although PCV2 infection had been diagnosed. Viral DNA extraction and nPCR were carried out to detect and differentiate PCV2 genotypes as described above.

3A.4. Results

3A.4.1. Specificity of nested polymerase chain reaction

The primer pairs designed to differentiate between 2 genotypes of PCV2 specifically amplified each PCV2 genotype without any nonspecific reaction. As shown in Fig. 3A.3a, viral DNA of PCV2b (EF452350) yielded a positive reaction with the 2b-F

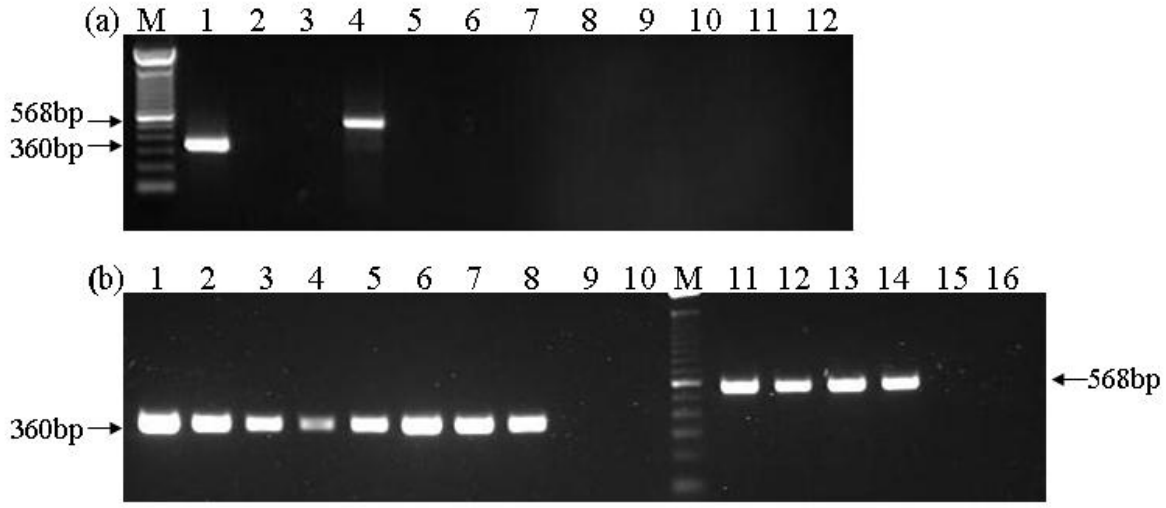


Fig. 3A.2. Specificity of nested polymerase chain reaction. (a) M: 100-bp marker; lane 1: Porcine circovirus 2 (PCV2) genotype 2b (EF452350) by PCV2b–specific primer set; lane 2: PCV-2b (EF452350) by PCV-2a–specific primer set; lane 3: PCV2a (EF452353) by PCV2b–specific primer set; lane 4: PCV2a (EF452353) by PCV2a–specific primer set; lanes 5, 6: Porcine circovirus 1 (PCV1); lanes 7, 8: Porcine parvovirus; lanes 9, 10: uninfected porcine kidney (PK)-15 cells; lanes 11, 12: distilled water by PCV2a– and PCV2b–specific primer sets. (b) Lanes 1–10: 8 PCV2b isolates of EF452351, EF452354, EF452356, EF452357, EF452359, EF452360, EF452361, EF452363, uninfected PK-15 cells, and distilled water by PCV2b–specific primer set; lanes 11–16: 4 PCV2a isolates of EF452352, EF452364, EF452365, EF452367, uninfected PK-15 cells, and distilled water by PCV2a–specific primer set.

and 2b-R primer set, but not with the PCV2a primer set. Porcine circovirus 2, genotype 2a (EF452353) was amplified only with the 2a-F and 2a-R primer set. PCV1 and PPV were not detected by any primer set, and neither the first, nor the second round primer sets cross reacted with any PCR reaction (Fig. 3A.2a). Four Minnesota PCV2a isolates (EF452352, EF452364, EF452365, and EF452367) were detected by the PCV2a–specific nPCR assay, and 8 Minnesota PCV2b isolates (EF452351, EF452354, EF452356, EF452358, EF452359, EF452360, EF452361, and EF452363) were detected by the PCV2b–specific nPCR (Fig. 3A.2b). There was no known nonspecific PCR product with these PCV2 isolates by cross reaction of the primer sets (data not shown).

3A.4.2. Sensitivity of nested polymerase chain reaction

The analytical sensitivity of the nPCR was examined by comparing the results by one-step and nPCR assays using viral DNA extracts from 10-fold serially diluted samples of the reference PCV2a and PCV2b. Both genotypes of PCV2 with an infectivity titer of $10^{4.0}$ $TCID_{50}$/ml were detected up to 10^{-5} dilution by 1-step PCR, whereas the viruses were detected up to 10^{-9} dilution by nPCR (Fig. 3A.3). When the nPCR was performed on 12 sera from 2 different farms with known clinical history, PCV2a was detected in 5 sera from a farm with no history of PMWS, while PCV2b was detected in 7 sera from another farm with clinical PMWS. However, in the 1-step PCR, only 5 of the 12 sera were positive (Fig. 3A.4.). One positive and 1 weak-positive band were detected with the 5 PCV2a–positive sera, and 1 positive and 2 weak-positive bands were detected with 7 PCV2b–positive sera. All of the 12 PCR-positive samples were sequenced, and their

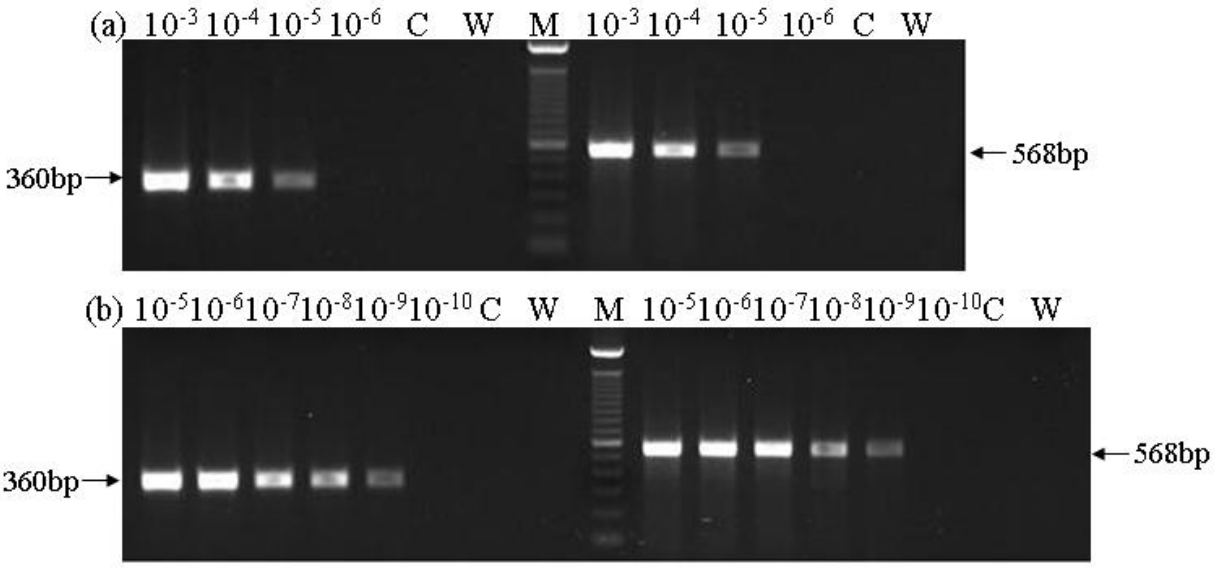


Fig.3A.3. Detection of PCV2 genotypes 2a and 2b in different dilutions. Both PCV2a (EF452353) and PCV2b (EF452350) were preadjusted to be $10^{4.0}$ $TCID_{50}$/ml. (a) One-step polymerase chain reaction (PCR); left 4 lanes: 10^{-3}–10^{-6} dilution of PCV2b (EF452350); right 4 lanes: 10^{-3}–10^{-6} dilution of PCV2a (EF452353). (b) Nested PCR; left 6 lanes: 10^{-5}–10^{-10} dilution of PCV2b (EF452350); right 6 lanes: 10^{-5}–10^{-10} dilution of PCV2a (EF452353). Lanes C and W in panels (A) and (B): uninfected porcine kidney (PK)-15 cells and distilled water by PCV2a– and PCV2b–specific primer sets.

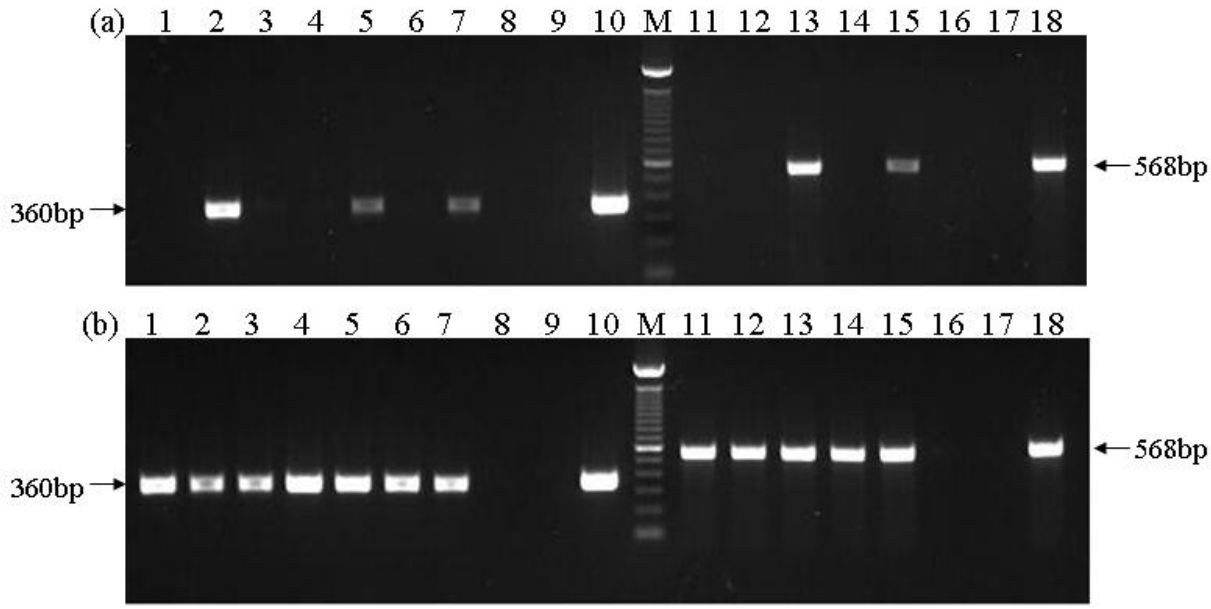


Fig.3A.4. Comparison of PCV2 detection by 1-step (a) and nested polymerase chain reaction (b) in serum samples. In panels (a) and (b), lanes 1–7: PCV2b–positive serum samples from a farm with postweaning multisystemic wasting syndrome (PMWS); lanes 11–15: PCV2a–positive samples from another farm without PMWS. Lanes 8, 16 and 9, 17 are uninfected porcine kidney (PK)-15 cells and distilled water, respectively. Lanes 10 and 18 are positive controls for PCV2b (EF452350) and PCV2a (EF452353), respectively.

genotypes were confirmed.

3A.4.3. Examination of field serum samples

Table 3A.1 summarizes the detection of PCV2 genotypes for 80 serum samples from 6 different swine farms. Of the 60 samples from farms A, B, C, and D with clinical PMWS, PCV2b was detected from 49 (81.7%) samples. Six samples from farm C were positive for both PCV2a and -2b. Of the 20 serum samples from farms D and E without PMWS, 11 samples were positive for PCV2a and none were positive for PCV2b.

3A.5. Discussion

Porcine circovirus–associated disease is now considered a global disease causing significant economic losses in the swine industry. In a case of PCVAD at the University of Minnesota research farm during mid-2005, 48 of 340 grower pigs (14.1%) died or were culled due to severe clinical signs of PMWS. PCV2 was isolated from the samples of pigs with PMWS signs. Since that time, samples from many farms both with and without PMWS have been tested to isolate PCV2 and to examine their genetic characteristics. Using a phylogenetic analysis, genetic similarity has been observed between the Minnesota isolates and various European strains in the Chapter 2 study. The PCV2 isolates and PCR-positive samples were classified into 2 genetic clusters. All of the 9 Minnesota PCV2 isolates in the lower cluster were from pigs with clinical PMWS. This finding led to an investigation of an association between the isolation of PCV2b and the presence of clinical PMWS on farms. The first step for the investigation was to develop a sensitive, specific, and differential PCR assay for the 2 genotypes

Table 3A.1. Differentiation between PCV2 genotypes 2a and 2b by nested polymerase chain reaction in serum samples collected from swine farms with a known clinical history of postweaning multisystemic wasting syndrome (PMWS).

Pig farms	Clinical PMWS	Year sample collected	No. of sera tested	PCV2 genotype detected		
				PCV2a	PCV2b	PCV2a+2b
A	Yes	2006	30	0	23	0
B	Yes	2006	10	0	10	0
C	Yes	2006	10	6	10	6
D	Yes	2006	10	0	6	0
E	No	2004	10	5	0	0
F	No	2004	10	6	0	0

Developing a differential PCR assay between PCV1 and PCV2 would be relatively easy as compared to developing an assay between PCV2a and PCV2b. PCV1 and PCV2 have higher genetic heterogeneity. For differential PCR between PCV2a and PCV2b, the main problem was that the choices of optimal positions for the primers were highly limited. Also, the designed primer set was able to amplify various nonspecific products due to the high level of genetic identity even with modifications in PCR conditions. Initially, nested differential PCR was performed in one tube, but the PCR reaction showed more than 2 product bands. The additional nonspecific band was believed to be due to cross reaction of the primers between 2 types of viral DNA. Therefore, the subsequent assays were carried out in 2 separate PCR tubes, each containing a specific primer set with 2 groups of viral DNA.

To develop a nPCR in the current study, a primer set for the first round PCR was designed on the outside positions of each group-specific primer and the designed primer sequences were further modified after comparing optimal PCR conditions. However, although the sensitivity of PCR was improved, nonspecific PCR products were amplified by both PCV2a– and PCV2b–specific primer sets in PCV-1 viral DNA, which had not been previously detected by conventional differential PCR. Therefore, the forward primer of first round PCR was replaced with another primer that can differentiate PCV-1 and -2, as found in a previous study (Lyoo et al., 2001).

The nPCR as compared to conventional PCR was more sensitive in the detection of PCV2 from serum samples as described by others (Larochelle et al., 2000; McIntosh et al., 2006b). The serum samples from PCV2–infected pigs were often negative by conventional PCR assay. The quantity of viral DNA in the sera was lower than in lungs

or lymph nodes by up to 1,000 times (Chung et al., 2005). The nPCR assay in the present study was highly sensitive, detecting up to 10^4 times more than conventional PCR. In the current study, conventional PCR detected PCV2 in only 5 (42%) of the 12 samples that tested positive by nPCR. This result agreed with a previous report showing that PCV2 from serum samples of 54% were detected by conventional PCR as compared to those with nPCR (Larochelle et al., 2000).

One great advantage of the nPCR described in the present study is that it can not only differentiate between the genotypes but can also detect dual infection of both genotypes (Table 3A.1.). In addition, the assay was found to be highly specific and no false-positive band was observed with PCV-1 or PPV (Fig. 3A.2.). Two genotypes of PCV2 were clearly differentiated by the nPCR, and there was complete agreement between the results by the nPCR and phylogenetic analyses with PCV2 isolates from the Minnesota swine farms in Chapter 2 study.

It is interesting to note that PCV2b was not detected in the farms without clinical signs of PMWS. In the farms with clinical PMWS, PCV2b alone or dual genotypes of PCV2 were detected. These findings were similar to the previous reports (Cheung et al., 2007; Gagnon et al., 2007; Horlen et al., 2007) of which observed that sudden appearance of PCV2b was associated with an increase in PMWS farms in North Carolina, Iowa, and Kansas, and suggested that the outbreaks of PCV2b was a concern for the swine industry (Cheung et al., 2007). Certainly there have been debates within the scientific community on the difference in virulence between the 2 genotypes of PCV2. Comparative experimental infection of PCV2a and 2b in susceptible pigs will be necessary to conclude any relation between the virulence and the genotype of PCV2.

In the present study, the main goal was to develop a nPCR assay for differentiation of the 2 genotypes of PCV2. It is believed that nPCR combines specificity and sensitivity, and reduces the labor and time-intensiveness of diagnosing PCV2a and -2b. It is interesting that, although limited serum sample was used in this study, PCV2b was detected in the sera from swine farms that were diagnosed with PMWS in 2006, while PCV2a was mainly detected in samples from the swine farms without PMWS in 2004. This finding could potentially initiate different studies using the nested differential PCR. An epidemiologic study with a large sample size will be needed to determine the relationship between the detection of PCV2b and the presence of clinical PMWS in swine farms. The prevalence of PCV2 genotypes can also be compared in sera from swine farms during PMWS outbreak and after the recovery. The nested differential PCR can be used in the pathogenesis studies with pigs following inoculation of 2 different genotypes of PCV2. Finally, nested differential PCR could be useful for examining semen samples in boar studs. In conclusion, nPCR promises to be a valuable tool for diagnosing PCV2 in pigs.

CHAPTER 3

SECTION 3B

Evaluation of a SYBR green real-time polymerase chain reaction assay for the quantification of porcine circovirus 2 genotype b in swine serum

3B.1. Summary

A real-time polymerase chain reaction (PCR) assay based on SYBR green was developed for the detection and quantification of *porcine circovirus-2* genotype b (PCV2b) infection in pigs, and the assay was evaluated by comparing the results with those of a nested polymerase chain reaction (nPCR) assay. Quantification of PCV2b was accomplished by a standard curve plotting cycle threshold values (Ct) against each dilution of the constructed plasmid DNA. The standard curve was obtained from 4.3 $x10^1$ to 4.3 x 10^9 copies per serum sample. The real-time PCR assay was performed to evaluate using 120 sera that were collected randomly from 20 pigs each at 5, 7, 9, 11, 13 and 15 weeks of age in a farm with severe clinical PMWS. Of the 120 sera, PCV2b was detected in 98 (82%) and 79 sera (66%) by real-time PCR and nPCR assays, respectively. These results indicate that the real-time PCR assay was more sensitive than nPCR and can be a useful diagnostic method for the detection and quantification of PCV2b in swine serum samples.

3B.2. Introduction

Porcine circovirus 2 (PCV2) is a very small, non-enveloped DNA virus, and is the major causative agent for post-weaning multi-systemic wasting syndrome (PMWS) in pigs (Allan and Ellis, 2000; Segales et al., 2005; Opriessnig et al., 2007). It has been demonstrated that quantity of PCV2 genome in tissue samples from the affected pigs has been highly associated with the severity of PMWS, and the use of a viral genomic quantification test has become important in the diagnosis of PMWS (Brunborg et al., 2004; Olvera et al., 2004). Real-time polymerase chain reaction (PCR) assay could estimate the numbers of the genomic copies for microorganisms, and it is generally considered to be more sensitive, rapid, and less labor required than conventional PCR assay (Mackay et al., 2002; Olvera et al., 2004). Previously, several quantitative PCR methods for PCV2 were developed based on the specific oligonucleotide probes labeled with fluorescent dyes or SYBR green (Brunborg et al., 2004; Olvera et al., 2004; Chung et al., 2005; McIntosh et al., 2009).

Both genotypes of PCV2a and PCV2b have been reported in association with clinical PMWS in pigs. However, PCV2b has been involved more commonly in pigs with PMWS and showed higher virulent than PCV2a (Gagnon et al., 2007; Dupont et al., 2008; Grau-Roma et al., 2008; Lyoo et al., 2008). Therefore, swine practitioners have requested frequently to differentiate between the two PCV2 genotypes for their samples from PMWS-affected farms. A nested PCR (nPCR) assay was previously developed and evaluated to differentiate between the two genotypes (Lyoo et al., 2008). In this study, a SYBR green real-time PCR specific for PCV2b genotype was developed, and the results were compared with those by the nPCR assay using the serum samples from a swine farm

during severe clinical PMWS. Although a PCV2 SYBR green real-time PCR assay was reported recently (McIntosh et al., 2009), the assay was not aimed to differentiate between the two genotypes of PCV2.

3B.3. Material and Methods

3B.3.1 Samples

A panel of sera was collected, for this study, from pigs of a farm located in a Minnesota during a severe clinical PMWS outbreak in 2006. The farm had mortality ranges of 4-6% in the nursery and 8-20% in the grow/finish pigs. Twenty sera each, that had been collected on a same day from randomly selected pigs at 5, 7, 9, 11, 13, and 15 weeks of age and stored at -80℃, were used in the evaluation.

3B.3.2 Nested PCR

The nPCR assay was conducted by the method as described previously (Lyoo et al., 2008). The first round PCR was performed using VF-2 (forward) and nest-R (reverse) primer set. A final volume of 25 contained 2 of extracted viral DNA, 5 5X GoTaq® Green Mater Mix (Promega Corp., Medison, WI) containing 7.5 mM $MgCl_2$, 1 pM of each primer, 0.25 mM dNTPs, and 1U of GoTaq polymerase (Promega Corp., Medison, WI), were adjusted with DNase free distilled water to make the final volume. The amplification was undertaken using a thermal cycler, and the cycling profile was in the following order of 5 min denaturation at 94℃, 30 repetitive cycles of 30 sec of

denaturation at 94℃, 30 sec of annealing at 54℃, and 40 sec of elongation at 72℃. Elongation was extended for 7 additional min in the last cycle.

For PCV2a and 2b specific nPCR, the amplified product of the first round PCR was reacted with PCV2a specific primer set; 2a-F (forward) and 2a-R (reverse), and PCV2b specific primer set; 2b-F2 (forward) and 2b-R (reverse). The primary amplification product of 1 was added to a new PCR formation described above in separated individual tube. The second round PCR step was performed with the conditions: 5 min denaturation at 94℃, 30 repetitive cycles of 30 sec of denaturation at 94℃, 30 sec of annealing at 57℃, and 30 sec of elongation at 72℃. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis and an UV transmitter.

3B.3.3. Quantitative real-time PCR

For PCV2b specific real-time PCR, a PCV2b strain (GenBank #EF452350) was propagated in PCV free PK-15 cells, and genomic DNA was extracted using DNeasy blood and tissue kit (Qiagen, Inc., Valecncia, CA). The primer set described above for 2b-F2; 5'-CTGTTTTCGAACGCAGTG-3', and 2b-R; 5'-CTCAAACCCCCGCTCTG-3', targeting the ORF2 gene was used, and DNA fragment of 361 base-pair was amplified by above procedure for nPCR assay. The amplified products were subcloned into pGEM-T Easy vector (Promega Corp., Medison, WI), and the plasmid was transformed into JM109 cells according to the manufacture's protocols. Nucleotide sequence analysis of the positive clones was performed on both strands by automated DNA sequencing (BioMedical Genomic Center, University of Minnesota, St. Paul, MN).

The concentration of the plasmid was determined by measuring the optical density (OD) at 260 nm. Conversion to genome equivalents was calculated with 1 OD at 260 nm equaling 50 g/ml and 1 bp equaling 660 g/mol, and the molecular weight for the plasmid (3,376 bp) was 2.2 x 10^6 g/mol. The copy numbers/ were determined by using the following formula: copies/ = concentration of plasmid in g per / (plasmid length x 660) x 6.022 x 10^{23}.

The constructed plasmid was then serially diluted by 10-fold, and real-time PCR reaction was performed to establish a standard curve. Two of the DNA plasmid stock or DNA of the same sera used in nPCR and 4 pmol of each primer (2b-F2 and 2b-R) were reacted with 15 of 2X real-time PCR master mix; PerfeCTa SYBR Green SuperMix (Quanta Bioscience, Gaithersburg, MD), UNG, Low ROX containing $MgCl_2$, dNTPs, AccuStart Taq Polymerase (Quanta Bioscience, Gaithersburg, MD), UNG, SYBR Green I dye, and ROX reference dye. Final volume of 30 was adjusted with DNase free distilled water. The mixture was reacted following conditions; 10 min denaturation at 95 ℃, followed by 40 cycles of 30 sec denaturation at 95℃, 30 sec annealing at 55℃, and 30 sec extension at 72℃. A dissociation curve was performed after amplification by a gradual rise in one-degree increments temperature from 54℃ to 95℃, and fluorescence signal was measured every 0.5℃. Real-time PCR reactions were performed in a thermal cycler and fluorescence detection system; Mx3005P (Stratagene, La Jolla, CA). Fluorescence normalization and data analysis were performed by thermal cycler program software; MxPro-Mx3005P v.3.00 (Stratagene, La Jolla, CA).

All real-time PCR reactions were performed in triplicate in neighboring wells. In order to examine the specificity, the real-time PCR was performed for porcine parvovirus (PPV), porcine circovirus 1, and PCV2a (EF452353), and uninfected PK-15 cells by the above procedure. The 10-fold diluted plasmid was reacted by nPCR testing comparative sensitivity with real-time PCR assay.

3B.4. Results

The recombinant plasmid was constructed as the standard of PCV2 genomic DNA to establish the standard curve calculation in quantitation assays. Based on the molecular weight of the constructed plasmid (3,376bp), the amount of input template copy number was calculated and the over range of from 4.3×10^1 to 4.3×10^9 copies was obtained from the linear standard curve per reaction mixture for PCV2b, which resulted in cycle threshold (Ct) values from 38.5 to 10.8 cycles (Fig. 3B.1.). The calculated real-time PCR efficiency, based on the slope value of -3.49 with the correlation value (R^2) of 0.989, was 93.4%. Specific peaks of the melting temperature were occurred between 81.5℃ and 82.1℃. The dilution factor from the original serum sample to real-time PCR reaction was taken into consideration when calculating PCV2b copy numbers. In comparison between real-time PCR and nPCR, detection limit of nPCR method and real-time PCR were 4.3×10^4 and 43 copies of the constructed plasmid, respectively (Fig. 3B.2). There was no reaction when the specificity was evaluated with PPV, PCV-1, PCV2a, and PK-15 cells under the same real-time PCR conditions. Seventy nine (66%) and 13 (11%) of 120 sera were positive for PCV2b and 2a genotype by nPCR, respectively, whereas 98 (82%) sera shown as PCV2b positive by real-time PCR assays. A comparative result between the

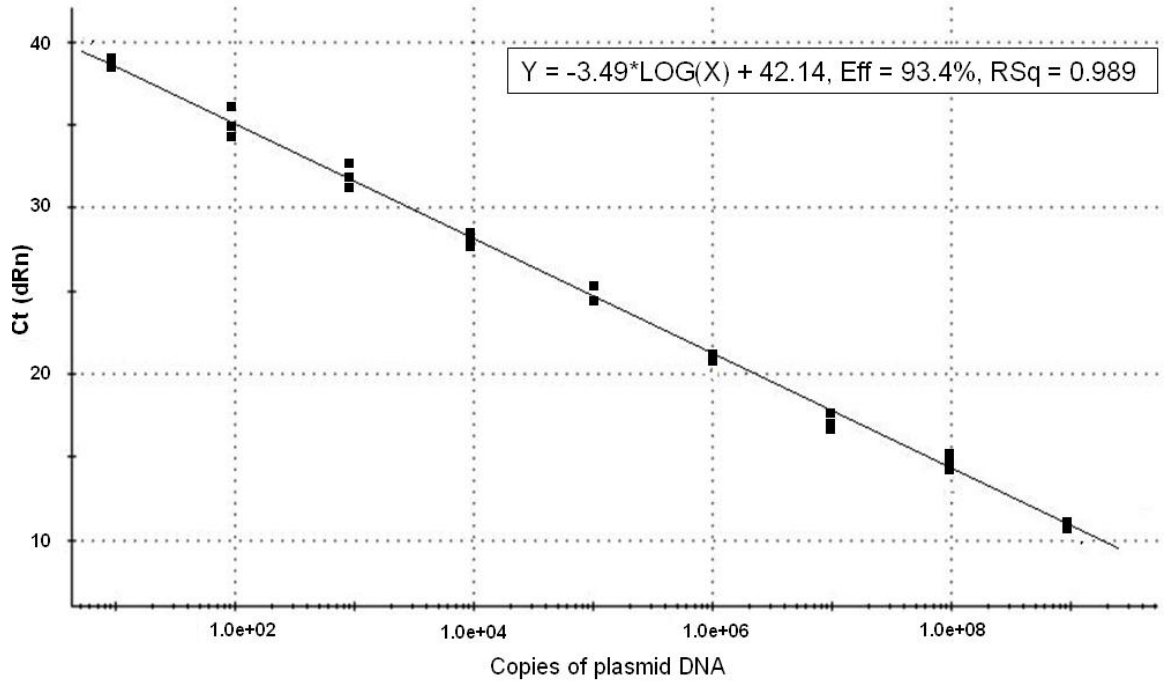


Fig. 3B.1. A standard curve plot using PCV2b 10-fold serially diluted plasmid DNA by SYBR green real-time PCR reactions. The x-axis represents copies of plasmid DNA in 10-fold dilutions and the y-axis the fluorescence data used for cycle threshold (Ct) determinations in dRn (baseline-corrected normalized fluorescence). The assays were linear with R2 values (square of the correlation coefficient) of 0.989 and reaction.

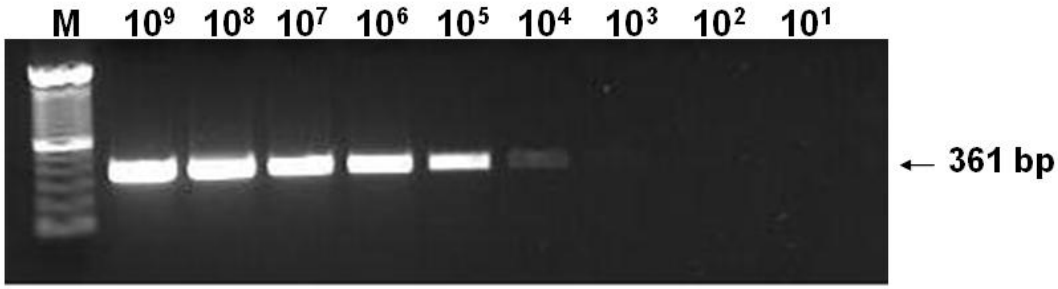


Fig. 3B.2. Sensitivity of nPCR for detection of the 10-fold diluted plasmid. The detection limit for the assay was 4.3 x 10^4 copy numbers in a serial 10-fold dilution pattern as observed by using a 361 bp amplicon. Lane M; 100-bp DNA ladder.

.

percentage of PCV2 viremic pigs by nPCR or real-time PCR, and the mean genomic copy number of PCV2b by real-time PCR using the same samples is shown in Table. 3B.1. The copy number of PCV2b DNA ranged from 3.66 $\log_{10}$ to 10.01 $\log_{10}$ in 1ml. serum sample. All negative samples by real-time PCR assay were also negative by nPCR reaction

3B.5. Discussion

Different epidemiologic studies for PCV2 infection showed that the prevalence of PCV2b was higher than PCV2a in swine farms with clinical PMWS (Gagnon et al., 2007; Olvera et al., 2007; Dupont et al., 2008; Grau-Roma et al., 2008; Takahagi et al., 2008; Wiederkehr et al., 2008). Higher detection rates with PCV2b (≥70%) than PCV2a (<10%) infection were also observed in sera from Minnesota pig farms between 2006 and 2008 in a different study by this laboratory (*data not shown*). In addition, some researchers suggested that PCV2b might be more virulent than PCV2a genotype in developing PMWS (Gagnon et al., 2007; Dupont et al., 2008; Grau-Roma et al., 2008). In the current study, the difference of infection rate between PCV2 genotypes was also examined in sera from a PMWS diagnosed farm. The higher prevalence of PCV2b in association with PMWS had initially led to develop a quantitative real-time PCR assay for specific detection of PCV2b genotype.

Most published real-time PCR assays for PCV2 were aimed at quantification of PCV2 without differentiation between the two genotypes. A multiplex real-time PCR assay was recently developed to differentiate the two genotypes (Gagnon et al., 2008). However, the present SYBR green real-time PCR is known to be relatively simple to

Table 3B. 1. Number and percentage of PCV2a and 2b viremic pigs detected by nested PCR (nPCR) or real-time PCR (qPCR), and mean DNA copy number per serum sample of PCV2b detected by qPCR in the sera. The sera were from 20 pigs with different age groups from a farm during the outbreaks of clinical PMWS

	Age of pigs (weeks)						Total	
	5	7	9	11	13	15	Positive (%)	Negative
nPCR-PCV2a	0	1	3	4	2	3	13 (11)	107
nPCR-PCV2b	9	13	14	17	15	11	79 (66)	40
qPCR-PCV2b	14	17	18	18	16	15	98 (82)	22
Mean DNA copy number (log_{10})	2.9	5.5	5.3	5.9	4.1	3.6	Total =120	

perform and less expensive when compared to the probe type in the multiplex PCR. In this real-time PCR, the primer set used was identical to those of the nPCR assay (Lyoo et al., 2008), and the same PCR conditions were applied in the comparative sensitivity study.

The results showed that the real-time PCR was found to be more sensitive than the nPCR assay. The PCV2 loads were increased in the sera of pigs from the age of 5 weeks to 11 weeks, and then decreased in the pigs after 11 weeks of age in a previous study (Carasova et al., 2007). A similar tendency of the viral load and the percent of viremic pigs were observed in this study. The real-time PCR, however, detected more positive samples (82%) than nPCR did (66%) in all sera tested. The higher sensitivity of the real-time PCR is believed to be due to the higher sensitivity of 1.000 times then nPCR. In order to make a diagnosis for clinical PMWS, it was suggested to detect PCV2 with $\geq 10^7$ $\log_{10}$ genomic copies per one milliliter in the serum (Olvera et al., 2004). Although the minimum viral genomic copies for the diagnosis of clinical PMWS were discussed in different studies (Segales et al., 2005; Carasova et al., 2007; Opriessnig et al., 2007), the copy values in those studies were estimated by genomic copies from the combination of both PCV2a and PCV2b. Therefore, it may be necessary to reevaluate the diagnostic values with only PCV2b infection for the PMWS diagnosis in the future.

The PCV2 genomes have been tested using sera as well as different tissue samples from pigs with clinical PMWS. In general, the PCV2 genome copies were higher in sera, lymph nodes, and feces than other tissues from PCV2 infected pigs (McIntosh et al., 2009). Therefore, only serum samples were used to test in this study. It is believed that serum alone could routinely be used as a diagnostic sample for PCV2b infection and

that the detection of PCV2b alone might be significant in the diagnosis for clinical PMWS.

In conclusion, a SYBR green based real-time PCR assay was developed for the detection and quantification of PCV2b in swine sera. The assay was found to be more sensitive than nPCR when compared each other using the same PCR primer set. The real-time PCR assay specific for PCV2b appears to be more useful in the diagnosis of PMWS because PCV2b is more closely associated with clinical PMWS.

CHAPTER 3

SECTION 3C

Standardization of serum neutralization assay and indirect fluorescent antibody assay for the detection of antibody to porcine circovirus type 2 in pig sera

3C.1 Summary

Different serologic assays have been developed and modified to improve the sensitivity and the specificity for detection of PCV2 antibody in pigs. However, little information is available on the test procedure for serum neutralizing (SN) antibody and its antibody response in PCV2 infected pigs. The purposes of the present study were to standardize the SN test along with the indirect fluorescent antibody (IFA) assay and to examine the humoral antibody response in pigs. The SN test was standardized by comparing different conditions on the virus genotype used, infectivity of the virus input in the test, incubation time for virus-serum mixture and interpretation of the SN titers. In order to understand the antibody response, 16 PCV2 free piglets were divided into 4 equal groups, and 4 pigs in each group were inoculated with one of the 3 different tissue extracts from PCV2 infected pigs. The remaining 4 pigs were served as controls. Both SN and IFA antibodies were detected in the pigs from 14 days post inoculation (dpi). Then, high levels of the IFA titer were maintained until the end of the study, while the SN antibody titers were peaked at 42 dpi and were decreased from 56 dpi. These results indicated that the standardized SN method is useful in detecting neutralizing antibody to PCV2.

3C.2. Introduction

Porcine circovirus type 2 (PCV2) has been recognized as an important agent of postweaning multisystemic wasting syndrome (PMWS) in pigs (Segales et al., 2005; Opriessnig et al., 2007). The PMWS affected pigs show wasting, respiratory distress, occasional diarrhea, enlarged lymph nodes, and icterus (Allan et al., 1998; Allan and Ellis, 2000; Segales et al., 2005). The detection of PCV2 antibody has been commonly performed by different serologic methods including immunoperoxidase monolayer assay (IPMA), indirect fluorescent antibody (IFA) test, enzyme-linked immunosorbent assay (ELISA), and serum neutralization (SN) test (Allan et al., 1998; Ellis et al., 1998; Pogranichnyy et al., 2000; Blanchard et al., 2003; Liu et al., 2004; Meerts et al., 2006; Opriessnig et al., 2007;).

PCV2-infected PK-15 cell monolayer that was fixed with an equal volume of acetone and ethanol was used for IFA and IPMA. The ELISA can be performed by using purified and expressed proteins of PCV2 (Vincent et al., 2003). A SN test was developed and has been used in different studies in Spain (Fort et al., 2007). The role of SN antibody against PCV2 appeared to be important for understanding the clinical development of PMWS (Meerts et al., 2006). In the experimental studies, significantly higher SN titers were detected in subclinical pigs, while PMWS affected pigs had low SN titers (Fort et al., 2007).

It is important to establish a standard protocol for the PCV2 SN test because limited information is available on each test procedure. Only a few groups of researchers have extensively investigated the SN test and its antibody response in pigs (Meerts et al., 2006; Fort et al., 2007). The objectives of this study were to modify and standardize the

SN and IFA methods and to apply the tests to understand the antibody response in experimentally inoculated pigs. In addition, an antibody test for PCV1 was evaluated using the sera of pigs inoculated with PCV2, since there was a low level of cross-reaction of the antibodies between PCV1 and PCV2 on the both assays (Pogranichnyy et al., 2000).

3C.3. Materials and Methods

3C.3.1. Test sera

Experimental sera were collected from pigs that were inoculated with a tissue extract containing PCV2 in a previous study. In the study, a group of pigs were farrowed from PCV2 antibody positive sows and raised in an isolated farm without PCV2 infection until their maternal antibody became undetectable. Sixteen pigs were randomly divided into 4 treatment groups A, B, C, and sentinel. All pigs were confirmed to be negative for PCV2 and its antibody before inoculation. The tissue extracts were used because PCV2 culture was not available at the time of experiment. Three different tissue extracts were prepared by pooling of lung, spleen, and lymph nodes from each of the 3 pigs that were collected from 3 different farms with history of PMWS, and 10% tissue homogenate was prepared from each pooled tissue sample.

Pigs in group A, B, C (n = 4 each) were inoculated directly to sub-inguinal lymph nodes of pigs with 1ml each of the 3 tissue extract preparations containing PCV2. A sentinel group (n = 4) was maintained without any treatment and served as controls. Blood samples were collected from each pig for the detection of PCV2 and its antibodies

at 0, 14, 28, 42 and 56 dpi. The tissue extracts were confirmed positive for PCV2 but negative for PCV1 and PRRSV by PCR assays.

3C.3.2. IFA and serum neutralizing test

PCV2 IgG antibody titers were measured by an IFA assay by a routine method. Briefly, the test plates were prepared by inoculating PK-15 cells with a PCV2 reference strain (GenBank session number EF452350) in 96-well microtiter plates. The plates were incubated at 37℃ for 72 hours and fixed with ethanol and acetone mixture of 1:1. Each of the test sera was diluted in 4-fold serially from 1:4 to 1: 16,384 and transferred to the test plate and incubated at 37℃ for 45 min. After washing 3 times with PBS, the plate was reacted with rabbit anti-swine IgG conjugated with FITC at 37℃ for 45 min. The plate was washed again and examined under a fluorescent microscope.

The procedure of the SN test reported by Fort et al., 2007 was slightly modified. In the modified test, test sera were not heat inactivated, the challenge virus used was 400 $TCID_{50}$/well, and the results were read 72 hours after the incubation. Serum samples were serially diluted in 2-fold from 1:2 to 1:1024 in a 96-well microtiter plate, and an equal amount of a PCV2b (Genbank accession number EF452350) reference strain (400 $TCID_{50}$/well) was added. The serum and virus mixtures were incubated at 37℃ for 1 hour, and PK-15 cells (2×10^5 cells /ml) were added to each well of the plate. The plate was fixed after 72 hours of the incubation, and a high PCV2 antibody positive reference serum (1:20 dilution in PBS) was reacted to all wells. Then, PCV2 positive cells were detected by IFA method. The SN antibody titers were calculated by the 50 percentage

reduction method. PCV2 positive cells were counted in 3 randomly selected fields in each well under a fluorescent microscope (Fig. 3C.1.). The calculating formula was % SN = [1- (mean number of positive cells in each serum dilution level / mean number of positive cells in negative control wells)] 100. The SN titer was determined at the last dilution when the number of positive cells of a given serum sample was reduced by 50% in a well compared to the numbers in the negative control wells.

3C.3.3. Standardization of serum neutralizing assay

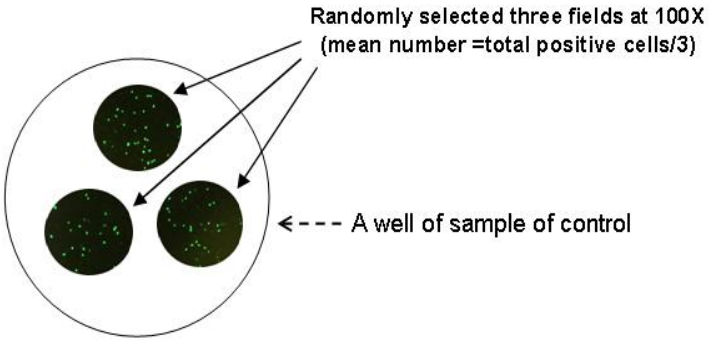
The SN method was based on a previous protocol by Fort et al., 2007, and the method was evaluated and compared using different conditions including heat inactivation of the test sera, virus genotypes (PCV2a - EF452353 and PCV2b - EF452350) used in the test, input of the challenge virus titers (200, 400 or 800 $TCID_{50}$), incubation time of the virus-serum mixture, and determination of the titer under fluorescent microscope.

3C.4. Results

3C.4.1. Standardization of serum neutralization assay

An illustration of the SN titer determination using the modified method is shown in Fig 3C.1. Comparisons of the SN titers using different PCV2 genotypes, input of PCV2 infectivity titers and the incubation times of serum-virus mixtures are shown in Tables 3C.1 and 3C.2. In the standardization of the test, PCV2 SN reference sera of 6 positive (2 sera each of 1:16, 1:64 and 1:256) and 2 negative sera were used. For the

(A)



(B)

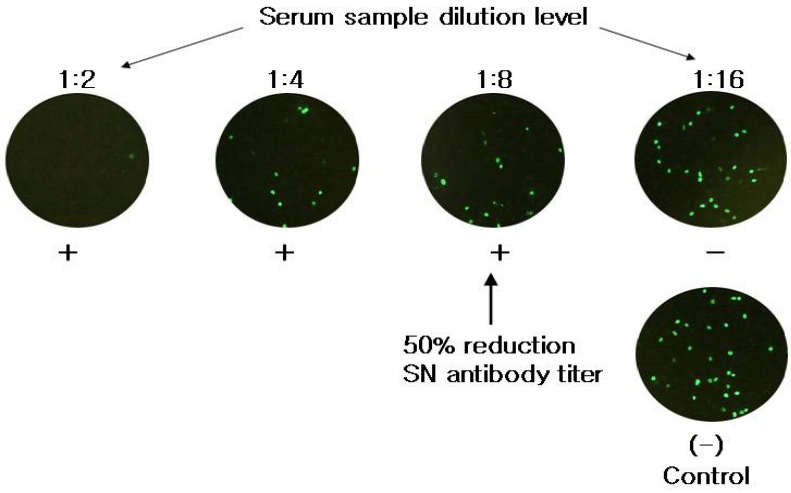


Fig.3C.1. An example of PCV2 SN titer determination by counting mean numbers of PCV2 positive cells. (A) Three fields were randomly chosen in a well, and the mean numbers between test sample and control wells were counted for a SN titer. (B) An example of SN titer determination. The numbers of positive cells were less than 50% of the control well at the dilution of 1:8. Therefore, the SN titer is determined to be 1:8.

Table 3C.1. Comparison of PCV2 SN titer between different virus inputs and incubation for 1 hour at 37℃

PCV2 genotype & virus input ($TCID_{50}$)		PCV2 SN antibody reference sera 1[a]	2	3	4	5	6	7	8
PCV2a	200	7.0	6.5	7.0	8.0	8.0	8.0	-	-
	400	4.0	3.5	5.5	6.0	8.0	8.0	-	-
	800	3.5	4.0	6.0	5.0	7.5	8.0	-	-
PCV2b	200	6.0	8.0	8.0	7.5	8.0	8.0	-	-
	400	4.0	4.0	6.0	6.5	8.0	7.5	-	-
	800	3.0	3.0	6.5	5.5	7.0	8.0	-	-

a: PCV2 SN reference sera (1- 6; SN antibody positive, 7-8; SN antibody negative sera).

Table 3C.2. Comparison of PCV2 SN titers among incubation times of the serum-virus mixture using virus input of 400 $TCID_{50}$

Serum-virus mixture	PCV2 SN antibody reference sera							
incubation time	1[a]	2	3	4	5	6	7	8
PCV2a 1 hour at 37℃	3.5	3.0	6.0	6.0	7.0	8.0	-	-
2 hours at 37℃	4.0	4.0	6.5	7.0	8.0	8.0	-	-
12 hours at 4℃	3.0	4.5	6.5	5.0	7.5	8.0	-	-
PCV2b 1 hour at 37℃	4.5	3.0	5.5	6.5	8.0	7.0	-	-
2 hours at 37℃	3.5	4.5	6.0	6.0	7.5	8.0	-	-
12 hours at 4℃	4.0	4.0	6.0	6.0	8.0	8.0	-	-

a: PCV2 SN reference sera (1- 6; SN antibody positive, 7-8; SN antibody negative sera).

37℃, and 12 hours at 4℃. No difference in the SN titers was observed when PCV2a or PCV2b genotype was used in the test. Also, there was no difference in the SN titers when a group of sera was tested before and after heat inactivation at 56℃ for 1 hour. Therefore, the use of PCV2a or 2b with 400 $TCID_{50}$/well and incubation time of 1 hour at 37℃ for serum-virus mixture was the choice in the standardized test.

challenge virus titer in the SN test, 200 $TCID_{50}$/well yielded too few positive cells to be counted, while similar numbers of positive cells were counted when PCV2 of 400 or 800 $TCID_{50}$/well were used. In regard to the incubation times of serum-virus mixtures, there were no differences in the SN titers among incubation times of 1 hour at 37℃, 2 hours at

3C.4.2. Antibody response by IFA and SN tests

Antibody responses by IFA and SN tests after an inoculation in PCV2-free pigs with each of the tissue extracts containing PCV2 are shown in Table 3C.3 and Fig. 3C.2. The IFA and SN antibodies were detected from 14 days post-inoculation (dpi) in the inoculated groups A, B, and C, and from 28 dpi in the sentinel group. There was no difference in the mean IFA and SN titers among the 3 inoculated groups of A, B, and C. The highest IFA titers in groups A, B and C were recoded at 28 dpi, and the high antibody levels were maintained until the end of the study, while SN antibody titers increased until 42 dpi and then decreased through to 56 dpi.

A comparison of IFA titers to PCV2 and PCV1 is illustrated in Fig 3C.3. The results show that PCV2 specific antibodies were detectable in pigs at 14 dpi, while PCV1 specific antibodies were detectable in pigs at 28 dpi. Average PCV1 specific IgG antibodies remained lower than those of PCV2 during the experimental period. These results indicate that there was a partial serologic relationship between PCV2 and PCV1.

3C.5. Discussion

PCV2 has now been accepted as the main causative agent of PMWS. However, the disease mechanism has not been fully characterized (Segales et al., 2005). Recently,

Table 3C.3. Detection of antibody to PCV2 in challenged and sentinel pigs by IFA and SN assay

Group	Pig No.	Days post inoculation 0	14	28	42	56
Cont	1	-/-*	-/-	6/1	>14/4	10/6
	2	-/-	-/-	-/-	12/3	>14/4
	3	-/-	-/-	-/-	12/3	nt/nt
	4	-/-	-/-	-/-	12/2	>14/5
A	5	-/-	6/2	12/3	12/4	>14/3
	6	-/-	10/2	>14/4	12/6	>14/3
	7	-/-	-/-	12/5	10/8	10/2
	8	-/-	6/3	10/2	>14/5	12/3
B	9	-/-	4/1	12/4	12/6	10/2
	10	-/-	10/2	10/4	nt/nt	12/5
	11	-/-	10/2	>14/3	>14/5	>14/4
	12	-/-	-/-	12/4	10/6	8/3
C	13	-/-	-/1	8/4	10/6	8/4
	14	-/-	6/3	8/5	>14/8	12/5
	15	-/-	8/2	10/2	8/4	>14/3
	16	-/-	10/3	>14/4	>14/8	>14/5

* Reciprocals of IFA/SN antibody titers (log 2) nt = not tested

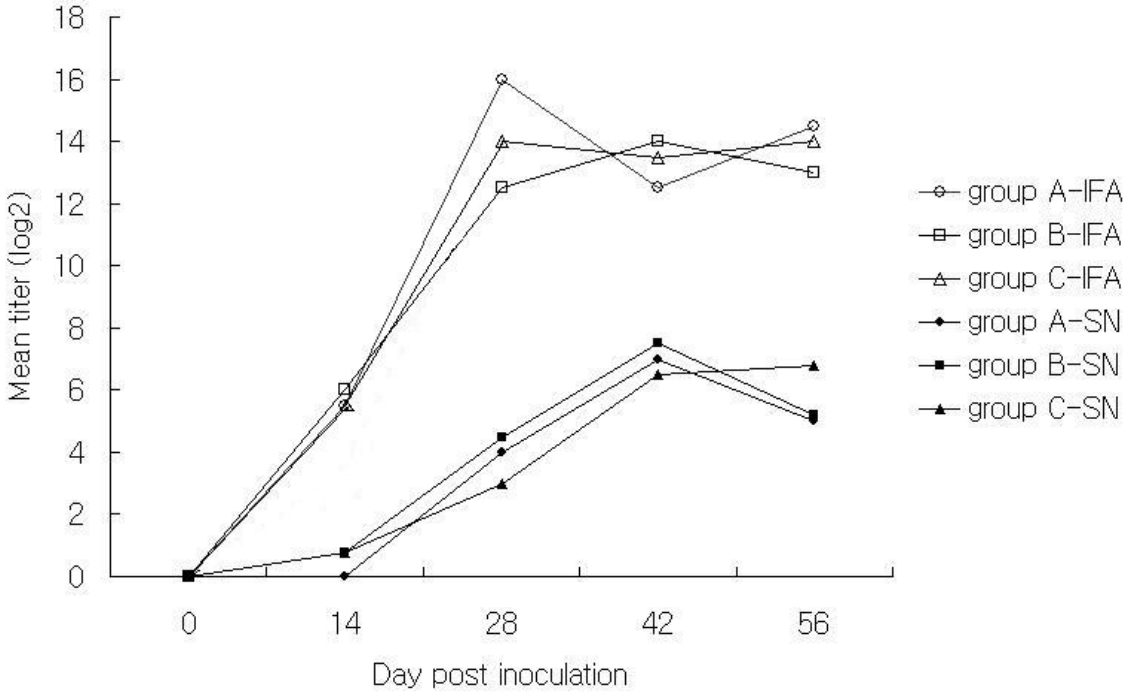


Fig. 3C.2. Mean IgG IFA titer and SN antibody titer against PCV2 in experimentally inoculated pigs. Blood was collected from pigs from 0 to 56 DPI. Antibody titers for each group (A, B & C) of pigs inoculated with PCV2 tissue extract were determined by IFA and SN assay. Number of pigs in each group was 4.

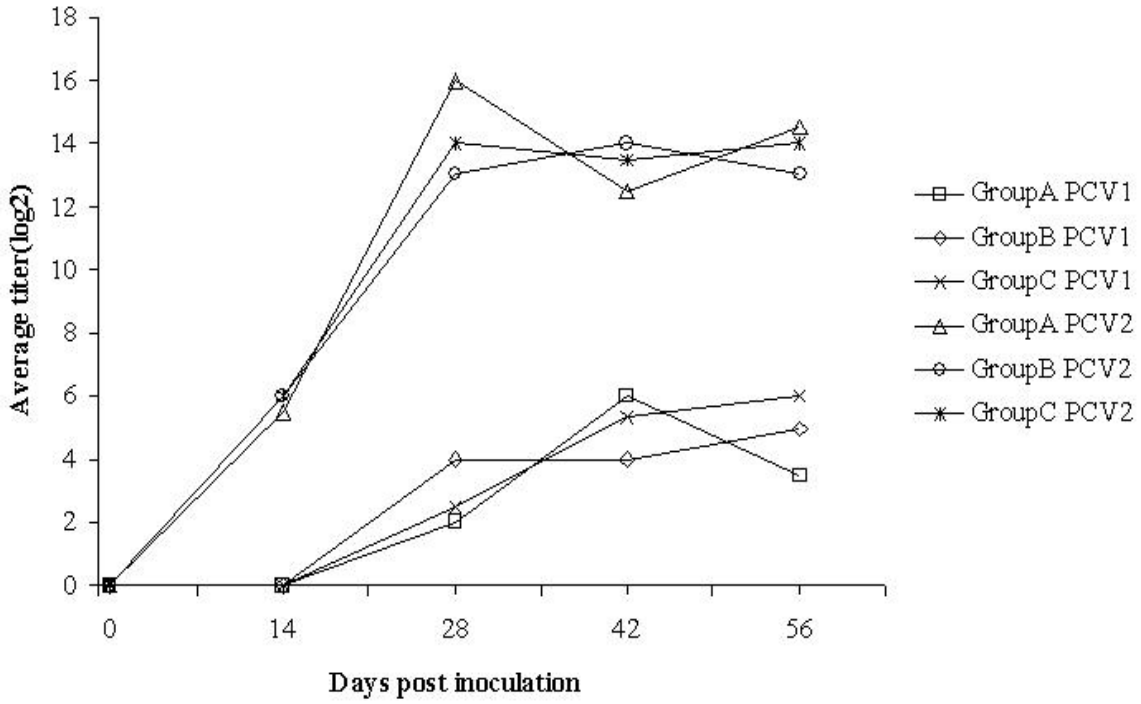


Fig. 3C.3. Mean IFA antibody titers (log2) against PCV1 and PCV2 in pigs inoculated with each of 3 different PCV2 tissue extracts. Blood was collected from pigs from 0 to 56 days post-inoculation. Number of pigs in each group was 4.

the role of neutralizing antibody was demonstrated in that there was a correlation between the SN antibody titers and PCV2 viremia levels in PMWS affected pigs (Meerts et al., 2006). Therefore, a PCV2 SN antibody assay using IPMA method was developed and has been used in Europe (Fort et al., 2007). In addition, it has been shown that PMWS affected pigs lack or have low neutralizing antibody titers to PCV2, while high levels of the antibody titer are observed in subclinically infected animals (Fort et al., 2007). Therefore, routine use of the SN test would help in understanding the epidemiology of PCV2 infection. However, there has been no standard protocol to perform the test, and the antibody response in pigs following PCV2 infection has not been evaluated by standard IFA or SN method.

In this study, we attempted to modify and standardize the SN assay that was initially described by Fort et al. 2007. The modification was made using the virus input of 400 $TCID_{50}$/well and the incubation time of 1 hour at 37℃. The end point SN results were read by IFA rather than IPMA method. The test sera were not heat inactivated, although there may be a concern that the SN results could be falsely calculated if a test serum was contaminated with live PCV2. However, replication of the PCV2 in the test sera and detectable by IFA after incubation for 72 hours would be almost impossible, because at least several passages for PCV2 viremic sera were necessary to be detectable by a fluorescence according to our experiences. This was also discussed by a personal communication with Dr. Fort in Spain, and the author agreed with our opinion. The author also described that serum samples with the highest PCV2 DNA copy number were incubated into PK-15 cells but the cells were not infected and there was no significant difference in the SN titers in a comparison between heated and non-heated sera.

Therefore, PCV2 in the test will not be inactivated by heat at 56℃ for 30 minutes. In conclusion, the modified SN method was successfully developed in the present study and evaluated to be useful in the serodiagnosis for PCV2 infection in swine.

CHAPTER 4.

EPIDEMIOLOGIC OBSERVATION OF PCV2 GENOTYPE INFECTION AND SEROPREVALENCE IN SWINE FARMS DURING AND AFTER CLINICAL OUTBREAKS OF PMWS

4.1. Summary

Commercial vaccines against PCV2 have now been widely used in pig farms in the United States, and the vaccines have been demonstrated to remarkably reduce the pig mortality associated with PMWS. It has been suggested that PCV2 viremia and serum neutralizing (SN) antibody can be markers for the protection against PMWS. The objectives of this study were to compare the viremia and antibody responses in pigs of the farms during the outbreak of clinical PMWS and after the outbreak with no clinical disease. Totals of 230 and 322 sera from 4 to 16 weeks age pigs were collected from 5 different farms during PMWS outbreak in 2006 and after the outbreak in 2008, respectively. The viremia was tested by a differential nested PCR assay, and the antibody levels were tested by both SN and IFA tests. From the sera of 230 in 2006 with clinical PMWS, PCV2a and 2b were detected in 25 (10.9%) and 150 sera (65.2%), respectively. From the sera of 322 in 2008 without clinical PMWS, PCV2a and 2b were detected in 8 (2.5%) and 35 sera (10.8%), respectively. Mean log 2 SN titers of the sera in 2006 and 2008 were 1:2.3 and 1:12.0, respectively. These results indicated that farms with clinical PMWS showed high percentages of PCV2 viremia, especially PCV2b, and low SN titers. These results suggested that either the levels of viremia or the SN antibody level could be used as a risk factor for clinical development of PMWS in swine farms.

4.2. Introduction

Postweaning multisystem wasting syndrome (PMWS) has been demonstrated as an economically important disease. The syndrome has been clinically manifested by wasting, respiratory complication, and high mortality (Opriessnig et al., 2007; Madec et al., 2008). Porcine circovirus type 2 (PCV2) has been identified as the main cause for the PMWS (Allan et al., 1998).

Commercial vaccines against PCV2 have now been widely used in pig farms in the United States (Ramamoorthy and Meng, 2008). The vaccines have been demonstrated in reducing the mortality and protecting the development of PMWS. The vaccines also caused significant improvement of the growth performance in pigs (Blanchard et al., 2003; Fenaux et al., 2004b; Opriessnig et al., 2004; Horlen et al., 2008;). In PCV2 experimental challenge studies, the vaccines reduced viremia and induced a strong antibody response (Blanchard et al., 2003; Fenaux et al., 2004a). The serum neutralization (SN) antibody, particularly, of the vaccinated animals was increased when compared to that of non-vaccinated animals, although the seroconversion of maternal antibody might be influenced in the antibody response (Meerts et al., 2006; Fort et al., 2007).

It has been demonstrated, therefore, that PCV2 SN antibody titer could be used as a marker for the protection of PMWS in pigs. PCV2 SN antibody assay was developed using immunoperoxidase monolayer assay (IPMA) and determining the end point by calculating 50% reduction in the infected cells (Fort et al., 2007). Because the results of PCV2 SN assay could be vary by different protocols, it was necessary to standardize the

assay protocol in each laboratory. Based on the method by Fort et al 2007, we have modified and standardized the SN assay in Chapter 3C.

Viremia has been examined in most experimental models for PCV2 vaccine efficacy, and a real-time PCR assay has been commonly applied to measure the quantity of PCV2 in tissue samples (Brunborg et al., 2004). That is why we previously developed a nested differential PCR (nPCR) and a quantitative RT-PCR assay in the previous Chapter. The nPCR was found to be highly sensitive and specific to detect PCV2a and 2b genotypes, and very useful to test a large number of the samples.

In a previous study with small number of the samples, PCV2b was detected in the sera from swine farms during the clinical PMWS in 2006, whereas PCV2a was mainly detected in samples from farms without PMWS (Lyoo et al., 2008). This finding led us to this investigation to determine any association between high percentage of PCV2b viremia and clinical severity of PMWS in swine farms. It was hypothesized that the viremia and SN antibody levels are closely related to the PMWS development in association with PCV2 infection. It was demonstrated that the lower PCV2 SN titers were associated with higher viremic pigs facilitating better for the disease development. In order to confirm these findings, we compared PCV2 antibody levels by both IFA and SN tests and the number of viremic pigs during and after the clinical PMWS in five selected farms in Minnesota.

4.3. Materials and Methods

4.3.1. Sample collection

Five commercial pig farms in Minnesota were selected based on the severe clinical PMWS in 2006 and no clinical signs in 2008 with routine PCV2 vaccination. The farms suffered from PMWS and high mortality up to 30% during the wean to finishing period and diagnosed as a typical PMWS by a veterinary diagnostic laboratory in 2006. A total of 230 serum samples from pigs between 4 and 16 weeks age were collected in 2006, and stored in -70℃. Since the mid to late 2006, PCV2 vaccines have been routinely used in all of the 5 farms, and no clinical PMWS has been confirmed by attending veterinarians. In 2008, blood samples were collected from 322 pigs of the similar age groups in the same 5 farms.

4.3.2. Serological test

PCV2 antibody titers were tested using indirect fluorescent antibody (IFA) assay as described previously in Chapter 3C. Briefly, PK-15 cells infected with a reference PCV2 (EF452350) in 96-well microtiter plates were prepared. The plate was fixed with ethanol and acetone mixture of 50:50. Each test serum was diluted in 4-fold serially from 1:4 to 1:16384, and each dilution was transferred to the test plate. After incubation at 37C for 45 min, and washing, the plate was reacted with rabbit anti-swine IgG conjugated with FITC for another 45 min. After washing again, the plates were examined under a fluorescent microscope. For the SN test, a standardized test protocol was described in Chapter 3C. Each test serum was serially diluted in 2-fold from 1:2 to 1:256 in a 96-well microtiter plate, and the equal amount of a PCV2 (EF452350, 400 $TCID_{50}$) was added. After incubation for 1 hour at 37℃, PK-15 cells were added to the plate and incubated for 72 hours. The plate was fixed and the positive cells infected by non-neutralizing virus

were detected by IFA using a PCV2 immune pig serum. For the SN titer calculation, the 50 percentage reduction method was used as described previously.

4.3.3. Genotype differential nested PCR

Differential nPCR for PCV2 genotypes was performed as described in Chapter 3A. Briefly, the primer sets were designed to detect two genotypes of PCV2. Genomic viral DNA was extracted and purified using DNeasy blood and tissue kit. The first round PCR was performed with VF-2 and nest-R primer set. A final volume of 25 contained 2 of extracted viral DNA, 2.5 5x reaction buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), 2.0 mM $MgCl_2$, 1 of 1 pM each primer, 0.25 mM dNTPs, and 1U of Taq polymerase, adjusted with DNase free distilled water to make the final volume. Amplification was undertaken using a thermal cycler, and the cycling profile was in the following order of 5 min denaturation at 94℃, 30 repetitive cycles of 30 sec of denaturation at 94℃, 30 sec of annealing at 54℃, and 40 sec of elongation at 72℃. Elongation was extended for 7 additional min in the last cycle. The amplified product of the first round PCR was reacted with each group specific primer set in separate tube. One of the primary amplification product was added to a new PCR mixture of 5X reaction buffer (200mM Tris–HCl pH 8.4, 500mM KCl), 2.0 mM $MgCl_2$, 1 of 3 pM each primer, 0.25mM dNTPs, and 1U of Taq polymerase, and adjusted with DNase free distilled water to make the final volume of 25 . The nested PCR steps for both group specific reactions were performed as described above for the amplification. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis and a UV transmitter.

4.3.4. Statistical analysis

The percentage of PCV2 viremic pigs, mean IFA and SN antibody levels of nursery (4-9 weeks of age) and grower (10-16 weeks of age) pigs were analyzed by parametric and non-parametric test, respectively, to test the differences during and after the clinical PMWS in the 5 farms. The comparisons of mean IFA and SN antibody titers were implemented by Student *t*-test. However, the data related to viremia during and after the clinical PMWS was categorized by 2 nominal indices, such as negative or positive sign. Unlike the sample type of viremia, although the sample types of IFA titer and SN titer were proportional in scale, the conditions (Homogeneity of Error Variance and Normality) for parametric test are not satisfied. Therefore, non-parametric test, such as Wilcoxon and Mann–Whitney test, was used to analyze the differences within the group age, serologic data, and PCV2 genotype parameters. Statistical analyses were performed using SPSS software student version 15.0.

4.4. Results

4.4.1. Percentage of viremic pigs

The profiles of PCV2 viremic pig percentage are illustrated in Fig. 4.1. The percentages of PCV2a viremic pigs in 2008 were significantly lower in 4 of the 5 farms (Fig. 4.1.A). Although the percentage of PCV2a viremic pigs in 1 (farm B) of the 5 farms was higher in 2008, the extent was statistically insignificant. In the comparison between the genotypes 2a and 2b, percentages of PCV2a viremic pigs were decreased from 11.1% in 2006 to 2.5% in 2008. PCV2b viremic pigs were markedly decreased from 66.6% in

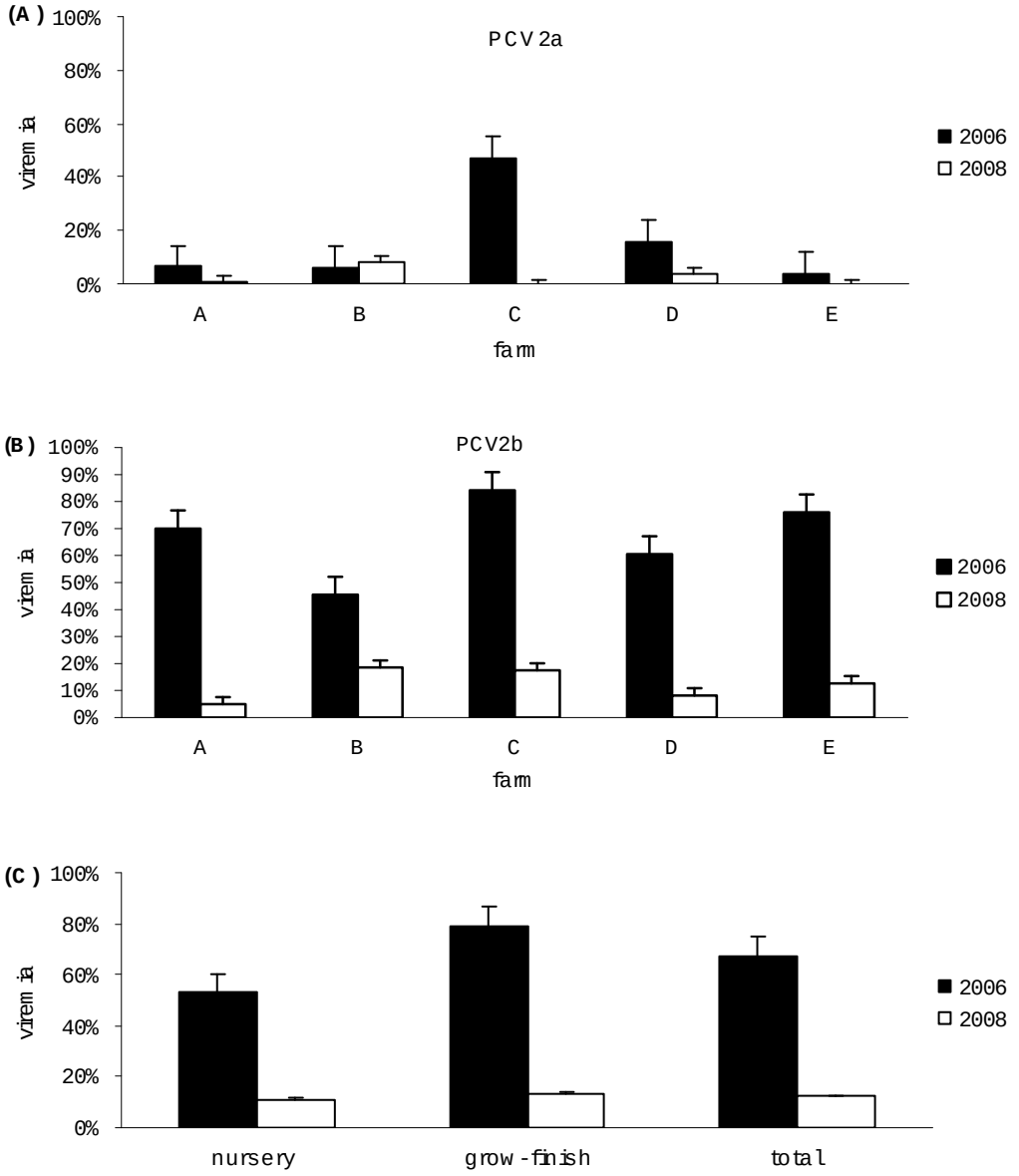


Fig. 4.1. Comparison of the percentages of viremic pigs with PCV2a (A) and PCV2b (B) in 5 different farms and different phage of production (C) between 2006 and in 2008. The percentages of PCV2a viremic pigs except for farm B and PCV2b viremic pigs were significantly (P <0.05) lower in 2008. Percentages of PCV2 viremic pigs with both 2a and 2b genotypes in nursery and grow-finish phages (C) was also significantly (P <0.05) lower in 2008.

2006 to 11.0% in 2008 in the 5 farms (Fig. 4.1.B.). The decreases were statistically significant for both genotypes 2a and 2b but PCV2b was more obvious. In the comparison by production phage (Fig. 4.1.C.), the percentages of viremic pigs during the nursery and the grow-finish phages were 52.8% and 78.9% in 2006, respectively. The viremic pigs were significantly lower to 11.3% in the nursery and 13.2% in the grow-finish phages in 2008.

Mean viremic pigs for PCV2a and PCV2b were significantly ($P< 0.001$) lower in 2008 when compared to those in 2006, and the difference was more obvious with PCV2b (Fig. 4.2.A). In order to verify the differences, a non-parametric test was used to determine the estimated marginal for the means of PCV2a and 2b in 2006 and 2008 and for the mean of PCV2b in nursery and grow-finish phases, and the results are illustrated in Fig. 4.2 B and C. The first null hypothesis was rejected that the decreasing rates of two genotypes between 2006 and 2008 were not different. Therefore, the decreasing rate of PCV2b was more significant than that of PCV2a (Fig. 4.2.B). The second null hypothesis was also rejected. It was concluded that the decreasing rate of PCV2b viremia was more significant in the grow-finish than the nursery production phage (Fig. 4.2.C).

4.4.2. Comparison of PCV2 IFA antibody titers

Mean PCV2 IFA antibody titers of each farm and production phage was illustrated in Fig. 4.3. The mean IFA antibody titers of farm A, B, C, and E in 2008 without PMWS were significantly ($P<0.001$) lower than those of the pigs from the same farms with PMWS in 2006 (Fig. 4.3.A). Although the mean IFA titer in farm D in 2008 was higher, there was no significant ($P>0.130$) difference. Fig. 4.3 B compared the mean

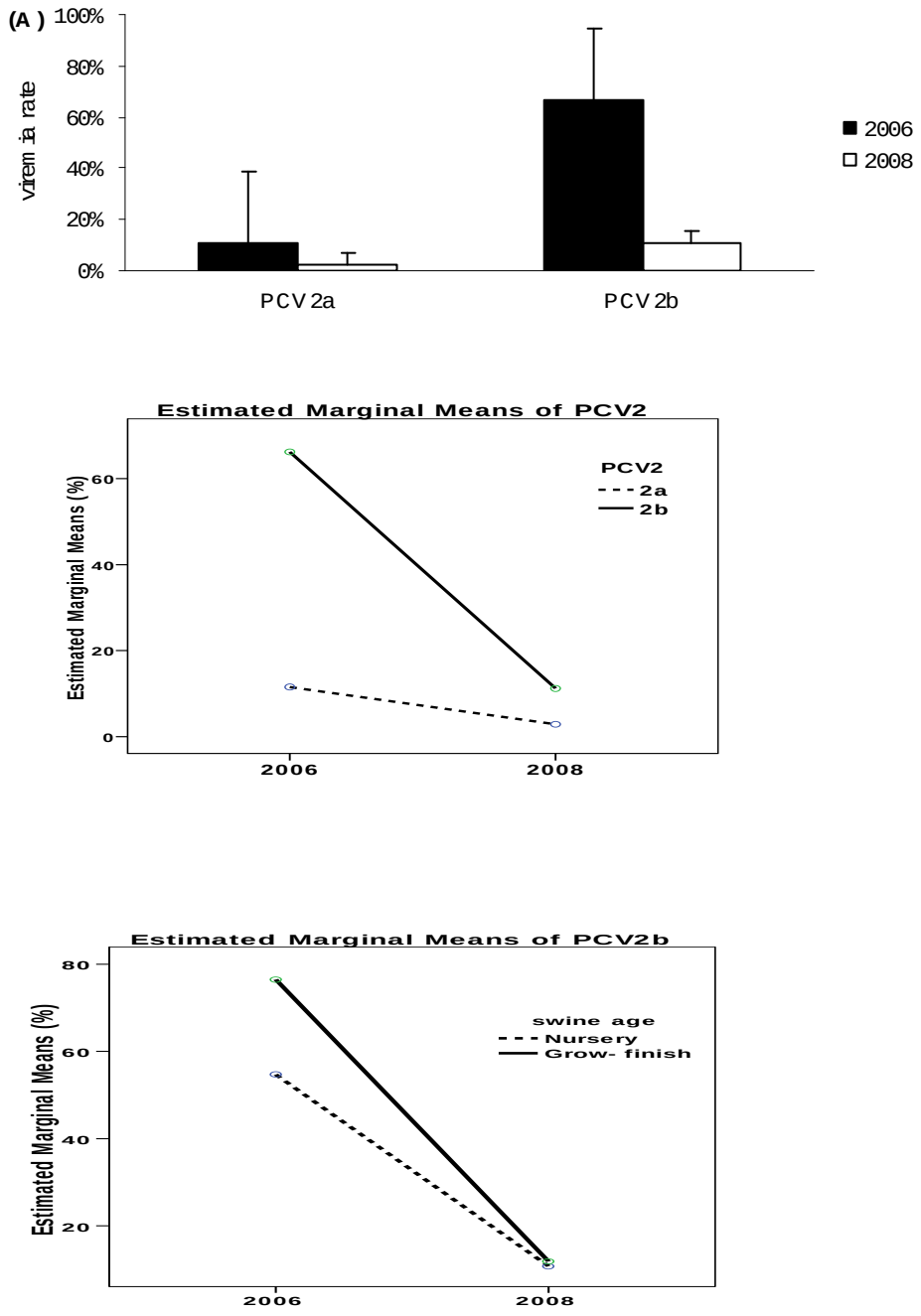


Fig 4.2. Comparison of the percentage of viremic pigs with PCV2a and 2b in 20006 and 2008 (A), and the results of non-parametric test. PCV2a and 2b viremia in 2006 were lower than those in 2008 (P <0.05) (A). The difference for PCV2b viremia was more significant than that of PCV2a viremia (P <0.001) (B), and the difference for PCV2 viremia in the grow-finish phase was more significant than that of the nursery phase by non-parametric test (P <0.001) (C).

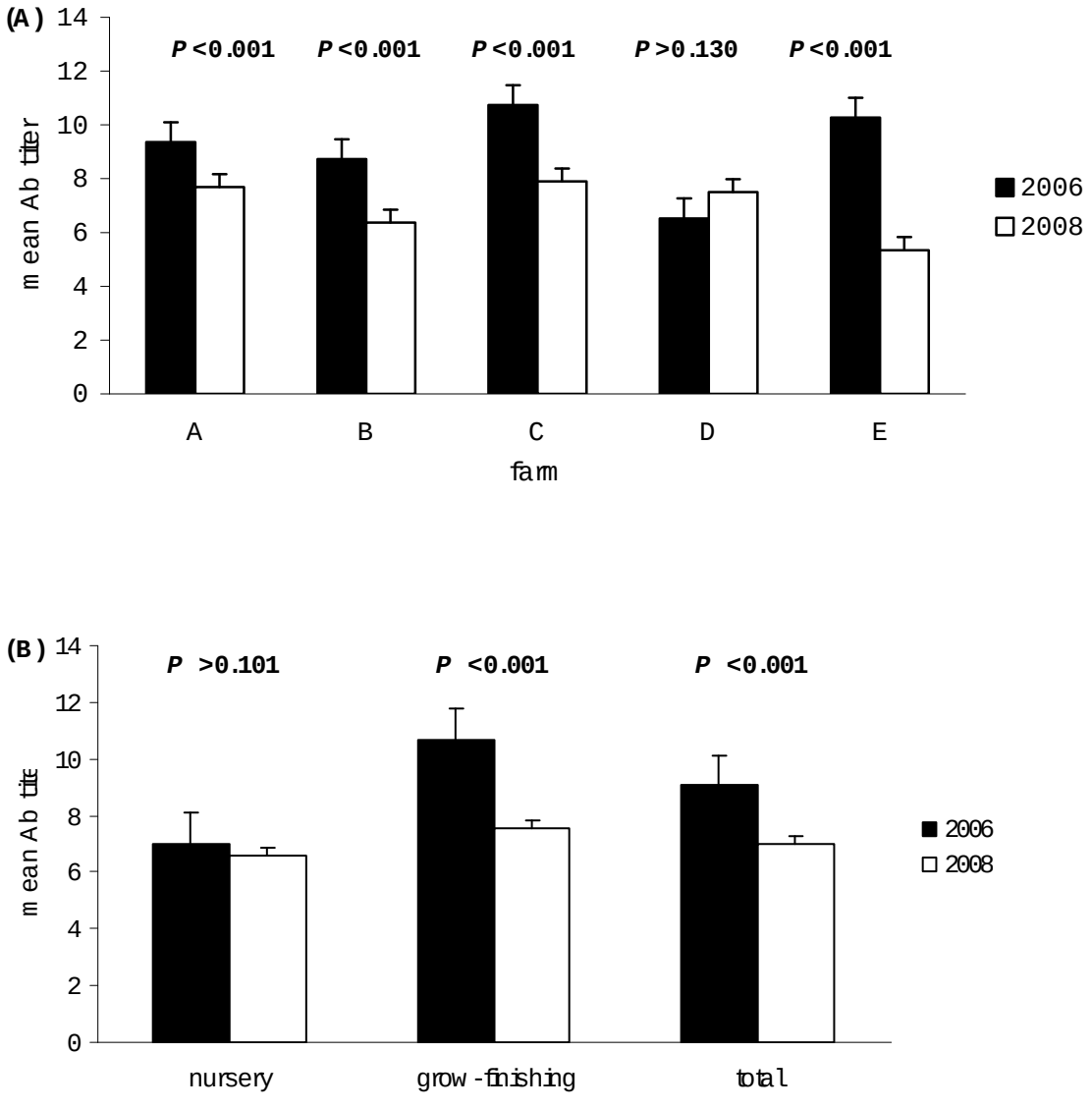


Fig. 4.3. Comparison of mean PCV2 IFA titers in 5 different farms (A) and different production phases (B) between 2006 and 2008.

IFA titers of the all 5 farms between the nursery phase (4-9 weeks age) and grow-finish (10-16 weeks age). The mean IFA titer of the grow-finish pigs was significantly (P<0.001) lower in 2008 (7.6 log2) than that in 2006 (10.7 log2) in 2006. However, the mean titer of the nursery pigs in 2006 (7.0 log2) was not significantly different as compared to that in 2008 (6.6 log2).

4.4.3. Comparison of PCV2 SN antibody titer

The mean SN antibody titers of each farm and production phage were summarized in Fig. 4.4 and Fig. 4.5. The mean SN antibody titers of farm A, B, C, and D in 2008 were significantly (P<0.001) higher than those in 2006 (Fig. 4.4.A). The SN titer difference of farm E, however, was not significant (P>0.389) between 2006 and 2008. The mean SN titers for all 5 farms classified by the production phages and compared between 2008 and 2009 (Fig. 4.4.B). Comparison of the mean SN titers for the nursery and grow-finish phages in 2008 were significantly higher than that in 2006 with *t*-test values of P<0.001 and P<0.005, respectively.

4.5. Discussion

According to the Minnesota Veterinary Diagnostic Laboratory database, clinical PMWS cases were dramatically increased from late 2005, and the highest case reports in 2006 (Torrison, 2008). The PMWS cases were, however, gradually decreased in 2007, and the decrease was coincided with the period that commercial PCV2 vaccines became available in the US. It has been now reported in swine industry that pig mortality by PMWS and PCV2 viremia were markedly reduced since late 2007. Another interesting

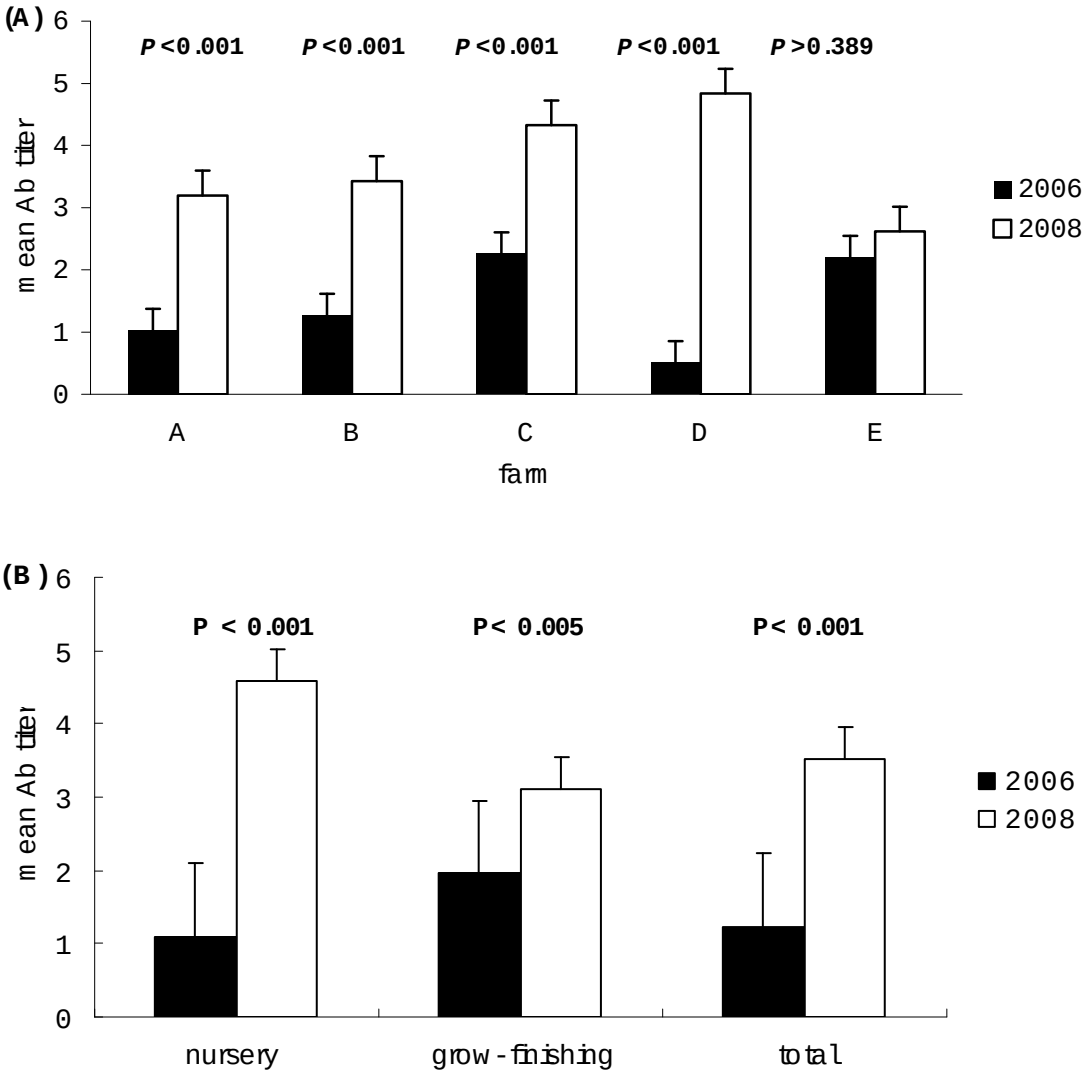


Fig. 4.4. Comparison of mean PCV2 SN antibody titers in 5 different farms (A) and different production phages (B) between 2006 and 2008.

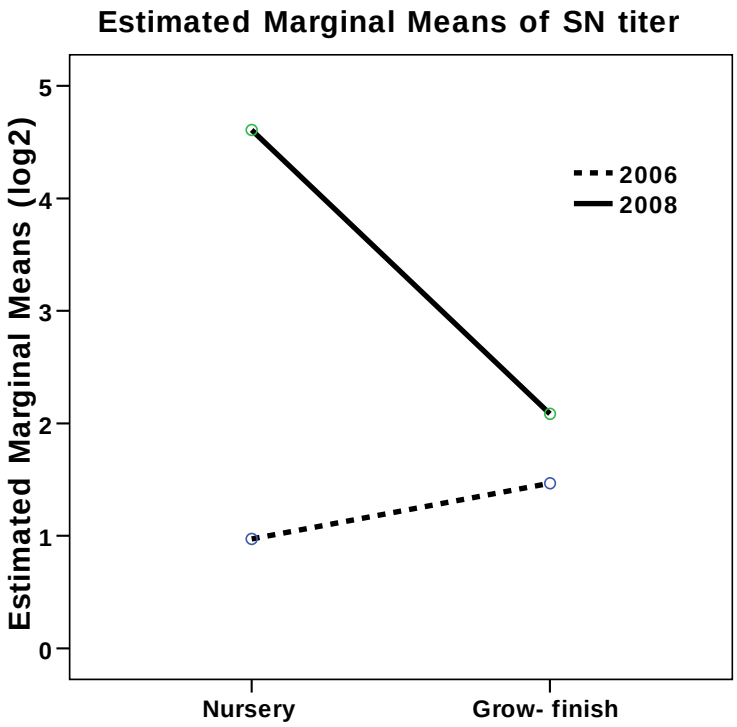


Fig. 4.5. Comparison of mean PCV2 SN antibody titers between the nursery and the grow-finish pig populations in 2006 and 2008 using non-parametric test. The mean SN titer of grow-finish pigs in 2008 was significantly (P <0.001) lower than that of nursery pigs, while the mean SN titer of the grow-finish pigs in 2006 was significantly (P <0.001) higher that that of nursery pigs.

finding was that a new PCV2 genotype of PCV2b was identified during the years whensevere PMWS was experienced in North America. Consequently, the present study was designed to compare the numbers of PCV2 viremic pigs with different genotypes and antibody levels during and after the clinical PMWS using the sera collected from the same 5 farms.

The 5 study farms were selected because of the high clinical severity among other PMWS affected farms, and there were no clinical sign and mortality associated with PCV2 infection in 2008 following routine PCV2 vaccination. The presence of high percentages of PCV2 viremia especially with PCV2b genotype was confirmed using a nested PCR in the farms in 2006. The 5 farms selected in the present study have maintained their same production management since 2006; therefore it is considered to be a valuable comparison.

In the previous studies, higher percentage of PCV2 viremic pigs with higher load of viral genome in the serum were demonstrated in PMWS-affected groups as compare to subclinical PMWS cases (Olvera et al., 2004; McIntosh et al., 2009). The present results not only confirmed the results of previous reports that pig groups without PCV2 vaccination and with high mortality showed more viremic pigs than the vaccinated groups (Kixmoller et al., 2008), but also extended the observation that the percentage of viremic pigs with PCV2b genotype was significantly higher than that with PCV2a genotype.

In the past, both PCV2a and 2b infection was observed in pig farms with or without clinical PMWS (Sibila et al., 2004). However, PCV2b has been dominantly detected in clinical PMWS cases, while the viral sequences from subclinical PMWS

farms mainly observed with PCV2a genotype (Carman et al., 2008; Dupont et al., 2008; Lohse et al., 2008). Those studies suggested that PCV2b genotype could be higher pathogenicity in pigs. In the present result, it was observed that PCV2b infection showed significantly higher proportion in clinical PMWS cases, but the PMWS case was markedly reduced in the vaccinated pigs in the same farms. Although both PCV2 genotypes were simultaneously decreased in total sera of the five farms in 2008, decreasing rate of PCV2b viremia percentage was more significant than that of 2a viremia by a statistic analysis (Fig 4.2B). Therefore, it could be speculated that vaccination against PCV2 would significantly decrease the viremia level of PCV2b comparing to PCV2a in PMWS-affected animals.

In the present study, the comparative results for PCV2 IFA and SN antibody levels during and after clinical PMWS were rather interesting. The IFA antibody levels were lower but the SN titers were higher in 2008 without clinical PMWS than those in 2006 with clinical PMWS. These results may indicate that the IFA antibody is an evidence of PCV2 infection, while the SN antibody is associated with protection of clinical PMWS. This is supported by a field observation that pigs died or showed worse growth performance with PMWS had very high IFA antibody titer (Horlen et al., 2008). More significant difference with mean IFA titers was observed in the grow-finish pigs, while the difference with mean SN titers was obvious in the nursery population. The higher SN antibody levels in the nursery pigs could be due to high maternal antibody and/or recent PCV2 vaccination. In fact, many farms vaccinated with a PCV2 vaccine a few days before the weaning to reduce stress after weaning and moving.

Difference in the pathogenicity between two PCV2 genotypes has not been well demonstrated in experimental or field studies (Opriessnig et al., 2008), and it is still debatable whether PCV2b genotype is more pathogenic in developing clinical PMWS. In a comparative pathogenicity study, there was no significant difference in the virulence between the 2 genotypes (Opriessnig et al., 2008). Nevertheless, the study confirmed the results in previous field studies (Horlen et al., 2007; Dupont et al., 2008) showing that PCV2b may be more related to PMWS development. This explanation was based on that the proportion of PCV2b viremic pigs was significantly higher than that of 2a in PMWS-affected cases and the viremia proportion was dramatically dropped by PCV vaccination.

Additionally, it was observed that infection rate of PCV2b was higher in pigs at grow-finish age than in nursery age. This finding was examined whether PCV2b viremia in grow-finish age was significantly lower than that in nursery age. Accordingly, it is speculated that PCV2b would be maintained as a risk factor until grow-finish age, even though pigs were exposed by PCV2 in post-weaning period.

CHAPTER 5

COMPARATIVE EFFICACY OF THREE COMMERCIAL PCV2 VACCINES IN CONVENTIOAL PIGS

5.1. Summary

Porcine circovirus type 2 (PCV2) is the primary agent of porcine multisystemic wasting syndrome (PMWS) in pigs. Several commercial PCV2 vaccines have been developed to prevent PMWS in swine herds. The objective of this study was to compare the efficacy of three PCV2 vaccines available in the United States. On a commercial farm in Minnesota, U.S.A., eighty pigs (3 weeks of age) were randomly divided into four equal groups. Twenty pigs each in three of the groups were injected with vaccines A, B, or C, and the remaining 20 pigs were unvaccinated control animals. Pigs in group A were revaccinated 3 weeks after the initial vaccination according to the manufacturer's recommendation. The presence of viremia and antibody responses to PCV2 was examined, and all pigs were weighed at 3 and 24 weeks of age. There were no noticeable clinical signs in any pigs in the vaccinated and control groups. Antibody titers in all pigs declined to their lowest levels at 15-18 weeks of age, and then increased sharply by 22 weeks. Mean viral genomic copies in blood of pigs in the 3 vaccinated groups at 22 weeks of age were significantly less than in the control group. The average daily gain (ADG) of pigs in groups A, B, C, and control were 0.81 kg, 0.80 kg, 0.78 kg, and 0.74 kg, respectively. The present results indicate that PCV2 vaccine reduced viremia and improved the ADG of pigs, although weight gain varied significantly between the vaccinated groups.

5.2. Introduction

Porcine circovirus type 2 (PCV2) is non-enveloped, approximately 17 nm in diameter and the smallest known DNA virus (Hamel et al., 1998). The virus has 2 functional proteins expressed by open reading frames 1 and 2 (Hamel et al., 1998; Segales et al., 2005). It is now accepted that PCV2 is the primary agent of postweaning multisystemic wasting syndrome (PMWS) in pigs, although other factors influence the severity of clinical signs (Opriessnig et al., 2007; Segales et al., 2005). Major clinical losses due to PMWS in US swine farms have been recognized since the late 2005, with mortality rate in growing pigs ranging from 10 to 40% on affected farms.

Among PCV2 isolates, a new genotype of PCV2 was reported from swine farms with or without clinical PMWS (Cheung et al., 2007; Horlen et al., 2008). The new genotype was differentiated in a separate cluster by phylogenetic analyses and is now named PCV2b as compared to PCV2a for the previously known genotype (Horlen et al., 2008). In a molecular evolution study, PCV2 strains in different geographical areas were clustered in two genetic groups, and PCV2a and PCV2b were found to contain 1,768 and 1,767 nucleotides, respectively (Olvera et al., 2007). It is interesting to note that all of the PCV2 isolates reported from North America before 2004 were categorized in the PCV2a genotype (Lyoo et al., 2008; Olvera et al., 2007). From 2005, both PCV2a and PCV2b were associated with clinical PMWS in pigs but PCV2b has been more frequently isolated from pigs with PMWS and shown higher virulence than PCV2a (Dupont et al., 2008; Horlen et al., 2008; Lyoo et al., 2008). The isolation of PCV2b coincided with the initial experiences with severe clinical signs of PMWS in swine farms in the USA and Canada (Gagnon et al., 2007; Horlen et al., 2008; Lyoo et al., 2008).

Three commercial PCV2 vaccines have been licensed in the U.S., and all of them are widely used on commercial swine farms. The efficacy of these vaccines was demonstrated in specific pathogen free (SPF) pigs following experimental challenge and examination of gross and microscopic lesions in the pigs (Fenaux et al., 2003; Opriessnig et al., 2009). All of the vaccines reduced mortality and improved growth performance of growing pigs under field conditions (Ritzmann and Kixmoeller, 2007; Thacker, 2008; Urniza, 2008). However, the three vaccines vary in the nature and concentration of the antigen, the type of adjuvant, and the dose of administration. At present, no report is available on the comparative efficacy side by side among the 3 vaccines under conventional farm condition.

The purpose of this study was to compare clinical sign, viremia, antibody response, and weight gain of conventional pigs following vaccination at 3-weeks of age with the respective commercial PCV2 vaccines.

5.3. Materials and Methods

5.3.1. Experiment design

The study farm was located in southern Minnesota, U.S.A. On July 30, 2008, four pigs of similar body weight were selected from each of 20 litters and individually indentified with an ear tag when weaned at 3 weeks of age. One pig from each litter was inoculated intramuscularly with PCV2 vaccine A, B, or C. The fourth pig from each sow remained unvaccinated and served as controls. All 80 pigs were moved into one pen of a wean-to-finish barn with 520 other pigs in different pens. The 20 pigs inoculated with

vaccine A were revaccinated at 6 weeks of age (3 weeks after the first vaccination) according to the manufacturer's recommendation. All pigs in the barn were monitored daily for any clinical abnormality by the farm manager. Blood samples from each pig were collected by jugular venipuncture at 3, 6, 9, 12, 15, 18, and 22 weeks of age, and the sera were stored at -20℃. All pigs were individually weighed at 3 weeks and 24 weeks of age using a platform scale. Antibody responses of each pig were measured by both indirect fluorescent antibody (IFA) and serum neutralization (SN) tests. The half-life of PCV2 maternal antibodies was determined and compared between groups as described previously (Paul et al., 1982) . A linear regression curve was fitted to the natural logarithms (ln) of the IFA and SN antibody titers. The equation used was $h = -(\ln 2) \div b$, where h is antibody half-life and b is the slope of the regression line. The antibody half-life for each pig was calculated, and the mean half-life with 95% confidence intervals (CI) was calculated for each group. Serum samples were tested for viremia using a differential nested polymerase chain reaction (nPCR) assay. The samples with PCV2b genotype positive were also tested by a quantitative real-time PCR (qPCR) assay.

5.3.2. Indirect fluorescent antibody (IFA) test

For preparation of PCV2 IFA test plate, PK-15 cells were inoculated with a PCV2 reference strain (GenBank accession no.EF452350) and fixed with an equal volume of ethanol and acetone in 96-well microtiter plates. Test sera were diluted serially in 4-fold (1:4 to 1:4096), transferred to the test plates, and incubated at 37℃ for 45 minutes. After washing 3 times with phosphate buffered saline (PBS, pH 7.2), the plate was reacted with

rabbit anti-swine IgG conjugated with FITC at 37℃ for 45 minutes. The plates were washed again and examined under a fluorescent microscope.

5.5.3. Serum neutralization (SN) test

The SN test was performed as previously described (Fort et al., 2007). Each serum was serially diluted 2-fold in a 96-well microtiter plate, and an equal volume of a PCV2b (GenBank accession no.EF452350, 400 $TCID_{50}$) was added. After 1 hour of incubation at 37℃, PK-15 cells were added to the plate and incubated for 60 hours. The plate was fixed and PCV2 positive cells infected by non-neutralizing virus were detected by reacting with a reference PCV2 swine antiserum in all wells and subsequently with rabbit anti-swine IgG conjugated with FITC. To calculate the SN titers, the numbers of PCV2 infected cells with fluorescence were counted in 3 randomly selected fields in the serially diluted wells with a reference negative serum and each test serum. The SN titer was determined as the highest dilution level of a serum at which the number of positive cells was less than the half the number of positive cells in the negative control wells.

5.3.4. Examination for viremia

A differential nPCR assay for PCV2 genotypes 2a and 2b was conducted as previously described (Lyoo et al., 2008). Briefly, primer sets were designed to detect both 2a and 2b genotypes of PCV2. Genomic viral DNA was extracted and purified using DNeasy (Qiagen, Inc., Valecncia, CA). The first round PCR was performed with the VF-2 and nest-R primer set. A final volume of 25 contained 2 of extracted viral DNA, 5 5x GoTaq® Green Mater Mix (Promega Corp., Medison, WI) containing 7.5 mM

$MgCl_2$, 1 of 1 pM each primer, 0.25 mM dNTPs, and 1U of GoTaq polymerase (Promega Corp., Medison, WI), adjusted with DNase free distilled water to make the final volume. Amplification was undertaken using a thermal cycler, and the cycling profile was in the following order of 5 min denaturation at 94℃, 30 repetitive cycles of 30 sec of denaturation at 94℃, 30 sec of annealing at 54℃, and 40 sec of elongation at 72℃. Elongation was extended for 7 additional min in the last cycle. The amplified product of the first round PCR was reacted in two separate tubes with PCV2a and PCV2b specific primer sets, respectively. One of the primary amplification product was added to a new PCR mixture as described above. The nested-PCR step was performed with the conditions; 5 min denaturation at 94℃, 30 repetitive cycles of 30 sec of denaturation at 94℃, 30 min of annealing at 57℃, and 30 min of elongation at 72℃. Elongation was extended for 7 additional min in the last cycle. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis and a UV transmitter.

To quantify the amount of the PCV2b viral genome in sera, a genotype specific qPCR assay was performed. A PCV2b strain (GenBank no. EF452350) was propagated in PK-15 cells, and genomic DNA was extracted using DNeasy blood and tissue kit. The primer set used in the nPCR for 2b-F and 2b-R targeting ORF2 gene of PCV2 was used, and DNA fragment of 361 base-pair was amplified by the above nPCR procedure. The amplified products were subcloned into pGEM-T Easy vector (Promega Corp., Medison, WI) and transformed into JM109 cells according to established methods. Nucleotide sequence analysis of positive clones was performed on both strands by automated DNA sequencing (BioMedical Genomic Center, University of Minnesota, St. Paul, MN).

Subsequently, the constructed plasmid was serially diluted in 10-fold, and real-time PCR reaction was performed to establish a standard curve. Two of the DNA plasmid stock or DNA of the same sera used in nPCR and 4 pmol of each primer were reacted with 15 of 2X real-time PCR master mix; PerfeCta SYBR Green SuperMix (Quanta Bioscience, Gaithersburg, MD), UNG, Low ROX containing $MgCl_2$, dNTPs, AccuStart Taq polymerase (Quanta Bioscience, Gaithersburg, MD), UNG, SYBR Green I dye, and ROX reference dye. DNase free distilled water was added to yield a final volume of 30 . The mixture was reacted following conditions; 10 min denaturation at 95℃, followed by 70 cycles of 30 sec denaturation at 95℃, 30 sec annealing at 55℃, and 30 sec extension at 72℃. Real-time PCR reactions were performed in a thermal cycler and fluorescence detection system; Mx3005P (Stratagene, La Jolla, CA). Fluorescence normalization and data analysis were performed by thermal cycler program software; MxPro-Mx3005P v.3.00. All real time reactions were performed in triplicate in neighboring wells.

5.3.5. Statistical analysis

Individual pigs were the experimental unit. Log_2 transformation was performed prior to statistical analysis of serological data, and assumptions of normality and homoscedasticity of the transformed data were tested using the Shapiro-Wilks test and Levene test, respectively. Since mean IFA and SN antibody (log_2) titer and quantity of the viral load were examined in the sample repeatedly collected from a same animal, one-way repeated measure ANOVA was used to prove whether the groups had significant

difference. Then, the antibody data, viral load, and body weight between the groups were analyzed by student *t-test* as a parametric test. The data for the number of viremic pigs were analyzed by a non-parametric method of Wilcoxon and Mann–Whitney test. Statistical analyses of the parametric and non-parametric data were performed using SPSS software (SPSS Inc., student version 15.0. Chicago, IL). An alpha value of <0.05 was used to infer statistical significance.

5.4. Results

5.4.1. PCV2 antibody response in pigs

The groups showed significantly different mean IFA and SN antibody titer by one-way repeated measure ANOVA. In this test, there was no difference among vaccine groups, but control group was significant differently from groups A, B, and C (P <0.03). Mean IFA antibody titers (log_2) of the pigs at 3 weeks of age in the groups A, B, C and control were 10.2, 9.8, 9.6, and 10.4, respectively (Fig. 5.1). The IFA titers of control pigs gradually decreased until 15-18 weeks of age, with the lowest titers (2.53 log_2 ± 1.58, 95% CI)) observed at 15 weeks of age. These control pigs showed high antibody titers at 22 weeks of age (mean IFA titer 9.05 log_2 ± 0.99, 95% CI). The mean IFA antibody half-life of the control group was 18.3 days (95% CI: 15.5 to 21.1 days). Among the vaccinated groups, the mean IFA half-lives (group A: 32.9 ± 6.2 days; group B: 25.1 ± 3.8 days; group C: 24.5 ± 5.1, 95% CI) were significantly longer (P <0.03) than the control group. The half-life of group A was significantly longer (P <0.05) than group B or C in the comparison between three vaccinated groups. The IFA titers of pigs in the

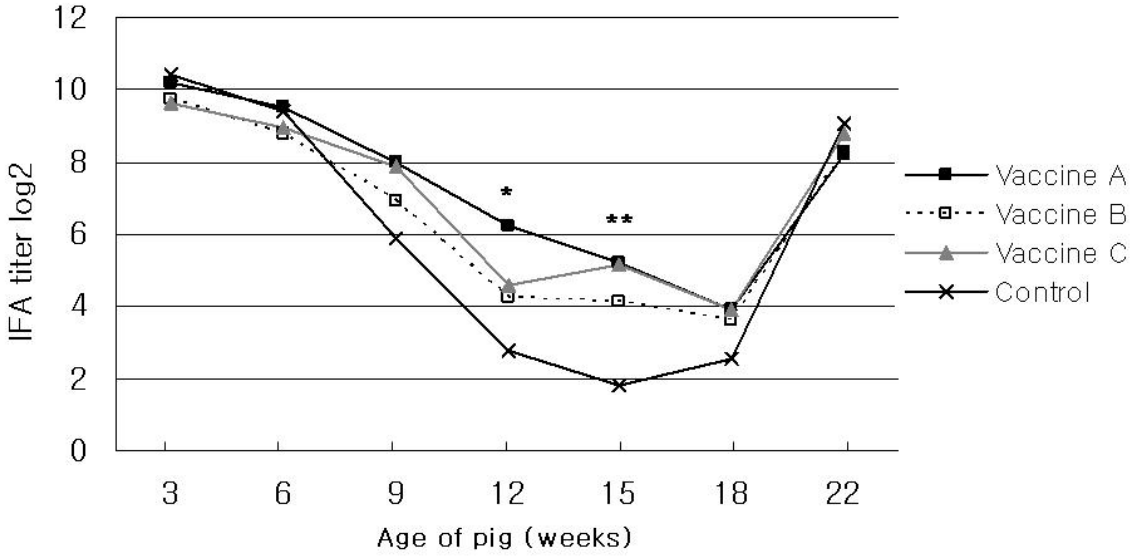


Fig. 5.1. Mean IFA titers to PCV2 in pig groups inoculated with vaccines A, B, C, and without a vaccine (control), respectively, at 3 weeks of age and at different weeks after the vaccination.

* Vaccinated groups A, B, and C at 12 and 15 weeks of age were significantly ($P < 0.05$) higher than the control group, and group A were significant ($P < 0.05$) higher than groups B and C.

3 vaccinated groups also decreased gradually but more slowly than in the control group. Mean IFA titers of the 3 vaccinated groups were significantly higher (P <0.05) than the control group at 12 and 15 weeks of age. The IFA titers in group A were significantly higher than those in groups B (P <0.001) and C (P <0.019) at 12 weeks of age. All vaccinated pigs showed high antibody titers at 22 weeks of age.

The mean $\log_2$ SN titers of all 4 groups were between 6.60 – 6.94 at 3 weeks of age and gradually decreased until 15-18 weeks of age (Fig. 5.2.). The mean SN antibody half-life of the control group was 24.9 days (95% CI: 21.3 to 28.5 days). In comparison between the vaccinated groups, the mean SN half-lives (95% CI) were; group A: 36.5 ± 5.6 days; group B: 32.9 ± 5.6 days; group C: 31.9 ± 7.1. The half-lives of the 3 vaccinated groups were significantly longer (P <0.05) than the control group. The SN antibody titers of pigs in the 3 vaccinated groups declined more slowly than in the control group, and the low SN antibody titers of all 4 groups at 18 weeks of age became high titers at 22 weeks of age. Mean SN titers of the groups vaccinated with A, B, or C were significantly higher (P <0.05) than in the control group at 6, 9 and 12 weeks of age. Among the vaccinated groups, the mean titer of group A was significantly higher (P <0.045) than those of groups B and C at 9 and 12 weeks of age.

5.4.2. PCV2 viremia in pigs

Viremia was evident in a few pigs of all 4 groups from the time of vaccination throughout the study (Table 5.1). There was no significant difference between the vaccinated and control groups until the age of 15 weeks. The numbers of the PCV2b viremic pigs increased after 15 weeks of age, and at 22 weeks of age the proportions of

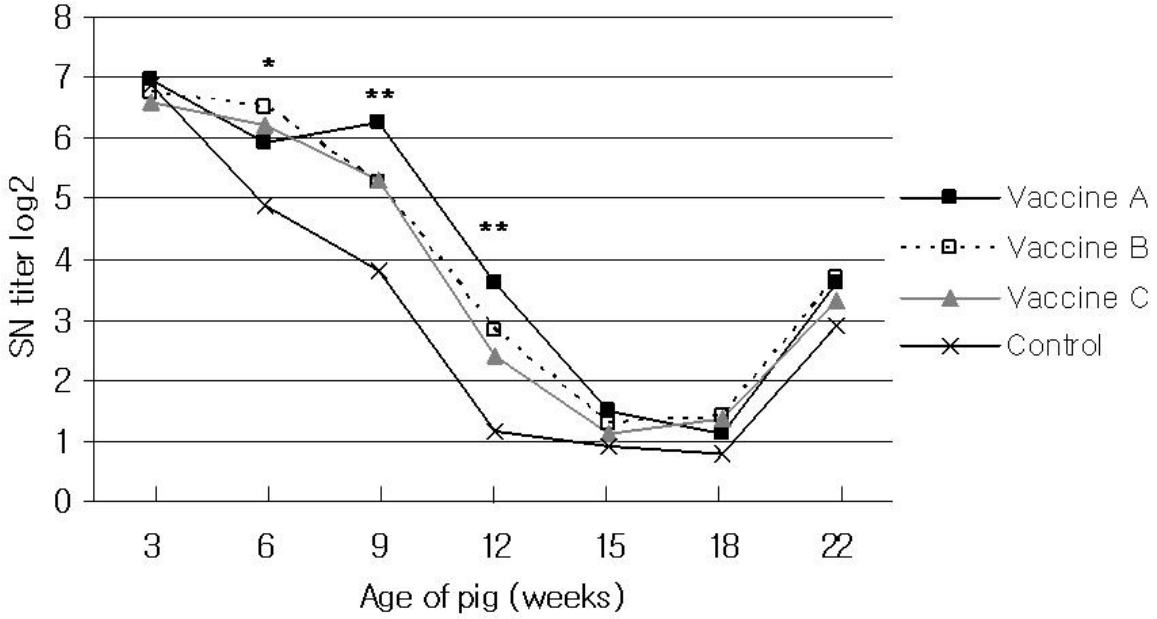


Fig. 5.2**.** Mean SN antibody titer to PCV2 in pig groups inoculated with vaccines A, B, C and without a vaccine (control), respectively, at 3 weeks of age and at different weeks after the vaccination.

* Vaccinated groups A, B, and C at 6, 9 and 12 weeks of age were significantly higher than control group. Vaccinated group A was significantly ($P < 0.05$) higher than the vaccinated groups B and C at 9 and 12 weeks of age.

Table 5.1. Numbers of PCV2 viremic pigs following vaccination with A, B, C at the age of 3 weeks and without vaccination (control)

Group	Genotype	Age (weeks) 3	6	9	12	15	18	22	Total
A	PCV2a	0/20(0)*	0/20 (0)	1/20 (5)	1/19 (5)	1/19 (5)	2/19 (11)	4/19 (21)	9/136 (6.6)
	PCV2b	1/20 (5)	1/20 (5)	2/20 (10)	1/19 (5)	3/19 (16)	5/19 (26)	10/19 (53)	23/136 (16.9)
B	PCV2a	0/19 (0)	0/16 (0)	0/17 (0)	0/16 (0)	0/16 (0)	2/16 (13)	3/16 (19)	5/116 (4.3)
	PCV2b	2/19(11)	1/16 (6)	0/17 (0)	1/16 (6)	3/16 (19)	5/16 (31)	9/16 (56)	21/116 (18.1)
C	PCV2a	0/20 (0)	0/19 (0)	0/20 (0)	1/20 (5)	1/18 (6)	1/17 (6)	3/18 (17)	6/132 (4.5)
	PCV2b	1/20 (5)	0/19 (0)	0/20 (0)	3/20 (15)	3/18 (17)	4/17 (24)	9/18 (50)	20/132 (15.2)
Control	PCV2a	0/19 (0)	0/19 (0)	0/19 (0)	1/19 (5)	1/18 (6)	4/19 (21)	5/18 (28)	11/131 (8.4)
	PCV2b	0/19 (0)	1/19 (5)	1/19 (5)	1/19 (5)	7/18 (39)	9/19 (47)	17/18 (94)	36/131 (27.5)

* Number of PCV2 viremic pigs/number of tested pigs (%)

viremic pigs were 53%, 56%, 50%, and 94% in groups A, B, C, and control, respectively. The proportions of PCV2b viremic pigs in group A were significantly lower than those of the control group at 18 (P <0.037) and 22 (P <0.020) weeks of age. The PCV2b specific qPCR assay was carried out for the sera collected at 15, 18 and 22 weeks of age (Fig. 5.3). The amount of PCV2b genomic DNA increased in all 4 groups from 15 weeks of age, but the mean DNA copies for the control group were higher than those of the 3 vaccinated groups at 15, 18 and 22 weeks of ages. At 15 weeks age, mean genomic DNA copies of the pigs in group A (2.35 $\log_{10} \pm 1.64$) were significantly lower (P <0.016) than that in control group (3.73 $\log_{10} \pm 1.21$). At 18 weeks of age, the copies of group A (2.75 $\log_{10} \pm 1.85$, P <0.021) and B (2.89 $\log_{10} \pm 1.87$, P <0.044) were significantly lower than those in control group (4.2 $\log_{10} \pm 1.83$). At 22 weeks of age, the copies of group A (4.92 $\log_{10} \pm 1.91$, P <0.001), group B (5.15 $\log_{10} \pm 1.84$, P <0.007) and group C (5.01 $\log_{10} \pm 1.13$, P <0.001) were significantly lower than that in the control group (6.75 $\log_{10} \pm 1.33$).

5.4.3. Clinical signs and weight gain

There were no noticeable clinical signs in any of the pigs in the vaccinated and control groups, although 2 pigs in group B were found dead overnight from unknown causes during the first 3 weeks of the experiment. No medication was used for pigs during the study. Average daily weight gain (ADG) for all 4 study groups was calculated for the interval from 3 to 24 weeks of age (Table 5.2). There was no significant difference in the mean body weights between the 4 groups at 3 weeks of age. The ADG of pigs in groups A (0.81kg ± 0.03, 95% CI, P <0.016)

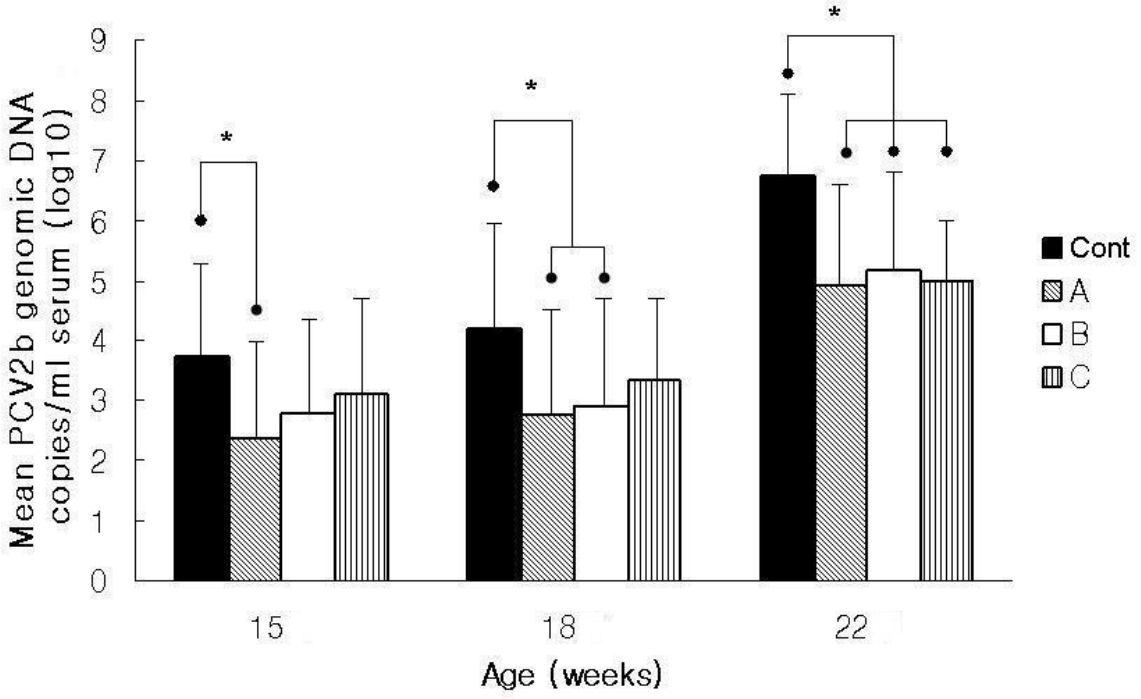


Fig. 5.3. Comparison of the mean PCV2b genomic copies between pig groups following vaccination with A, B, C, and control. Viral DNA copies were measured by a quantitative real-time PCR at 15, 18 and 22 weeks of age. * Statistically significance (*P* <0.05).

Table 5.2. Comparison of the mean body weights between the pig groups at 3 and 24 weeks of age and average daily gain (ADG) between the groups

Age of pigs	Group (weight kg ± 95% CI) [a]			
	A	B	C	Control
3 weeks	7.09 ± 0.46 (n=20)	7.42 ± 0.39 (n=19)	7.32 ± 0.45 (n=20)	7.05 ± 0.59 (n=18)
24 weeks	126.41 ± 3.84 [b] (n=19)	126.95 ± 7.20 [b] (n=16)	123.21 ± 6.0 (n=19)	117.98 ± 6.79 [b] (n=18)
ADG	**0.81 ± 0.03 [b]**	**0.80 ± 0.05 [b]**	**0.78 ± 0.04**	**0.74 ± 0.05 [b]**

[a] Pig groups were inoculated with vaccine A, B, and C, respectively. [b] Statistically significance of groups A and B at 24 weeks of age and average daily gain were $P < 0.05$ when compared to those in control group. n = number of pig.

ADG of pigs of the group C (0.78kg ± 0.04, 95% CI, P = 0.132) did not differ significantly from the controls.

5.5. Discussion

PMWS and other circovirus-associated diseases have caused devastating losses on affected farms and are among the most economically significant problems in the US swine industry. Fortunately, clinical PMWS cases and their associated economic impact have been significantly decreased since the introduction of PCV2 vaccines. Previous efficacy studies were conducted in SPF pigs or PCV2 antibody free pigs following an experimental challenge (Blanchard et al., 2003; Fenaux et al., 2003; Opriessnig et al., 2007; Opriessnig et al., 2009). There have been a few investigations of the efficacy of PCV2 vaccination under natural conditions on commercial farms (Horlen et al., 2008; Kixmoller et al., 2008) and no published study comparing the 3 approved PCV2 vaccines side by side under conventional conditions.

The potential value of vaccination of pigs against PCV2 is not questioned by veterinarians, but some uncertainty remains about optimizing vaccination protocols. Important parameters to consider are mortality rate, growth performance, and the vaccine costs. It is in general agreed that all of the 3 vaccines reduce the mortality equally and the vaccine costs are similar (Fort et al., 2008; Kixmoller et al., 2008; Opriessnig et al., 2008). However, limited data are available comparing the effects of the three vaccines on growth performance. The present results showed an obvious difference in growth performance and body weight at marketing age among the study groups (Table 5.2). Mean ADG and body weight just before the marketing of all 3 vaccinated groups were

higher than those of the control group. However, statistically significant differences were observed in the vaccinated groups A and B when compared to the control group. The differences were supported by the results of viremia in the pigs. For example, the numbers of viremic pigs in group A were significantly lower than those of the control group at 18 and 22 weeks of age. The viral DNA copies of group A and B were significantly lower than those in control group at 18 weeks of age.

All of the pigs used in this study had high maternal PCV2 antibody levels. Most sows in the U.S. were seropositive for PCV2 antibody due to natural infection and/or vaccination (Opriessnig et al., 2004; Walker et al., 2000). Maternal antibodies acquired passively from immune sows provide variable protection of newborn pigs against many infectious organisms. High maternal PCV2 antibodies prevent or reduce PCV2 viremia and protect pigs from PCV2 infection, while in piglets with no or low levels of maternal antibody protection are susceptible (McKeown et al., 2005). In addition, pigs with high maternal antibody titers at the time of PCV2 vaccination showed significant reduction of the viremia and PMWS-like lesions following experimental PCV2 challenge (Fort et al., 2008; Kixmoller et al., 2008; Opriessnig et al., 2008). However, high levels of maternal antibody could also inhibit the development of immunity from vaccination.

In previous reports (Fachinger et al., 2008; Kixmoller et al., 2008), a criterion for antibody titer classification used to differentiate between 'low' (<1:1000) and 'high' (≥1:1000) titer classes, and vaccine efficacy (based on growth performance and viremia), was lower in the animals with high maternal antibody than in those with low maternal antibody. In the current study, the maternal PCV2 antibody levels of the pigs were 'high' at the time of vaccination and declined steadily in both vaccinated and non-vaccinated

pigs. However, the decrease of PCV2 antibody in the vaccinated pigs was significantly slower than that in the control pigs. In addition, the pigs with two-dose of the vaccine A maintained significantly longer half-life of IFA antibody levels than one-dose vaccine groups B or C.

All pigs in the vaccinated and control groups developed high antibody response by 22 weeks of age. This indicates that widespread natural exposure to PCV2 had occurred in the pigs when PCV2 antibody titers were 1:4 - 1:16 by IFA and <1:4 by SN test. Previous investigators suggested that the decay of PCV2 SN antibody could be associated with the presence of viremia and development of PMWS in pigs (Fort et al., 2007; Meerts et al., 2005). It is not known if the development of viremia is associated more with the reduction of the SN antibody titers than IFA titers. PCV2 viremic pigs were intermittently detected in the pigs even with high PCV2 antibody titers, and marked increase of PCV2b viremic pigs was observed in pigs after 15 weeks of age.

This field study has some limitations. Clinical signs were not closely monitored by a veterinarian, and the causes for death of the 2 pigs in group B during the first 3 weeks were not known. The study focused only on PCV2, and no examinations of possible concurrent infections with other pathogens were conducted. In addition, 8 pigs were lost to follow up (6 lost ear tags; 2 found dead), and blood sampling and weighing events were incomplete for some pigs. The size of the study was limited and larger studies on under various field conditions are required to make firm conclusions regarding comparative efficacy of vaccines.

In conclusion, pigs vaccinated with any of the 3 PCV2 vaccines showed delayed decay of PCV2 maternal antibodies, reduced numbers of viremic pigs and less PCV2b

viremia compared with control pigs. Although no clinical signs were observed any of the groups, there were statistical differences in the growth performance between the vaccinated and control pigs. In this experiment with the 3 vaccines, vaccine A appears to be better in the overall evaluations of the viremia, antibody response and growth performance. However, the vaccine A requires two doses according to the manufacturer's recommendation.

CHAPTER 6

A PRELIMINARY STUDY ON THE COMPARISON OF PORCINE CIRCOVIRUS TYPE 2 VIREMIA IN THE VACCINATED PIGS BETWEEN HEAVEY AND LIGHT WEIGHT PIGS AT THE MARKETING AGE

6.1. Summary

Porcine circovirus type 2 (PCV2) is considered to be an essential agent for postweaning multisystemic wasting syndrome (PMWS), and the syndrome has now been recognized as a global disease with significant economic losses. Currently commercial PCV2 vaccines are widely used in swine farms. However, marked body weight variation at the marketing age has been a problem in commercial farms even with routine PCV2 vaccination. The purpose of this study was to investigate whether PCV2 infection could be a factor for the body weight variation. Seven conventional farms in Minnesota with routine PCV2 vaccination were selected, and 30 sera each were collected from the heavy and the light body weight pigs in the 7 farms and tested for PCV2 indirect fluorescent antibody (IFA) titer and viremia. Of the 7 farms, mean PCV2 IFA titer in 3 farms, number of PCV2 viremic pigs in 4 farms and mean PCV2b genomic DNA copy number in 4 farms were significantly different between the heavy and light weight pigs. These preliminary results suggest that PCV2 infection could be one of the factors for the light weight marketing pigs.

6.2. Introduction

Porcine circovirus type 2 (PCV2) is single-strand DNA virus having circular genome of 1.7 kbp and is regarded as an essential causative agent of postweaning multisystemic wasting syndrome (PMWS) in pigs (Ellis et al., 1998; Hamel et al., 1998; Allan and Ellis, 2000). The PMWS has now been recognized as a global epizootic disease that causes significant economic losses in swine industry (Allan et al., 1998; Segales et al., 2005). The disease is characterized by high mortality with weight loss, enlargement of lymphoid node, interstitial pneumonia, and jaundice in affected animals (Allan and Ellis, 2000; Segales et al., 2005; Opriessnig et al., 2007).

The pathogenesis of PMWS is not fully understood, and high mortality is not common if there are no additional factors such as co-infection. PCV2 is known to be largely associated in the pathogenesis, while the virus is ubiquitous and is not a new virus according to retrospective epidemiological studies (Rodriguez-Arrioja et al., 2003; Allan and McNeilly, 2006). Recent observation of a new genotype of PCV2b with higher pathogenicity may help better in understanding the PMWS pathogenesis. In addition, prevalence of PCV2b has been found to be dramatically increased in the farms with clinical PMWS (Olvera et al., 2007; Dupont et al., 2008; Grau-Roma et al., 2008). In North America, PCV2 isolates before 2004 were identified as PCV2a genotype, while PCV2b genotype was dominantly detected and widely spread in association with clinical PMWS after 2005 (Cheung et al., 2007; Gagnon et al., 2007; Horlen et al., 2008; Torrison, 2008).

To prevent or minimize the impact by PCV2 infection, several PCV2 vaccines have been applied. The vaccines were developed by expressing viral capsid protein into

a vector or PCV1 and PCV2 chimeric vaccine (Blanchard et al., 2003; Fenaux et al., 2004; Charreyre et al., 2005). In the United States, commercial PCV2 vaccines with one or two doses have been available since 2006. In the most experimental efficacy studies, the vaccines actively induced immune response against PCV2 and successfully reduced the viral replication and shedding, clinical signs and lesions. The efficacy of the vaccines was also demonstrated by reducing mortality of the growing pigs and improving growth performance under field conditions (Grau, 2007; Kixmoller et al., 2008; Thacker, 2008).

Although the vaccinated pigs performed significantly better in terms of survivability and growth performance, small proportions of light weight pigs in late finishing phase have been observed in the farms with routine PCV2 vaccination. Several veterinarians have expressed concern over this problem and questioned whether protection by the PCV2 vaccine is adequate. Therefore, it is hypothesized that the light weight pigs could be due to PCV2 infection. The light weight pigs at the marketing age will be most likely PCV2 viremic and show higher IFA antibody. This preliminary study was designed to compare PCV2 viremia and the antibody status between the bottom light weight and the top heavy weight pigs at the marketing age in the farms with a routine PCV2 vaccination.

6.3. Materials and Methods

6.3.1. Experimental design

Seven different finishing farms with average of 2400-5000 pigs each were selected in Minnesota in 2008 (Table 6.1.). All of the pigs had been vaccinated with a

Table 6.1. Description of PCV2 vaccination history and blood sample collection in the finishing farms

Farm	Site pig population	PCV2 vaccination	Pig age at sampling	Approximate body weight (Kg)***	
				Light	Heavy
A	4000	BI (6 wks)*	21 wks	73	109
B	4000	IV (3 & 5 wks)**	20 wks	73	109
C	3000	IV (3 & 5 wks)	22-23 wks	71	105
D	4000	IV (3 & 5 wks)	23 wks	75	109
E	5000	BI (4 wks)	22 wks	73	118
F		BI (3 wks)		95	122
G	2400	IV (3 & 5 wks)	23 wks	80	120

* BI: One dose of Ingelvac® CircoFLEXTM; Boehringer Ingelheim Vetmedica Inc. (Approximate vaccination age)

** IV: Two doses of CircumventTM PCV2; Intervet Inc. (Approximate vaccination age)

*** Approximate visual weight judgments by each farm manager

commercial PCV2 vaccine around weaning age, and there were age differences of 3-7 days within the room mates. All farms had no clinical problem related with PMWS based on herd veterinarian assessments. At less than 2 weeks before the marketing, each farm manager identified 30 pigs each of the lightest and the heaviest pigs by their visual judgments in each farm. Blood samples were collected from the 60 pigs in each farm, and all sera were stored in -20℃ until laboratory analysis.

6.3.2. Indirect immunofluorescence antibody (IFA) test to PCV2

PCV2 IFA antibody titers were tested using PCV2-infected PK-15 cells inoculated in 96-well microtiter plates as described in Chapter 3C. Briefly, all sera were diluted in 4-fold serially from 1:4 to 1: 16,384 and were transferred to the test plate. After incubation and washing, the plate was reacted with rabbit anti-swine IgG conjugated with FITC. The plate was washed again and examined under a fluorescent microscope.

6.3.3. Genotype differential nested PCR and PCV2b specific quantitative real-time PCR

A differential nPCR for PCV2 genotypes and a PCV2b specific quantitative real-time PCR (qPCR) was performed as described in Chapter 3A and 3B. Briefly for nPCR, genomic viral DNA was extracted and purified using DNeasy blood & tissue kit (Qiagen, Inc., Valecncia, CA). The first round PCR was performed with VF-2 and nest-R primer set. A final volume of 25 contained 2 of extracted viral DNA was reacted with GoTaq® polymerase and Green Mater Mix (Promega Corp., Medison, WI). The amplification was undertaken with previously described PCR conditions using a thermal cycler. The amplified product of the first round PCR was reacted with PCV2a specific

primer set (2a-F and 2a-R) and PCV2b specific primer set (2b-F2 and 2b-R) with previously described PCR conditions in separate tube. For the qPCR, PCV2b specific primer set was used to construct the cloning plasmid using pGEM-T Easy vector (Promega Corp., Medison, WI), and then a standard curve was established for PCV2b quantification. Two of sample DNA 4 pmol of each primer were reacted with 15 of 2X real-time PCR master mix; PerfeCta SYBR Green SuperMix (Quanta Bioscience, Gaithersburg, MD). The mixture was reacted following the same PCR conditions with that of nPCR. Real-time PCR reactions were performed in a thermal cycler and fluorescence detection system; Mx3005P (Stratagene, La Jolla, CA). All real-time reactions were performed in triplicate in neighboring wells.

6.3.4. Statistic analysis

The statistic analysis was based on the individual pig. Mean PCV2 IFA antibody titers and quantity of the viral load in all samples were analyzed and compared between the light and the heavy weight pigs by parametric test (ANOVA) and student t-test. Viremia of PCV2a and 2b was compared between the light and the heavy weight pigs by percentage in each group. A non-parametric test, Wilcoxon test, was used in order to analyze the differences of PCV2 viremia between the heavy and the light groups using SPSS software student version 15.0.

6.4. Results

6.4.1. Indirect immunofluorescent antibody (IFA) assay

A comparative result for mean IFA antibody titers between the heavy and the light weight pigs in the 7 finishing farms is illustrated in Fig. 6.1. Different PCV2 IFA antibody titers ranging the highest titer with 9.97 $\log_2$ in the light weight group of farm E and the lowest titer with 1.13 $\log_2$ in the heavy weight group of farm G were detected. Overall, the IFA titers of the light weight pigs were higher than those of the heavy weight pigs. Three of the 7 farms showed that mean IFA titers of the light weight groups were significantly higher than the heavy weight groups; farm A - 8.14 $\log_2 \pm 2.14$ vs. 5.86 $\log_2 \pm 3.51$; P <0.048, farm B - 5.53 $\log_2 \pm 2.76$ vs. 2.83 $\log_2 \pm 3.51$; P <0.014, and farm D - 7.82 $\log_2 \pm 2.60$ vs. 5.67 $\log_2 \pm 2.85$; P <0.047.

6.4.2. Genotype differential nested PCR and quantitative real-time PCR

Percentages of PCV2a and PCV2b viremic pigs in the 7 different finishing units tested by nPCR are shown in Table 6.2. No or few PCV2a viremic pigs were detected in all of the tested farms, and there was no statistically significant difference in PCV2a viremic pigs between the light and the heavy weight groups. In contrast, percentages of PCV2b viremic pigs were higher in the light weight pigs than the heavy weight pigs in 6 of the 7 farms. An equal number of PCV2b viremic pigs was detected only in 1 (farm C) of the 7 farms. In the non-parametric test, there was no difference in PCV2a viremic pigs between the two weight groups, but 4 of the 7 farms showed significantly difference (farm A; $P < 0.014$, farm D; $P < 0.001$, farm E; $P < 0.001$, farm F; $P < 0.046$) of PCV2b viremia between the heavy and light weight groups.

The real-time PCR was performed for quantification of the PCV2b genomic copy number for all test sera, and the results for the heavy and light weight pigs in each farm

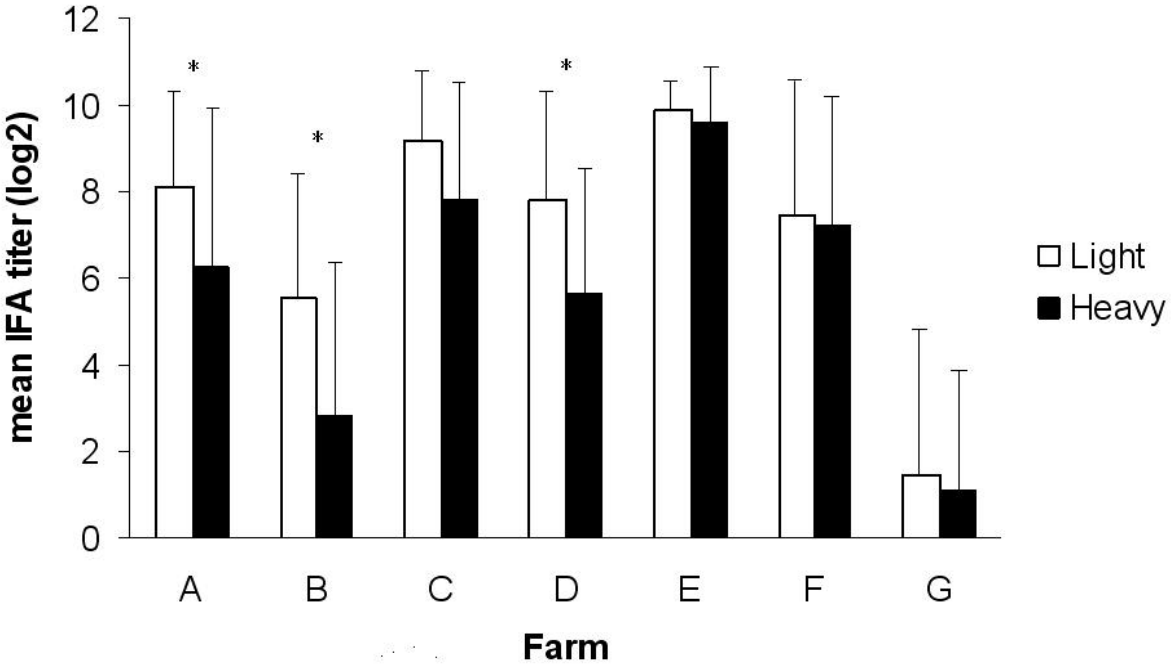


Fig. 6.1. Comparison of the mean PCV2 IFA titers between the light and the heavy weight pigs in each finishing farm. The mean antibody titers were calculated in $\log_2$ for the light and heavy weight groups. The error bars indicate standard deviation. Asterisks present statistical significance between the groups (p <0.05)

Table 6.2. Numbers and percentages of PCV2a and 2b viremic pigs in the light and heavy body weight groups in finishing farms

	PCV2a genotype viremic			PCV2b genotype viremic		
Farm	Light weight pigs	Heavy weight pigs	*P*-value	Light weight pigs	Heavy weight pigs	*P*-value
A	3 (10) /30*	5 (17) /30	0.157	8 (27)/30	2 (7)/30	**0.014**
B	1 (3) /30	1 (3) /30	1.0	10 (33)/30	7 (23)/30	0.083
C	3 (10) /30	4 (13) /30	0.317	28 (93)/30	28 (93)/30	1.0
D	3 (10) /28	0 (0) /28	0.083	27 (96)/28	16 (57)/28	**0.001**
E	2 (7) /29	0 (0)/29	0.157	28 (97)/29	3 (10)/29	**0.001**
F	2 (7) /28	2 (7)/28	1.0	16 (57)/28	12 (43)/28	**0.046**
G	0 (0) /30	0 (0)/30	1.0	6 (20)/30	4 (13)/30	0.157
Total	14(7)/205	12(6)/205	0.157	123(60)/205	72(35)/205	0.001

* No. of viremic pigs (%) / no. of pigs tested

Letters in bold – Statistical significance

are shown in Table 6.3. Overall, the average amount of PCV2b genomic DNA was higher in the light weight pigs than in the heavy weight pigs. The differences between the heavy and the light weight groups varied in each farm ranging from 0.13 $\log_{10}$ to1.4 $\log_{10}$ genomes /ml. Three (farms B, D, and E) of the 7 farms showed significantly higher mean PCV2b genomic copy number in the light weight pigs than the heavy weight pigs (P <0.05).

6.5. Discussion

Since PMWS was first recognized, this disease has been considered to cause severe economic problem in the swine industry throughout the world. Therefore, PCV2 vaccines have been routinely applied in commercial farms, and the use of the vaccines significantly improved weight gain and reduced mortality. In a comparative study, the administration of a commercial PCV2 vaccine was significantly effective for weight gain, and mean body weight was heavier at the marketing age when compared to non-vaccinated pigs (Horlen et al., 2008). However, it has been reported by swine practitioners and producers that there have been some farms with marked difference in the body weight among the pigs at marketing age despite of the routine use of PCV2 vaccine. Another observation showed that PCV2 viremic pigs were continuously detected in the vaccinated pigs even with high titers of PCV2 antibody were maintained until the marketing age (Horlen et al., 2008; Kixmoller et al., 2008).

Similarly, high percentages of PCV2 viremic pigs under the presence of high PCV2 antibody levels were observed in the vaccinated pigs when they reached to the marketing age (Chapter 5). In this study, the pigs of the 7 farms had routinely been

Table 6.3. Mean genomic DNA copy numbers per 1ml serum of PCV2b viremic pigs in light and heavy weight groups in finishing farms

Farm	Light weight pig	Heavy weight pig	*P*-value
A	2.70 ± 1.46*	2.53 ± 1.56	0.363
B	4.28 ± 2.32	2.94 ± 2.10	**0.042**
C	6.05 ± 2.11	5.23 ± 2.13	0.117
D	5.04 ± 0.92	3.65 ± 1.56	**0.0003**
E	5.64 ± 0.75	4.12 ± 1.72	**0.0004**
F	2.99 ± 2.09	2.83 ± 2.36	0.446
G	1.28 ± 1.17	1.15 ± 1.03	0.473
Total	4.02 ± 2.29	3.37 ± 2.25	**0.012**

* genomic DNA numbers (log_{10}) and standard deviation

Letters in bold – Statistical significance

vaccinated with one of the PCV2 vaccines and did not suffer from any clinical wasting syndrome and unusual motility. However, high number of the underweight pigs in the same barn or pen at finishing age was unacceptable and the weight difference over 80 lbs was commonly observed. There was a speculation that poor growth of the light weight pigs could be associated with PCV2 infection, because different factors may involve in the protection by PCV2 vaccination.

Variations in weight gain are caused by numerous factors including nutritional, environmental, genetic, infectious agents and others. Interestingly in this study, however, higher numbers of PCV2b viremic pigs were observed with the light weight than the heavy weight pigs in 6 of the 7 farms. Differences in PCV2b load between the light and heavy weight pig groups were statistically significant in 3 of the 7 farms. Therefore, the role of PCV2 infection should be considered highly for the cause of light weight pigs at marketing age, although the light weight pigs due to other pathogenic agents were not investigated in this study.

The present study was performed as a preliminary investigation. However, the results showed a high association between PCV2 infection and the marketing weight. Although statistical significance was not always observed between the light and the heavy weight groups, higher levels of PCV2 viremia and antibody were generally evident in the light weight pigs. In addition, an important finding was that the viremia was predominantly due to PCV2b genotype. This finding confirmed the previous suggestion that the genetic shift of PCV2 has been progressed in global scale (Dupont et al., 2008). Therefore, an investigation for the use of PCV2b genotype vaccine may be required since all of the current vaccines are based on PCV2a genotype.

In this study, the farms were selected randomly, and the investigation was carried out only in 7 finishers. Also, there were many variables within and between the farms for the market weights. The vaccine used, the age of pigs at the time of the vaccination and the examination were different on each farm. A well controlled experiment with fewer variables should be designed to evaluate the effects of PCV2 vaccine on the marketing weight. The end points should be based on the total weight gain and feed efficiency in the comparative studies. Then, the best vaccination program for PCV2 infection should be employed by the farmers for a maximum performance.

GENERAL DISCUSSION AND CONCLUSIONS

Postweaning multisystemic wasting syndrome (PMWS) is now considered a global endemic disease causing significant economic losses in the swine industry. Porcine circovirus type 2 (PCV2) is the major causative agent for PMWS and also for dermatitis and nephropathy syndrome, respiratory disease complex and reproductive abnormalities in swine. However, the disease mechanism for PMWS has still not been well understood, and there are different scientific views within the scientific community. Nevertheless, PCV2 vaccines work remarkably well protecting from the development of PMWS and other related syndromes.

In Minnesota, the first case of clinical PMWS was reported in mid 2005. Two PCV2 strains were initially isolated from pigs of the first case, and the isolates replicated well in PK-15 cell culture reaching the infectivity titers to $10^{6.0}$ $TCID_{50}$/ml. It has been described that PCV2 infectivity has not always been high in PK-15 cell culture system. Because of the high permissiveness with the first isolate (EF452350), the virus was continuously used as the reference strain in the thesis research.

Immediately after virus isolation, molecular and physico-chemical characteristics of the isolates were examined and compared with previous reports. When the PCV2 isolates were sequenced, it was found that some of the Minnesota isolates were not been previously reported in the US. Therefore, attempts were made to isolate more PCV2 strains not only from clinical PMWS farms in 2006, but also from subclinical PMWS farms in 2004. At the similar time, studies on PCV2 genetic evolution were reported by some of the European and Canadian research groups, and PCV2 strains were genetically divided into two groups of PCV2a (European group 2; classically detected in the US) and PCV2b (European group 1; classically detected in European countries). In this study, a

total of 14 PCV2b strains and 6 PCV2a stains were detected and propagated in PK-15 cell culture. All of the PCV2b strains were confirmed to have originated from farms with clinical PMWS in 2006. This coincided with field observations of marked increase of the mortality with wasting syndrome in Minnesota. In addition, it was found that PCV2 isolates were unusually resistant to high temperature and low pH during the investigation of the physicochemical properties.

For the next step in Chapter 3, different laboratory methods (nested PCR, real-time PCR, and SN antibody assay) for the diagnosis of PCV infection were developed and evaluated. In order to differentiate PCV2 genotypes, viral genomic sequencing or restriction fragment length polymorphism (RFLP) had been initial method of choice during that time. However, these methods were difficult and time consuming to perform. It was necessary to develop a sensitive and specific method that could differentiate PCV2 genotypes. Such test was necessary especially since the new genotype of PCV2b could be associated with more severe forms of PMWS. Therefore, a nested PCR (nPCR) assay was developed and applied to detect and differentiate PCV2 genotypes. For the differential PCR, one of the main problems was that the choices of optimal positions for the primers were highly limited due to the high genetic identity between the 2 genotypes. The nPCR assay developed was found to be highly sensitive, detecting up to 10^4 times more than a conventional PCR. One great advantage of the nPCR was to differentiate between the genotypes with ability of detecting dual infection of the both genotypes.

Secondly, the numbers of genomic copies for each microorganism can be estimated by a real-time PCR assay, and the assay is generally considered to be more sensitive, rapid, and requiring less labor than the conventional PCR assay. Although

several qPCR methods for PCV2 were previously published, most of them were aimed at only for the quantification of PCV2 without differentiation between PCV2a and 2b. Therefore, a real-time PCR specific for PCV2b genotype was developed, because PCV2b was predominantly detected in pigs with clinical PMWS. In this study, a SYBR green based real-time PCR assay was developed for the detection and quantification of PCV2b in swine sera. The PCV2b primer set used in nPCR assay was applied to compare the results between nPCR and qPCR. The qPCR assay was found to be more sensitive than nPCR when compared each other under the same reaction conditions.

Thirdly, detection of PCV2 antibody has been widely used to diagnose PCV2 infection. Among the different serology for PCV2 infection, serum neutralization (SN) antibody was of particular interest because the roll of neutralizing antibody appeared to be important in understanding the pathogenesis of PMWS. However, there was no standard procedure for PCV2 SN test. Different conditions in the SN assay including optimal virus titer, incubation time and temperature for neutralization were standardized.

According to preliminary data in this study, it was found to be rather interesting that PCV2b was mainly detected in the sera from farms that were diagnosed with severe PMWS cases. Therefore, an epidemiologic study for a comparison of PCV2 genotype prevalence in the farms during PMWS outbreak and after the recovery was carried out in the next chapter. So far, vaccination has been suggested as the most effective method to prevent PCV2 infection in pigs. Three commercial PCV2 vaccines became available in the United States since 2006, and the vaccines have been widely used in the swine industry. Efficacy of the vaccines has been satisfactory in demonstrating reduction of the mortality and improving weight gain. Production values in commercial farms with PCV2

vaccination returned to the normal levels that had shown before the PMWS outbreaks. It was then interesting to investigate the differences in the serum profiles of the farms during and after severe clinical PMWS. That is why an epidemiological observation in Chapter 4 was designed. The results indicated that farms during clinical PMWS showed high percentages of PCV2 viremia, especially PCV2b, and low SN titers. These results suggested that either the levels of viremia or the SN antibody level could be used as a risk factor for clinical development of PMWS in swine farms.

At present, three commercial PCV2 vaccines have been licensed in the U.S. and the efficacy of each vaccine was well demonstrated under experimental and field conditions by different researchers. The purpose of the study in Chapter 5 was to compare the efficacy of the 3 vaccines side by side in conventional pigs, because of a lack of comparative data especially for weight gain. High levels of PCV2 maternal antibody showed delayed decay in the vaccinated groups as compared to the control group. Interestingly, although clinical signs related with PMWS were not shown in the control pigs, significant reduction of the viremic pigs and improvement of the weight gain were observed in the vaccinated pigs. In the overall evaluation for viremia, antibody level and weight gain, at least one of the 3 vaccines was found to be less satisfactory.

During the comparative vaccine efficacy experiment, acute PCV2 infection without noticeable clinical sign was observed in pigs at the finishing age regardless the vaccination history. This finding led a hypothesis that PCV2 infection in finishing age was due to the loss of vaccine or maternal immunity and may affect in the growth performance during the last stage of the finishing. At the same time, some veterinarians and producers reported too much weight variation as a common problem even in the

farms with routine PCV2 vaccination. That is why the study in the last Chapter was designed to compare any difference in the profiles of PCV2 viremia and antibodies. The study was considered to be a preliminary one, and the study farms were selected based on the owner's willingness to participate. It was interesting that 4 of the 7 farms showed significantly different number of PCV2 viremic pigs between the heavy and light weight groups. It is not believed that the weight differences in the 4 farms were solely due to PCV2 infection because many other factors could influence in the weight loss. However, in this study, significantly higher levels of PCV2 infection along with lower antibody titer in the light weight pigs implied that PCV2 infection would be influenced to growth performance. If a modification of PCV2 vaccination program such as vaccination at older age, the problem with weight variation could be eliminated.

In summary, this thesis research was started when clinical PMWS was observed for the first time in Minnesota. It was suspected that an emerging strain of PCV2 in the United States would be related with severe clinical signs of PMWS in pigs, because PCV2a genotype had been dominant and the disease condition was mild to moderate until the time period in the US swine industry. Although PCV2a and 2b are indistinguishable in the cross serology and low heterogeneity of <5% between the 2 genotypes in whole genome sequence, the genetic difference would probably be significant in the pathogenesis in pigs. The hypothesis, therefore, was generally covered in the thesis background. The development of diagnostic methods was attempted, and then SN assay standardization and the differentiation between two genotypes overcoming high genetic identity were established. The introduction of commercial vaccines in 2006 provided the improvement of the disease problems related with PMWS in the US swine herds. In these

studies; epidemiological investigation in conventional farms and comparison of vaccine efficacy, PCV2b was more widely and significantly infected than PCV2a in the US pig farms. Although it is still debatable whether a PCV2 genotype is more virulent in pigs according to several experimental and field studies, it was demonstrated that the genetic shifting about prevalence from PCV2a to 2b has been emergently proceeded in the United States. This is important, because current PCV2 vaccines were originally designed with PCV2a and the difference of the viral prevalence between PCV2a and 2b would be due to the vaccine strain. Moreover, it is questionable whether the weight differences of finishing pigs vaccinated at nursery age are significantly related with vaccine failure or inappropriate vaccine strain.

Future studies include continuous monitoring of PCV2 genetic variation and disease phase in field, because PCV2 has existed since at least 1969, but PCV associated disease was recently recognized in pigs. PCV2 is ubiquitous and extremely stable in various environments. For the virus eradication, advanced vaccination programs combined with several production cycles and bio-security strategies need to be developed. It is suggested that a PCV2 vaccine designed with PCV2b needs to be developed in future. Although current commercial vaccines used PCV2a are effective in pig farms, it is not certain that how long the vaccines will be stable and protective against PCV2b infection. It would be expected that the use of new vaccines using PCV2b could improve protection or growth performance in swine industry.

REFERENCES

Allan G.M., Ellis J.A., 2000, Porcine circoviruses: a review. J Vet Diagn Invest 12: 3-14.

Allan, G.M., Kennedy, S., McNeilly, F., Foster, J.C., Ellis, J.A., Krakowka, S.J., Meehan, B.M., Adair, B.M., 1999, Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J Comp Pathol 121, 1-11.

Allan, G.M., McNeilly, F., 2006, PMWS/PCVD: Diagnosis, disease, and control: What we know? . Proc 19th Congress Inter Pig Vet Soc p1-9.

Allan G.M., McNeilly F., Kennedy S., Daft B., Clarke E.G., Ellis J.A., Haines D.M., Meehan B.M., Adair B.M., 1998, Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J Vet Diagn Invest 10: 3-10.

Allan, G.M., McNeilly, F., McMenamy, M., McNair, I., Krakowka, S.G., Timmusk, S., Walls, D., Donnelly, M., Minahin, D., Ellis, J., Wallgren, P., Fossum, C., 2007, Temporal distribution of porcine circovirus 2 genogroups recovered from postweaning multisystemic wasting syndrome affected and nonaffected farms in Ireland and Northern Ireland. J Vet Diagn Invest 19: 368-375.

Allan G.M., Mc Neilly F., Meehan B.M., Kennedy S., Mackie D.P., Ellis J.A., Clark E.G., Espuna E., Saubi N., Riera P., Botner A., Charreyre C.E., 1999, Isolation and characterization of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. Vet Microbiol 66: 115-123.

Allan G.M., Phenix K.V., Todd D., McNulty M.S., 1994, Some biological and physico-chemical properties of porcine circovirus. Zentralbl Veterinarmed B 41: 17-26.
Allan, G.M., McNeilly, F., 2006, PMWS/PCVD: Diagnosis, disease, and control: What we know? Proc 19th Congress Inter Pig Vet Soc p1-9.

Blanchard, P., Mahe, D., Cariolet, R., Keranflec'h, A., Baudouard, M.A., Cordioli, P., Albina, E., Jestin, A., 2003, Protection of swine against post-weaning multisystemic wasting syndrome (PMWS) by porcine circovirus type 2 (PCV2) proteins. Vaccine 21: 4565-4575.

Blanchard, P., Mahe, D., Cariolet, R., Truong, C., Le Dimna, M., Arnauld, C., Rose, N., Eveno, E., Albina, E., Madec, F., Jestin, A., 2003, An ORF2 protein-based ELISA for porcine circovirus type 2 antibodies in post-weaning multisystemic wasting syndrome. Vet Microbiol 94: 183-194.

Bogdan, J., West, K., Clark, E., Konoby, C., Haines, D., Allan, G., McNeilly, F., Meehan, B., Krakowka, S., Ellis, J.A., 2001, Association of porcine circovirus 2 with reproductive failure in pigs: a retrospective study, 1995-1998. Can Vet J 42: 548-550.

Brunborg, I.M., Moldal, T., Jonassen, C.M., 2004, Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a TaqMan-based real-time PCR. J Virol Methods 122: 171-178.

Calsamiglia, M., Segales, J., Quintana, J., Rosell, C., Domingo, M., 2002, Detection of porcine circovirus types 1 and 2 in serum and tissue samples of pigs with and without postweaning multisystemic wasting syndrome. J Clin Microbiol 40: 1848-1850.

Carman, S., Cai, H.Y., DeLay, J., Youssef, S.A., McEwen, B.J., Gagnon, C.A., Tremblay, D., Hazlett, M., Lusis, P., Fairles, J., Alexander, H.S., van Dreumel, T., 2008, The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease--2004-2006. Can J Vet Res 72: 259-268.

Carman S., McEwen B., DeLay J., van Dreumel T., Lusis P., Cai H., Fairles J., 2006 Porcine circovirus-2 associated disease in swine in Ontario (2004 to 2005). Can Vet J 47: 761-762.

Carasova, P., Celer, V., Takacova, K., Trundova, M., Molinkova, D., Lobova, D., Smola, J., 2007, The levels of PCV2 specific antibodies and viremia in pigs. Res Vet Sci 83: 274-278.

Charreyre, C., Bésème, S., Brun, A., Bublot, M., Joisel, F., Lapostolle, B., Sierra, P., Vaganay, A., 2005, Protection of piglets against a PCV2 experimental challenge by vaccinating gilts with inactivated adjuvanted PCV2 vaccine. Proc Inter Conf Animal Circoviruses and Associated Disease, p26-30.

Cheung A.K., 2003, The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. Virology 313: 452-459.

Cheung, A., Lager, K., Gauger, P., Vincent, A., Opriessnig, T., 2007, Comparison of the pathogenicity of porcine circovirus type 2 group1 and group 2 isolates Proc 5th Inter Symp Emerg and Re-emerg Pig Disease p273.

Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007, Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Arch Virol 152: 1035-1044.

Chung, W.B., Chan, W.H., Chaung, H.C., Lien, Y., Wu, C.C., Huang, Y.L., 2005, Real-time PCR for quantitation of porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 in naturally-infected and challenged pigs. J Virol Methods 124: 11-19.

Clark, E.G., 1997, Post-Weaning Multisystemic Wasting Syndrome. Proc Am Assoc Swine Prac p499.

de Boisseson, C., Beven, V., Bigarre, L., Thiery, R., Rose, N., Eveno, E., Madec, F., Jestin, A., 2004, Molecular characterization of Porcine circovirus type 2 isolates from post-weaning multisystemic wasting syndrome-affected and non-affected pigs. J Gen Virol 85: 293-304.

Deboer C.J., Barber T.L., 1964, pH and thermal stability of rinderpest virus. Arch Gesamte Virusforsch 15: 98-108.

Farnham M.W., Choi Y.K., Goyal S.M., Joo H.S., 2003, Isolation and characterization of porcine circovirus type-2 from sera of stillborn fetuses. Can J Vet Res 67: 108-113.

Delay, J., McEwen, B., Carman, S., van Dreuel, T., Fairles, J., 2005, Porcine circovirus type 2-associated disease is increasing. AHL Newsletter 9: 22.

Dorr, P.M., Baker, R.B., Almond, G.W., Wayne, S.R., Gebreyes, W.A., 2007, Epidemiologic assessment of porcine circovirus type 2 co-infection with other pathogens in swine. J Am Vet Med Assoc 230: 244-250.

Dupont, K., Nielsen, E.O., Baekbo, P., Larsen, L.E., 2008, Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. Vet Microbiol 128: 56-64.

Ellis J., Clark E., Haines D., West K., Krakowka S., Kennedy S., Allan G..M., 2004, Porcine circovirus-2 and concurrent infections in the field. Vet Microbiol 98: 159-163.

Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin, K., McNeilly, F., Meehan, B., Todd, D., Haines, D., 1998, Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can Vet J 39: 44-51.

Ellis, J., Krakowka, S., Lairmore, M., Haines, D., Bratanich, A., Clark, E., Allan, G., Konoby, C., Hassard, L., Meehan, B., Martin, K., Harding, J., Kennedy, S., McNeilly, F., 1999, Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. J Vet Diagn Invest 11: 3-14.

Fachinger, V., Bischoff, R., Jedidia, S.B., Saalmuller, A., Elbers, K., 2008, The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. Vaccine 26: 1488-1499.

Fenaux M., Halbur P.G., Gill M., Toth T.E., Meng X.J., 2000, Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J Clin Microbiol 38: 2494-2503.

Fenaux, M., Halbur, P.G., Haqshenas, G., Royer, R., Thomas, P., Nawagitgul, P., Gill, M., Toth, T.E., Meng, X.J., 2002, Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J Virol 76: 541-551.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004, A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J Virol 78: 6297-6303.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004b, Two amino acid mutations in the capsid protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and attenuated the virus in vivo. J Virol 78: 13440-13446.

Fenaux, M., Opriessnig, T., Halbur, P.G., Meng, X.J., 2003, Immunogenicity and pathogenicity of chimeric infectious DNA clones of pathogenic porcine circovirus type 2 (PCV2) and nonpathogenic PCV1 in weanling pigs. J Virol 77: 11232-11243.

Fort, M., Olvera, A., Sibila, M., Segales, J., Mateu, E., 2007, Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. Vet Microbiol 125: 244-255.

Fort, M., Sibila, M., Allepuz, A., Mateu, E., Roerink, F., Segales, J., 2008, Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. Vaccine 26: 1063-1071.

Gagnon, C.A., del Castillo, J.R., Music, N., Fontaine, G., Harel, J., Tremblay, D., 2008, Development and use of a multiplex real-time quantitative polymerase chain reaction assay for detection and differentiation of Porcine circovirus-2 genotypes 2a and 2b in an epidemiological survey. J Vet Diagn Invest 20: 545-558.

Gagnon C.A., Tremblay D., Tijssen P., Venne M.H., Houde A., Elahi S.M., 2007, The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. Can Vet J 48: 811-819.

Grau-Roma, L., Crisci, E., Sibila, M., Lopez-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A., Segales, J., 2008, A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. Vet Microbiol 128: 23-35.

Grasland, B., Loizel, C., Blanchard, P., Oger, A., Nignol, A.C., Bigarre, L., Morvan, H., Cariolet, R., Jestin, A., 2005, Reproduction of PMWS in immunostimulated SPF piglets transfected with infectious cloned genomic DNA of type 2 porcine circovirus. Vet Res 36: 685-697.

Grau, F., Thacker, B., Francisco, C., Wilson, W., Schlueter, R.,Eggen, A.,, 2007, Filed trials to assess the performance of a conditionally licensed PCV2 vaccine in Canada. Proc. 5th Inter Sympo on Emerg and Re-emerg Pig Disease p 120.

Grau-Roma, L., Crisci, E., Olvera, A., Sibila, M., López-Soria, S., Nofrarias, M., Fraile, L., Segalés, J., 2007, Are specific porcine circovirus type 2 (PCV2) genotypes related with postweaning mutisystemic wasting syndrome (PMWS) occurrence? Proc 5th Inter Symp Emerg and Re-emerg Pig Disease p42.

Grierson S.S., King D.P., Wellenberg G.J., Banks M., 2004 Genome sequence analysis of 10 Dutch porcine circovirus type 2 (PCV-2) isolates from a PMWS case-control study. Res Vet Sci 77: 265-258.

Hamel, A.L., Lin, L.L., Nayar, G.P., 1998, Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J Virol 72, 5262-5267.

Harding, J.C., 1997, Post-weaning multisystemicwasting syndrome (PMWS): preliminary epidemiology and clinical presentation. Proc Ame Associ Swine Prac p503.

Harms, P.A., Sorden, S.D., Halbur, P.G., Bolin, S.R., Lager, K.M., Morozov, I., Paul, P.S., 2001, Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet Pathol 38: 528-539.

Hesse, R., Kerrigan, M., Rowland, R.R., 2008, Evidence for recombination between PCV2a and PCV2b in the field. Virus Res 132: 201-207.

Horlen, K.P., Dritz, S.S., Nietfeld, J.C., Henry, S.C., Hesse, R.A., Oberst, R., Hays, M., Anderson, J., Rowland, R.R., 2008, A field evaluation of mortality rate and growth performance in pigs vaccinated against porcine circovirus type 2. J Am Vet Med Assoc 232: 906-912.

Horlen, K.P., Schneider, P., Anderson, J., Nietfeld, J.C., Henry, S.C., Tokach, L.M., Rowland, R.R., 2007, A cluster of farms experiencing severe porcine circovirus associated disease: Clinical features and association with the PCV2b genotype. J Swine Health and Production 15: 270-278.

Johnson, C.S., Joo, H.S., Direksin, K., Yoon, K.J., Choi, Y.K., 2002, Experimental in utero inoculation of late-term swine fetuses with porcine circovirus type 2. J Vet Diagn Invest 14: 507-512.

Kennedy, S., Moffett, D., McNeilly, F., Meehan, B., Ellis, J., Krakowka, S., Allan, G.M., 2000, Reproduction of lesions of postweaning multisystemic wasting syndrome by infection of conventional pigs with porcine circovirus type 2 alone or in combination with porcine parvovirus. J Comp Pathol 122: 9-24.

Kim, J., Chung, H.K., Chae, C., 2003, Association of porcine circovirus 2 with porcine respiratory disease complex. Vet J 166: 251-256.

Kixmoller, M., Ritzmann, M., Eddicks, M., Saalmuller, A., Elbers, K., Fachinger, V., 2008, Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. Vaccine 26: 3443-3451.

Krakowka, S., Ellis, J.A., McNeilly, F., Ringler, S., Rings, D.M., Allan, G., 2001, Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Vet Pathol 38: 31-42.

Kyriakis, S.C., Saoulidis, K., Lekkas, S., Miliotis Ch, C., Papoutsis, P.A., Kennedy, S., 2002, The effects of immuno-modulation on the clinical and pathological expression of postweaning multisystemic wasting syndrome. J Comp Pathol 126: 38-46.

Ladekjaer-Mikkelsen, A.S., Nielsen, J., Stadejek, T., Storgaard, T., Krakowka, S., Ellis, J., McNeilly, F., Allan, G., Botner, A., 2002, Reproduction of postweaning multisystemic wasting syndrome (PMWS) in immunostimulated and non-immunostimulated 3-week-old piglets experimentally infected with porcine circovirus type 2 (PCV2). Vet Microbiol 89: 97-114.

Larochelle, R., Antaya, M., Morin, M., Magar, R., 1999, Typing of porcine circovirus in clinical specimens by multiplex PCR. J Virol Methods 80: 69-75.

Larochelle, R., Bielanski, A., Muller, P., Magar, R., 2000, PCR detection and evidence of shedding of porcine circovirus type 2 in boar semen. J Clin Microbiol 38: 4629-4632.

Larochelle, R., Magar, R., D'Allaire, S., 2002, Genetic characterization and phylogenetic analysis of porcine circovirus type 2 (PCV2) strains from cases presenting various clinical conditions. Virus Res 90: 101-112.

Larochelle, R., Magar, R., D'Allaire, S., 2003, Comparative serologic and virologic study of commercial swine herds with and without postweaning multisystemic wasting syndrome. Can J Vet Res 67: 114-120.

Larochelle R., Morin M., Antaya M., Magar R., 1999, Identification and incidence of porcine circovirus in routine field cases in Quebec as determined by PCR. Vet Rec 145: 140-142

Liu, J., Chen, I., Du, Q., Chua, H., Kwang, J., 2006, The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. J Virol 80, 5065-5073.

Liu, C., Ihara, T., Nunoya, T., Ueda, S., 2004, Development of an ELISA based on the baculovirus-expressed capsid protein of porcine circovirus type 2 as antigen. J Vet Med Sci 66: 237-242.

Liu, Q., Wang, L., Willson, P., Babiuk, L.A., 2000, Quantitative, competitive PCR analysis of porcine circovirus DNA in serum from pigs with postweaning multisystemic wasting syndrome. J Clin Microbiol 38: 3474-3477.

Lohse, L., Botner, A., Hansen, A.S., Frederiksen, T., Dupont, K., Christensen, C.S., Baekbo, P., Nielsen, J., 2008, Examination for a viral co-factor in postweaning multisystemic wasting syndrome (PMWS). Vet Microbiol 129: 97-107.

Lopez-Soria, S., Segales, J., Nofrarias, M., Calsamiglia, M., Ramirez, H., Minguez, A., Serrano, I.M., Marin, O., Callen, A., 2004, Genetic influence on the expression of PCV disease. Vet Rec 155: 504.

Lyoo, K.S., Kim, H.B., Joo, H.S., 2008, Evaluation of a nested polymerase chain reaction assay to differentiate between two genotypes of Porcine circovirus-2. J Vet Diagn Invest 20: 283-288.

Lyoo, K.S., Park, Y.H., Park, B.K., 2001, Prevalence of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2 and porcine parvovirus from aborted fetuses and pigs with respiratory problems in Korea. J Vet Sci 2: 201-207.

Mackay, I.M., Arden, K.E., Nitsche, A., 2002, Real-time PCR in virology. Nucleic Acids Res 30: 1292-1305.

Madec, F., Eveno, E., Morvan, P., Hamon, L., Blanchard, P., Cariolet, R., Amenna, N., Morvan, H., Truong, C., Mahé, D., Albina, E., Jesting, A., 2000, Post-weaning multisystemic wasting syndrome (PMWS) in pigs in France: clinical observations from follow-up studies on affected farms. Livestock Prod Sci 63: 223-233.

Madec, F., Rose, N., Grasland, B., Cariolet, R., Jestin, A., 2008, Post-weaning multisystemic wasting syndrome and other PCV2-related problems in pigs: a 12-year experience. Transbound Emerg Dis 55: 273-283.

Magar, R., Larochelle, R., Thibault, S., Lamontagne, L., 2000, Experimental transmission of porcine circovirus type 2 (PCV2) in weaned pigs: a sequential study. J Comp Pathol 123: 258-269.

Maldonado, J., Segales, J., Martinez-Puig, D., Calsamiglia, M., Riera, P., Domingo, M., Artigas, C., 2005, Identification of viral pathogens in aborted fetuses and stillborn piglets from cases of swine reproductive failure in Spain. Vet J 169: 454-456.

Mankertz, A., Caliskan, R., Hattermann, K., Hillenbrand, B., Kurzendoerfer, P., Mueller, B., Schmitt, C., Steinfeldt, T., Finsterbusch, T., 2004, Molecular biology of Porcine circovirus: analyses of gene expression and viral replication. Vet Microbiol 98: 81-88.

Mankertz A., Domingo M., Folch J.M., LeCann P., Jestin A., Segales J., Chmielewicz B., Plana-Duran J., Soike D., 2000 Characterization of PCV-2 isolates from Spain, Germany and France. Virus Res 66: 65-77.

Martin, H., Le Potier, M.F., Maris, P., 2008, Virucidal efficacy of nine commercial disinfectants against porcine circovirus type 2. Vet J 177:388-393.

McIntosh, K.A., Harding, J.C., Ellis, J.A., Appleyard, G.D., 2006a, Detection of Porcine circovirus type 2 viremia and seroconversion in naturally infected pigs in a farrow-to-finish barn. Can J Vet Res 70: 58-61.

McIntosh, K.A., Harding, J.C., Parker, S., Ellis, J.A., Appleyard, G.D., 2006b, Nested polymerase chain reaction detection and duration of porcine circovirus type 2 in semen with sperm morphological analysis from naturally infected boars. J Vet Diagn Invest 18: 380-384.

McIntosh, K.A., Tumber, A., Harding, J.C., Krakowka, S., Ellis, J.A., Hill, J.E., 2009, Development and validation of a SYBR green real-time PCR for the quantification of porcine circovirus type 2 in serum, buffy coat, feces, and multiple tissues. Vet Microbiol 133: 23-33.

McKeown, N.E., Opriessnig, T., Thomas, P., Guenette, D.K., Elvinger, F., Fenaux, M., Halbur, P.G., Meng, X.J., 2005, Effects of porcine circovirus type 2 (PCV2) maternal antibodies on experimental infection of piglets with PCV2. Clin Diagn Lab Immunol 12: 1347-1351.

Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998, Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J Gen Virol 79: 2171-2179.

Meehan B.M., McNeilly F., McNair I., Walker I., Ellis J.A., Krakowka S.,Allan G.M., 2001, Isolation and characterization of porcine circovirus 2 from cases of sow abortion and porcine dermatitis and nephropathy syndrome. Arch Virol 146: 835-842.

Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Botner, A., Kristensen, C.S., Nauwynck, H.J., 2006, Correlation between the presence of neutralizing antibodies

against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Vet Res 2: 6.

Meerts, P., Misinzo, G., McNeilly, F., Nauwynck, H.J., 2005, Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, fetal cardiomyocytes and macrophages. Arch Virol 150: 427-441.

Meerts, P., Van Gucht, S., Cox, E., Vandebosch, A., Nauwynck, H.J., 2005, Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. Viral Immunol 18: 333-341.

Nawagitgul, P., Harms, P.A., Morozov, I., Thacker, B.J., Sorden, S.D., Lekcharoensuk, C., Paul, P.S., 2002, Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. Clin Diagn Lab Immunol 9: 33-40.

Nawagitgul P., Morozov I., Bolin S.R., Harms P.A., Sorden S.D., Paul P.S., 2000, Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J Gen Virol 81: 2281-2287.

Olvera, A., Sibila, M., Calsamiglia, M., Segales, J., Domingo, M., 2004, Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. J Virol Methods 117: 75-80.

Olvera, A., Cortey, M., Segales, J., 2007, Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. Virology 357: 175-185.

Opriessnig, T., Meng, X.J., Halbur, P.G., 2007, Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J Vet Diagn Invest 19: 591-615.

Opriessnig, T., Patterson, A.R., Elsener, J., Meng, X.J., Halbur, P.G., 2008, Influence of maternal antibodies on efficacy of porcine circovirus type 2 (PCV2) vaccination to protect pigs from experimental infection with PCV2. Clin Vaccine Immunol 15: 397-401.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Halbur, P.G., 2009, Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2-3-months post vaccination. Vaccine 27: 1002-1007.

Opriessnig, T., Ramamoorthy, S., Madson, D.M., Patterson, A.R., Pal, N., Carman, S., Meng, X.J., Halbur, P.G., 2008, Differences in virulence among porcine circovirus type 2 isolates are unrelated to cluster type 2a or 2b and prior infection provides heterologous protection. J Gen Virol 89: 2482-2491.

Opriessnig, T., Thacker, E.L., Yu, S., Fenaux, M., Meng, X.J., Halbur, P.G., 2004, Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with Mycoplasma hyopneumoniae and porcine circovirus type 2. Vet Pathol 41: 624-640.

Opriessnig, T., Yu, S., Thacker, E.L., Halbur, P.G., 2004, Derivation of porcine circovirus type 2-negative pigs from positive breeding herds. J Swine Health Prod 12: 186-191.

Ostanello, F., Caprioli, A., Di Francesco, A., Battilani, M., Sala, G., Sarli, G., Mandrioli, L., McNeilly, F., Allan, G.M., Prosperi, S., 2005, Experimental infection of 3-week-old conventional colostrum-fed pigs with porcine circovirus type 2 and porcine parvovirus. Vet Microbiol 108: 179-186.

Paul, P.S., Mengeling, W.L., Pirtle, E.C., 1982, Duration and biological half-life of passively acquired colostral antibodies to porcine parvovirus. Am J Vet Res 43: 1376-1379.

Pesch, S., Ohlinger, V.F., 2007, Two subtypes of PCV2 are widely spread in European pig population causing PCVAD. Proc 5th Inter Symp Emerg and Re-emerg Pig Disease p81.

Pogranichniy, R.M., Yoon, K.J., Harms, P.A., Sorden, S.D., Daniels, M., 2002, Case-control study on the association of porcine circovirus type 2 and other swine viral pathogens with postweaning multisystemic wasting syndrome. J Vet Diagn Invest 14: 449-456.

Pogranichnyy, R.M., Yoon, K.J., Harms, P.A., Swenson, S.L., Zimmerman, J.J., Sorden, S.D., 2000, Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunol 13: 143-153.

Quintana, J., Segales, J., Rosell, C., Calsamiglia, M., Rodriguez-Arrioja, G.M., Chianini, F., Folch, J.M., Maldonado, J., Canal, M., Plana-Duran, J., Domingo, M., 2001, Clinical and pathological observations on pigs with postweaning multisystemic wasting syndrome. Vet Rec 149: 357-361.

Ramamoorthy, S., Meng, X.J., 2008, Porcine circoviruses: a minuscule yet mammoth paradox. Anim Health Res Rev, 10: 1-20.

Resendes, A., Segales, J., Balasch, M., Calsamiglia, M., Sibila, M., Ellerbrok, H., Mateu, E., Plana-Duran, J., Mankertz, A., Domingo, M., 2004, Lack of an effect of a commercial vaccine adjuvant on the development of postweaning multisystemic wasting syndrome (PMWS) in porcine circovirus type 2 (PCV2) experimentally infected conventional pigs. Vet Res 35: 83-90.

Ritzmann, M., Kixmoeller, M., 2007, Field efficacy study of a PCV2 vaccine in three week old piglets in southern Germany. In: Proceedings of the 5th international symposium on emerging and re-emerging pig diseases. p 118.

Rodriguez-Arrioja, G.M., Segales, J., Rosell, C., Rovira, A., Pujols, J., Plana-Duran, J., Domingo, M., 2003, Retrospective study on porcine circovirus type 2 infection in pigs from 1985 to 1997 in Spain. J Vet Med B Infect Dis Vet Public Health 50: 99-101

Rosell, C., Segales, J., Plana-Duran, J., Balasch, M., Rodriguez-Arrioja, G.M., Kennedy, S., Allan, G.M., McNeilly, F., Latimer, K.S., Domingo, M., 1999, Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. J Comp Pathol 120: 59-78.

Rosell, C., Segales, J., Ramos-Vara, J.A., Folch, J.M., Rodriguez-Arrioja, G.M., Duran, C.O., Balasch, M., Plana-Duran, J., Domingo, M., 2000, Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet Rec 146: 40-43.

Rovira, A., Balasch, M., Segales, J., Garcia, L., Plana-Duran, J., Rosell, C., Ellerbrok, H., Mankertz, A., Domingo, M., 2002, Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. J Virol 76: 3232-3239.

Segales, J., Allan, G.M., Domingo, M., 2005, Porcine circovirus diseases. Anim Health Res Rev 6: 119-142.

Sibila, M., Calsamiglia, M., Segales, J., Blanchard, P., Badiella, L., Le Dimna, M., Jestin, A., Domingo, M., 2004, Use of a polymerase chain reaction assay and an ELISA to

monitor porcine circovirus type 2 infection in pigs from farms with and without postweaning multisystemic wasting syndrome. Am J Vet Res 65: 88-92

Sorden, S.D., 2000, Update on porcine circovirus and postweaning multisystemic wasting syndrome (PMWS). J Swine Health and Production 8: 133-136.

Takahagi, Y., Nishiyama, Y., Toki, S., Yonekita, T., Morimatsu, F., Murakami, H., 2008, Genotypic change of porcine circovirus type 2 on Japanese pig farms as revealed by restriction fragment length polymorphism analysis. J Vet Med Sci 70: 603-606.

Thacker, B., Wilson, W., Johnsom, J., Francisco, C., Schlueter, R., 2008, Circumvent PCV vaccine: Performance and serology evaluation. 20th Congress Inter Pig Vet Soci p 90.

Tischer, I., Buhk, H.J., 1988 Viral DNA from cells infected with porcine circovirus. Zentralbl Bakteriol Mikrobiol Hyg [A] 270: 280-287.

Tischer, I., Gelderblom, H., Vettermann, W., Koch, M.A., 1982, A very small porcine virus with circular single-stranded DNA. Nature 295: 64-66.

Tischer, I., Peters, D., Rasch, R., Pociuli, S., 1987, Replication of porcine circovirus: induction by glucosamine and cell cycle dependence. Arch Virol 96: 39-57.

Torrison, J., 2008, PCV2 diagnostic and clinical trends. in Proceedings. 39th Annu Meet Am Assoc Swine Vet, p 457-461.

Urniza, A., Segales, J., Balasch, M., Bru, T., Fraile, L., Nofrarias, M., Alegre, A., Lopez-Soria, S., Sibila, M., Xu, Z., Chu, H. J., Plana-Duran, J., 2008, Efficacy of the vaccine Suvaxyn Circo One shot for the prophylaxis of PMWS under field conditions Pcoc. 20th Inter Pig Vet Soci p 55.

Vincent, I.E., Carrasco, C.P., Herrmann, B., Meehan, B.M., Allan, G.M., Summerfield, A., McCullough, K.C., 2003, Dendritic cells harbor infectious porcine circovirus type 2 in the absence of apparent cell modulation or replication of the virus. J Virol 77: 13288-13300.

Walker, I.W., Konoby, C.A., Jewhurst, V.A., McNair, I., McNeilly, F., Meehan, B.M., Cottrell, T.S., Ellis, J.A., Allan, G.M., 2000, Development and application of a competitive enzyme-linked immunosorbent assay for the detection of serum antibodies to porcine circovirus type 2. J Vet Diagn Invest 12: 400-405.

Wiederkehr, D., Sydler, T., Brugnera, E., Buergi, E., Sidler, X., 2007, Different genotypes of porcine circovirus type 2 in immunohistologically positive Swiss pigs from 1986 to 2005. Proc 5th Inter Symp Emerg and Re-emerg Pig Disease p84.

Wiederkehr, D.D., Sydler, T., Buergi, E., Haessig, M., Zimmermann, D., Pospischil, A., Brugnera, E., Sidler, X., 2009, A new emerging genotype subgroup within PCV-2b dominates the PMWS epizooty in Switzerland. Vet Microbiol 136: 27-35.

West, K.H., Bystrom, J.M., Wojnarowicz, C., Shantz, N., Jacobson, M., Allan, G.M., Haines, D.M., Clark, E.G., Krakowka, S., McNeilly, F., Konoby, C., Martin, K., Ellis, J.A., 1999, Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus 2. J Vet Diagn Invest 11: 530-532.

Yoon, K.J., Jepsen, R.J., Pogranichniy, R.M., Sorden, S., Stammer, R., Evans, L.E., 2004, A novel approach to intrauterine viral inoculation of swine using PCV type 2 as a model. Theriogenology 61: 1025-1037.