UNIVERSITI PUTRA MALAYSIA

EFFICACY OF PORCINE CIRCOVIRUS VACCINE IN REDUCING IRAEMIA STATUS IN PIGS

SIA BANG WEN

EFFICACY OF PORCINE CIRCOVIRUS VACCINE IN REDUCING VIRAEMIA STATUS IN PIGS

BY

SIA BANG WEN

A project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfillment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE
Universiti Putra Malaysia
Serdang, Selangor Darul Ehsan

MARCH 2015

It is hereby certified that we have read this project paper entitled "Efficacy of Porcine Circovirus Vaccine in Reducing Viraemia Status in Pigs", by Sia Bang Wen and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Final Year Project.

ASSOC. PROF. DR. SITI SURI ARSHAD
DVM (UPM), MSc (UPM), PhD (LONDON)
Deputy Dean of Academic and Student Affairs
Faculty of Veterinary Malaysia
Universiti Putra Malaysia
(Supervisor)

DR. OOI PECK TOUNG
DVM (UPM), PhD (GLASGOW)
Lecturer
Faculty of Veterinary Malaysia
Universiti Putra Malaysia
(Co-Supervisor)

DEDICATION

This report is dedicated to all who work in the swine industry, members of Faculty of Veterinary Medicine, Universiti Putra Malaysia, my family and friends.

ACKNOWLEGEMENTS

I would like to pledge my gratitude and deep obligation to those who have contributed time, effort and aid to the completion of this project paper:

Associate Professor Dr. Siti Suri Arshad

Dr. Ooi Peck Toung

Dr. Tan Sheau Wei

Dr. Michelle Fong

Dr. Moo Kar Shen

Dr. Ernest Khoo

Tan Lai Ting

Daniel Mohan Jacob

Victor Kuiek Ah Meng

Tan Yi Wei

Family and Friends

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

Page

LIST OF ABBREVIATIONS

AA	Amino Acid
ADG	Average Daily Gain
CP	Capsid Protein
DNA	Deoxyribonucleic Acid
IACUC	Institutional Animal Care and Use Committee
IBS	Institute Bioscience
IFN– γ –SCs	Interferon- γ-Secreting Cell
IHC	Immunohistochemistry
ISH	In situ Hybridization
Kbp	Kilo-base Pair
MDA	Maternally Derived Antibodies
NA	Neutralising Antibodies
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction- Restriction Fragment Length Polymorphism

PCV1	Type 1 Porcine Circovirus
PCV2	Type 2 Porcine Circovirus
PCVAD	Porcine Circovirus Associated Disease
PCVD	Porcine Circovirus Diseases
PMWS	Post Weaning Multisystemic Wasting Syndrome
PPV	Porcine Parvovirus
PRRS	Porcine Reproductive and Respiratory Disease
qPCR	Quantitative Polymerase Chain Reaction
UPM	University Putra Malaysia
%	Percent
mL	Millilitre
g	Gram
Kg	Kilogram
G	Gauge
g	g Force
µl	Microlitre
℃	Degree Celsius

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Ilmiah Tahun Akhir.

Keberkesanan Vaksin Circovirus Porsin untuk Mengurangkan Tahap Viremia dalam Porsin

oleh

Sia Bang Wen

2015

Penyelia : Prof. Madya Dr. Siti Suri Arshad

Penyelia Bersama : Dr. Ooi Peck Toung

Post-weaning multisystemic wasting syndrome (PMWS) merupakan sejenis penyakit porsin yang berkaitan dengan jangkitan circovirus babi jenis 2 (PCV2). Pada tahun 2007, laporan kes PMWS menunjukkan bahawa penyakit ini telah menyebabkan kerugian yang serius dalam bidang ekonomi industri porsin Malaysia. PCV2 vaksin telah dicadangkan sebagai strategi yang berkesan untuk mengurangkan kes PMWS. Oleh itu, vaksin percubaan telah dijalankan untuk mengkaji keberkesanannya bagi mengurangkan tahap viremia dalam porsin. Sebanyak 30 ekor porsin berusia 3 minggu telah dipilih daripada 5 pariti dan seterusnya dibahagikan kepada dua kumpulan, iaitu kumpulan rawatan dan kawalan. Selepas itu, porsin dari kumpulan rawatan disuntik dengan 2 mL vaksin PCV2 ke dalam otot. Berat badan dan sampel darah diambil pada hari ke-0 (pra-vaksinasi), hari ke-20, dan hari ke-40 selepas vaksinasi. Seterusnya, sampel darah di uji untuk ujian kuantitatif PCR (qPCR). Kajian ini menunjukkan pada hari ke-40 selepas vaksinasi, kumpulan rawatan menunjukkan bilangan salinan PCV2 viral DNA (5.01 $\log_{10}$ salinan/mL of serum) yang lebih rendah berbanding dengan kumpulan kawalan (6.18 $\log_{10}$ salinan/mL of serum). Manakala, seekor porsin dari setiap kumpulan yang di korbankan dan dibuat pemeriksaan post-mortem pada hari ke-40 menunjukkan tiada perbezaan yang ketara daripada tisu limfa secara mikroskopik antara kedua-dua kumpulan tersebut. Tambahan pula, purata berat badan dalam kumpulan rawatan dan kawalan juga menunjukkan perbezaan yang tidak ketara. Kesimpulannya, penggunaan PCV2 vaksin dapat mengurangkan tahap viremia dalam porsin dengan berkesan.

Kata kunci: Post-weaning multisystemic wasting syndrome, porsin circovirus 2, PCV2 vaksin, kuantitatif PCR (qPCR)

ABSTRACT

Abstract of a project paper submitted to the Faculty of Veterinary Medicine, Universiti Putra Malaysia in partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

Efficacy of Killed Porcine Circovirus Type 2 Vaccine

In Reducing Viraemia Status In Pigs

by

Sia Bang Wen

2015

Supervisor : Associate Professor Dr.Siti Suri Arshad

Co-supervisor : Dr. Ooi Peck Toung

Post-weaning multisystemic wasting syndrome (PMWS) in pigs is a disease associated with porcine circovirus type 2 (PCV2) infections. In 2007, sporadic cases of PMWS had caused major economic losses in Malaysia swine industry. PCV2 vaccines were suggested as an important strategy in the prevention of PMWS. Thus, field trial of PCV2 vaccine was performed in order to evaluate the efficacy of the vaccine in reducing the PCV2 viraemia in pigs. Thirty piglets of three weeks old were randomly selected from 5 litters and equally divided into two groups of 15 pigs (treatment and control group). The treatment group was administered with 2 mL of PCV2 vaccine intramuscularly. Blood samples and body weight were obtained on days 0 (pre-vaccination), 20 and 40 post-vaccination. Samples were tested using quantitative PCR (qPCR) assay to evaluate the viral load in blood. This study revealed that on day 40 post-vaccination, vaccinated pigs showed lower PCV2 viral DNA copies number (5.01 log_{10} copies/mL of serum) compared to control pigs (6.18 log_{10} copies/mL of serum). One pig was sacrificed from each treatment and control groups for post-mortem examination at day 40 and it revealed no significant findings on lymphoid tissues from both groups. In addition to that, results on average body weight showed no significant difference between groups. This study suggested that PCV2 vaccine able to reduce PCV2 viraemia in vaccinated pigs.

Keywords: Post-weaning multisystemic wasting syndrome, porcine circovirus type 2, PCV2 vaccine, quantitative PCR (qPCR)

1.0 INTRODUCTION

Postweaning multisystemic wasting syndrome (PMWS) is a disease associated with type 2 porcine circovirus (PCV2) infection which commonly affect nursery and grower pigs (Sorden, 2000). Porcine circovirus (PCV) was first described in 1974 as a contaminant of the porcine kidney cell line PK-15. It is a very small, non-enveloped, single stranded DNA virus with a genome of about 1.76 kbp (Tischer *et al*., 1982). Two types of PCV have been characterized and subsequently named PCV1 and PCV2 (Meehan *et al*., 1998). The initially recognized PCV1 was not thought to be pathogenic (Tisher *et al*., 1986) while PCV2 was proved to be the pathogenic PCV (Meehan *et al*., 1998) and the virus was successfully isolated from pigs affected by PMWS (Allan *et al*., 1998). The common presenting clinical signs of PMWS include wasting or unthriftiness, dyspnea, pallor, rough hair coat, diarrhea, and jaundice (Harding *et al*., 1998). The likely route of infection of PCV2 is through oronasal route, but PCV2 can also be shed in nasal, tonsillar, bronchial and ocular secretions, as well as in saliva, feces, urine, milk and semen (Krakowka *et al*., 2000). PMWS was first described in Saskatchewan (Canada) in 1991 as a sporadic disease characterized by wasting and jaundice (Harding, 1996). Since then, PMWS was reported in most parts of the pig producing countries, which include Europe, Northern American and Asia (Chae, 2004). PMWS is now considered as a multifactorial new emerging disease and one of the most costly diseases currently faced by swine industry (Gillespie *et al*., 2009).

In Malaysia, PCV2 was first reported in 2004 by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) methods (Hassuzana *et al.*, 2004), followed by the first case study of PMWS in 2007 which based on clinical signs, histopathology findings and PCR screenings of PCV2 infected pigs (Ooi *et al.*, 2007). In one of the study which involved 42 farms from six states include Penang, Perak, Selangor, Melaka, Johor and Sarawak, the prevalence of PCV infection was estimated to be 88% (Jaganathan *et al.*, 2011). This showed that PCV2 is a ubiquitous virus present in nearly all pig herds in Malaysia. According to sporadic cases reported in Malaysia in 2007, PMWS had caused major economic losses of 2.2 billion Malaysia ringgit (MYR) (Seetha *et al.*, 2011).

In general, viral diseases in swine can be diagnosed based on the detection of virus by culture, in situ hybridization, immunohistochemistry, PCR, or detection of antibodies by serology. However, diagnosis of PMWS is different from the general approaches as PCV2 can be commonly detected even in normal healthy pigs (Allan and Ellis 2000). Therefore, the detection of PCV2 alone does not necessarily confirm a diagnosis of PMWS (Chae, 2004). The diagnosis of PMWS requires that a pig or group of pigs to meet three criteria: (i) the presence of clinical signs include wasting/ weight loss/ ill thrift, with or without dyspnea or icterus, (ii) the presence of histological lesions include depletion of lymphoid organs or tissues and lymphohistiocytic to granulomatous inflammation in any organ (typically in lymphoid tissues, lungs and less often liver, kidney, intestine), (iii) the presence of PCV2 infection within characteristic lesions (Sorden, 2000).

Although detection of PCV2 in tissues does not necessarily confirm a case of PMWS, however study had found out that increased severity of the characteristic PMWS histological lesions is associated with increased PCV 2 antigens or DNA within tissues (Rosell *et al*., 1999). Based on this correlation, threshold quantitative PCR (qPCR) values from blood samples had been proposed to diagnose PMWS in pigs (Brunborg *et al*., 2004; Olvera *et al*., 2004). In one of the study, it showed that no healthy pigs had viral load greater than 10^6 PCV2 genomes per ml serum sample, while all clinically sick PMWS pigs had PCV2 loads above 10^7 in both serum or plasma and in tissue samples (Brunborg *et al*., 2004). Even though low viraemia status with viral load of below 10^4 viruses per mL may not trigger clinical outbreaks of PMWS, but the low level of PCV2 infection can also negatively impact production by decreased average daily gain, increased morbidity and mortality (Olvera *et al*., 2004). When there are subclinical PCV2 infections, energy and body resources will be directed to boost the immune system rather than growth and thus causing reduced average daily weight gain and increased mortality (Colditz, 2002). During subclinical infection, pigs can harbour the virus and spread PCV2 to others in the herd (Patterson *et al*., 2002).

PCV2 is highly resistant to inactivation by common detergents and disinfectants, making decontamination of infected premises difficult (Allan and Ellis 2000). By considering PCV2 as a multifactorial disease and is a ubiquitous virus present in nearly all pig herds, prevention and control of PMWS should include management practices, control of co-infections, breeding and semen quality control, herd nutrition, as well as sounds vaccination program (Grau-Roma *et al*., 2011).

Recently, a PCV2 vaccine based on inactivated recombinant PCV1 expressing the PCV2 ORF2 protein has been commercialized. This vaccine was claimed to be able to reduce viral load in blood and lymphoid tissues, and the lesions in lymphoid tissues associated with PCV2 infection, as well as to reduce clinical signs including reduction of daily weight gain and mortality associated with PMWS. This vaccine is suitable to be used in healthy pigs of three weeks of age or older with administration of single 2-mLdose intramuscularly. In 2014, field trial had been done at a commercial pig farm Penang, Malaysia to evaluate efficacy of the inactivated PCV2 vaccine in decreasing the PCV2 viraemia in pigs. Results concluded that inactivated PCV2 vaccine was able to reduce PCV2 viraemia in vaccinated pigs, but both treatment and control group had relatively low viral load. The low viraemia in control group was believed to be due to the history of farm had using PCV2 vaccine for more than two years. Besides that, there was no significant (p>0.05) different in their mean body weight throughout the experiment as well as significant organ lesions (Moo *et al*., 2014).

Therefore, a similar field trial will be carried out to measure the efficacy of inactivated PCV2 vaccine in reducing the PCV2 viraemia in pigs at another pig farm with no history of using PCV2 vaccine in the farm. The selected farm is located at a remote area and isolate from other. In this study, body weight and blood samples were collected before and after vaccination. Samples were tested using quantitative PCR (qPCR) assay to detect virus load in the blood. Soon later, tissues samples were collected on suspected PCV2 infected pigs and sent for histopathology to identify the microscopic lesions.

Objective

The objective of this study is to determine the PCV2 viraemia status in vaccinated pigs compare to control following PCV vaccination and also to compare the mean body weight between treatment and control group.

Hypothesis

Vaccinated pigs with inactivated PCV2 vaccine will have lower PCV2 viraemia status and better performance in term of higher mean body weight in compared to the control unvaccinated group.

2.0 LITERATURE REVIEW

2.1 Postweaning Multisystemic Wasting Syndrome

Postweaning multisystemic wasting syndrome (PMWS) was first described in Saskatchewan (Canada) in 1991, a disease characterized by wasting and jaundice (Harding, 1996). The syndrome is caused by a DNA virus referred to a type 2 porcine circovirus (PCV2), which is classified in the family Circoviridae. PMWS primarily affect pigs from two to four months of age, the late-nursery and early-fattening phases of production (Harding *et al*., 1998; Segale and Domingo, 2002). The common presenting clinical signs of PMWS include wasting or unthriftiness, dyspnea, pallor, rough hair coat, diarrhea, and jaundice (Harding *et al*., 1998). As this syndrome was characterized by progressive weight loss condition with involvement of multi organs infection, the term 'postweaning multisystemic wasting syndrome' (PMWS) had been proposed to describe the clinical condition (Clark, 1996; Harding, 1996). Apart from PMWS, PCV2 is associated with a number of conditions collectively known as porcine circocirus-associated diseases (PCVAD) in North America (Opriessnig *et al*., 2007) or porcine circovirus diseases (PCVD) in Europe (Allan *et al*., 2002).

The diagnosis of PMWS requires that a pig or group of pigs to meet three criteria: (i) clinical signs include wasting/ weight loss/ ill thrift, with or without dyspnea or icterus, (ii) histological lesions include depletion of lymphoid organs or tissues and lymphohistiocytic to granulomatous inflammation in any organ (typically in lymphoid tissues, lungs and less often liver, kidney, intestine), (iii) demonstration of PCV2 infection within characteristic lesions (Sorden, 2000).

The detection of PCV2 alone does not necessarily confirm a diagnosis of PMWS as the virus is present in nearly all pig herd including normal healthy pigs (Allan and Ellis, 2000). In Malaysia, PCV2 was first reported in 2004 by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) methods (Hassuzana *et al.*, 2004), followed by the first case study of PMWS in 2007 which based on clinical signs, histopathology findings and PCR screenings of PCV2 infected pigs (Ooi *et al.*, 2007). PMWS is now considered an endemic disease which strike many swine producing countries and continues to be a major cause of wasting disease in swine (Chae, 2004). Depending on the stage of outbreak and management, the morbidity and mortality associated with PMWS can be varying between pig herds. During acute outbreak, the overall mortality can reach 10%, but both morbidity and mortality are less in endemically infected herds (Allan and Ellis, 2000).

PMWS affected pigs often exhibit a wide spectrum of concomitant infections including viral and bacterial pathogens, such as porcine parvovirus (PPV), porcine reproductive respiratory syndrome virus (PRRS), swine influenza, *Haemophilus parasius*, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae* and *Streptococcus suis* (Kim *et al.*, 2002). These co-infection agents may confuse or complicate the clinical presentation and the prevalent co-infecting agents appeared to vary in different countries. PCV2 infection alone was found in only 15% of PMWS cases (Chae, 2004).

2.2 Etiology

Porcine circovirus (PCV) was first described in 1974 as a contaminant of the porcine kidney cell line PK-15. It is a very small, nonenveloped, single stranded DNA virus with a genome of about 1.76 kbp (Tischer *et al*., 1982). PCV is the smallest virus to date that replicates autonomously in mammalian cells (Mankertz *et al*., 1997). Two types of PCV have been characterized and subsequently named PCV1 and PCV2 (Meehan *et al*., 1998). The initially recognized PCV1 was not thought to be pathogenic (Tisher *et al*., 1986) while PCV2 was proved to be the pathogenic PCV (Meehan *et al*., 1998) and the latter virus was successfully isolated from pigs affected by PMWS (Allan *et al*., 1998). PCV1 and PCV2 may have a common evolutionary origin, yet a common ancestor has not been identified (Olvera *et al*., 2007).PCV2 genes are arranged in 11 putative open reading frames (ORFs) (Hamel *et al*., 1998). ORF1 encodes a nonstructural replicase protein essential for replication of viral DNA (Ilyina and Konin, 1992). ORF2 encodes for the major structural proteins, 233 – 234AA virus capsid protein (CP) and oriented in the antisense direction (Meehan *et al*., 2001). ORF3 codes for a non-structural protein 105AA in length. In vitro, the ORF3 protein induces apoptosis in PK-15 cells (Liu *et al*., 2005). An ORF3-deficient PCV2 mutant was shown to have less virulent if compared with wild-type PCV2 (Karuppannan *et al*., 2009). The overall DNA sequence homology between PCV1 and PCV2 is 68-76% (Meehan *et al*., 1998). Between PCV1 and PCV2, ORF1 has more homology than ORF2, with 83% nucleotide homology and 86% amino acid homology. ORF2 with nucleotide homology of 67% and amino acid homology of 65% seems to be more variable (Morozov *et al*., 1998). A recombinant virus which containing PCV1 ORF1 and PCV2a ORF2 was identified in Quebac (Canada), but it's exact origin is still under debate due to its resemblance to a commercial killed vaccine strain (Gagnon *et al*., 2010). To date, three genotypes of PCV2 had been identified including PCV2a and PCV2b, the two main phylogenetic groups and a third genotype PCV2c (Dupont *et al*., 2008). PCV2c has only been reported in Denmark. Phylogenetic assessment suggests that PCV2a is older in evolutionary terms, than PCV2b (Grau-Roma *et al*., 2008) and a shift from the PCV2a to 2b genotype has been implicated in outbreak of PMWS (Dupont *et al*., 2008). PCV2a was the most prevalent in clinically affected pigs from 1996 to the early 2000s, whereas PCV2b currently be predominates. The emergence of PCV2b in North America and Europe was associated with the appearance of more severe clinical disease (Carman *et al*., 2006). According to one study on genetic characterization of PCV2 found in Malaysia, all isolates fell into the same clade as PCV2b isolates from other countries and these isolates shared 98.3-99.2% similarities with sequence of isolates from the Netherlands (Seetha *et al*., 2011). Amino acid sequence analysis of the putative capsid protein (ORF2) of the PCV2 revealed that there are three clusters found in Malaysia, namely cluster 1C and 1A/1B. This finding was found to be similar to the neighbouring countries, such as Thailand and Indonesia suggesting that similar PCV2b clusters are circulating in these few Asian countries.

2.3 Epidemiology of Porcine Circovirus disease

PCV1 and PCV2 are ubiquitous viral agents that present in most of the pig herds where both domestic and feral swine are the natural host (Allan and Ellis, 2000). It is currently thought that non-porcine species (other than the mouse), are not susceptible to PCV2 infection (Ellis *et al*., 2001). In one study, it showed that PCV2 can replicate in and transmit between mice to a limited degree (Opriessnig *et al*., 2009). PCV2 also has been found in rats and mice from pig farms, but not in other rodents collected outside the swine herds (Lőrincz *et al*., 2010). This results in suggestive of possible role of rats and mice in the pig farms as alternate hosts or mechanical vectors for PCV2 infection.

Oronasal route is considered the most likely route of PCV2 infection, but the virus has also been found to shed in nasal, tonsillar, bronchial and ocular secretions, as well as in faeces, saliva, semen, urine and milk (Krakowka *et al*., 2000; Larochelle *et al*., 2000). Pigs can also become infected via oral route by eating raw tissues from viraemic animals (Opriessnig *et al*., 2009). The horizontal transmission of PMWS has been demonstrated experimentally by inter-mingling of affected and healthy pigs (Dupont *et al*., 2009). Although PCV2 transmission is favoured by direct contact, virus transmission can occur indirectly where pigs were held in adjacent pens (Kristensen *et al*., 2009).

2.4 Pathogenesis of PMWS

Various articles and studies have been carried out to investigate the role of PCV2 in porcine circovirus diseases (PCVDs), which including PMWS, but the exact mechanism of pathogenesis due to PCV2 infection are still not fully understood. It was found out that PCV2 does not code for its own DNA polymerase. To complete its infectious cycle, the virus required cells in S phase for replication (Tischer *et al*., 1987). One consistent feature in pigs which developed clinical PMWS was lymphoid depletion and lymphopenia in peripheral blood. Immunohistochemistry(IHC) test has demonstrated presence of large amount of PCV2 antigens in the cytoplasm of macrophages and dendritic cells which replacing lymphocytes in the depleted follicles in lymphoid tissues (Sorden, 2000), but PCV2 antigens was only sporadically detected in lymphocytes (Chianini *et al*., 2001). The reason for reduction of lymphocytes in PMWS affected pigs was still unknown (Opriessnig *et al*., 2007). Besides that, the persistence of PCV2 in dendritic cells without loss of infectivity has given the thought that; dendritic cells can act as vehicle for virus transportation throughout the host (Vincent *et al*., 2003). In one study, highest concentration of virus was found in the cytoplasm of monocyte or macrophage lineage cells of PCV2 infected pigs (Rosell *et al*., 1999) and these cells become persistently infected with little or no virus replication (Gilpin *et al*., 2003). Thus, monocytic cells may serve as a mechanism to disseminate PCV2 within host, rather than a primary target for PCV2 replication (Vincent *et al*., 2003).

Field study has showed that PMWS is a multifactorial disease, and not all pigs that were infected with PCV2 will develop clinical case. Experimental work has confirmed at least four components are the key building blocks to influence the outcome of PCV2 infection, which including the virus, host, coinfections, and immune modulation (Opriessnig *et al*., 2007).

Pigs of all breeds seem to be susceptible to PCV2 infection (Opriessnig *et al*., 2007). Pietrain pig was initially thought to be less susceptible to PCVDs, but later field studies by using Pietrain boar line have reported contradictory results (Rose *et al*., 2005). Host susceptibility and the effect of various breeds on the outcome of PCV2 infection was investigated by compared three commercial breeds: Duroc, Landrace, and Large White. Results showed that the purebred Landrace pigs were clearly more susceptible to PCVDs as measured by severity of clinical signs and microscopic lesions associated with PCV2 infection (Opressnig *et al*., 2006b).

PCV2 infection alone was found in only 15% of PMWS cases (Chae *et al*, 2004). PMWS affected pigs often exhibit a wide spectrum of concomitant infections including viral and bacterial pathogens, such as porcine parvovirus (PPV), porcine reproductive respiratory syndrome virus (PRRS), swine influenza, *Haemophilus parasius*, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae* and *Streptococcus suis* (Kim *et al*., 2002). The prevalence of co-infecting agents appeared to vary in different countries and these co-infection agents may confuse or complicate the clinical presentation. However, there is no single agents can solely attributed to enhancing the severity and incidence of PCVDs.

2.5 Diagnosis of PMWS

PMWS primarily affect pigs from two to four months of age, the late-nursery and early-fattening phases of production (Segale and Domingo, 2002). The showings of clinical signs alone are not diagnostic as the poor performance in nursery and grower pigs can be due to many other causes. Besides that, presence of other gross lesions, such as interstitial pneumonia (mottled red and tan, rubbery, noncollapsed lungs) and lymph nodes enlargement are also not diagnostic as these lesions also have been found in other diseases, especially porcine reproductive and respiratory syndrome (PRRS) and septicemic salmonellosis. To diagnose PMWS through histological lesions, the tissue samples that required to be submitted including lung, lymphoid tissues (tonsil, spleen, thymus), enlarged lymph nodes, liver, kidney and pancreas. Those tissue samples should be formalin-fixed. Preferred tools that used for demonstration of PCV2 within histological lesions are immunohistochemistry (IHC) for PCV2 antigen or in situ hybridization (ISH) for PCV2 nucleic acid (Sorden, 2000). Microscopic finding in lymphatic tissues include histiocytic infiltration, lymphatic depletion, multinucleated giant cells, and grape-like basophilic intracytoplasmic inclusion bodies. The pulmonary lesions are characterized by thickening of alveolar septa due to mononuclear cells infiltration and type II pneumocyte hyperplasia. Kidney lesions will include multifocal lymphohistiocytic interstitial nephritis and pyelitis (Chae *et al*., 2004).

Real time or quantitative PCR (qPCR) thresholds in the serum also had been proposed as diagnostic method for PMWS (Harding *et al*., 2008). The qPCR thresholds are supported by strong correlation observed between the amount of PCV2 antigen and the severity of PMWS histopathological lesions. It is considered as an alternative diagnostic method to avoid the euthanasia of pigs (Segalés, 2012). A threshold of more than 10^7 of PCV2 genomes per ml of serum has been suggested as indicating for the presence of PMWS lesions (Olvera *et al*., 2004). Threshold of less than 10^6 of PCV2 genomes per ml of serum indicating of negative PMWS infection, while threshold between $10^6 – 10^7$ of PCV2 genomes per ml of serum indicative of positive PCV2 infection and PMWS should be suspected (Opriessnig *et al*., 2007). For herd diagnosis of PMWS, two diagnostic criteria should be fulfilled: (1) a significant increase in postweaning mortality and wasting syndrome, and (2) at least 1/5 of pigs undergoes post mortem should demonstrate the diagnostic criteria of PMWS using the above individual animal diagnostic criteria as described by Sorden (Grau-Roma *et al*., 2011).

2.6 PCV Vaccines

PCV2 is one of the most economically important swine pathogens faced by the global swine industry. The diseases associated with PCV2 infection such as

PMWS has caused major economic lost worldwide including Malaysia. Commercial PCV2 vaccines were first introduced in Europe and North America countries in the period of 2004 – 2006. The introduction of PCV2 vaccines has significantly changed the impact of PCV2 infection on global pig production due to the effectiveness of the vaccines in controlling the disease outbreak and reduce incidence of PMWS in the farms. Currently, at least five type commercial vaccines against PCV2 are available worldwide but with different antigen content. One inactivated, oil-adjuvanted vaccine (Circovac®, Merial) has been licensed for used in breeding age animals and is indicated for the passive immunization of piglets via the colostrum following vaccination of sows or gilts. Three subunit vaccines (Circoflex®, Boehringer Ingelheim; Circumvent PCV®, Merck; Porcilis PCV®, Intervet) are based on PCV2 ORF2 protein expressed in baculovirus system, licensed for pig of three weeks and older animals. Another vaccine (Suvaxyn PCV2®, Pfizer) is based on inactivated recombinant PCV1 expressing the PCV2 ORF2 protein, also licensed for pig of three weeks and older animals. This vaccine is indicated for the active immunisation of piglets from 21 days of age in order to reduce PCV2 viraemia and to reduce PCV2-associated lymphoid tissue lesions. All current PCV2 vaccines are based on the PCV2a isolates against infection with PCV2b (Fort *et al*., 2008). One meta-analysis to review the published literature from 2006 to 2008 concerning the efficacy of PCV2 vaccines on average daily weight gain (ADG) and mortality rate in pigs from weaning to slaughter has been carried out using computerized literature databases (Kristensen *et al*., 2011). Out of 170 studies identified, only 24 studies fulfilled the inclusion criteria and included in the analysis. The results demonstrate significant effect of PCV2 vaccination on ADG for pigs in all production phases. The increased in ADG for finishing pigs was 41.5g, nursery-finishing pigs was 33.6g and only 10.6g increased in the nursery pigs. Mortality rate was reduced for 4.4% in finishing pigs, 5.4 % in nursery-finishing pigs, but was not significantly reduced for nursery pigs (0.25%). Besides that, there were no differences in effect on ADG and mortality rate among the PCV2 vaccines.

The timing of vaccination in piglets is often the topic for argument due to the possible interference of maternal derived antibodies (MDA) with PCV2 vaccines induced immunity. One experimental to study the efficacy of immunizing pigs against PCV2 with different group of pigs vaccinated at three and six weeks of age, indicating no significant impact of MDA (Cline *et al*., 2008). Although field and experimental data indicate PCV2 vaccines partly overcome the MDA effects, but it is recommended to avoid vaccination of too young piglets.

Neutralizing antibodies (NA) against PCV2 has been suggested as the key element in protection against PMWS as few studies have associated the lack of PCV2 neutralizing antibodies with the development of PMWS (Fort *et al*., 2007; Meerts *et al*., 2006). Researcher also reported that increased of NA coincided with the drop in viral load (Fort *et al*., 2007). In one study using the reformulated inactivated chimeric PCV1-2 vaccine has proved to be able to induce PCV2 specific NA and interferon- γ -secreting cells (IFN- γ -SCs), and the induced immunity was correlated with the reduction of PCV2 viremia in pigs (Seo *et al*., 2012). The IFN- γ, which is the main product of IFN- γ -SCs has play important role in regulation of innate and adaptive immunity against viral infections (Schroder *et al*., 2003). Besides that, IFN- γ is also a key immunoregulatory cytokine that controls the differentiation of naïve $CD4^+$ into $CD4^+$ cells, and thus promote the specific cytotoxic immunity by indirect mechanisms, such as up-regulation of antigen processing and presentation. The study described earlier using inactivated chimeric PCV 1-2 also shows result of significant elevation on number of $CD4^+$ cells in those vaccinated animals, but only transient increased of $CD4^+$ cells in non-vaccinated animals. Other studies also reported the selective loss of $CD4^+$ cells was observed in those pigs affected by PMWS (Segale *et al*., 2001). The impaired immunity showed in PMWS affected pigs might explained why PMWS pigs were commonly associated with coinfections with other viral or bacterial pathogens (Seo *et al*., 2012). The same reformulated inactivated chimeric PCV1-2 vaccines were used as well in this study.

In short, commercial PCV2 vaccines able to induce both humoral and cell mediated immunity by stimulate the production of NA and IFN- γ -SC specifically against PCV2. The cell mediated immunity together with NA is able to reduce PCV2 viremia status in pigs and thus improve the pig production. As PMWS and those porcine circovirus associated diseases have multifactorial etiology, it is vital to remember that control and prevention strategies must be implemented at different levels despite the successes of PCV2 vaccines in controlling the diseases.

3.0 MATERIALS AND METHODS

3.1 Animals

This study was conducted at a commercial pig farm located in Tapah, Perak. The selected pig farm is located at a remote area and isolated from others farms. This is an open house pig farm, using the farrow-to-finish, intensive farming system. This pig farm had 200 sows head. Total of thirty 3 weeks old piglets from five farrowed sows were randomly selected as the study objects.

The sows were selected based on the farrowed and parity status. In this study, all sows were farrowed three weeks ago and they were either gilts or with parity status of first-parity up to sixth-parity respectively. All sows were kept were kept in the farrowing pens. The farm has no history of vaccination against porcine circovirus. The experiment protocol was approved by Institutional Animal Care and Use Committee (IACUC), UPM, (No.: FYP- 2014/FYP.042).

3.2 PCV2 vaccine

Commercial PCV2 vaccines were used in this study. The vaccine formulation is an inactivated chimeric PCV1-2 vaccine with sulfolipo-cyclodextrin in squalene-in-water emulsion used as the vaccine adjuvant. The chimeric PCV1-2 virus containing the genomic backbone of the non-pathogenic PCV1, with the ORF2 capsid gene replaced by that of PCV2. PCV2 vaccines were administered according to the manufacturer's instructions with regards to route (intramuscularly) and time of injection (three weeks old piglet).

3.3 Vaccinated Animal

In this study, thirty of 3 weeks old piglets from five farrowed sows were randomly selected and divided into two groups (treatment and control group), each group with 15 animals. For the treatment group, one dose of 2 ml PCV2 vaccine was given intramuscularly, while placebo, 2 ml of sterile water was delivered to control group. All the piglets were fed daily ad libitum with commercial swine feed.

Table 3.1: Total of animals used in this study

	Treatment group (with PCV2 vaccination)	Control group (without PCV2 vaccination)
Number of animals	15	15

3.4 Sampling and Sample handling

Blood sampling and body weight of each animals were measured before and several days after vaccination, where day 0 was referred as before vaccination, followed by day 20 and day 40 post vaccination. The body weight was measured using electronic weighing machine. For blood sampling technique, all piglets of three weeks old (less than 20 kg) were restrained by holding vertically and blood was collected via anterior vena cava by using Vacutainer holder (Vacutainer® Brand Holder, France) and 21G needles (BD Vacutainer® Precision Glide™, UK) and later blood was transferred to plain tubes (Vacutest®, Italy). For weaner (more than 20 kg), the pigs were restrained in their own pens in standing position by using a pig snare (Appendix 3). Bloods were collected via jugular vein by using Vacutainers and 18G needles and later transferred into plain tubes.

Blood samples were leave at room temperature until blood clot formation before immediately stored in an ice box at 4 ℃ and were sent to laboratory. Next, the serums were collected from each blood samples by using a pipette (Eppendorf Research®, Germany). Serums were later transferred into individual 1.5ml micro-centrifuge tube (Copolymer Graduated microtubes, USA), labeled and stored at -20 ℃ (Acson, Malaysia). Serum samples were sent for quatitative polymerase chain reaction (qPCR) test, which were performed with the assistance of laboratory staff.

3.5 Real Time or Quantitative Polymerase Chain Reaction (qPCR)

All serum samples were sent to Institute of BioScience (IBS), UPM for detection of PCV2 virus antigen and evaluation of the PCV2 viremia status by using the qPCR assay. DNA extractions were performed on the serum samples before running of qPCR assay.

3.5.1 DNA Extraction

DNA extraction from the serum samples were performed using commercial NucleoSpin® Blood QuickPure kit (Macherey-Nagel, Germany). Firstly, 200 μL of serum was added into 1.5 mL microcentrifuge tube. Twenty-five μL of proteinase K and 200 μL of Lysis Buffer BQ1 were then added to the tube and the mixture was mixed thoroughly by using a vortex mixer. Proteinase K is a type of broad spectrum serine protease which able to digest the contaminating proteins and nucleases (enzyme that degrade nucleic acids) in serum sample, thus protects the nucleic acids from nuclease attack. The function of Lysis Buffer BQ 1 is to break the cell and nuclear membranes that present in the mixture. Mixture was then incubated at 70 ℃ for 10 – 15 mins. After that, 200 μL of ethanol was added to the sample to adjust the DNA binding conditions. The mixture was then vortex again before transferred to the NucleoSpin® Blood QuickPure Column (Macherey-Nagel, Germany) which placed in a collection tube. The sample was centrifuged for 1 min at 11,000 x g (Eppendorf, Germany) and then the collection tube with flow-through was discarded. Next, the NucleoSpin® Blood QuickPure Column was placed in a new 2 mL collection tube and 350 μL of Buffer BQ2 was added. The mixture was centrifuged again for 3 mins at 11,000 x g, followed by discard of the collection tube with flow-through. Function of Buffer BQ2 is to wash the DNA attached in the column membrane of contaminants and purify the DNA materials. The drying of the silica membrane in the column was performed by centrifugation for 3 mins at 11,000 x g. Finally, the NucleoSpin® Blood QuickPure Column was placed in a new 1.5 mL microcentrifuge tube and 50 µL of Buffer BE was added directly onto the silica membrane to elute the highly pure DNA. The column was incubated at room temperature for 1 min and the final product was obtained by centrifugation of the tube for 1 min at 11,000 x g.

3.5.2 Quantitative or Real-Time Polymerase Chain Reaction (qPCR)

Firstly, a PCR mastermix with standard volume of 20 µL were prepared. The PCR mastermix comprised 10 µL of 2x SensiFAST SYBR No-Rox Mix (Bioline, UK) (containing all the components necessary for real-time PCR, including the SYBR® Green I dye, dNTPs, stabalisers and enhancers), forward and reverse primer each with 400 nM final concentration, 3 µL of template and 5 µL of sterile water. Positive control and no template control (NTC) were included to check the validity of the real-time PCR assay. Real time PCR reactions were performed using CFX96 Real-Time PCR Detection System (Bio-Rad, USA). A 2-step cycling method was used for the q-PCR reactions. The cycling condition included the first 3 mins at 95 ℃ for polymerase activation, followed by 40 cycles of 10 seconds denaturation at 95 ℃ and 30 second of annealing/ extension at 60 ℃. The fluorescence acquisition was performed at every cycle after the annealing or extension. Upon completion of PCR amplification, melt curve analysis was performed. The PCR product was gradually heated from 65 ℃ to 95 ℃ with increment of 0.5 ℃ for every 5 seconds. Fluorescent signal was recorded at each increment of temperature. All samples were analyzed using the CFX96 Manager Version 3.0 (Bio-Rad, USA).

3.5.3 Standard Curve

The amount of virus shedding was measured based on absolute concentration using a standard curve. The standard cure was generated via CFX manager software (Bio-Rad, USA) and shown in Figure 3.1. Efficacy of Real-Time RT-PCR for measuring the load of PCV was 102.7 % with R^2 = 0.999. Slope for the standard curve was -3.258 and y-intercept was 48.508.

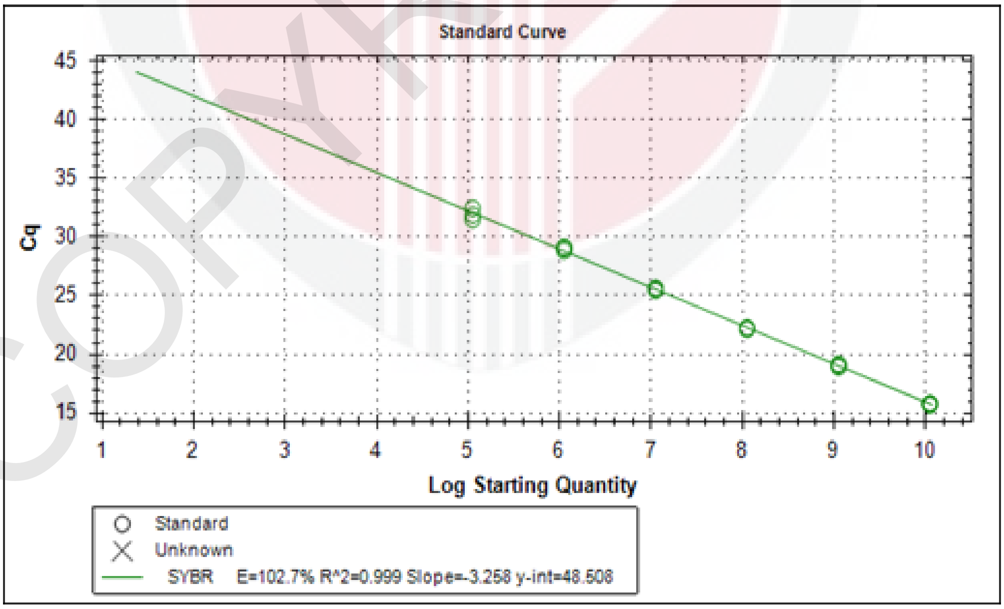

Figure 3.1: A linear relationship between quantification cycle (Cq) and 10 fold serial dilution of DNA. Standard cure was generated using serially diluted DNA of PCV2

3.6 Post-mortem and Histopathology

On day 40 post vaccination, one animal from each vaccination and control group were selected and sacrificed for identification of possible PMWS infection through gross and histological lesions. Pig was sacrificed by using over dose barbiturate (Vétoquinol, UK) and post-mortem was performed. Several tissues samples, such as spleen, inguinal lymph nodes and mesenteric lymph nodes were collected and sent to histopathology laboratory for preparation of histological slides through routine tissues processing and staining with Haematoxylin and Eosin.

4.0 RESULTS AND DISCUSSION

PMWS is one of the most economically important diseases currently faced by the swine industry. Although field studies have showed that PMWS is a multifactorial disease and several co-pathogens might be necessary for the development of clinical disease, PCV2 is considered as the essential etiological agent (Ellis *et al*., 1998). In Malaysia, case study of PMWS has been carried out in 2007 and PCV2 was demonstrated to present in all clinically affected pigs (Ooi *et al*., 2007). The prevalence of PCV infection in Malaysia was estimated to be 88% (Seetha *et al.*, 2011). Quantitation of PCV2 viraemia has been suggested to be used for the prediction of PCV2 infection status as several studies have shown that PCV2 antigens amount were correlated with the severity of PMWS histopathological lesions (Segalés, 2012). Hence, the ability of PCV2 vaccine to cause reduction of PCV2 viraemia plays a critical role in controlling PCV2 infections (Seo *et al*., 2012). In this study, the reformulated inactivated chimeric PCV1-2 vaccines were used to immunize the pigs and efficacy of the vaccine was measured through quantitation of PCV2 viraemia before and after vaccination.

A threshold of more than $10^7$of PCV2 genomes per ml of serum has been suggested as indicating for the presence of PMWS lesions (Olvera *et al*., 2004). Threshold of less than 10^6 of PCV2 genomes per ml of serum indicating of negative PMWS infection, while threshold between 10^6 – 10^7 of PCV2 genomes per ml of serum indicative of positive PCV2 infection and PMWS should be suspected (Opriessnig *et al*., 2007).

The viral PCV2 DNA copies in treated group at Day 0, Day 20 and Day 40 are 5.21 log_{10} copies/mL, 6.25 log_{10} copies/mL and 5.01 log_{10} copies/mL respectively. Whereas in control group, the viral PCV2 DNA copies are 5.15 log_{10} copies/mL, 6.04 log_{10} copies/mL and 6.18 log_{10} copies/mL respectively (Table 4.1). At Day 0, PCV2 DNA was detected in serum from both vaccinated and non-vaccinated pigs. This result has proved that there was PCV2 field challenge in this farm of current study. The mean value of PCV2 virus load in serum is shown in Figure 1.

Table 4.1: Mean value of the genomic copy number of PCV2 DNA in serum from vaccinated and control groups of pigs as determined by real-time PCR assay

Groups	**Viral DNA Copies Number (log_{10})**		
	Day 0	Day 20	Day 40
Treatment	5.21 ±0.18	6.25 ±0.09	5.01 ±0.24
Control	5.15 ±0.21	6.04 ±0.12	6.18 ±0.10

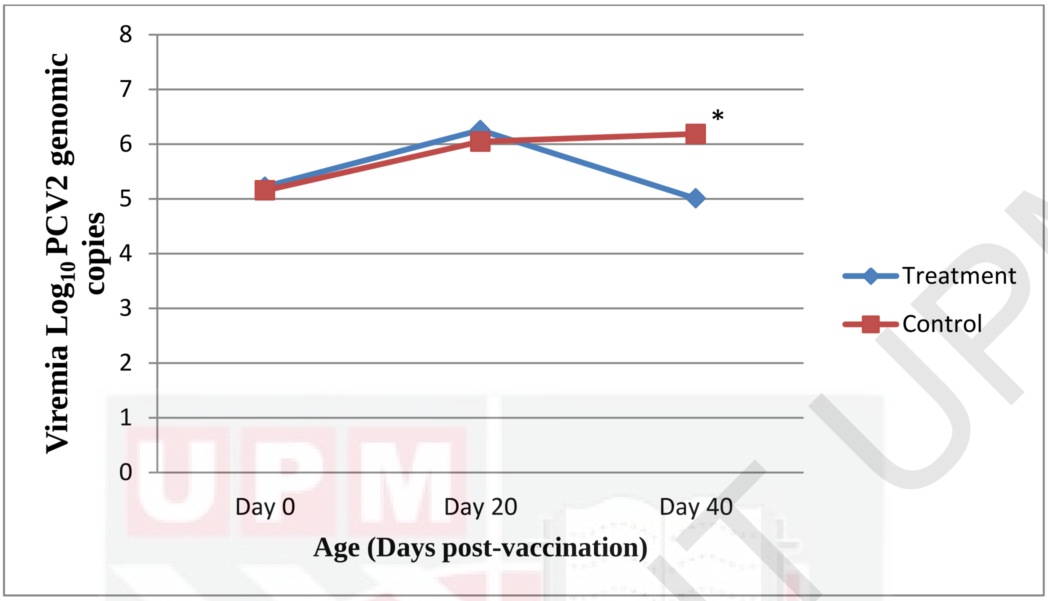

Figure 4.1: Mean value of the genomic copy number of PCV2 DNA in serum from vaccinated and control groups on Day 0, Day 20 and Day 40 post-vaccination as detected by real-time PCR assay. *Significant difference between treatment and control groups is indicated at p value <0.05

On day 20, there was marked elevation of PCV2 viraemia status for both vaccinated and non-vaccinated groups. This was followed by dropped in PCV2 viraemia in vaccinated pigs on day 40 post-vaccination (from 6.25 $\log_{10}$ copies/mL on day 20 to 5.01 $\log_{10}$ copies/mL on day 40), but continuous rising in PCV2 viraemia status was observed in the non-vaccinated pigs. Besides that, there was statistically difference in PCV2 viraemia status between treatment and control groups on day 40 post-vaccination, where treatment group has lower PCV2 viraemia status than the control group.

The initial increment of PCV2 viral load from day 0 to day 20 as seen in both treatment and control groups were believed to be due to the result of shifting of the piglets from farrowing pens to weaner pens have resulted the exposure of pigs to higher environmental PCV2 viral load. At the same time, the maternal antibodies level was decreasing gradually during the nursery periods. PCV2 viraemia usually occurred when maternal derived antibody levels waned (Grau-Roma *et al*., 2009). Besides that, the viraemia status in treatment group was started to drop on day 20 post-vaccination. This finding has fulfilled the indication of the vaccine product that was used in this study where onset of immunity will occur from 3 weeks post-vaccination. The induction of immunity is correlated with reduction of PCV2 viraemia status.

The result from this study support the previous research which showed that one dose of PCV2 vaccine can cause reduction in PCV2 viraemia status in vaccinated animals (Opriessnig *et al*., 2009). Throughout this study, there was no clinically sick animal was detected, and it was supported by the real-time PCR results of all pigs which were below threshold of less than 10^7 PCV2 genomes/mL of serum. In summary, vaccination of pigs with PCV2 vaccine at 3 weeks of age was able to reduce the viraemia status in compared to non-vaccinated pigs.

The mean value of body weight of pigs in treated group at Day 0, Day 20 and Day 40 are 4.58 Kg, 10.45 Kg and 22.29 Kg respectively. Whereas in control group, the mean value of body weight are 4.33 Kg, 9.93 Kg and 21.72 Kg respectively (Table 4.2). This study reveals that statistically there no significant difference between vaccinated and non-vaccinated pigs for mean values of body weight as examined on Day 0, Day 20 and Day 40. The mean value of body weight is shown in Figure 4.2.

Table 4.2: Mean values of body weight of pigs from the treatment and control group on Day 0, Day 20 and Day 40 post vaccination with inactivated PCV2 vaccines

Groups	**Mean Body Weight (Kg)**		
	Day 0	Day 20	Day 40
Treatment	4.58 ±0.18	10.45 ±0.35	22.29 ±0.70
Control	4.33 ±0.17	9.93 ±0.40	21.72 ±0.63

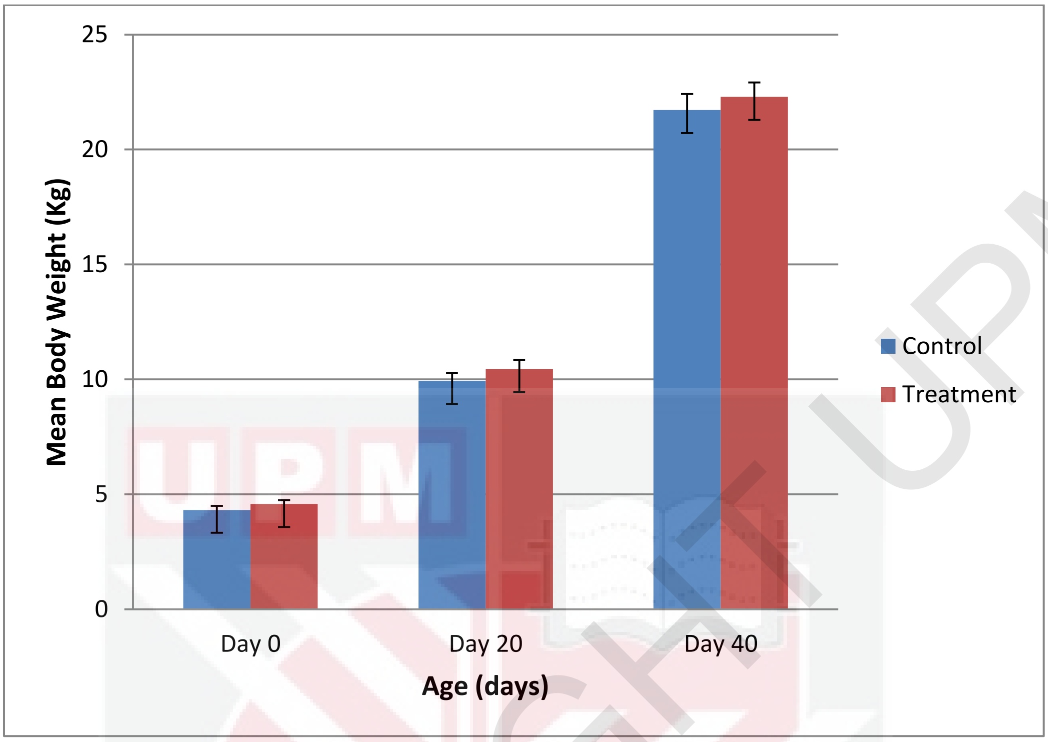

Figure 4.2: Mean values of body weight of pigs from the treatment (vaccinated pig) and control (non-vaccinated pig) group on day 0, day 20 and day 40 post-vaccination

Even though statistically there were no significant difference between vaccinated and non-vaccinated pigs for mean values of body weight, however results showed that vaccinated pigs has slightly higher mean values of body weight than non-vaccinated pigs by 0.52 Kg and 0.57 Kg respectively for day 20 and day 40 post-vaccination. The possible reason that contributed to this result is because PMWS will clinically affect pigs from 2 to 4 months of age, the late-nursery and early-fattening phases of production (Segalés and Domingo, 2002). However, our experiment was ended when pigs were just reached around 2 months of age. This suggests that longer period of experimental study might be required for pigs to show significant results for mean values of body weight of pigs.

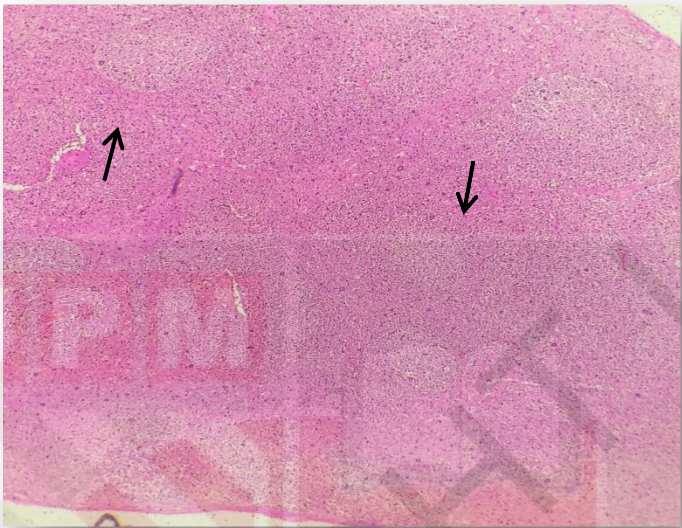

Figure 4.3: Histology section of pig mesenteric lymph node from a vaccinated pig with PCV2 vaccine showed normal lymphoid tissue with presence of spheroidal lymphoid follicles. Haematoxylin and Eosin, magnification 4X

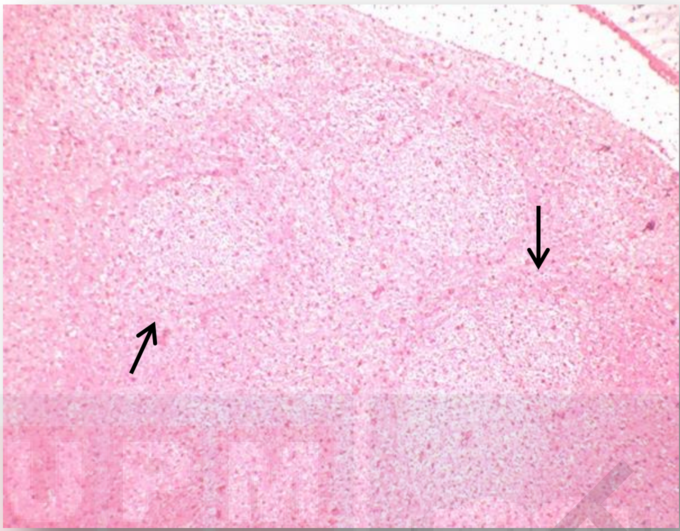

Figure 4.4: Histology section of pig mesenteric lymph node from a pig without PCV2 vaccine shows normal lymphoid tissue with presence of spheroidal lymphoid follicles. Haematoxylin and Eosin, magnification 4X

In this study, one animal from each group was selected and sacrificed for tissues samples for histopathology on day 40. The histology findings from the section of mesenteric lymph nodes of both vaccinated and non-vaccinated animal show normal lymphoid tissue with presence of spheroidal lymphoid follicles. Histology result for other lymphoid tissues, such as spleen and inguinal lymph nodes for both selected animals were also found to be normal. The possibility of the normal histological findings might be due to the subclinical PCV2 infection or animals are not subjected to field challenge. In this study, the PCV2 virus copies number for both vaccinated and non-vaccinated groups were below 10^7 DNA copies per mL of serum. According to one experimental study, results showed that low amounts of PCV2 antigens associated with no to minimal lesions (Opriessnig *et al*., 2007). Microscopic finding in lymphatic tissues for PMWS affected pigs should include histiocytic infiltration, lymphatic depletion, multinucleated giant cells, and grape-like basophilic intracytoplasmic inclusion bodies (Chae, 2004).

5.0 CONCLUSION

As a conclusion, results from this study suggest that PCV2 vaccine given at recommended age of 3 weeks old piglets, able to reduce the PCV2 viraemia level in vaccinated pigs compared to non-vaccinated pigs. By reducing the viraemia status, it is suggested that PCV2 vaccine has the potential to be able to reduce the risk of PMWS infections in pig population.

6.0 RECOMMENDATIONS

To improve the study in future, it is recommended to extend the period of experiment until the pigs reach 100 days old, so that more data and results can be obtained. PMWS will clinically show signs in affected pigs from 2 to 4 months of age, during late-nursery and early fattening phases of production (Segalé and Domingo, 2002). In addition, the farm selected didn't encounter any PCV2 infections problems. It will be interesting to study the result from a PCV2 infected field farms, as to investigate how the vaccine able to manipulate the viraemia of sick pigs. Besides that, larger sample sizes and more number of pig farms (from different states of Malaysia) should be included in the future study as to represent the real scenario of PCV2 infection in Malaysia.

REFERENCES

Allan, G., McNeilly, F., Kennedy, S., Daft, B., Ellis, J. Haines, D., Meehan, B. & Adair, B. (1998). Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. *Journal of Veterinary Diagnostic Investigation*, *10*(1), 3-10. doi:10.1177/104063879801000102

Allan, G., Kennedy, S., McNeilly, F., Foster, J., Ellis, J., Krakowka, S., Meehan, B. & Adair, B. (1999). Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. *Journal of Comparative Pathology*, *121*(1), 1-11. doi:10.1053/jcpa.1998.0295

Allan, G. & Ellis, J. (2000). Porcine circoviruses: A Review. *Journal of Veterinary Diagnostic Investigation*, *12*(1), 3-14. doi:10.1177/104063870001200102

Allan, G., Krakowka, S. & Ellis, J. (2002). PCV2: ticking time bomb. *Pig Progress*, *18*(2).

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. *Journal of Virological Methods* 122, 171–178.

Brunborg, I., Jonassen, C., Moldal, T., Bratberg, B., Lium, B., Koenen, F. & Schonheit, J. (2007). Association of myocarditis with high viral load of porcine circovirus type 2 in several tissues in cases of fetal death and high mortality in piglets. A Case Study. *Journal of Veterinary Diagnostic Investigation*, *19*(4), 368-375. doi:10.1177/104063870701900405

Carman, S., McEwan, B. & DeLay, J. (2006). Porcine circovirus-2 associated disease in swine in Ontario (2004 to 2005). Canada. *Canadian Veterinary Journal*, *47*, 761-762.

Chae, C. (2004). Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. *The Veterinary Journal*, *168*(1), 41-49. doi:10.1016/j.tvjl.2003.09.018

Chang, H., Jeng, C., Lin, T., Liu, J., Chiou, M., Tsai, Y., Chia, M., Jan, T. & Pang, V. (2006). Immunopathological effects of porcine circovirus type 2 (PCV2) on swine alveolar macrophages by in vitro inoculation.*Veterinary Immunology and Immunopathology*, *110*(3-4), 207-219. doi:10.1016/j.vetimm.2005.09.016

Chianini, F., Majo, N., Segalé, J., Dominguez, J. & Domingo, M. (2001). Immunohistological study of the immune system cells in paraffin-embedded tissues of conventional pigs. *Veterinary Immunology and Immunopathology*, *82*(3-4), 245-255. doi:10.1016/s0165-2427(01)00364-6

Clark. & E.G. (1996). Post-weaning multisystemic wasting syndrome. *In:* Proceedings*:* Western Canadian Association *of* Swine Practitioners, pp. 19–20.

Cline, G., Wilt, V., Diaz, E. & Edler, R. (2008). Efficacy of immunising pigs against porcine circovirus type 2 at three or six weeks of age. *Veterinary Record. 163*, 737-740.

Colditz, I. (2002). Effects of the immune system on metabolism: implications for production and disease resistance in livestock. *Livestock Production Science*, *75*(3), 257-268.

Dupont, K., Nielsen, E., Bækbo, P. & Larsen, L. (2008). Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case–control study supports a shift in genotypes with time.*Veterinary Microbiology*, *128*(1-2), 56-64. doi:10.1016/j.vetmic.2007.09.016

Dupont, K., Hjulsager, C., Kristensen, C., Baekbo, P. & Larsen, L. (2009). Transmission of different variants of PCV2 and viral dynamics in a research facility with pigs mingled from PMWS-affected herds and non-affected herds. *Veterinary Microbiology*, *139*(3-4), 219-226. doi:10.1016/j.vetmic.2009.06.001

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. Canada. *Canadian Veterinary Journal*, *39*(1), 44-51.

Ellis, J.A., Konoby, C., West, K.H., Allan, G.M., Krakowka, S., McNeilly, F., Meehan, B. & Walker, I. (2001). Lack of antibodies to porcine circovirus type 2 virus in beef and dairy cattle and horses in western Canada. *Canadian Veterinary Journal* 42, 461–464.

Fort, M., Olvera, A., Sibila, M., Segalés, J. & Mateu, E. (2007). Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs.*Veterinary Microbiology*, *125*, 244-255.

Fort, M., Sibila, M., Allepuz, A., Mateu, E., Roerink, F. & Segalés, 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., Music, N., Fontaine, G., Tremblay, D. & Harel, J. (2010). Emergence of a new type of porcine circovirus in swine (PCV): A type 1 and type 2 PCV recombinant. *Veterinary Microbiology*, *144*(1-2), 18-23. doi:10.1016/j.vetmic.2009.09.072

Gilpin, D., McCullough, K., Meehan, B., McNeilly, F., McNair, I., Stevenson, L., Foster, J., Ellis, J., Krakowka, S., dair, B. & Allan, G. (2003). In vitro studies on the infection and replication of porcine circovirus type 2 in cells of the porcine immune system. *Veterinary Immunology and Immunopathology*, *94*(3-4), 149-161. doi:10.1016/s0165-2427(03)00087-4

Grau-Roma, L., Crisci, E., Sibila, M., López-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A. & Segalés, J. (2008). A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. *Veterinary Microbiology* 128, 23–35.

Grau-Roma, L., Hjulsager, C.K., Sibila, M., Kristensen, C.S., López-Soria, S., Enoe, C., Casal, J., Botner, A., Nofrarias, M., Bille-Hansen, V., Fraile, L., Baekbo, P., Segalés, J. & Larsen, L.E. (2009). Infection, excretion and seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome (PMWS) affected farms in Spain and Denmark. *Veterinary Microbiology* 135, 272–282.

Grau-Roma, L., Fraile, L. & Segalés, J. (2011). Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. *The Veterinary Journal*, *187*(1), 23-32. doi:10.1016/j.tvjl.2010.01.018

Gillespie, J., Opriessnig, T., Meng, X.J., Pelzer, K. & Buechner-Maxwell, V. (2009). Porcine circovirus type 2 and porcine circovirus-associated disease. *Journal of Veterinary Internal Medicine* 23 (6), 1151–1163.

Hamel, A., Lin, Andre, L. & Nayar. (1998). Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *Journal of Vorology*, *72*, 5262-5267.

Harding, J. (1996). Post-weaning multisystemic wasting syndrome (PMWS): preliminary epidemiology and clinical presentation. *Proceedings*: *Western Canadian Association* of *Swine Practitioners*, 21.

Harding, J., Clark, E., Strokappe, J., Willson, P. & Ellis, J. (1998). Postweaning multisystemic wasting syndrome: Epidemiology and clinical presentation. *Swine Health and Production*, *6*(6), 249-254.

Harding, J., Baker, C., Tumber, A., McIntosh, K., Parker, S., Middleton, D., Hill, J. & Krakowka, S. (2008). Porcine circovirus-2 DNA concentration distinguishes wasting from nonwasting pigs and is correlated with lesion distribution, severity, and nucleocapsid staining intensity. *Journal of Veterinary Diagnostic Investigation*, *20*(3), 274-282. doi:10.1177/104063870802000303

Hasslung, F., Wallgren, P., Ladekjarhansen, A., Botner, A., Nielsen, J., Wattrang, E., Allan, G., McNeilly, F., Ellis, J., Timmusk, S., Belak, K., Segall, T., Melin, L., Berg, M. & Fossum, C. (2005). Experimental reproduction of postweaning multisystemic wasting syndrome (PMWS) in pigs in Sweden and Denmark with a Swedish isolate of porcine circovirus type 2. *Veterinary Microbiology*, *106*(1-2), 49-60. doi:10.1016/j.vetmic.2004.12.011

Hassuzana, K., Sharifah, S.H., Siah, M.H., Zuraidah, O. & Fauad, T.R. (2004). The use of restriction fragment length polymorphism technique to identify PMWS in pigs. *In the Proceedings of the 14th International Conference of the Association of Institutes for Tropical Veterinary Medicine and 16th Veterinary Association Malaysia Congress*.p. 238-239.

Hoogland, M., Opriessnig, T. & Halbur, P. (2006). Effects of adjuvants on porcine circovirus type 2-associated lesions. *Journal of Swine Health and Production*, *14*, 133-139.

Ilyina, T. & Koonin, E. (1992). Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria.*Nucleic Acid Research*, *20*(13), 3279-3285. doi:10.1093/nar/20.13.3279

Karuppannan, A., Jong, M., Lee, S., Zhu, Y., Selvaraj, M. Lau, J., Jia, Q. & Kwang, J. (2009). Attenuation of porcine circovirus 2 in SPF piglets by abrogation of ORF3 function. *Virology*, *383*(2), 338-347. doi:10.1016/j.virol.2008.10.024

Kekarainen, T., McCullough, K., Fort, M., Fossum, C., Segalés, J. & Allan, G.M. (2010). Immune responses and vaccine-induced immunity against porcine circovirus type 2. Vet. *Immunol. Immunopathol*. 136 (3–4), 185–193.

Kim, J., Chung, H., Jung, T., Cho, W., Choi, C. & Chae, C. (2002). Postweaning multisystemic wasting syndrome of pigs in Korea: prevalence, microscopic lesions and coexisting microorganisms. *Journal of Veterinary Medical Science*, *64*(1), 57-62. doi:10.1292/jvms.64.57

Krakowka, S., Ellis, J., Meehan, B., Kennedy, S., McNeilly, F. & Allan, G. (2000). Viral wasting syndrome of swine: Experimental reproduction of postweaning multisystemic wasting syndrome in gnotobiotic Swine by coinfection with porcine circovirus 2 and porcine parvovirus.*Veterinary Pathology*, *37*(3), 254-263. doi:10.1354/vp.37-3-254

Krakowka, S., Ellis, J., McNeilly, F., Ringler, S., Rings, D. & 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). *Veterinary Pathology*, *38*(1), 31-42. doi:10.1354/vp.38-1-31

Kristensen, C., Bækbo, P., Bille-Hansen, V., Bøtner, A., Vigre, H., Enøe, C. & Larsen, L. (2009). Induction of porcine post-weaning multisystemic wasting syndrome (PMWS) in pigs from PMWS unaffected herds following mingling with pigs from PMWS-affected herds. *Veterinary Microbiology*, *138*(3-4), 244-250. doi:10.1016/j.vetmic.2009.04.004

Kristensen, C., Baadsgaard, N. & Toft, N. (2011). A meta-analysis comparing the effect of PCV2 vaccines on average daily weight gain and mortality rate in pigs from weaning to slaughter.*Preventive Veterinary Medicine*, *98*(4), 250-258. doi:10.1016/j.prevetmed.2010.11.015

Larochelle, Bielanski, Muller. & Magar. (2000). PCR detection and evidence of shedding of porcine circovirus type 2 in boar semen. *Journal of Clinical Microbiology*, *38*, 4629-4632.

Larochelle, R., Magar, R., D'Allaire. & S. (2003). Comparative serologic and virologic study of commercial swine herds with and without postweaning multisystemic wasting syndrome.*Canadian Journal of Veterinary Research*, *67*, 114-120.

Liu, J., Chen, I. & Kwang, J. (2005). Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. *Journal of Virology*, *79*(13), 8262-8274. doi:10.1128/jvi.79.13.8262-8274.2005

Lőrincz M., Cságola A., Biksi I., Szeredi L., Dán A. & Tuboly T. (2010) Detection of porcine circovirus in rodents - short communication. Acta Vet Hung. 58. 265-268. *Journal Impact Factor*: 1,264

Madson, D., Ramamoorthy, S., Kuster, C., Pal, N., Meng, X., Halbur, P. & Opriessnig, T. (2009). Infectivity of porcine circovirus type 2 DNA in semen from experimentally-infected boars. *Veterinary Research*, *40*(10).

Mankertz, A., Persson, F., Mankertz, J., Blaess, G. & Buhk, H.-J. (1997). Mapping and characterization of the origin of DNA replication of porcine circovirus. *Journal of Virology* 71, 2562–2566.

Meehan, B., McNeilly, F. & Todd, D. (1998). Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *Journal of General Virology*, *79*, 2171 - 2179.

Meehan, B., McNeilly, F., McNair, I., Walker, I., Ellis, J., Krakowka, S. & Allan, G. (2001). Isolation and characterization of porcine circovirus 2 from cases of sow abortion and porcine dermatitis and nephropathy syndrome. *Archives of Virology*, *146*(4), 835-842. doi:10.1007/s007050170152

Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Bøtner, A., Kristensen, C. & Nauwynck, H. (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 Veterinary Research*, *2*(1), 6. doi:10.1186/1746-6148-2-6

Moo, K.S., Arshad, S.S. & Ooi, P.T. (2014). Efficacy of killed porcine circovirus vaccine in reducing viraemia status in pigs. *In: 9th Proceedings of* *the Seminar on Veterinary Sciences, Faculty of Veterinary Medicine, University Putra Malaysia*, 62-65.

Morozov, I., Sirinarumitr, T., Sorden, S., Halbur, P., Morgan, M., Yoon, K. & Paul, P. (1998). Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. *Journal of Clinical Microbiol*, *36*(9), 2535-2541.

Olvera, A., Sibila, M., Calsamiglia, M., Segalés, 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. *Journal of Virological Methods* 117, 75–80.

Olvera, A., Cortey, M. & Segalés, J. (2007). Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. *Virology* 357, 175–185.

Ooi, P.T., Choo, P.Y., Hii, Do., Shahirudin, S., Seetha, J. & Lim, B.K. (2007). Identification of porcine circovirus type 2 from pigs with postweaning multisystemic wasting syndrome in Malaysia. *In: Proceedings of the 19th Veterinary Malaysia Association Scientific Congress*, 109-111.

Opriessnig, T. (2006a). Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. *Journal of General Virology*, *87*(10), 2923-2932. doi:10.1099/vir.0.82099-0

Opriessnig, T., Fenaux, M., Thomas, P., Hoogland, M., Rothschild, M., Meng, X. & Halbur, P. (2006b). Evidence of breed-dependent differences in susceptibility to porcine circovirus type-2-associated disease and lesions. *Veterinary Pathology*, *43*(3), 281-293. doi:10.1354/vp.43-3-281

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. *Journal of Veterinary Diagnostic Investigation* 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. *Clinical and Vaccine Immunology* 15, 397–401.

Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N. & Halbur, P.G. (2009). Comparisonof 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.

Park, J., Kim, J., Ha, Y., Jung, K., Choi, C. Lim, J., Kim, S. & Chae, C. (2005). Birth abnormalities in pregnant sows infected intranasally with porcine circovirus 2. *Journal of Comparative Pathology, 132*(2-3), 139-144. doi:10.1016/j.jcpa.2004.09.003

Patterson AR, Ramamoorthy S, Madson DM, Meng XJ, Halbur PG, Opriessnig T. (2011). Shedding and infection dynamics of porcine circovirus type 2 (PCV2) after experimental infection. Vet Microbiology. 2011. 149:91-98.

Rose, N., Abhervé-Guéguen, A., Le Diguerher, G., Eveno, E., Jolly, J., Blanchard, P., Aurelie, O., Andre, J. & Francois, M. (2005). Effect of the Pietrain breed used as terminal boar on post-weaning multisystemic wasting syndrome (PMWS) in the offspring in four PMWS-affected farms. *Livestock Production Science,95*(3), 177-186. doi:10.1016/j.livprodsci.2004.12.020

Rosell, C., Segalés, J., Plana-Duran, J., Balasch, M., Rodríguez-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. *Journal of Comparative Pathology, 120*(1), 59-78. doi:10.1053/jcpa.1998.0258

Schroder, K., Hertzog, P., Ravasi, T. & Hume, D. (2003). Interferon- : an overview of signals, mechanisms and functions. *Journal Of Leukocyte Biology, 75*(2), 163-189. doi:10.1189/jlb.0603252

Seetha, J., Ooi, P.T., Tan, D.Y. & Choo, P.Y. (2011). Molecular characterization of porcine circovirus in Malaysia. *In: Proceedings of the 22th Veterinary Association Malaysia Congress.*

Segalés, J., Alonso, F., Rosell, C., Pastor, J., Chianini, F. Campos, E. Lopez-Fuertes, L., Quintana, J., Rodriquez-Arrioja, G., Calsamiglia, M., Pujols, J., Dominguez, J. & Domingo, M. (2001). Changes in peripheral blood leukocyte populations in pigs with natural postweaning multisystemic wasting syndrome (PMWS). *Veterinary Immunology and Immunopathology, 81*(1-2), 37-44. doi:10.1016/s0165-2427(01)00326-9

Segalés, J. & Domingo, M. (2002). Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. *Veterinary Quarterly* 24, 109–124.

Segalés, J., Allan, G.M. & Domingo, M. (2005). Porcine circovirus diseases. *Animal Health Research Reviews* 6, 119–142.

Segalés, J. (2012). Porcine circovirus type 2 (PCV2) infections: Clinical signs, pathology and laboratory diagnosis. *Virus Research, 164*(1-2), 10-19. doi:10.1016/j.virusres.2011.10.007

Seo, H., Oh, Y., Han, K., Park, C. & Chae, C. (2012). Reduction of porcine circovirus type 2 (PCV2) viremia by a reformulated inactivated chimeric PCV1-2 vaccine-induced humoral and cellular immunity after experimental PCV2 challenge. *BMC Veterinary Research*, *8*(1), 194. doi:10.1186/1746-6148-8-194

Sorden, S. (2000). Journal of Swine Health and Production. *Update on Porcine Circovirus and Postweaning Multisystemic Wasting Syndrome (PMWS)*, *8*(3).

Tischer, I., Gelderblom, H., Vettermann, W. & Koch, M. (1982). A very small porcine virus with circular single-stranded DNA. *Nature*, *295*(5844), 64-66. doi:10.1038/295064a0

Tischer, I., Mields, W., Wolff, D., Vagt, M. & Griem, W. (1986). Studies on epidemiology and pathogenicity of porcine circovirus. *Archives of Virology*, *91*(3-4), 271-276. doi:10.1007/bf01314286

Tischer, I., Peters, D., Rasch, R. & Pociuli, S. (1987). Replication of porcine circovirus: induction by glucosamine and cell cycle dependence. *Archives of Virology*, *96*(1-2), 39-57. doi:10.1007/bf01310989

Vincent, I., Carrasco, C., Herrmann, B., Meehan, B., Allan, G., Summerfield, A. & McCullough, K. (2003). Dendritic cells harbor infectious porcine circovirus type 2 in the absence of apparent cell modulation or replication of the virus. *Journal of Virology*, *77*(24), 13288-13300. doi:10.1128/jvi.77.24.13288-13300.2003

APPENDIX 1

Result of the mean PCV2 copies number in treatment and control group of pigs as determined by q-PCR assay at different age

Treatment Group

Tag Number	Viral DNA Copies Number ($1og_{10}$)		
	Day 0	Day 20	Day 40
1922	6.44	6.67	6.14
1924	6.02	5.91	4.54
1925	4.05	5.84	5.36
1929	4.24	5.89	5.57
1930	4.36	6.80	4.21
1932	5.15	5.90	4.03
1935	5.95	6.06	5.31
1939	5.62	5.89	5.82
1940	6.28	6.57	5.70
1944	5.49	6.18	5.83
1946	4.66	6.58	3.86
1947	5.37	5.79	6.19
1950	4.90	6.60	4.19
1953	4.46	6.20	5.19
1955	5.12	6.78	2.92

Control Group

Tag Number	Viral DNA Copies Number ($1og_{10}$)		
	Day 0	Day 20	Day 40
1921	4.47	6.56	6.14
1926	5.80	5.80	6.16
1927	5.30	5.77	6.30
1931	5.87	6.22	5.33
1933	4.05	6.18	5.83
1934	5.11	6.51	6.62
1936	5.73	6.47	5.66
1941	5.01	6.00	6.44
1942	4.88	4.81	6.03
1943	5.84	6.24	6.82
1948	5.97	5.97	5.90
1951	5.89	5.91	6.61
1952	4.88	6.42	6.44
1954	3.31	5.74	6.30

APPENDIX 2

Result for mean body weight in treatment and control group of pigs at different age

Treatment Group

Tag Number	Mean Body Weight (Kg)		
	Day 0	Day 20	Day 40
1922	4.36	10.24	19.70
1924	4.54	8.74	19.30
1925	5.24	10.68	25.00
1929	4.62	11.36	23.00
1930	4.54	10.32	22.70
1932	4.92	12.02	28.0
1935	3.64	10.46	22.10
1939	4.32	10.08	20.30
1940	3.28	7.36	18.50
1944	4.58	9.94	20.20
1946	5.96	11.78	24.0
1947	5.62	12.78	25.90
1950	4.28	9.20	22.50
1953	4.16	10.54	19.80
1955	4.68	11.30	23.30

Control Group

Tag Number	Mean Body Weight (Kg)		
	Day 0	Day 20	Day 40
1921	3.50	7.80	17.80
1926	5.16	11.52	23.20
1927	4.38	11.12	21.70
1931	4.54	10.10	22.30
1933	3.86	8.86	19.70
1934	4.32	9.26	24.30
1936	3.76	9.52	22.00
1941	4.50	9.84	19.20
1942	3.58	7.88	19.20
1943	3.58	8.84	18.70
1948	6.06	13.88	26.70
1951	4.64	10.60	23.70
1952	4.68	10.80	22.70
1954	4.18	9.88	23.40
1956	4.18	9.12	21.20

APPENDIX 3

APPENDIX 3.1: Blood sampling through anterior vena cava in three weeks old piglet

APPENDIX 3.2: Blood sampling in two month old pig via jugular vein with standing restraining method

APPENDIX 3.3: Post-mortem on day 40 pig from control group, showing mild enlargement of inguinal lymph node