An investigation of the aetiology and natural transmission of postweaning multisystemic wasting syndrome in pigs

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Abstract

Postweaning multisystemic wasting syndrome (PMWS) is a wasting disease primarily affecting weaned pigs. The disease causes significant production and financial losses through increased mortality rates and reduced daily weight gain. The aetiology is controversial although reports commonly suggest that PMWS is associated with the presence of porcine circovirus type 2 (PCV2) with disease expression modified by a range of infectious and non-infectious factors. While PCV2 infection in New Zealand is ubiquitous, PMWS has behaved as a propagating epidemic since its first incursion beginning in about 1999. The initial outbreak of PMWS in New Zealand was limited to a small cluster of farms near Auckland, which were epidemiologically linked to a possible entry mechanism.

A transmission study was conducted in 2005 to critically evaluate alternative hypotheses which have been proposed for the causation and epidemiology of PMWS. The study set out to investigate the natural transmission of PMWS by direct contact between PMWS-affected and susceptible pigs, while managing the influence of proposed co-factors. Six different groups, comprised of pigs from PCV2-negative and positive herds were directly exposed to possible PMWS agents at 4 and 12-weeks-of-age and compared with two groups of unexposed pigs. All experimental groups were observed daily for 8 weeks or longer and evaluated clinically and pathologically.

After exposure to PMWS-affected pigs, disease characterised by wasting, dyspnoea and high case fatality rates occurred in both PCV2-positive and PCV2-negative pigs of four-weeks-of-age, but not in pigs older than 12 weeks. Histopathological lesions found in experimental groups with clinical cases were consistent with those previously reported for PMWS. A range of infectious pathogens proposed to have a modifying influence on PCV2 and to contribute to disease causation were absent as determined by molecular and serological test methods. In addition, there was not sufficient molecular evidence to explain the genomic difference between PCV2 isolates from healthy and PMWS-

affected pigs. Taking this, and supporting evidence from the other experimental groups into account, the findings of this study strongly support the conclusion that a transmissible agent other than PCV2 is involved in the causality of PMWS.

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Contents

List of Figures

List of Tables

Chapter 1

Introduction

The first cases of postweaning multisystemic wasting syndrome (PMWS) were identified in pig herds of high health status in western Canada in 1991 (Harding & Clark 1997, Ellis et al. 1998). The pigs had been raised in total confinement and were free of the common major porcine respiratory and enteric diseases (*Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, progressive atrophic rhinitis, salmonellosis, swine dysentery, transmissible gastroenteritis virus, and Aujezsky's disease), but not free of porcine reproductive and respiratory syndrome virus (PRRSV) (Harding & Clark 1997, Ellis et al. 1998). PMWS was named due to severe losses in body condition typically confined to pigs in the postweaning stage of production. Dyspnoea was also a consistent and characteristic clinical sign, while pallor, rough hair coat, jaundice and diarrhoea were recorded infrequently (Harding et al. 1998).

PMWS has behaved as a propagating epidemic on an international level since 1991 (Morris et al. 2002, Lawton et al. 2004a, Vigre et al. 2005). It has spread to many countries worldwide including the USA, UK, Asia and most of Europe (Allan & Ellis 2000, Segalés & Domingo 2002) and causes significant production and financial loss to a large number of pig farms. At farm level, the economic impact of PMWS infection depends on the severity of disease with morbidity rates reported from 4% to 60%, mortality rates reported from 4% to 20% and case fatality rates of 70% to 80% (Segalés & Domingo 2002). The most significant economic effects are seen in direct production loss through increased mortality rates and reduced daily weight gain. Post weaning mortality increased by 27 percentage points (from 3% to 30%) in a batch of growers on a finishing farm of 1,000 places resulted in a deficit of >$40 (Canadian dollars) per pig (Leblanc & Morin 2005). On a large multi-site production system with 15,000 sows, an estimated loss in gross margin of between €3.9 and €4.1 (Euros) per pig was associated with PMWS (Hardge et al. 2003). PMWS also has a considerable economic impact at the international level where the overall losses for countries of the European Union

(EU) are estimated to be between €562 million and €900 million per year (Segalés et al. 2006, Anonymous 2007d).

The expression of PMWS has remained relatively consistent, although clinically, PMWS can be difficult to recognise and differentiate from other pig diseases. Post mortem examination is required to diagnose PMWS with confidence. Although necropsy and histopathological investigation of a PMWS case reveals a range of pathological lesions affecting multiple organs (lymph nodes, lung, kidney, spleen, and gastro-intestinal tract) they are not pathognomonic (Harding & Clark 1997). Common microscopic findings are interstitial pneumonia and nephritis, hepatocellular apoptosis, lymphohistiocytic infiltration of gastric, caecal and colonic mucosa, lymphocyte depletion and histiocytic infiltration of lymphoid tissues (Harding et al. 1998). The presence of intensely basophilic stained clusters of intracytoplasmic inclusion bodies is a particular histological feature of PMWS (Harding & Clark 1997). This is most frequently recorded in lymphatic tissue such as the lymph nodes, ileal Peyer's patches and tonsils of PMWS-affected pigs but is not always present. These inclusion bodies are identified as clusters of porcine circovirus type 2 (PCV2) by diagnostic test methods such as *in situ* hybridisation and immunohistochemistry.

Developing a useful case definition of PMWS is challenging given uncertainty of the aetiological agent and the absence of a diagnostic gold standard. The first formal case definition of PMWS was published in 2000 (Sorden 2000) and is known as Sorden's criteria for PMWS. It is the most commonly used case definition and requires that a pig or a group of pigs exhibit the following three criteria: (1) clinical signs characterised by wasting, with or without dyspnoea or icterus; (2) histologic lesions characterised by depletion of lymphoid tissues with or without lymphohistiocytic to granulomatous inflammation in any organ; and (3) PCV2 infection within characteristic lesions. Not all PMWS cases fulfil the Sorden's criteria, mainly because the clinical signs and severity of disease seen in individual pigs can be variable. Some affected countries have developed their own definition of PMWS due to slight variation of disease manifestation between countries.

The detection of porcine circovirus (PCV) in tissues of PMWS-affected pigs suggests that it plays some role in the pathogenesis of PMWS (Harding & Clark 1997).

Although PCV has not been confirmed as the sole aetiologic agent of PMWS, the virus has commonly been implicated as the causative agent for the disease (Ellis et al. 1998, Allan et al. 1998a). Antigenic and genomic differences have been identified between PCV isolates from field cases of PMWS and the previously detected PCV contaminant of PK-15 cell cultures (porcine kidney cell line routinely used in virology laboratories). These findings have strongly pointed to the existence of two distinct PCV genotypes (Allan et al. 1998b, Meehan et al. 1998, Hamel et al. 1998, Allan et al. 1999b). The PCV contaminant of the PK-15 cell line has consequently been designated as type 1 PCV (PCV1) and is known to be non-pathogenic in pigs, while viral strains associated with PMWS cases in the field and potentially pathogenic in pigs have been termed type 2 PCV (PCV2) (Allan et al. 1998b, Meehan et al. 1998).

There has been much speculation about the possible origins of PMWS. Multiple hypotheses have been formulated in an effort to explain its aetiology with most assuming PCV2 as the causal agent of PMWS. It has been suggested that PCV2 is a mutated strain of PCV1 that has emerged in the pig populations of several countries during the last decade (Ellis et al. 1998). However, retrospective studies on archived tissues and serum samples have revealed that PCV2 was prevalent and widespread prior to the occurrence of the recent PMWS epidemics in Canada, Northern Ireland, Spain, England and Wales (Larochelle et al. 1999a, Magar et al. 2000b, Walker et al. 2000, Rodriguez-Arrioja et al. 2003, Grierson et al. 2004a). This suggests the involvement of additional co-factors of an infectious or non-infectious nature, which have contributed to the development of PMWS.

Research is ongoing in an effort to further elucidate the pathogenesis of the disease and to determine the causality of PMWS. There are divergent views of what agents cause PMWS, each supported by research and field experience. The most widely recognised position is that PMWS is caused by PCV2 with disease expression modified by the presence of co-infections with PRRSV, PPV (porcine parvovirus) (Kennedy et al. 2000, Ellis et al. 2000, Harms et al. 2001, Hasslung et al. 2005) or other porcine pathogens (Rodriguez-Arrioja et al. 1999, Pallarés et al. 2002, Ellis et al. 2004, Wellenberg et al. 2004); immunostimulation (Krakowka et al. 2001, Kyriakis et al. 2002); genetics (López-Soria et al. 2004, Opriessnig et al. 2006a) or management effects (Allan et al.

2003). The association between the presence of these factors and the occurrence of PMWS is weak and can commonly be contradicted with findings of other studies.

A recent hypothesis on the role of PCV2 in the aetiology of PMWS consists of the possible existence of different PCV2 strains which vary in virulence. This hypothesis may have merit in explaining PMWS occurrence despite the lack of evidence identifying significant differences between PCV2 strains on affected and unaffected farms (Grierson et al. 2004b, de Boisséson et al. 2004). A number of studies have associated changes in PCV2 genomes with a difference in the pathogen's virulence. Respiratory signs, enlarged lymph nodes, and lymphoid depletion among inoculated pigs were associated with changes in the capsid protein encoded segment of the PCV2 genome (Fenaux et al. 2004, McKeown et al. 2006). Opriessnig et al. (2006c) demonstrated the first evidence of a clear difference in histopathological lesions when comparing two strains of PCV2. The study, however, failed to reproduce clinical signs consistent with PMWS despite the use of an original PCV2 isolate which was collected from a confirmed case of PMWS. In summary, there is insufficient molecular evidence to explain the difference between PMWS-affected farms and non-affected farms by an infection of PCV2 alone. It appears that an additional co-factor or unknown agent is required to trigger clinical PMWS under field conditions.

The presence of clinical PMWS may be better explained by an unidentified agent because PCV2 infection is widespread and frequently found on PMWS unaffected farms (Larochelle et al. 1999a, Rodriguez-Arrioja et al. 2000, Pogranichniy et al. 2002). This hypothesis is strongly supported by the epidemiological behaviour of PMWS being consistent with that of a novel infectious agent (Morris et al. 2002, Lawton et al. 2004a). Vigre et al. (2005) described the spatial and temporal pattern of PMWS-positive Danish pig herds over two years following the introduction (from October 2001) of the disease into the country. The number of herds affected with PMWS increased markedly, spreading to most parts of Denmark after the local outbreak. Two geographical clusters were identified in areas with the highest density of pig herds. PMWS has spread between pig farms in New Zealand in an identical epidemiological pattern. New Zealand's first PMWS-infected pig herd was identified in the Waikato region (North Island) in September 2003 (Lawton et al. 2004b). Additional farms were diagnosed as PMWS-positive during the outbreak investigation. All herds were geographically

clustered near Auckland and epidemiologically linked through frequent movements of live pigs, feed, equipment, vehicles and people (Lawton et al. 2004a). Movement controls between the North Island and South Island and quarantine measures on PMWS-affected premises were legally enforced in late 2003/early 2004 to avoid nationwide spread of the disease (Rawdon et al. 2004, Stone 2004).

It appears that PMWS has behaved like a propagating epidemic in the New Zealand pig population with similarity to the pattern of spread in other countries. Porcine circovirus was endemic in New Zealand's pig population at the time of the PMWS epidemic without distinction between PCV1 and PCV2 (Horner 1991). Later studies also confirmed that PCV2 was ubiquitous in New Zealand's pig population at that time (Tham & Hansen 2003, Garkavenko et al. 2005). The geographical cluster of farms with a higher risk of PMWS and the evidence of linkages between most farms indicate that a novel agent may have been transmitted between PMWS-affected farms.

Scientific knowledge regarding the pathogenesis and aetiology of PMWS is still incomplete and some findings contradict previous work. Further research and experimental studies focus on resolving the remaining questions of what the causal agent of PMWS is, the specific pathogenesis of the disease and its modes of transmission.

The following chapters describe the investigation of the natural transmission of PMWS and the clinical course of the disease with the pathological and molecular findings of a transmission study in New Zealand, undertaken in 2005. An additional objective of the study was to produce evidence for the existence of an unknown causal agent.

Chapter 2

Literature review

2.1 Introduction

Porcine multisystemic wasting syndrome (PMWS) was detected as a new emerging disease in weaned pigs in Canada more than a decade ago (Harding & Clark 1997, Ellis et al. 1998) and has since behaved as a propagating epidemic and spread worldwide. Research in different parts of the world has focused on the pathogenesis of the disease with particular effort to find the causal agent. The most frequently suggested causal agent for PMWS is porcine circovirus type 2 (PCV2) but this agent can be found in almost all pig herds throughout the world regardless of PMWS occurrence. There are still many unresolved questions about the causality of PMWS which serve as objectives for our studies.

2.2 History

2.2.1 Detection of PMWS

The first cases of PMWS in recently weaned pigs (weaners) were identified in high health SPF[1] pig herds in Saskatchewan (western Canada), in 1991, and reported later in 1996 (Harding & Clark 1997, Ellis et al. 1998). These authors suggested naming the disease 'postweaning multisystemic wasting syndrome' due to its clinical conditions. Pigs were most commonly affected at two to three weeks post weaning (five to six-weeks-old). The most consistent and characteristic clinical signs were wasting and dyspnoea; the expression and severity of the disease being exacerbated by non-infectious factors such as poor air quality and drafts, commingling, and overcrowding. Other more infrequent clinical signs such as pallor, rough hair coat, jaundice and diarrhoea were recorded as well (Harding et al. 1998). PMWS has been difficult to recognise and differentiate from other diseases and infectious pathogens causing similar

[1] SPF: Specific-pathogen-free means a herd or animal is free of defined pathogens.

symptoms due to its non-specific clinical signs. Porcine reproductive and respiratory syndrome (PRRS), swine influenza, porcine proliferative enteropathy (*Lawsonia intracellularis*), *Mycoplasma hyopneumoniae*, and Glässer's disease (*Haemophilus parasuis*) were common diseases in western Canada known to cause clinical wasting. PMWS could not easily be diagnosed based on clinical signs alone and therefore required the addition of post mortem examination. Post mortem examinations of PMWS-affected pigs revealed a diversity of lesions similar to the variability seen in the clinical presentation of the disease. Pathology was found in multiple organs and in particular affected lungs, lymph nodes, the kidney, the spleen, and the gastro-intestinal tract (Harding & Clark 1997). Microscopic lesions have frequently been reported including systemic lymphadenopathy, interstitial pneumonia, hepatocellular apoptosis, lymphohistiocytic infiltration of gastric, caecal and colonic mucosa, lymphocyte depletion and histiocytic cell infiltration of lymphoid tissues (Harding et al. 1998). These authors considered the presence of these lesions to be strongly suggestive of PMWS, although not pathognomonic. A more unique feature of PMWS was the presence of intensely basophilic stained clusters of intracytoplasmic inclusion bodies noted in different lymphatic tissues (lymph nodes, Peyer's patches of the ileum, and tonsils). When viewed through electron microscopy, these inclusion bodies were identified as clusters of porcine circovirus (PCV) (Harding & Clark 1997). Their identity was confirmed by PCV nucleic acid and PCV antigen being found in large quantities within the tissue lesions of PMWS-affected pigs. These findings provided the first suggestion that PCV might have been the causal agent.

2.2.2 PCV virus as a causal agent

Porcine circovirus infection has been associated with PMWS and the virus implicated as the causative agent for the disease. Different diagnostic methods have been used to detect PCV in tissues of clinically PMWS-affected pigs. Immunohistochemical staining, electron microscopy, and virus isolation have demonstrated the presence of porcine circovirus-like (PCV-like) particles in porcine tissues from Canada, California (USA) and Brittany (France) (Ellis et al. 1998, Allan et al. 1998a). However, the researchers questioned whether this PCV-like virus was identical to the well-known PCV contaminant of a continuous pig kidney cell line (PK-15), known to be non-pathogenic in pigs.

2.2.3 Differentiation between PCV types

The PCV contaminant was identified by German researchers in 1974 (Tischer et al. 1974) and described as a small spherical virus, morphologically resembling the picornavirus. The same research group published the morphological details of the virus 10 years later and proposed to name it porcine circovirus due to its circular deoxyribonucleic acid (DNA) structure (Tischer et al. 1982). Further studies were conducted to examine the pathogenicity and epidemiology of this PCV (Tischer et al. 1986). Six PCV-negative miniature pigs (mini-pigs) were experimentally infected intranasally with a purified suspension of PCV at nine months of age. The pigs did not develop clinical signs of disease over 39 weeks and there was no pathological or histopathological evidence of viral infection at post mortem. Virus was, however, isolated from nasal and faecal samples from days 3 – 6, 13 and 14 post infection, indicating that pigs had been infected with PCV. This provided evidence that PCV was not pathogenic in pigs but did cause an apparent infection.

Demonstration of PCV-like antigen and nucleic acid in tissue lesions of PMWS cases led to speculation among scientists that a novel, potentially pathogenic porcine circovirus may have emerged in pig populations of several countries. Results of studies by Nayar et al. (1997) and Ellis et al. (1998) suggested significant antigenic differences between PCV-like viruses from field cases with PMWS and PCV from PK-15 cells. This finding was strongly supported by Allan et al. (1998b), indicating that virus isolates from field cases were closely related in size and morphology but antigenically distinct from the viral contaminant of PK-15 cells. Following studies on genotyping of the original PCV contaminant of PK-15, and different PCV isolates from field cases, have verified the preceding assumptions that these two agents are distinct genotypes (Meehan et al. 1998, Hamel et al. 1998, Morozov et al. 1998, Allan et al. 1999b). Allan et al. (1998b) and Meehan et al. (1998) proposed to designate the non-pathogenic original PCV contaminant of PK-15 cell line as type 1 PCV (PCV1) and the potentially pathogenic novel PCV as type 2 PCV (PCV2) for reasons of simplification and clear differentiation between the two genotypes. Today, these viruses are commonly referred to as PCV1 and PCV2. Ongoing research has confirmed the existence of at least two genogroups of PCV2 and proposed the classification of two PCV2 genotypes (1 and 2) (Grau-Roma et al. 2007, Lager et al. 2007, Cheung et al. 2007).

2.2.4 The origin of PMWS

There have been multiple hypotheses and speculation about the origin of PMWS. It has been suggested that due to its high similarity with PCV1, PCV2 is simply a mutated strain of PCV1 that emerged in pig populations of several countries during the last decade (Ellis et al. 1998). Given their identical host cell tropism and similar genomic structure, organisation, and nucleotide sequence, PCV1 and PCV2 could possibly have a common ancestor (Hamel et al. 1998, Olvera et al. 2007). Other causes of PMWS have also been hypothesised. Interactions between immunosuppressive agents such as porcine reproductive and respiratory syndrome virus (PRRSV) and unidentified agents have been proposed. Changes in non-infectious factors such as management or environment may have altered the host's immune response to PCV2 which is prevalent in pig populations worldwide, creating a situation where the circovirus has become more virulent (Ellis et al. 1998).

Although no causal association has been confirmed between PMWS and PCV2, porcine circovirus has been implicated as the causative agent for the disease and further studies have been focused primarily on this pathogen. More investigations are required to better elucidate the aetiology of PMWS and to bring insights into the pathogenesis of this complex disease.

2.3 Epidemiology

2.3.1 Relationship between PCV2 and PMWS prevalence

Researchers and epidemiologists attempted to clarify whether PMWS was truly a newly emerged disease after the discovery of the first few cases of PMWS. Retrospective studies on archived tissues and serum samples revealed that PCV2 was prevalent prior to the occurrence of the PMWS epidemic. Larochelle et al. (1999a) identified PCV2 DNA in archived tissues dating back to 1994, indicating that PCV2 was circulating in pigs several years before clinical PMWS occurred in the province of Québec (Canada) in 1997. Similar findings were confirmed in a study of archived samples from the Canadian national pig serum bank. Magar et al. (2000b) demonstrated that PCV2 was present in the Canadian pig population for at least 10 years before the first cases of PMWS were reported in 1996.

Retrospective studies on archived samples were also conducted in different European countries to investigate the prevalence of PCV2 prior to the PMWS epidemic and to estimate the date of virus introduction into the pig population. Walker et al. (2000) identified antibodies specific to PCV2 in archived porcine sera from Northern Ireland, dating back to 1973, indicating that the virus was introduced more than 20 years before the first case of PMWS occurred. Rodriguez-Arrioja et al. (2003) studied archived tissues and sera of selected 4 to 16-week-old pigs in Spain between 1985 and 1997. According to their findings, the first case with pathological lesions characteristic of PMWS occurred in 1996, whereas PCV2 had been circulating in the pig population since at least 1985, which was 12 years prior to the first case of PMWS being reported. It was possible that diagnostic laboratories in the country might have failed to report cases of PMWS prior to 1996, due to a lack of central data collection in Spain (Joaquim Segalés, personal communication). In Grierson's study (2004a), archived tissue samples were selected from pigs between 5 and 12 weeks-of-age from Wales (1995 – 1997) and England (1970 – 1995). The findings of this study were consistent with the situation previously documented in Northern Ireland and Spain. PCV2 DNA was detected in archived pig tissues from 1970, demonstrating that England's pig population was exposed to the virus at least 30 years before the PMWS epidemic started in 1999.

In summary, PCV2 was circulating well before the PMWS epidemic. Consequently, this would suggest the involvement of co-factors of infectious or non-infectious nature in the development of PMWS. It could also be argued that genetic mutation of PCV2 from a non-pathogenic to pathogenic form might have caused the worldwide occurrence of PMWS.

2.3.2 Prevalence of PCV2 at farm, national and international level

Serological surveys have shown that PCV2 was distributed worldwide since at least the 1980's (Allan & Ellis 2000). Further studies were required to investigate the prevalence of PCV2 on current PMWS-affected and non-affected pig farms to better understand the epidemiology and aetiology of PMWS. Rodriguez-Arrioja et al. (2000) compared serum samples of necropsied pigs with and without PMWS between 1997 and 1998. The 4 to 20-week-old pigs used originated from 37 different Spanish farms. Antibody titres against PCV2 were present in 88/90 serum samples with two pigs being sero-

negative. The majority of sero-positive samples corresponded to PMWS-affected pigs and PMWS-positive farms but high antibody levels to PCV2 were also observed in animals and farms without a history of PMWS, indicating that subclinical PCV2 infections were occurring on these farms. These findings (Rodriguez-Arrioja et al. 2000) were supported by the results of a field-based case-control study by Pogranichniy et al. (2002), where PCV2 was found in 63% of pigs that had no clinical signs or histopathological lesions typical of PMWS. Comparable results were also observed in a Canadian study (Larochelle et al. 1999a) on archived tissue from 1997 to 1998. PCV2 DNA was detected in 40/42 field cases submitted but only 15 cases demonstrated clinical signs compatible with PMWS. This suggested that PCV2 was even more prevalent than previously expected and that subclinical or asymptomatic PCV2 infection may occur in the absence of clinical signs of PMWS. To summarise the findings of these studies, subclinical PCV2 infection has been shown to be prevalent in pig herds without PMWS, providing no evidence for a causal link between PCV2 prevalence and the occurrence of PMWS on affected farms. This led to new hypotheses that differences in virulence or pathogenicity exist between PCV2 strains.

It is important to look at the incidence of PMWS on an international scale with reference to findings of serological surveys on the prevalence of PCV2 at farm and national level. Publications of the first reported cases of PMWS from individual countries are detailed in Table 2.1.

Table 2.1: Publication details of 'first case' reports of PMWS for several countries.

Year	Country	Reference
1991	Canada	(Harding & Clark 1997)
1995	France	(LeCann et al. 1997)
1996	USA	(Daft et al. 1996)
1997	Spain	(Segalés et al. 1997)
1998	Northern Ireland	(Kennedy et al. 1998)
1999	England Japan	(Potter 2000b, Gresham et al. 2000) (Onuki et al. 1999)
2000	Netherlands	(Wellenberg et al. 2000)
2000	Switzerland	(Borel et al. 2001)
2001	South Africa Mexico Denmark	(Drew et al. 2004) (Trujano et al. 2001) (Hassing et al. 2002)
2003	New Zealand	(Rawdon et al. 2004)
2005	Venezuela	(Cano et al. 2005)

2.3.3 Introduction of a novel agent

PMWS epidemic in the United Kingdom

Publications of observations and surveys of the PMWS epidemic in the UK illustrate the spatial epidemiology of the disease and provide useful information about its transmission. A marked increase of cases was noticed between December 1999 and mid-April 2000 (Gresham et al. 2000), after reporting the first cases of PMWS in early 1999. The majority of cases occurred in East Anglia (eastern region of England), where extensive corporate pig farming existed (Mackinnon 2000). The higher incidence of PMWS in this region was associated with increased movements of pigs between corporate farms, implicating direct contact between animals as a means of disease transmission. A number of independent pig farms were also affected, however, and these farms had not bought in any livestock for many years. Vectors such as semen, feed, equipment, clothing and birds were considered as possible routes of disease transmission. In consequence, strict biosecurity measures have been recommended but despite attempts to control the disease, PMWS spread throughout the UK subsequent to 1999/2000 (Muirhead 2002). This situation was surveyed in December 2001, involving a non-random selection of 62 veterinary practitioners' clients (Gresham et al. 2003).

Information was collected on disease expression and implemented control measures on PMWS-affected farms during the preceding 12-month-period. No explanation for the differences in the expression of PMWS throughout the UK was found despite comparisons of different control measures that were applied. At this stage, further research is required to determine the means of PMWS transmission.

Findings of a recent study by Woodbine et al. (2007) indicate that PMWS behaved as an infectious epidemic in Great Britain in 2000 – 2003. This retrospective cohort study investigated risk factors for herd breakdown due to PMWS and potential risks preceding the evidence of disease in 93 pig herds and pig farming companies. Risk factors associated with PMWS breakdown were large herd size, purchasing stock, human biosecurity and local spread from neighbouring infected premises. The conclusions of the study were that the pathogen of PMWS was very resistant with a long infectious period and low transmissibility and that transmission can occur directly between pigs or indirectly between pigs and humans.

PMWS epidemic in Denmark

Vigre et al. (2005) described the spatial, temporal and spatio-temporal pattern of PMWS-affected Danish pig herds for the two years period after the first disease was diagnosed (October 2001/September 2003). The study population consisted of 6,724 pig herds, which involved 277 herds diagnosed with PMWS. Two geographically independent areas were identified with a significantly higher risk for PMWS when compared to the rest of the study population. Not surprisingly, the two clusters coincided with the areas highest in pig herd density in Denmark. The authors' conclusions were that (1) transmission of PMWS was due to movement of live pigs between herds, and (2) the PMWS epidemic was caused by the introduction of an unidentified pathogen rather than changes in management.

PMWS epidemics in New Zealand

North Island of New Zealand

New Zealand's first PMWS case was diagnosed in the Waikato district (North Island) in September 2003 (Lawton et al. 2004b). Additional infected farms were identified in

2003/2004 which formed a geographical cluster near Auckland and farms were epidemiologically linked through frequent movements of live pigs, feed, equipment, vehicles and people (Lawton et al. 2004a). Movement controls between the North and South Island and quarantine measures on PMWS infected premises were legally imposed late 2003/early 2004 to limit nationwide spread of the disease (Rawdon et al. 2004, Stone 2004). The route of entry of PMWS to New Zealand has still not been fully confirmed but the most likely pathway was through the legal importation of pig meat around 1999. Kitchen waste of human food could have been fed to pigs without prior processing during this period (Stone 2004). New Zealand is free from most major viral pig diseases such as PRRS, foot and mouth disease (FMD), swine influenza, and classical swine fever (CSF) (Anonymous 2007b) so their involvement as viral co-infections is questionable. Porcine circovirus was already endemic in New Zealand's pig population in 1991 (Horner 1991), although there was no distinction between PCV1 and PCV2 at that time. It was later confirmed that PCV2 was ubiquitous in New Zealand's pig population (Tham & Hansen 2003, Garkavenko et al. 2005).

South Island of New Zealand

A survey was conducted on 48 purposively selected pig farms in the South Island during the PMWS outbreak investigations on the North Island, to determine whether the South island was free of PMWS (Stone 2004). None of the herds examined were infected at that time, and no subsequent cases of PMWS were detected through surveillance until January 2006, when clinical signs of PMWS were observed in the Canterbury region. The first clinical signs consistent with PMWS were reported from two outdoor pig breeding units (index farms) in 8 to 14-week-old pigs. These farms were five kilometres apart and were not linked by contact or exchange of live animals, or other fomites. Additional pig herds soon became infected through the purchase of weaners from one of the index farms. Other differential diagnoses were excluded during the outbreak investigation and the final diagnosis of PMWS was made early in March 2006. Investigations trying to identify the source of the PMWS incursion are currently in progress, with a specific focus on feeding of human waste food. Pig movements and birds are implicated as potential routes of transmission in the South Island outbreak (Neumann et al. 2007).

In the absence of major viral pig diseases, proposed as necessary co-factors for development of PMWS, it has behaved like a propagating epidemic on both the North and South Islands of New Zealand, although these outbreaks were spatially and temporally unrelated. The geographically clustered distribution of PMWS infection and evidence of linkages between most farms suggests that an infectious agent has been transmitted and circulates on PMWS-affected farms. The similar characteristics of PMWS epidemics in Denmark, New Zealand and the UK strongly support the hypothesis of an unidentified transmissible agent or a novel strain of agent being responsible for causing the disease.

2.4 Case definition of PMWS

2.4.1 General definition of PMWS

A case definition of PMWS was published by Sorden (2000), years after the first cases were identified in Canada in 1991. This case definition required that a pig or a group of pigs exhibit all of the following three criteria: (1) clinical signs characterised by wasting, with or without dyspnoea or icterus; (2) histologic lesions characterised by depletion of lymphoid organs/tissues with or without lymphohistiocytic to granulomatous inflammation in any organ (typically lungs and/or lymphoid tissues, and less often liver, kidney, pancreas, intestine); and (3) PCV2 infection within characteristic lesions.

Not all recognised PMWS cases fulfil the Sorden criteria, mainly because the clinical signs and severity seen in individual animals are quite variable. Some affected countries have developed their own definition of PMWS to reflect the variation of disease manifestation in their pig population. Developing a useful definition of PMWS is challenging given the current uncertainty regarding the aetiological agent and the absence of a diagnostic gold standard[2].

[2] Diagnostic gold standard is usually an internationally recognised diagnostic method or approach with best sensitivity and specificity.

2.4.2 International variations in the definition of PMWS

Europe

An international consortium project in the European Union (EU) focused on the control of porcine circovirus diseases with an aim towards improved food quality and safety. The consortium published its own PMWS herd case definition in October 2005 (Committee of PCVD consortium 2005) for reasons of clarification and uniform use of terminology and definitions. The definition is expected to change whenever new knowledge of aetiology, epidemiology or pathology of PMWS becomes available. The current definition is based on: (1) the clinical appearance in the herd; and (2) laboratory examination of necropsied pigs suffering from wasting as follows:

(1) Clinical appearance on herd level – The occurrence of PMWS in a herd is characterised by an excessive increase in mortality and wasting in pigs post weaning compared to historical levels. Two options are suggested for recognising an increase in mortality depending on availability of mortality records:

(1.1) If mortality has been recorded, then the increase in mortality may be recognised if CM $\geq$ Mean of HM + 1.66 $\times$ SD (where CM = current mortality, HM = historic mortality and SD = standard deviation), or a chi-square test of whether CM > HM. Mortality is defined as the prevalence of dead pigs within one or two months and the historical reference period should be at least three months.

(1.2) If there are no records on mortality, then an increase in mortality exceeding the national or regional level by 50% is considered indicative of PMWS.

(2) Pathological and histopathological diagnosis of PMWS – A herd is considered positive for PMWS when the pathological and histopathological findings, indicative for PMWS, are all present at the same time in at least one of the autopsied pigs (minimum of 5 pigs/herd). The pathological and histopathological findings include:

(2.1) Clinical signs of growth retardation and wasting, enlargement of inguinal lymph nodes, dyspnoea, diarrhoea with jaundice apparent sporadically.

(2.2) Presence of characteristic histopathological lesions in lymphoid tissues including lymphocyte depletion with histiocytic infiltration and/or inclusion bodies and/or giant cells.

(2.3) Detection of PCV2 within the lesions in lymphoid tissues of affected pigs (using antigen detection in tissue by immunostaining or *in situ* hybridisation) in moderate to large quantity.

To exclude other obvious reasons for high mortality (e.g. *Escherichia coli* post weaning diarrhoea or acute pleuropneumonia) relevant diagnostic procedures have to be carried out.

America

As information of an unrecognised disease becomes available, case definitions, classifications and terminology are continuously altered. In November 2006, the American Association of Swine Veterinarians (AASV) approved that the name 'Porcine Circovirus Associated Diseases' (PCVAD) be adopted rather than PMWS to account for the variability in clinical presentations currently described and associated with PCV2 infections in pigs (Anonymous 2006). PMWS is therefore no longer considered as a distinct disease, but more as one of several expressions of porcine circovirus infection. The AASV considers its PCVAD case definition to be dynamic and presents it as follows:

(1) PCVAD can be subclinical or include one or more of the following clinical manifestations concurrently:

(1.1) Multisystemic disease with weight loss (formerly known as PMWS).
(1.2) Doubling of historical mortality rate without the introduction of a new known pathogen.
(1.3) Respiratory signs including pneumonia.
(1.4) Porcine dermatitis and nephropathy syndrome (PDNS).
(1.5) Enteric signs including diarrhoea and weight loss.

(1.6) Reproductive disorders including abortions, stillbirths and fetal mummification with diagnosis requiring the presence of fetal myocarditis associated with PCV2 antigen in lesions.

(2) PCVAD is a broad categorisation of multisystemic diseases that are confirmed by documentation of the following histopathological findings in affected pigs:

(2.1) Depletion of lymphoid cells in lymphoid tissues of growing pigs.
(2.2) Disseminated granulomatous inflammation in one or more tissues (e.g. spleen, thymus, intestines, lymph nodes, lung, kidney, liver, tonsil).
(2.3) Detection of PCV2 within the lesions of growing pigs.
(2.4) PCV2 associated reproductive disease with diagnosis requiring the presence of PCV2 antigen in fetal myocarditis lesions.

New Zealand

The New Zealand Ministry of Agriculture and Forestry (MAF) developed a case definition for the diagnosis at the farm level in combination with the diagnostic criteria for pigs by Sorden (Stone 2004). The farm-level diagnosis had to fulfil the following criteria:

(1) Current or historical evidence of non-responsive wasting in 6 to 12-week-old pigs with a high case fatality rate; combined with
(2) Acute phase mortality rate in the target age group typically of about 15% or greater, but at least twice the pre-acute mortality; or, elevated mortality rate in the post acute phase.

Criteria to determine animals as affected by PMWS had to include the following:

(1) Clinical signs of wasting with or without dyspnoea or icterus

(2) Histopathological lesions in lymphoid tissues such as depletion of lymphoid cells and
(2.1) presence of infiltrative (eventually multinucleated) histiocytes in the cortex or,

(2.2) evidence of lympho-histiocytic inflammatory and degenerative epithelial changes in non-lymphoid organs (e.g. liver, lung, kidney) but definitively,

(2.3) evidence of botryoid inclusion bodies within lesions.

It was not always feasible to identify pig farms as PMWS affected after first investigations despite applying the case definition. Often the clinical picture of wasting in grower pigs was confounded with poor management and it was almost always possible to find six poorly performing pigs within herds. In addition, on some farms livestock records were deficient to verify current or historic mortality rates, or history of any disease incursion. A longitudinal surveillance of affected farms on a weekly basis over a few months by inspectors of MAF was imposed as a consequence (Loth & Stone 2005). If long-term observations had not clarified the suspicion of PMWS infection on certain farms, naïve sentinel pigs of high health status from a PMWS-free herd were introduced. Following the correction of major management deficiencies and sentinel pigs remaining free of disease the farm was declared as free of PMWS (Lachlan McIntyre, personal communication).

Australia

In June 2005, a disease investigation suggestive of PMWS at a South Australian growout unit necessitated the development of a case definition for diagnosis of PMWS within Australia. The Australian case definition relied on the following three key elements (Pope 2007):

(1) A herd syndrome involving elevated mortality and obvious wasting in pigs between weaning and 12 weeks of age, generally unresponsive to appropriate management interventions.

(2) Characteristic histopathological signs (particularly involving lymphoid tissues) present in laboratory submissions from affected/'suspect' pigs: and

(3) The presence of abundant Porcine Circovirus type 2 (PCV2) associated with the histological lesions.

Australia still claims freedom from PMWS but this might be due to the failure of suspect cases of PMWS to meet the stringent terms of the Australian definition.

2.5 Clinical diagnosis of PMWS

2.5.1 Differential diagnoses for PMWS

There are numerous causes for poor performance and wasting in nursery and grower pigs which can be infectious and non-infectious in nature, hence PMWS does not produce pathognomonic clinical signs. Common pig diseases and pathogens such as PRRS, swine influenza, porcine proliferative enteropathy (*Lawsonia intracellularis*), *Mycoplasma hyopneumoniae*, Glässer's disease (*Haemophilus parasuis*), and post weaning colibacillosis are all capable of mimicking the clinical picture of PMWS (Harding & Clark 1997). Wasting in growing pigs can also be caused by post weaning anorexia, starvation, substandard management, poor quality and/or mouldy feed, environmental stressors, and gastric ulcers. Post mortem examination, therefore, is crucial to differentiate PMWS from other diseases or non-infectious causes of wasting. A brief overview of the major viral and bacterial diseases with comparable clinical signs to PMWS is given in Table 2.2.

Table 2.2: Major viral and bacterial diseases with comparable clinical signs to PMWS.

Disease	Pathogen	Clinical signs	Reference
Viral			
PRRS	PRRSV	Dyspnoea, anorexia, wasting, morbidity (5%-75%), increased mortality (12%-20%)	(Zimmerman et al. 2006)
Swine influenza	Influenza A virus	Dyspnoea, anorexia, coughing, wasting, fever, high morbidity (near 100%), low mortality (<1%)	(Olsen et al. 2006)
Bacterial			
Proliferative enteropathy	*Lawsonia intracellularis*	Anorexia, wasting, diarrhoea	(McOrist & Gebhart 2006)
Mycoplasmal pneumonia	*Mycoplasma hyopneumoniae*	Coughing, decreased appetite, wasting, fever, high morbidity, low mortality	(Thacker 2006)
Glässer's disease	*Haemophilus parasuis*	Fever, anorexia, dyspnoea, wasting, rough hair coat	(Rapp-Gabrielson et al. 2006)
Enteric colibacillosis	*Escherichia coli*	Diarrhoea, reduced appetite	(Fairbrother & Gyles 2006)
Swine dysentery	*Brachyspira hyodysenteriae*	Diarrhoea, partial anorexia, elevated temperature (40.0°C-40.5°C)	(Hampson et al. 2006)

2.5.2 Clinical indications of PMWS

The early indications of PMWS incursion on a grower farm are an increased frequency of 'un-thriftiness' (wasting), dyspnoea, pallor, rough hair coat, jaundice and diarrhoea in weaned pigs. Affected pigs are generally non-responsive to various modes of antimicrobial therapy and higher than expected rates of post weaning mortality occur. Not all of the clinical signs will be observed in a single affected pig but collectively PMWS-affected groups will experience most of these signs over time (Harding 2004). It can be difficult to definitively arrive at a diagnosis of PMWS in practice, especially on small farms where the number of PMWS-affected animals might be too low to recognise or often there is a lack of adequate farm records to demonstrate changes in mortality rate. In addition to this, it is well documented that PMWS does not always

appear in the same form. Clinical symptoms can differ considerably between farms and there is variation in morbidity and mortality rates among infected herds depending on whether infection is acute or chronic. Early in the Canadian PMWS epidemic, Harding et al. (1998) reported wasting and dyspnoea as the most common and consistent clinical signs, closely followed by rough hair coat, pallor, jaundice and diarrhoea. Other researchers have repeatedly confirmed similar clinical findings (Sorden 2000, Segalés & Domingo 2002) and sometimes visibly enlarged inguinal superficial lymph nodes are noticed (Ellis et al. 1998, Segalés et al. 2004b).

2.5.3 Mortality rates

Mortality due to PMWS can vary considerably between farms. Mortality rate can reach a peak of up to 10% in the acute phase of a PMWS outbreak (Harding & Clark 1997). In an epidemiological survey of 15 PMWS-affected farms in Saskatchewan and Alberta (Canada), a slightly lower average mortality rate of 6.7% with a case fatality rate of 81.4% was documented (Harding et al. 1998). Other authors have reported morbidity rates to range from 4% to 60% and case fatality and mortality rates of 70% to 80% and 4% to 20%, respectively (Segalés & Domingo 2002). An increase of up to 40% in mortality rates on affected farms was seen in the rising epidemic in the UK in 2002 (Muirhead 2002). In New Zealand, early reports of mortality rates were confounded with poor management practices which exacerbated mortality. The first two farms in the PMWS outbreak on the North Island in 2003 reported weaner mortality rates of 20% to 50% (Rawdon et al. 2004).

2.5.4 Infections coinciding with PMWS

There is circumstantial evidence that severity and expression of disease symptoms depend on farm-specific factors such as husbandry, management, environment, nutrition and health status. Drafts, poor air quality, overcrowding, commingling pigs of different age groups and the presence of other infections increase the prevalence and severity of PMWS (Harding & Clark 1997, Allan & Ellis 2000). Concurrent diseases have been observed more frequently on PMWS-affected farms when compared to non-infected farms (Ellis et al. 2004). In a study of 484 field cases of PMWS in the United States large percentages of farms were co-infected with other pathogens (PRRSV (51.9%),

Mycoplasma hyopneumoniae (35.5%), bacterial septicaemia (14.0%), bacterial pneumonia (7.6%), and swine influenza (5.4%)) (Pallarés et al. 2002). Wellenberg et al. (2004) confirmed similar findings in a case-control study of 60 PMWS-affected pigs (cases) and 180 pigs without PMWS (controls) in the Netherlands. A concurrent PRRS infection was found in at least 83% of the cases as compared to 35% in controls. This illustrates not only the increased prevalence of co-infections on PMWS-affected farms, but also emphasises the importance and difficulty of differentiating PMWS from other diseases with similar clinical symptoms.

2.5.5 Duration of PMWS outbreaks

There are few published studies on the duration of PMWS persistence in infected herds. In a survey of 15 PMWS-positive Canadian pig farms (Harding et al. 1998), PMWS contributed to increased mortality rates for a period of 18 months. Similarly, in a survey conducted on 62 PMWS-affected farms with PDNS[3] in the UK (Gresham et al. 2003), 10 farms experienced an increased mortality rate for 274 days on average (95% CI: 201 to 347 days).

2.6 Pathology of PMWS

2.6.1 Macroscopic findings

Post mortem examination is essential to differentiate PMWS from other important diseases. Necropsy reveals a diversity of lesions with macroscopic lesions not always being present (Harding & Clark 1997, Segalés & Domingo 2002). Characteristic gross pathological findings of PMWS are summarised in Table 2.3.

[3] PDNS: Porcine dermatitis and nephropathy syndrome is a relatively recent disease in pigs and is characterised by multifocal skin lesions, weight loss, oedema of the limbs, vasculitis, and glomerulonephritis. The cause is unknown, but histopathological and immunological findings suggest the pathogenesis involves an immune-complex disorder possibly due to an infectious agent (Cameron 2006). It is speculated that PCV2 may be associated with the aetiology of PDNS (Grierson et al. 2004a).

Table 2.3: Typical macroscopic lesions of PMWS.

Condition/Organ	Lesion	Reference
Body condition	Wasting, marked spine, cachexia	(Segalés & Domingo 2002)
Skin	Moderate pallor, icterus	(Harding & Clark 1997)
Lung	(Diffusely) non-collapsed, rubbery, firm, tan-mottled, sometimes marked interstitial oedema, associated bacterial bronchopneumonia	(Harding & Clark 1997, Rosell et al. 1999, Segalés & Domingo 2002)
Lymph nodes	*Early clinical stage:* Marked enlargement (mainly superficial inguinal, submandibular, mesenteric, mediastinal), homogenous on cut surface *Advanced clinical stage:* Normal or even atrophic	(Harding & Clark 1997, Rosell et al. 1999, Segalés & Domingo 2002, Segalés et al. 2004b)
Stomach	Gastric ulceration of pars oesophagea, sometimes gastric wall oedema	(Harding & Clark 1997, Segalés & Domingo 2002)
Kidney	Normal, or multifocal white foci in cortex, or enlarged, oedematous	(Harding & Clark 1997, Segalés & Domingo 2002)
Liver	Yellowish-orange, mild to moderate mottling, diffuse atrophy	(Harding & Clark 1997, Segalés & Domingo 2002)
Spleen	Enlarged, meaty, non-congested	(Harding & Clark 1997, Segalés & Domingo 2002)
Intestine	Fluid-filled, thin-walled sections of lower intestine (particularly ileum and spiral colon), soft rectal faeces (catarrhal colitis associated with diarrhoea)	(Harding & Clark 1997, Segalés & Domingo 2002)

The most frequent gross pathological findings seen in PMWS-affected pigs are poor body condition, non-collapsed lungs with associated bacterial bronchopneumonia, and enlarged lymph nodes. Gastric ulcers are of multifactorial origin and not seen as a direct effect of the disease, although a few PMWS-associated deaths are caused by gastric ulcers and their internal haemorrhage and are related to the pale skin (Segalés & Domingo 2002).

2.6.2 Microscopic findings

Histopathology is required for the final diagnosis of PMWS as macroscopic lesions seen at necropsy are not sufficient to diagnose the disease with confidence. The most characteristic microscopic lesions attributable to PMWS are found in lymphoid tissues

(tonsils, lymph nodes, spleen and ileal Peyer's patches), although inflammatory infiltrates associated with PMWS are observed in a variety of other organs (Harding & Clark 1997, Rosell et al. 1999). Histopathological lymphoid lesions are distinctive where lymphocyte depletion with loss of follicular structure is found in almost all PMWS-affected pigs (Segalés & Domingo 2002). Large histiocytes and/or multinucleated histiocytic cells are detected in a lymphocyte depleted tissue in the early stage of disease, and a prominent network of stromal and accessory cells is found in an empty lymphoid tissue in the final stage (Segalés et al. 2005).

Other microscopic lesions associated with PMWS are found in a wide range of tissues with the most common being interstitial pneumonia found in lungs (Rosell et al. 1999). Changes in lung tissue are characterised by thickening of interalveolar walls and the presence of inflammatory cells in the alveoli. Mild to intense multifocal interstitial nephritis is often present and infiltrates of lympho-histiocytic inflammatory cells are detected within the renal cortex (Rosell et al. 1999). The most common hepatic lesions are described as lymphocytic-histiocytic inflammatory infiltration in portal zones. Multifocal necrosis of single hepatocytes is observed and sometimes generalised perilobular fibrosis with disruption of liver plates. Extensive changes of hepatocytes are associated with icterus and macroscopic lesions in the liver (Rosell et al. 1999, Segalés & Domingo 2002).

The presence of sharply demarcated, spherical, intensely basophilic, intracytoplasmic inclusions in histiocytic cells of lymphoid tissues is another prominent feature of PMWS. These inclusion bodies are associated with large amounts of intracytoplasmic PCV2 antigen (Rosell et al. 1999, Allan & Ellis 2000) and their presence in microscopy depends on the severity of disease (Krakowka et al. 2005). Methods such as *in situ* hybridisation (ISH) or immunohistochemistry (IHC) are used to detect PCV2 within tissue lesions. PCV2 can be observed in cells such as histiocytes, multinucleated giant cells, and in other monocyte/macrophage lineage cells, including alveolar macrophages, Kupffer cells and follicular dendritic cells of lymphoid tissues (Rosell et al. 1999, Allan & Ellis 2000).

2.6.3 Virus detection

In situ hybridisation and immunohistochemistry

A variety of techniques have been developed to detect PCV2 antigen in the tissues of pigs affected by PMWS. ISH and IHC are routinely used tests for detecting PCV2 nucleic acid and antigen, respectively. These diagnostic methods have led to the observation that there is a strong correlation between the amount of PCV2 nucleic acid or antigen and the severity of microscopic lymphoid lesions (Rosell et al. 1999, Quintana et al. 2001). In the study of Quintana et al. (2001), however, PCV2 nucleic acid and antigen was also found in tissues of clinically healthy pigs, albeit in smaller amounts with mild histopathological lesions. Positive results in clinically healthy pigs or diseased pigs without clinical signs and macroscopic lesions consistent with PMWS should be interpreted carefully (Segalés & Domingo 2002), because subclinical PCV2 infection with viremia occurs on almost all farms regardless of PMWS occurrence (Rodriguez-Arrioja et al. 2000, Pogranichniy et al. 2002).

Polymerase chain reaction

Polymerase chain reaction (PCR) technique has been described as a very sensitive test to detect PCV2 (Larochelle et al. 1999b, Mankertz et al. 2000, Quintana et al. 2001, Calsamiglia et al. 2002), therefore recently infected or subclinically infected animals and convalescent PMWS cases with very mild microscopic lesions and/or very little virus are more likely to be detected by PCR than by ISH (Calsamiglia et al. 2002). The sole use of non-quantitative PCR is not, however, suited for PMWS diagnosis as PCV2 is widely spread within the pig population. The quantitative real-time PCR method is recommended to quantify PCV2 load in tissues and/or serum samples as the amount of PCV2 within lesions is the major difference between clinically PMWS-affected pigs and subclinically PCV2 infected pigs (Rovira et al. 2002, Olvera et al. 2004, Brunborg et al. 2004). The studies of Olvera et al. (2004) and Brunborg et al. (2004) described quantitative PCR as a complementary test to histochemical PMWS diagnosis. These authors investigated the relationship between viral load in plasma and tissues, proposing that true PMWS cases will exceed 10^7 PCV2 genomes per ml of serum. It was concluded that PCR on serum was well suited to screening in live animals. This methodology is technically demanding and dependent on the availability of properly

designed probes and standards, limiting its application as a diagnostic and prognostic tool (Krakowka et al. 2005).

2.6.4 Antibody detection

PCV2 is ubiquitous in the pig population but no distinct pattern is seen between seroconversion to PCV2 infection in herds with PMWS and those without PMWS (Rose et al. 2002, Sibila et al. 2004). Several serological tests have been reported in the literature including ELISA[4] and fluourescent antibody techniques. These tests have been developed by research groups to measure PCV2 antibodies and investigate PCV2 infections in experimental and epidemiological studies (Segalés et al. 2005), but there is a commercial PCV2 serology test available for routine diagnostic use (Synbiotics Europe SAS 2005).

2.6.5 Diagnostic issues

A diagnostic gold standard for PMWS does not exist. Gold standards are defined as tests or procedures that diagnose with 100% sensitivity and specificity (Dohoo et al. 2003). In reality, no method is able to fulfil these idealistic criteria but some tests perform better than others and are officially recognised as gold standards in science and industry. Several methods and approaches have been used over the last decade to diagnose PMWS, but none of them have performed satisfactorily enough to be recognised as a gold standard.

Case definitions of PMWS have been reviewed over time and adjusted to correspond with scientific findings and an increased knowledge of PMWS. Diagnostic approaches and methods have also been evaluated to verify their validity as reassessments are made. For instance, in the early stages of PMWS investigation in New Zealand ISH and IHC were not readily available in national veterinary laboratories so tissue samples were sent overseas for IHC. High cost and the slow turnaround of results meant only limited numbers of tissue samples (one or two pigs/farm) were sent for testing. IHC was, however, abandoned early on due to poor specificity, timeliness, and high cost and was

[4] ELISA: Enzyme linked immunosorbent assay uses microtitre plates coated with a monolayer of PCV2 infected cell culture or baculovirus-expressed PCV2 capsid protein (Segalés et al. 2005).

replaced with a histopathological scoring system identifying key microscopic features of PMWS.

Another example of reassessment was demonstrated during a PMWS case-control study in Denmark from 2003 to 2004 (Jorsal et al. 2006). The objective of the study was to evaluate the usefulness of monitoring specific histopathological lesions and quantity of PCV2 as parameters for case identification. Case herds (n = 74) were selected if they met particular criteria[5] for PMWS and matched to control herds (n = 74) with no clinical signs of PMWS. Three unthrifty pigs from each herd were submitted for laboratory examinations including histopathology and IHC. PMWS was identified in 58/74 case herds but also in 19/74 control herds when at least one pig was diagnosed positive in histopathology. A poor correlation between clinical observations at herd-level and laboratory diagnosis of PMWS at the individual pig-level was noticed, although a high PCV2-load in lymphoid tissues was a good indicator of PMWS in individual animals. Consequently, previous declaration of PMWS within the Danish SPF-system was slightly changed in April 2005 to state that key histopathological changes and medium-to-high levels of PCV2 had to be present in individual pigs in order for the farm to be considered a PMWS case (Bækbo 2005).

2.7 Investigation of the disease process

2.7.1 Hypotheses about aetiology of PMWS

There are two very divergent views on the causation of PMWS supported by research and field experience despite extensive research on the disease. Firstly, there is the view that PMWS is caused by PCV2 with disease expression modified by the presence of other factors (viral co-infections, immunostimulation, genetics or management effects). Secondly, there is the view that PCV2 and these cofactors are widespread and do not match the distribution of PMWS, and the disease has behaved like a propagating epidemic, implying the existence of an unidentified agent.

[5] Danish case definition for PMWS included (1) increased and high prevalence of unthrifty, wasting pigs and mortality, (2) autopsy and histopathological examinations of lymph nodules in typical cases show (a) depletion of lymphocytes, (b) histocytic inflammation (giant cells and/or inclusion bodies), and (c) porcine circovirus type 2 (PCV2) antigens by immunochemistry (Bækbo 2005).

The evidence so far is inconsistent between studies and the following sections summarise evidence for and against the various hypotheses that have been put forward to explain the aetiology and epidemiology of PMWS.

PCV2 as causal agent for PMWS

A small number of successful transmission experiments have been published where the authors have argued that PCV2 is the primary aetiological agent for PMWS (Allan et al. 1999a, Magar et al. 2000a, Bolin et al. 2001, Okuda et al. 2003). Allan et al. (1999a) reported that intranasal inoculation of PCV2 to colostrum-deprived one to two-day-old piglets caused clinical signs and moderate histopathological lesions in lymphoid tissues in one out of three infected piglets that were consistent with PMWS. Interestingly, in a very similar study conducted by Krakowka et al. (2000), no clinical PMWS could be produced in the three piglets inoculated intranasally with PCV2, but there were mild histological inflammatory lesions consistent with PMWS. Okuda et al. (2003) induced clinical PMWS in 4/16 caesarean-derived, 14 day-old colostrum-deprived piglets. Most of the pigs remained healthy and asymptomatic despite PCV2 seroconversion. Magar et al. (2000a) suggested that experimental infection of colostrum-fed pigs results in disease that is more representative of infections that occur in field cases of PMWS. Eleven, three to four-week-old SPF weaners were inoculated intranasally with PCV2 and no clinical signs resulted but widespread distribution of PCV2 was detected in lymphoid tissues. Similar results were confirmed later (Fenaux et al. 2002). None of these findings are sufficient to confirm that PCV2 is the causal agent of PMWS.

PCV2 has been circulating in pig populations of many countries for decades (Walker et al. 2000), and PMWS has spread progressively within and between countries over this time. In most pig herds PMWS clinical disease is not present although most herds are infected with PCV2. Not all PCV2 infections are associated with clinical PMWS or with the histological lesions linked with PMWS in experimental studies (Allan & Ellis 2000). These facts cast serious doubt on the hypothesis that PCV2 alone is responsible for PMWS.

Immune modulation and PCV2

Observations of lymphocyte depletion in PMWS-affected pigs suggest that immunosuppression may be an underlying factor responsible for producing PMWS outbreaks in PCV2 infected herds (Segalés et al. 1997, Allan et al. 1998a). It has been proposed that PRRSV or PPV may be responsible for initiating the disease process (Rodriguez-Arrioja et al. 1999), but both agents are widespread with no clear association between their presence and the occurrence of PMWS.

Immunostimulation has also been suggested as a means of enhancing PCV2 replication. In the study of Krakowka et al. (2001), one-day-old gnotobiotic piglets were oronasally infected with PCV2, with or without immunostimulation by keyhole limpet haemocyanin (KLH). Piglets infected with PCV2 alone (n = 10) did not develop PMWS whereas all piglets receiving PCV2 plus KLH (n = 3) developed moderate to severe PMWS with extensive PCV2 replication. These authors proposed that activation of the immune system was a key component in the pathogenesis of PMWS. In an effort to test this hypothesis in a field setting, Kyriakis et al. (2002) conducted a trial during a PMWS outbreak on a commercial farm. Eighty-four weaners were allocated to three groups; an untreated control group (n = 28), a group vaccinated twice with a *Mycoplasma hyopneumoniae* bacterin (n = 28), and a group injected with a non-specific immune stimulant (n = 28). In the control group 3/28 pigs developed PMWS while 12/28 and 14/28 pigs of the vaccine group and immune stimulant group developed PMWS.

Resendes et al. (2004b) conducted a similar study on experimentally PCV2 infected eight to nine-week-old pigs to determine the effect of an immune stimulant (vaccine adjuvant) on development of PMWS. There was no PMWS in the control or adjuvant piglets although viraemia and seroconversion to PCV2 were detected. Findings of a recent vaccination study conducted under field conditions could not support immunostimulation significantly increasing the incidence of PMWS in pigs infected with PCV2 (Haruna et al. 2006). The obvious difference between the studies of Krakowka, Kyriakis and Resendes is in the age of the pigs that were infected. Bailey et al. (2001) provide an explanation for this result by the observation normal architecture of the mucosal immune system present in mature animals is reached by about six-

weeks-old, although the number of $CD8^+$ T-cells continues to increase. Therefore, pigs older than six weeks are better able to resist viral challenge than those that are younger.

Studies to determine the effect of immune stimulation or immune suppression on the clinical outcome of PCV2 infection have yielded equivocal evidence but both of the proposed hypotheses cannot be true. Immune modulators can substantially influence the development of PMWS but research has not provided a comprehensive explanation of their role and the epidemiological association between these factors and the disease is poor.

Effect of genotype and management on PMWS

Given the fact that immune modulation is related to the expression of PMWS, it follows that certain management practices thought to affect the immune status of pigs are also likely to influence the expression of PMWS. Allan et al. (2000b) suggested that changes in pig farming practices may have triggered the sudden worldwide emergence of PMWS. Management practices including early weaning, commingling of litters, introduction of intensive vaccination strategies and implementation of animal welfare husbandry practices have been suggested as predisposing factors for PMWS (Allan et al. 2003). In contrast, PMWS was reported in a group of wild Eurasian boars raised under free-range conditions (Ellis et al. 2003), providing an effective contradiction to this hypothesis. There is a weak association however, between these factors and disease.

Field observations suggest that certain genetic lines are at higher risk for PMWS than others. López-Soria et al. (2004) compared the effect of three genetically different boar lines (A: 100% Pietrain, B: 50% Large White × 50% Pietrain, C: 25% Large White × 75% Duroc) on the outcome of general postweaning mortality and that associated with PCV2 infection in their offspring on two 5,000-sow farms. Significant differences in PCV2-associated mortality were observed between the different genetic backgrounds. The first farm experienced mortality rates of 1.5%, 4.7% and 9.8% in boar lines A, B and C, respectively, whereas the second farm had rates of 2.1%, 5.9% and 26.3%. These findings clearly indicate a genetic effect on PMWS expression and suggest that boar line C (25% Large White × 75% Duroc) has a predisposition to PMWS. It could

not be clarified, however, whether the observed effects were due to the genetic characteristics of a particular breed or the boar line.

A study by Opriessnig et al. (2006a) evaluated the differences in susceptibility to PCV2 by comparing infection in three pure breeds (Landrace, Duroc and Large White). Five to seven-week-old pigs were inoculated intranasally and intramuscularly with PCV2. Based on gross and microscopic lesions the incidence of PMWS was 15.8% in Landrace and was not detected in Duroc and Large White, indicating that the Landrace was predisposed to PMWS. This contradicts the finding of Large White × Duroc being predisposed to PMWS in the study of López-Soria et al. (2004).

There has been little PMWS research conducted in feral pigs. Ellis et al. (2003) reported PCV2 infection in wild Eurasian boars in free-range conditions with clinical signs and lesions similar to PMWS in domestic pigs. Similar observations were reported in a wild European boar (Schulze et al. 2003) where PCV2 infection was associated with morphological lesions characteristic of PMWS. PMWS was also confirmed to be prevalent in Spanish wild boars (Vicente et al. 2004).

PCV2 and concurrent infection with another agent

The relationship between PCV2 and concurrent infection with PRRSV or PPV has been extensively reported. The experimental evidence strongly suggests that the clinical outcome of these dual infections is worse than infection with PRRSV or PPV alone (Kennedy et al. 2000, Harms et al. 2001, Hasslung et al. 2005). Similar evidence has been reported for numerous concurrent infections with other porcine pathogens in the field (Rodriguez-Arrioja et al. 1999, Pallarés et al. 2002, Ellis et al. 2004, Wellenberg et al. 2004). Conversely, there were no clinical symptoms or lesions typical of PMWS observed in conventional piglets dually infected with PCV2 and PPV (Ostanello et al. 2005).

Variation in pathogenicity between PCV2 strains

PCV2 genomes differing in virulence have been reported, despite a lack of difference between PCV2 strains on affected and unaffected farms that would be sufficient to

explain PMWS causation (Grierson et al. 2004b, de Boisséson et al. 2004). Fenaux et al. (2004) reported that two amino acid mutations in the capsid protein[6] of PCV2 slightly enhanced virus replication *in vitro*. In a follow up study (McKeown et al. 2006), differences between inoculated pigs were observed in respiratory signs, enlarged lymph nodes, and lymphoid depletion and were attributed to two additional mutations in the capsid protein of PCV2. The first study to show a clear difference in virulence between PCV2 strains was that of Opriessnig et al. (2006c). Significant differences in the immune response and severity of pathological lesions in inoculated pigs were found between two isolates from the USA. Despite successful reproduction of pathological lesions characteristic of PMWS, only mild respiratory disease was observed for a few days with no difference in mean daily weight gain between pigs inoculated with different PCV2 strains and no rectal temperatures were above 40°C. This study failed to reproduce clinical signs consistent with PMWS even though an original PCV2 isolate was used that was collected from a confirmed case of PMWS (Fenaux et al. 2000). There is not sufficient molecular evidence to explain differences between PMWS-affected and non-affected farms by genomic differences in PCV2 strains.

Novel infectious agent(s)

PMWS occurs as a propagating epidemic having characteristics consistent with those produced by a novel infectious agent (Morris et al. 2002, Lawton et al. 2004a, Vigre et al. 2005). Vigre et al. (2005) described the spatial and temporal pattern of Danish pig herds diagnosed with PMWS in the first two years after it was identified in Denmark from October 2001. The number of herds affected with PMWS increased markedly after a local outbreak when it spread to most parts of the country with two geographical clusters in areas with the most pig herds. The mode of transmission was not determined but the higher risk of infection in geographical areas with high pig herd density supports contagious transmission. Cook et al. (2001) presented potential risk factors for PMWS on UK pig farms and concluded that herds purchasing large numbers of replacement breeding stock were more at risk for PMWS. Transmission of PMWS followed the movement of pigs from affected to unaffected herds during the initial spread of the

[6] The protein coat that surrounds the infective nucleic acid in some virus particles and contains the main neutralising epitopes.

disease in the UK (Done et al. 2001). Commingling of pigs at weaning from multiple sources was also an important risk factor for the introduction of PMWS to a pig herd.

Australia is free of PMWS despite being endemically infected with PCV1 and PCV2 (Raye et al. 2005, Muhling et al. 2006), raising the question of the difference between Australia and PMWS-affected countries which are also endemically infected with PCV1 and PCV2. Genetic difference in PVC2 strains is one possible explanation, although this was largely excluded by Muhling et al. (2006) through sequencing of 7 strains of PCV2 from 41 samples from 3 Australian states. These authors found 94% to 99% genetic homology with PCV2 isolates of other countries. The absence of PMWS from Australia, therefore, leads to the theory that a novel agent causes the disease.

2.7.2 Pathogenesis of PMWS

Immunostimulation and immunosuppression

It is known that the immune system is involved in the pathogenesis of PMWS but several attempts to elucidate the interaction between PCV2 and the immune system were inconclusive. Experimental studies have demonstrated that an increased amount of PCV2 in diseased pigs follows stimulation of the immune system by infectious or non-infectious factors (Kennedy et al. 2000, Harms et al. 2001, Krakowka et al. 2001, Kyriakis et al. 2002, Hasslung et al. 2005). Consequently, PCV2 infection and immunostimulation were seen as important components in the development of PMWS. In contradiction, lymphocyte depletion commonly seen in lymphoid tissues of PMWS-affected pigs suggests an immunosuppressed status. Co-infections with opportunistic pathogens (Carrasco et al. 2000, Segalés et al. 2003), alterations in subpopulations of immune cells in lymphoid tissues and peripheral blood mononuclear cells (Segalés et al. 2001, Darwich et al. 2002, Chianini et al. 2003, Nielsen et al. 2003) in PMWS diseased pigs indicate that immunosuppression plays a role in pathogenesis. The mechanism responsible for lymphocyte depletion and its association with the expression of PMWS has not been fully explained. Apoptosis of B-lymphocytes was suggested as a possible mechanism for B-cell depletion in lymph nodes (Shibahara et al. 2000), but other researchers do not consider apoptosis to be a significant feature of PMWS (Mandrioli et al. 2004, Resendes et al. 2004a). Moreover, Krakowka et al. (2004) suggested that

apoptosis is not the primary mechanism for lymphocyte depletion but is attributable to pyrexia instead of the direct effects of the virus. Darwich et al. (2003) demonstrated that decreased cell proliferation is related to T-cell and B-cell depletion in the thymus and secondary lymphoid organs as opposed to increased apoptosis. Mandrioli et al. (2004) confirmed the hypothesis that lymphoid tissue depletion is related to decreased proliferative activity of cells. This is caused by a prolonged absence of cytokines (positive factors for growth) in lymph nodes arising through lymphocyte inactivation (Sarli et al. 2001). The prolonged inactivation of lymphocytes might originate from the inhibitory effect of PCV2 on the interaction between lymphocytes and macrophages, which stimulates B- and T-cell proliferation (McNeilly et al. 1996).

Many stages of the disease process remain unresolved and further experimental studies focussing on the immunological interactions between host and agent are crucial to understanding the pathogenesis of PMWS.

Passive immunity

Based on findings of field and experimental studies, the development of PMWS in weaned pigs is influenced by the passive transfer of maternal antibodies to PCV2. It has been suggested that the development of PMWS is dependent on the level of PCV2 antibodies at the time of PCV2 inoculation (Ostanello et al. 2005). Low serologic titres of PCV2 antibodies in piglets during lactation and nursery period have been associated with a higher prevalence of PMWS (Rodriguez-Arrioja et al. 2002, Calsamiglia et al. 2007), whereas piglets born of gilts or sows with high titres of serum antibodies to PCV2 have shown protection from PMWS but not from PCV2 infection (Allan et al. 2002a, McKeown et al. 2005). In contradiction, Hassing et al. (2004) demonstrated that offspring from sows with high antibody levels for PCV2 were at higher risk of post weaning mortality.

Transmission modes of PMWS

Despite comprehensive experimental research on PMWS and PCV2 infections, there are still uncertainties about disease introduction and modes of transmission. Relatively early during the investigations of PMWS, it was evident that transmission was possible

between PMWS-affected and non-affected herds by contact via feed, equipment and clothing (Mackinnon 2000). In experimental transmission trials, healthy pigs were exposed to PMWS-affected pigs through direct contact (housed in the same pen) and indirect contact (snout contact with neighbouring pen) (Kristensen et al. 2004, Kristensen et al. 2006) confirming that transmission through pig-to-pig contact is possible.

Another suspected means of direct transmission of PMWS is through infected semen (Mackinnon 2000). PCV2 transmission through infected semen has been shown to occur (Cook et al. 2001, Gresham et al. 2003). The first report of PMWS in South Africa (Drew et al. 2004) occurred on a farm which had used imported semen from Iowa (USA) to inseminate gilts supporting the hypothesis of direct transmission through semen. It was suggested from this study that transmission through infected semen was the cause of PMWS introduction to South Africa. Transmission of PCV2 via artificial insemination is possible as PCV2 nucleic acid has been detected in boar semen (Hamel et al. 2000, Kim et al. 2001) and PCV2 may be shed intermittently in semen of infected boars (Larochelle et al. 2000). Experimental evidence of PCV2 infection via the introduction of infected semen into naïve pigs has not been reported but a case-control study in Denmark did not find any association between PMWS and the use of artificial insemination (Enøe et al. 2006).

Transmission of PMWS through bird or wildlife vectors has been suggested (Potter 2000a, Mackinnon 2000), which is especially relevant to outdoor production farms. Seagulls have been suggested as a potential route of transmission in the recent PMWS outbreak on the South Island of New Zealand (Neumann et al. 2007).

Apart from other vectors such as rodents and fomites, feeding waste food containing cooked pork may also be a potential mode of transmission. PMWS contaminated waste food was seen as a likely source of incursion in both outbreaks of PMWS in New Zealand. Investigations continue to identify the source of infection in the recent outbreak on the South Island (Neumann et al. 2007). Preliminary results of a transmission study (Opriessnig & Halbur 2006) indicate that PCV2 can be transmitted to pigs by consumption of uncooked meat, bone marrow, or lymphoid tissues. The

study is ongoing and the final results along with further studies are required for confirmation.

Airborne spread of PMWS has not been excluded in transmission studies undertaken so far. Oronasal exposure has been suggested as the most likely and frequent natural route of PMWS transmission (Allan et al. 1999a, Balasch et al. 1999, Krakowka et al. 2000, Rovira et al. 2002, Calsamiglia et al. 2004). Further experimental research is required to confirm whether airborne transmission of PMWS is possible.

2.7.3 Prevention and control strategies for PMWS

General strategies

In general, prevention and control strategies are developed on the basis of scientific knowledge about the pathogenesis and epidemiology of a disease. Regarding PMWS, both are poorly understood and the causal agent remains uncertain. It appears that other infectious and non-infectious factors, possibly in combination with an unknown agent, are involved in the development of PMWS. Current preventive measures, therefore, are primarily focused on the understanding and control of influential co-factors on PMWS-affected farms such as husbandry practices, movement control and vaccination.

A broad range of viral and bacterial agents have been observed in association with the occurrence of PMWS, hence the control of concurrent infections has been proposed to reduce the incidence of PMWS. Options for control include vaccination against potential pathogens, appropriate use of antibiotics, high quality sanitation programmes and strict biosecurity policies. PMWS cases in western Canada, however, were most commonly diagnosed in herds of high health status where biosecurity was well maintained (Harding et al. 1998, Ellis et al. 1998). This suggests that conventional biosecurity may not prevent the entry of PMWS into a herd. Rapid, accurate diagnosis and removal of diseased animals in combination with good husbandry practices appears to be the best current method to control PMWS infection on affected farms (Allan & Ellis 2000).

Management measures

Other suggested control strategies have been directed toward improving farm management practices (Madec et al. 2000). Observations from a prospective study conducted on 12 PMWS-affected farms in France indicated that a poor quality environment was a pre-requisite for clinical expression of PMWS (Madec et al. 2000). These authors observed decreased mortality in severely PMWS-affected farms after implementing better hygiene, less over-crowding and mixing along with improved hygiene and management. Some of these affected farms adopted practices referred to as Madec's 20-point plan (Anonymous 2007c), which is a package with 20 technical recommendations designed to reduce the challenge of microbiological infections, improve hygiene and reduce stress at different production stages. The implementation of some strategies provides difficulty if herd managers do not fully comply, resulting in limited reduction of PMWS-associated mortality. Mortality rates do not usually return to pre-PMWS outbreak levels according to field observations despite implemented control measures during outbreaks (Gresham et al. 2003).

Movement control

Movement control of livestock between infected farms is generally an effective strategy to restrict the spread of infectious disease. The efficacy of this strategy is dependent on the level of compliance of affected farms and on the agent's transmissibility. Reports from the UK indicate that pig movements between farms contribute significantly to the spread of PMWS (Mackinnon 2000, Done et al. 2001). An efficient control of spread was also observed during the first PMWS outbreak in New Zealand, when movement controls between the North and South Island and quarantine measures on infected premises were legally enforced (Rawdon et al. 2004). PMWS suspect or confirmed PMWS-positive herds were placed under movement control and declared as restricted places with implementation of strict biosecurity measures. Government animal control officials visited these herds on a regular basis over a prolonged time to closely monitor the course of disease.

Vaccination against PMWS

Four commercial vaccines have been available since 2006/2007. Circovac® (Merial, Inc.) is an inactivated PCV2 vaccine administered intramuscularly to gilts and sows prior to farrowing to increase the amount of maternal antibodies against PCV2. Piglets are, consequently, better protected against PMWS due to passively acquired antibodies via colostrum. Data have not yet been published from large vaccination trials in the field but preceding experimental studies were very successful (Reynaud et al. 2004a, Reynaud et al. 2004b, Charreyre et al. 2006b, Sierra et al. 2006). Ingelvac® CircoFLEX™ (Boehringer Ingelheim) is a single-dose vaccine injected intramuscularly to piglets from three weeks of age prior to exposure to PCV2. This vaccine stimulates the piglet's immune system to produce antibodies for PCV2 and provides long-lasting protection. Suvaxyn® PCV2 One Dose (Fort Dodge) is an inactivated PCV1-2 chimera single-dose vaccine for pigs from four weeks of age and works on the same principle as Ingelvac® CircoFLEX™. The fourth vaccine (Porcilis PCV®) is available from Intervet. The antigen is PCV2 expressed in an inactived Baculovirus and is administered twice intramuscularly at a three week interval to piglets from three-weeks-of-age, stimulating their immune system to produce protective antibodies for PCV2.

2.8 Economic effects of PMWS

The economic impact of PMWS depends on the severity of disease on affected farms. Increased mortality rates and reduced daily weight gains cause dramatic reductions in the profitability of pig production. It is not surprising therefore, that some New Zealand farms were driven to ruin with post weaning mortality rates of up to 50% (Rawdon et al. 2004). The effect of increased mortality due to PMWS was calculated by Leblanc & Morin (2005). An increase in post weaning mortality rate from 3% to 8% in a batch of growers from a finishing farm of 1,000 places resulted in a financial loss of approximately $7.77 (Canadian dollars) per pig whereas increases from 3% to 30% caused deficits of >$40.00 (Canadian dollars) per pig. Hardge et al. (2003) estimated the financial loss in a large multi-site production system with more than 15,000 sows with a gross margin of €3.9 to €4.1 (Euros) per pig due to PMWS. The most significant economic effects were seen in direct production losses, increased antibiotic use due to increased secondary infection rates and the remaining increased mortality rates after outbreaks.

It is in the interest of the pig industry to fund further scientific research on PMWS as there are significant production and financial impacts on a large number of pig producing farms and countries. The overall losses for EU countries are estimated to be between €562 million and €900 million per year (Segalés et al. 2006, Anonymous 2007d) highlighting the vast financial consequence of PMWS.

2.9 Conclusions

In the past decade, an enormous amount of research has been conducted to elucidate the pathogenesis, epidemiology and aetiology of PMWS. Based on outbreak investigations on PMWS-affected farms in combination with pathological and histopathological analyses, PCV2 has been proposed as the causal agent of PMWS. This conclusion is questionable as PCV2 infection is widespread and most pig farms are endemically infected with PCV2 without the occurrence of PMWS. The evidence of PCV2's role in the aetiology of PMWS does not fully explain PMWS outbreaks or explain why Australia remains free of PMWS. These factors strongly indicate the involvement of an unidentified agent in the aetiology of PMWS. Scientific knowledge of PMWS is incomplete with contradictions evident in past research. Further research should focus on the causality of PMWS so effective control strategies can be implemented to reduce the economic impact of the disease on pig farms.

Chapter 3

An investigation into the natural transmission of postweaning multisystemic wasting syndrome and the clinical course of disease

3.1 Introduction

In 1991, a new syndrome termed postweaning multisystemic wasting syndrome (PMWS) was identified in pigs in western Canada (Harding & Clark 1997). Over the last decade, PMWS has been described in many countries worldwide including the USA, UK, Asia and most of Europe (Allan & Ellis 2000, Segalés & Domingo 2002). It is considered to have a significant economic impact on affected farms. PMWS affects recently weaned pigs at 5 to 12-weeks-old (Harding & Clark 1997, Allan & Ellis 2000) and is clinically characterised by progressive weight loss, with or without respiratory signs, diarrhoea, pallor, and jaundice (Harding & Clark 1997, Allan et al. 1999a, Allan & Ellis 2000). Morbidity can range from 10% to 50% in the at-risk age group of pigs with case fatality of up to 80% (Segalés & Domingo 2002).

Porcine circovirus type 2 (PCV2) is reported as a necessary agent for expression of PMWS (Allan et al. 2002b, Pogranichniy et al. 2002, Rodriguez-Arrioja et al. 2002). Nevertheless, the precise role of PCV2 in the pathogenesis is still poorly understood. Some studies suggest that a range of co-factors to PCV2 can be involved in the aetiology of PMWS, with no specific factor needed to cause the disease (Pogranichniy et al. 2002, Ellis et al. 2003, Wellenberg et al. 2004). Retrospective and prospective investigations of field cases of pig diseases have proposed that PCV2 also has a causal association with porcine respiratory disease complex (PRDC), porcine dermatitis and nephropathy syndrome (PDNS), and reproductive failure (Ellis et al. 2004, Harding 2004, Wellenberg et al. 2004).

The detection of PCV2 alone does not confirm a diagnosis of PMWS as this virus can be found in healthy pigs on almost all PMWS-free farms as well as infected farms (Allan & Ellis 2000, Calsamiglia et al. 2002). No consistent differences have been

identified between PCV2 strains on affected and unaffected farms that are sufficient to explain PMWS causation (Grierson et al. 2004a, de Boisséson et al. 2004). However, Opriessnig et al. (2006c) demonstrated that the severity of PCV2-associated lesions differed significantly between two isolates but did not report clinical signs or mortality typical of PMWS.

There are two very divergent views on the causation of PMWS (each supported by research and field experience), despite extensive research on the disease. Firstly, there is the view that PMWS is caused by PCV2 with disease expression modified by the presence of other factors (viral co-infections (PRRSV and PPV), immunostimulation, genetics and management effects). However, the evidence to support this is inconsistent between studies. Secondly, there is the view that PCV2 and these factors are widespread yet their distribution does not fit epidemiologically to the distribution, hence the disease has behaved like a propagating epidemic which implies the existence of another necessary causal agent but no aetiological pathogens have been identified despite extensive research.

PMWS can occur as a propagating epidemic having characteristics consistent with those produced by a novel infectious agent (Morris et al. 2002, Lawton et al. 2004a, Vigre et al. 2005). Vigre et al. (2005) described the spatial and temporal pattern of PMWS in Danish pig herds over 2 years after its introduction into the country in October 2001. After a localised outbreak, the number of herds affected with PMWS increased markedly and spread to most parts of Denmark and showed two geographical clusters in areas with the highest pig herd density. In their study, the mode of transmission could not be determined but the high risk of infection in geographical areas with high pig herd density is supportive of contagious transmission. In another study, Cook et al. (2001) analysed potential risk factors for PMWS and concluded that pig farms that purchased an increased number of replacement breeding stock were at greater risk for PMWS. Done et al. (2001) reported that transmission of PMWS in the UK followed the movement of pigs from affected herds to unaffected herds and the merging of pigs from multiple sources at weaning was an important risk factor for introduction of PMWS to a pig farm.

The emergence of PMWS in New Zealand has very similar characteristics to the outbreak described in Denmark. The first diagnosis of PMWS was confirmed in September 2003 in a farrow-to-finish pig herd in the Waikato region (North Island, New Zealand) (Lawton et al. 2004b). High mortality rates among pigs of 6 to 12-weeks-of-age and clinical signs characteristic of PMWS were reported. Laboratory examination of tissues confirmed histopathological lesions consistent with PMWS. Epidemiological investigation of herds on both the North and South Island revealed a single cluster of nine affected farms in the Waikato/South Auckland region (Stone 2004, Lawton et al. 2004a). The diagnosis was made on the basis of epidemic non-responsive wasting with high case fatality in 5 to 14-week-old pigs using the case definition described by Stone (2004). Other clinical signs included dyspnoea, enlarged lymph nodes and diarrhoea. Interviews with farmers and retrospective analysis of farmer's records revealed that PMWS behaved as a focal incursion with an introduction into the country estimated to have occurred in about 1999/2000 (Stone 2004). Spread beyond the initial outbreak was prevented by application of control measures (movement controls between the North Island and South Island, quarantine measures on infected premises) ordered by New Zealand's Ministry of Agriculture and Forestry (Rawdon et al. 2004, Stone 2004). Despite extensive surveillance, there were no new farms found infected from when movement restrictions were imposed in late 2003/early 2004 until January 2006. This was when a second spatial cluster was found near Christchurch, in the South Island. Epidemiological investigations during the 2006 outbreak suggested this was a new disease incursion with no known links to the infected North Island farms. This disease appearance followed the spatio-temporal pattern of a propagating epidemic.

Evidence of PCV in New Zealand pre-dated any clinical evidence of PMWS by a significant margin (Horner 1991). Of particular note in New Zealand was the distinctly clustered spatial distribution of PMWS-affected farms in the vicinity of Auckland, whereas PCV2 infection was ubiquitous in New Zealand herds as shown by serological surveillance (Tham & Hansen 2003). This would not be expected if the disease was caused by PCV2 alone. The infected farms could all be linked by known movements of young pigs, pig feed and fomites (e.g. transport vehicles, pig equipment and feeding of food waste). Food waste fed to growing pigs was an important risk factor for a farm becoming a case (odds ratio = 8.25, P = 0.10) (Stone 2004, 2005).

Since PCV2 infection alone cannot be responsible for expression of PMWS, there must either be an unidentified infectious agent (Lawton et al. 2004a, Lawton et al. 2004b), or a spectrum of known infectious agents and/or non-infectious factors modifying the expression of PCV2 infection to produce the disease. Various co-factors have been proposed; most of them are widespread in the global pig population and show no evidence of strong association with outbreaks of PMWS. A number of the putative co-factors for PMWS (porcine reproductive and respiratory syndrome virus (PRRSV), Aujeszky's disease virus (AujD), and swine influenza (Allan & Ellis 2000, Ellis et al. 2000)) are absent from New Zealand while others such as porcine parvovirus (PPV) are far more widespread than the disease. Other risk factors have also been proposed, such as use of highly immunogenic vaccines and use of modern genotypes of pigs. Reports of affected herds in New Zealand show that these postulated non-infectious factors were not present. Therefore, it has been difficult to explain the occurrence of disease in terms of the postulated risk factors. Although PCV2 is generally considered to be a necessary component of PMWS (Kennedy et al. 2000, Krakowka et al. 2001), the evidence from New Zealand strongly suggests that it is not a sufficient cause to trigger PMWS outbreaks on commercial pig farms.

The availability of susceptible pigs of an archaic genotype (free of PCV2 and a wide range of other pathogens common to commercially farmed pigs) has offered an opportunity to study the transmission of PMWS to pigs from PCV2-negative and positive herds at different ages.

The objectives of this study were: (1) to determine what factors were necessary to produce clinical PMWS in pigs of susceptible age and serological status and; (2) to collect ante mortem and post mortem samples for detailed microbiological investigation. The recording of detailed daily observations was expected to provide evidence of the clinical course of the disease.

3.2 Materials and Methods

3.2.1 Study design

The study was set out to evaluate the possibility of natural transmission of PMWS by direct contact between PMWS-affected and susceptible pigs, while managing the influence of proposed co-factors. The primary transmission routes of PMWS were assumed to be direct contact with PMWS-affected pigs and vectors such as feed, equipment and clothing.

3.2.2 Source farms

Pigs free of PCV2 and other most common pig pathogens were obtained from Farm A, a herd comprised of pigs of an archaic genotype. The initial breeding stock of this farm was removed from the Auckland Islands (part of the New Zealand Sub-Antarctic Islands, 50°42'South, 166°5'East) by a rare breeds conservation group several years earlier. They had been placed on the islands as a food source for ship-wrecked sailors in 1807 with further pigs added in 1842 and the 1890s. Currently, these pigs are maintained under strict biosecurity and health surveillance of Living Cell Technology Ltd, Auckland. Extensive testing by the company's molecular diagnostic laboratory has shown this herd to be free of the most common and exotic pig pathogens, including PCV2, PPV and PPRSV. Pigs were also obtained from a high health status commercial New Zealand piggery (Farm B), known to be endemically infected with PCV2, but free of PMWS and a range of other pathogens (PRRSV, AujD, and PPV). PMWS-affected pigs were obtained from two affected farms (Farm C and D), which had recent and historical post weaning mortality rates regularly in excess of 20%. A description of farms that provided pigs for this study is summarised in Table 3.1.

Table 3.1: Disease status of source farms which supplied pigs used to investigate the natural transmission of PMWS.

		Disease status			
Farm	Type of farm	PMWS	PCV2	Parvovirus	PRRSV
A	Private research herd Archaic genotype Fully-enclosed housing	–	–	–	–
B	Commercial piggery Modern genotype Fully-enclosed housing	–	+	+	–
C	Commercial piggery Composite genetic mix Outdoor and indoor housing	+	+	+	–
D	Commercial piggery Composite genetic mix Outdoor and indoor housing	+	+	+	–

PMWS – postweaning multisystemic wasting syndrome
PCV2 – porcine circovirus type 2
PRRSV – porcine reproductive and respiratory syndrome virus
– = negative
+ = positive

3.2.3 Animals

There were eight groups of pigs used in this trial. The exposure group was comprised of a combination of pigs with clinical signs of wasting from Farms C and D, diagnosed with PMWS as described by Stone (2004). Groups 1 to 4 were directly exposed to pigs of the exposure group for up to 56 days. Sixteen weaners from PMWS-affected herds (Farm C (n = 4) and D (n = 12)) were stratified by farm of origin and randomly allocated to Groups 1 to 4, ensuring that pigs from both affected farms were represented within each group. These exposure pigs were then rotated between Group 1 to 4 after the first week in an attempt to minimise any variation in shedding between PMWS-affected pigs within groups. Group 5 was not directly exposed to PMWS-affected pigs of the exposure group but held adjacent to Groups 1 to 4, serving as an on-site negative control group. Group 6 remained at its source farm (Farm B) for the duration of the exposure period, serving as an off-site control group. Groups 7a and 7b were comprised

of 12 pigs from Farms A and B, which were held in the same pen for 81 days. These two groups of pigs were exposed to a 12-week-old pig from Farm B and to faeces collected from 12-week-old pigs at Farm B. Blood and faecal samples collected from the source pig were PCV2-positive as determined by polymerase chain reaction (PCR). A summary of the experimental groups is shown in Table 3.2.

Table 3.2: Study design of natural transmission study of PMWS; set up of groups with animals of different age, number of animals within each group and their origin, including PCV2 and PMWS status.

Group	Age (weeks)	N	Farm of origin	PCV2 status	PMWS status	Exposure source farm
1	4	6	A	–	–	C and D
2	13	3	A	–	–	C and D
3	4	10	B	+	–	C and D
4	12	10	B	+	–	C and D
5	4	10	B	+	–	On-site control
6	4	6	B	+	–	Off-site control
7a	4	6	A	–	–	B
7b	4	6	B	+	–	B

– = negative
+ = positive

3.2.4 Housing, feeding and biosecurity measures

Study site 1

Groups 1 to 5 were housed in a purpose-built outdoor facility at Massey University (Palmerston North, New Zealand) in a location previously used for ruminant grazing only (Figure 3.1). This facility, with separate pens for each group, was constructed with plywood and straw bales and was fully meshed to prevent bird access. On top of the existing pasture a 40cm layer of sawdust was used for flooring with straw bedding on top. Group 5 was held 2 metres away from the main pens.

A

B

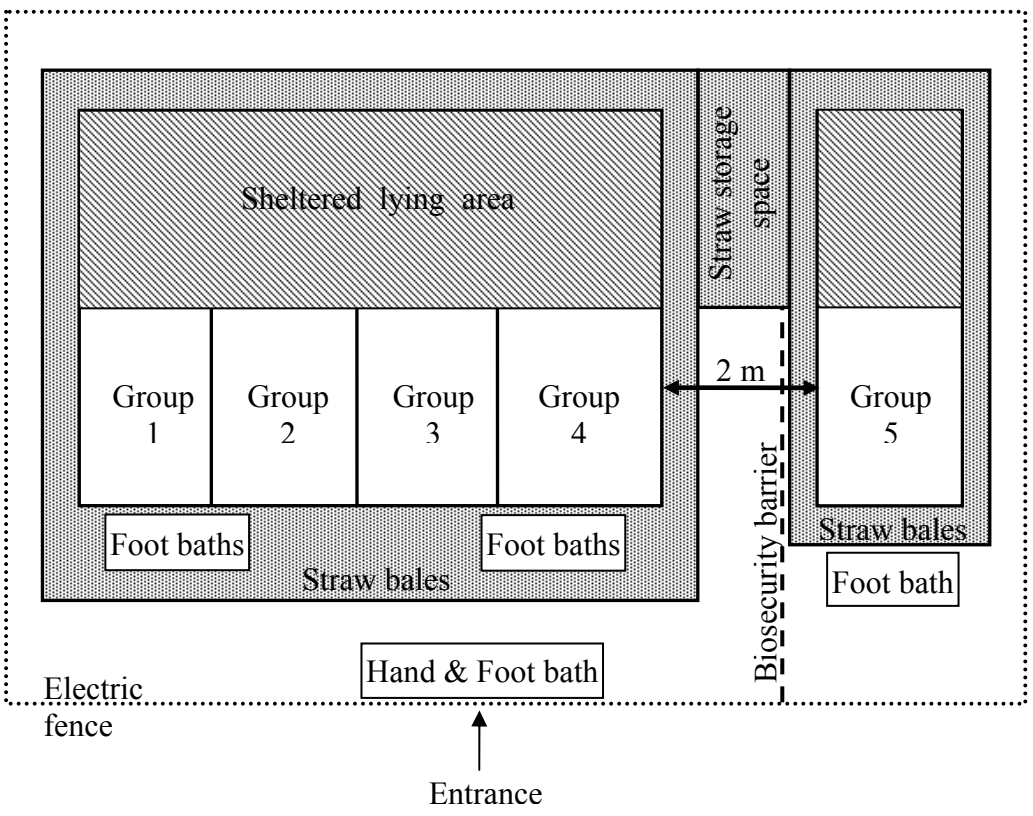


Figure 3.1: Study site 1 for the investigation of the natural transmission of PMWS. **A:** The temporary outdoor shelter with separate pens for Groups 1 to 5 constructed with plywood and straw bales, and **B:** The floor plan of the study site.

Animals were fed twice a day in concrete troughs with a commercially compounded feed (Pig Tucker PLUS, NRM). Feed levels were adjusted according to the appropriate quality and quantity of feed for normal growth. Concrete troughs in each pen supplied water *ad libitum*.

The pens were entered by research personnel in increasing order of expected infectious status (Group 5, Group 2 and 4, Group 3 then Group 1). Strict biosecurity measures were applied prior to entering pens such as changing protective clothing, disinfection of boots, hands and equipment with Virkon®S (Antec International). To minimise the risk of mechanical transmission of infectious agents from exposed groups (Group 1 to 4) to Group 5, separate examination equipment and sources of feed were used for this group's pen.

Group 6 pigs were housed off-site at their source farm (Farm B) with other commercial pigs of high health status. This group was housed and fed under commercial management and examined pathologically at 12-weeks-of-age for evidence of PMWS.

Study site 2

Groups 7a and 7b were housed in a room on a Massey University research farm without any other pigs (Figure 3.2). A 30cm layer of sawdust was used for flooring with straw bedding on top. The same feeding management was applied to this group as for Groups 1 to 5. Nipple drinkers supplied water *ad libitum*. Biosecurity measures applied included wearing of protective clothing and disinfection of boots, hands and equipment with Virkon®S (Antec International).

A

B

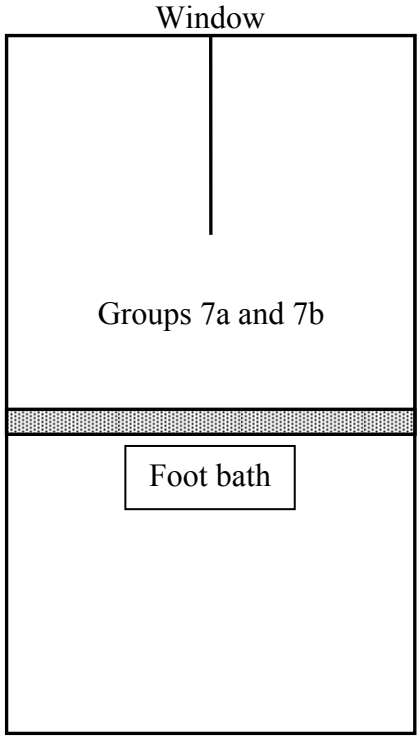


Figure 3.2: Study site 2 for the investigation of the natural transmission of PMWS. **A:** The indoor housing of Group 7a and 7b on a research farm without any other pigs, and **B:** The floor plan of the room.

3.2.5 Medical treatments

On the initial day of the study (day 0) all pigs from Group 1 to 5 were injected intramuscularly with 20mg/kg body weight (BW) oxytetracycline (Engemycin, CHEMAVET Pharmaco Ltd). Antibiotic prophylaxis was maintained by medicating water with 0.15g/L per day of soluble tylosin (Tylan soluble™, ELANCO Animal Health) for the early part of the trial, and pulsing it for the later part. This medication program was designed to avoid exposure of Group 1 to 4 pigs to other diseases unrelated to PMWS, which were potentially carried by pigs of the exposure group. In cases where individual antibiotic treatment was required, 20mg/kg BW oxytetracycline (Engemycin, CHEMAVET Pharmaco Ltd) was injected intramuscularly. All groups, with the exception of Group 6, were injected subcutaneously with 300µg/kg BW of Ivermectin parasiticide (Ivomec, MERIAL, New Zealand) on day 0 to ensure freedom from endoparasites and ectoparasites. Two pigs of Group 1 were injected intramuscularly with 1.1mg/kg BW flunixin meglumine (Flunixin, BOMAC

Laboratories Ltd) daily, for three days each (between day 8 and 13), following traumatic arthritis due to bullying. No vaccines were used in the pigs prior to, or during the study.

3.2.6 Clinical observations

All pigs were observed twice daily and rectal temperatures, respiratory and heart rates were recorded once daily. Rectal temperatures and heart rates were measured during the morning feeding. If a pig had an elevated temperature (>40˚C) at this time, a temperature was taken again in the afternoon. Respiratory rates were recorded while animals were resting in the early afternoon. Body condition scores of pigs were classified based on the amount of fat and/or muscle covering after first signs of weight loss became apparent, with scores recorded at three day intervals. Scores were based on a 1 to 5 (poor to fat body condition) according to the New Zealand Animal Welfare Code for pigs (National Animal Welfare Advisory Committee 2005) (Appendix I).

3.2.7 Definition of clinical cases of PMWS

Pigs with elevated temperature (≥40˚C) for at least two consecutive days prior to their first signs of weight loss, with or without development of scruffy hair coats, were defined as clinical cases of PMWS.

3.2.8 Sampling

Blood samples

A V-shaped cradle was used for restraint during blood collection for pigs less than eight-weeks-old. For the restraint of older or bigger pigs a wire snare was looped around the snout and blood was collected with the pigs in standing position. Blood samples were collected from Groups 1 to 5 and Groups 7a and 7b as presented in Table 3.3.

Table 3.3: Summary of sampling days for experimental groups stratified by collected serum, peripheral blood mononuclear cells (PBMCs), and whole blood samples.

Experimental group	Sampling days	
	Serum and PBMCs	Whole blood
1[a]	0, 3, 5, 8, 11, 15, 22	0, 8, 11
2[b]	0, 3, 5, 8, 11, 15, 22, 29, 36, 43, 50, 56	0, 8, 11, 29
3[c]	0, 3, 5, 8, 11, 15, 22, 29, 36, 43, 50, 56	0, 8, 11, 29
4[d]	0, 3, 5, 8, 11, 15, 22, 29, 36, 43, 50, 56	0, 8, 11, 29
5[e]	0, 3, 5, 8, 11, 15, 22, 29, 36, 43, 50, 56	0, 8, 11, 29
7a[f] and 7b[g]	0, 3, 5, 8, 11, 15, 22, 29, 36, 43, 50, 57, 64, 71, 81	0, 5, 8, 11, 15, 22, 29, 36, 43, 50, 57, 64, 71, 81

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Blood was collected from the jugular vein into specified vacutainer tubes as required for serum (no additives), peripheral blood mononuclear cells (PBMCs) (Acid Citrate Dextrose additive (ACD)) and whole blood (Ethylenediamine Tetraacetic Acid additive (EDTA)). Serum and PBMCs samples were cooled, centrifuged at 4000U/minute for 13 minutes (Labofuge 6000, Heraeus) and frozen at -84˚C (MDF-U50V, Sanyo) on the same day.

Nasal and faecal samples

Faecal samples and nasal swabs were collected from individual pigs at the same time as the collection of blood samples and were frozen at -84˚C (faecal samples) and -20˚C (nasal swabs) (SH236E, Shacklock). The individual faecal samples were collected by rectal manipulation. Cultiplast® (LP Italiana, Milano, Italy) swabs were used for collecting nasal swab samples of the external nares.

Testing for infection of PPV, PRRSV and PCV2

Selected samples of serum, PBMCs, faeces and tonsil tissues from pigs of Groups 1, 2, 3, and 7a on termination day were tested for PRRSV, PPV and PCV2 by PCR and

enzyme-linked immunosorbent assay (ELISA), to determine whether the pigs had become infected during the study.

3.2.9 Termination

Pigs were euthanased prior to the planned study termination day if mortality due to PMWS appeared to be close, to ensure a humane endpoint and suitable timing of pathological examination. A decision flow chart was developed for this purpose to define early termination by euthanasia (Appendix II). Crucial factors included in this decision flow chart were fever (>40˚C), general condition, respiratory distress and feed intake. Overdose of sodium pentobarbital by intravenous injection was used for euthanasia. At the end of the study remaining pigs from Groups 2 to 5 were also euthanased by this method, whereas pigs from Groups 6, 7a and 7b were euthanased by a captive bolt pistol, followed by immediate exsanguination.

This study was granted approval by the Animal Ethics Committee of Massey University, permit numbers 05/41 and 05/90.

3.2.10 Statistical analysis

Descriptive statistics were calculated for each study group to assess completeness and validity of data. Average rectal temperature and body condition scores were calculated for groups with clinical symptoms of PMWS and compared to equivalent control groups. Kaplan-Meier survival curves were created of cumulative proportion of survived pigs stratified by experimental group. Fisher's exact test was used to evaluate the association between clinical cases of Groups 1 to 4 and age and breed. Statistical analysis was undertaken in SPSS 13.0, where reference is made to statistical significance at p-value < 0.05.

3.3 Results

3.3.1 Clinical outcomes

Pigs from three groups (Group 1, 3, and 5) showed clinical evidence of PMWS and PMWS-associated mortality was evident in two groups (Group 1 and 3). There was no evidence of PMWS in any of the other groups.

Figures 3.3, 3.4 and 3.6 show the average rectal temperatures, average body condition scores and survival curves according to experimental study group.

Group 1 (at day 0: PCV2-negative, PMWS-negative, 4-weeks-old)

The earliest indication of a disease process in Group 1 (n = 6) was an increase in body temperature 3 days after merging with pigs from the exposure group (n = 16, PCV2-positive, PMWS-positive). A blood sampling accident occurred on day 5 in the only pig of this group not to die of PMWS. This animal was excluded from any further analysis. On day 6 when all pigs in Group 1 had temperatures of ≥40°C, the first peak of a biphasic temperature pattern in the group occurred and 4/5 pigs were coughing. Coughing continued among individuals in the group until they were euthanased. A second peak of fever occurred on day 17 (Figure 3.3).

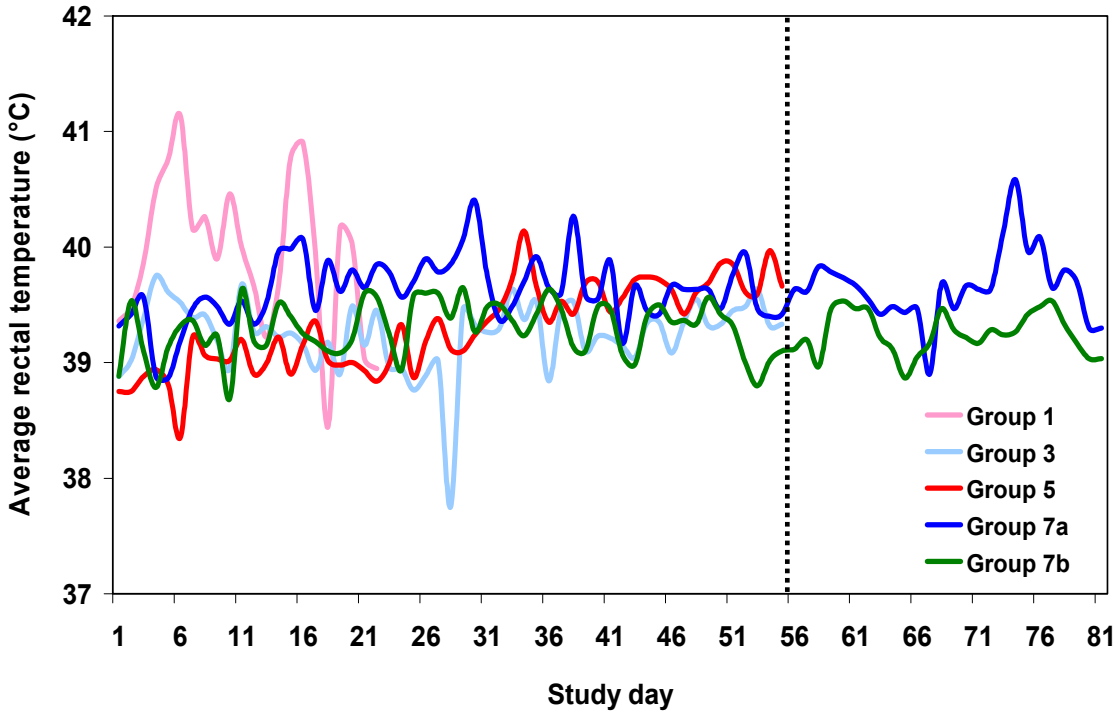


Figure 3.3: Average rectal temperatures of experimental groups of pigs (1, 3, 5, 7a, and 7b) during a natural transmission study on PMWS. The vertical dotted line at day 56 shows the end of the study for Groups 1 to 5.

One animal showed biphasic inspiration two days prior to euthanasia. An outbreak of diarrhoea started on day 10 in 4/5 pigs and within 5 days 5/5 pigs were affected. Over this period, faecal consistency changed from a soft scour to a watery scour, and faecal colour changed from grey to yellow. In one case a pig vomited bile on day 10. After the first week all animals began to rapidly lose body condition. Within 13 days post exposure the average body condition score decreased rapidly from 3 to 1 (Figures 3.4 and 3.5).

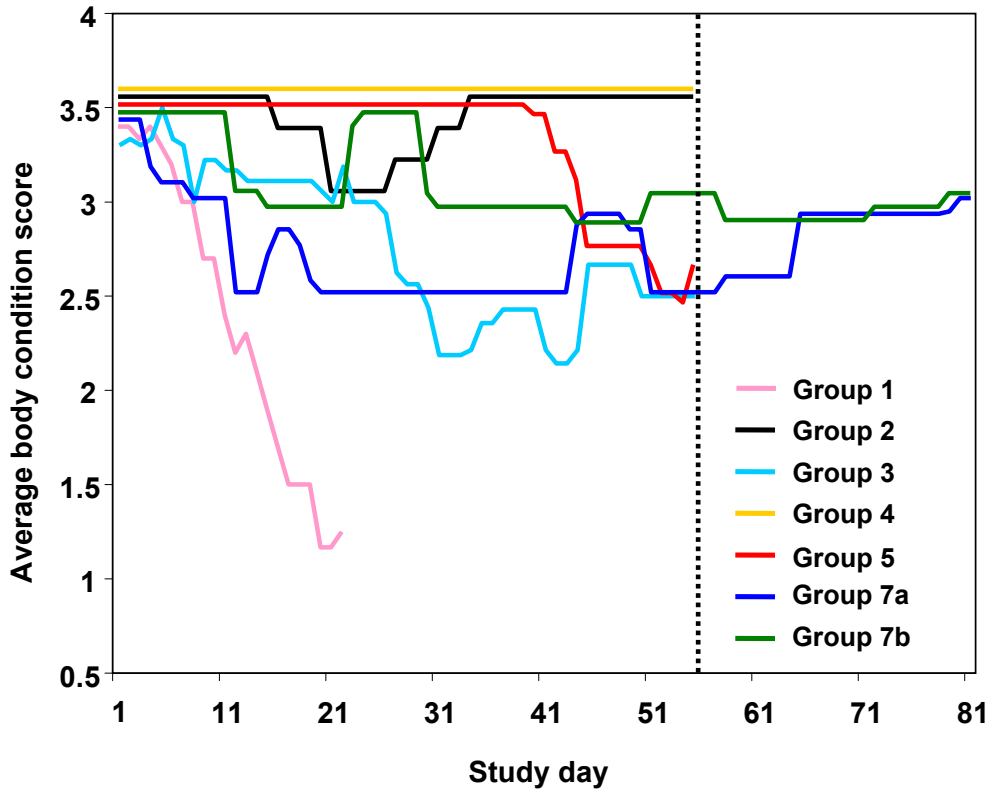


Figure 3.4: Average body condition scores in experimental groups of pigs (1 to 5, 7a and 7b) during a natural transmission study on PMWS. The vertical dotted line at day 56 shows the end of the trial for Groups 1 to 5.

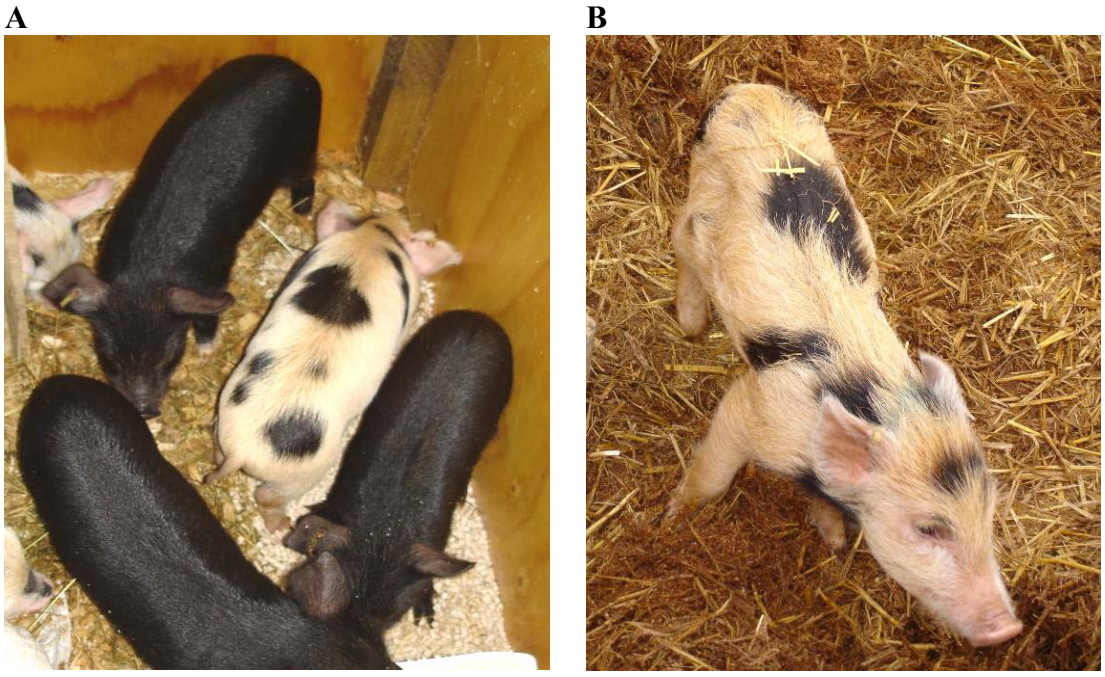


Figure 3.5: Photographs of pigs from Group 1. **A:** Prior to exposure to PMWS-affected pigs (exposure group) and **B:** At 18 days post exposure.

All pigs in this group died within 22 days post exposure so that both the mortality rate and clinical PMWS case fatality rate were 100% (Figure 3.6).

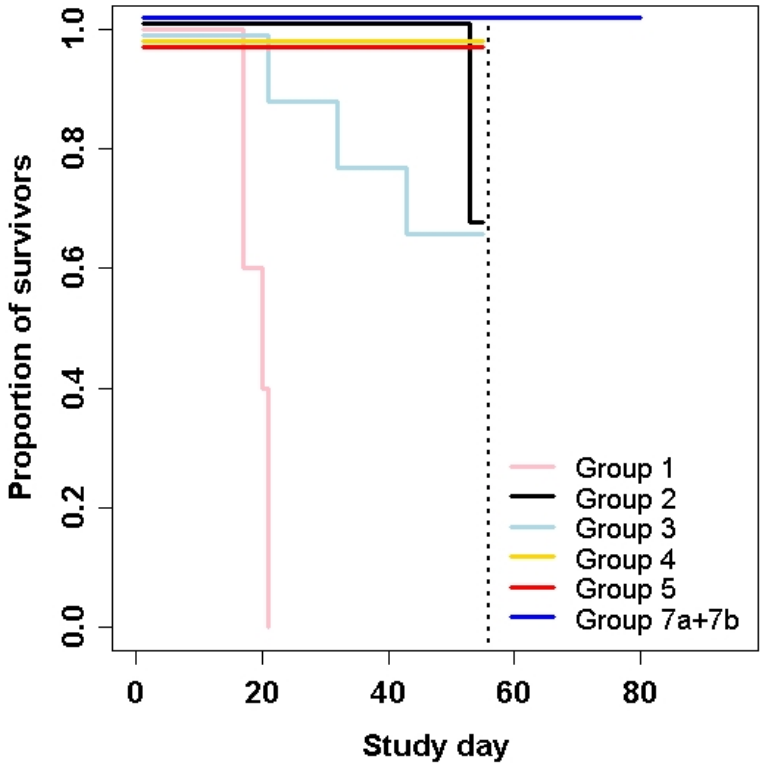


Figure 3.6: Kaplan-Meier survival curves of experimental groups of pigs (Groups 1 to 5, 7a and 7b) from a natural transmission study on PMWS. Stepped lines represent the proportion of animals in each group surviving at each day over the study period. The vertical dotted line at day 56 shows the end of the study for Groups 1 to 5.

ELISA results from terminal serum samples and PCR results from PBMCs and faecal samples showed there was no infection of PPV or PRRSV, and 4/5 pigs were positive for PCV2 on termination day as determined by PCR on faecal samples.

Group 2 (at day 0: PCV2-negative, PMWS-negative, 13-weeks-old)

In contrast to Group 1, the pigs from Group 2 (n = 3) did not show clinical signs of PMWS but did show some evidence of exposure to an infectious agent. There were episodes of pyrexia and temporary weight loss but no other clinical signs. The first temperature peak occurred on day 12 with 2/3 animals affected and an average rectal temperature of 40.1°C. Peaks in average temperature occurred at days 18 and 25 of

40.2°C and 40.3°C, respectively. Between these days all three pigs had a slight loss of body condition but had recovered this loss by the end of the study (Figures 3.4 and 3.7).

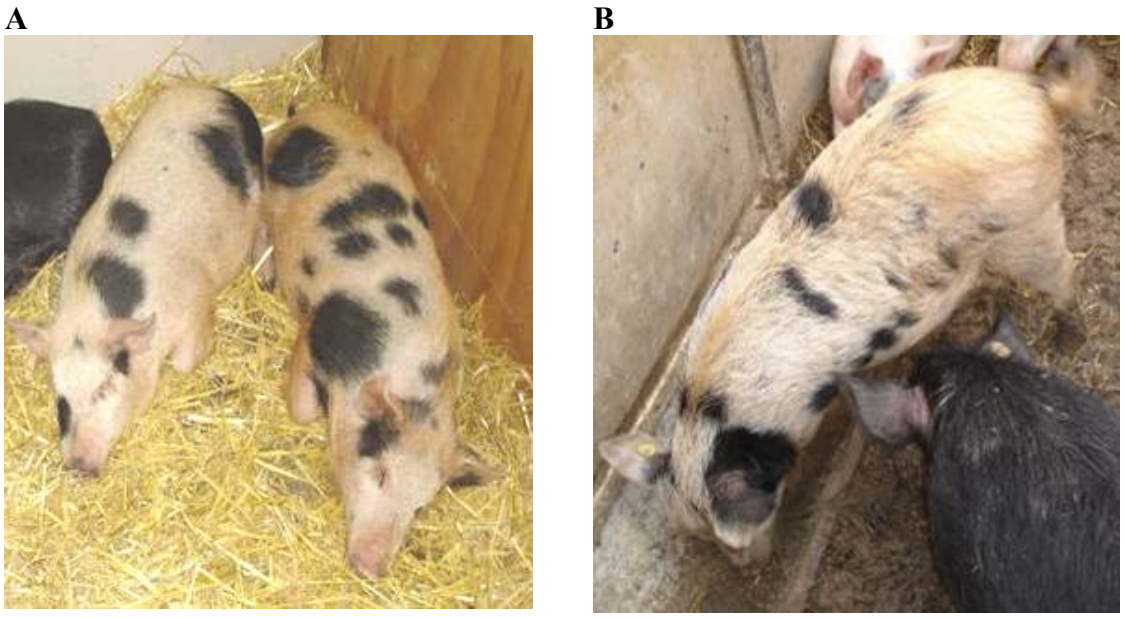


Figure 3.7: Photographs of pigs from Group 2. **A:** Prior to exposure to PMWS-affected pigs (exposure group) and **B:** 53 days post exposure.

One pig had faeces of watery consistency for 2 days from day 16. Another pig developed a cough on day 9, which progressed to a lasting dry harsh cough 14 days later, indicative of Mycoplasmal pneumonia. This pig expressed severe respiratory distress with biphasic inspiration and had to be euthanased one day before the planned termination date with no lesions of PMWS evident. It was the only PCV2-positive pig of this group as determined by PCR on terminal faecal samples.

Group 3 (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old)

Group 3 (n = 10) showed a slower and more variable development of clinical disease consistent with PMWS in comparison to Group 1. The average temperature curve of this group did not exceed 40°C during the study period (Figure 3.3). Two peaks with increased average temperatures, however, occurred on day 20 (3/9 pigs) and on day 33 (3/8 pigs) when a few pigs had temperatures of ≥40°C. Three animals (ID 18, 21, 24)

showed the first signs of pyrexia (above 41°C) on days 4, 7 and 10 with a second temperature peak recorded on days 16, 20 and 23. One pig (ID 22) had to be euthanased on day 3 after developing acute meningitis and was excluded from further analysis. Another pig (ID 20) died at day 21 after showing rapid wasting within the first week, a biphasic breathing pattern and scouring from day 19. The final cause of death was an incarcerated intussusception but the clinical signs were typical of PMWS. Two other pigs (ID 25, 27) had similar courses of disease with wasting apparent in the fourth week (Figure 3.8), coughing from days 13 and 31 and manifested respiratory distress with a biphasic breathing pattern 2 days before euthanasia (day 33, 44).

A **B**

Figure 3.8: Photographs of pigs from Group 3. **A:** Prior to exposure to PMWS-affected pigs (exposure group) and **B:** A PMWS infected animal (ID 27) at day 30 post exposure.

After day 29 the average body condition score of the surviving pigs remained at ≤2.5 (Figure 3.4). The first signs of scruffy coats appeared from day 31 and 4/6 pigs were affected by day 45. This group showed a morbidity rate of 66% (6/9 pigs) with a case fatality rate of 50% (3/6 pigs). The mortality rate was 33% (3/9 pigs) (Figure 3.6). None of the pigs in Group 3 had seroconverted for PPV or PRRSV during the study as

determined by ELISA. PCR results from PBMCs and faecal samples on termination day were all negative for PPV and PRRSV.

Group 4 (at day 0: PCV2-positive, PMWS-negative, 12-weeks-old)

Group 4 (n = 10) developed no clinical signs suggestive of any transmitted disease (Figure 3.9).

A **B**

Figure 3.9: Photographs of pigs from Group 4. **A:** Prior to exposure to PMWS-affected pigs (exposure group) and **B:** 54 days post exposure.

The average body condition score of Group 4 remained unchanged at level 3.5 (Figure 3.4) and the average temperature curve varied within a physiologically normal range. A maximum of 3/10 pigs had rectal temperatures ≥40°C on day 34, probably due to the onset of dry coughing which changed into mainly moist coughing after day 36. The survival rate of Group 4 until the termination of the study (day 56) was 100% (Figure 3.6).

Group 5 (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old)

There were no apparent clinical signs typical of a transmitted disease evident up to day 33 in Group 5 (n = 10). At day 34 the average rectal temperature of the group increased where 6/10 pigs had elevated temperatures of ≥40°C (Figure 3.3). After this initial phase (6 to 18 days later) 3 animals (ID 16, 19, 29) developed temperatures >41°C for 2 to 3 days. Antibiotic treatment with oxytetracycline failed to reduce these high temperatures and between day 40 and 54 the average body condition score dropped from 3.5 to 2.5 (Figure 3.4). The first signs of scruffy coats appeared from day 36 (Figure 3.10) and 4/10 pigs in the group were affected by day 45.

A **B**

Figure 3.10: Photographs of pigs from Group 5. **A:** At the beginning of the transmission study and **B:** With visible signs of scruffy hair coats at day 36.

The morbidity rate of this group was 70% (7/10 pigs) with a survival rate of 100% by the end of the study (day 56) (Figure 3.6).

Group 6 (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old)

Group 6 (n = 6) was examined pathologically at 12-weeks-of-age for evidence of PMWS. Farm records (from Farm B) indicated that the herd continued to be free of PMWS according to the definition used in the New Zealand outbreak investigation (Stone 2004), with low mortality and no clinical signs suggestive of PMWS.

Group 7a (at day 0: PCV2-negative, PMWS-negative, 4-weeks-old) and Group 7b (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old)

All pigs from Group 7b (n = 6) had normal average temperatures for the duration of the study period (Figure 3.3) and showed no clinical signs of any disease. In Group 7a (n = 6), all pigs had a slight loss of body condition during the first two weeks, but recovered the loss in the following seven weeks (Figure 3.4). Concurrently, there was vigorous fighting activity between the two genotypes. The average rectal temperature of Group 7a was slightly elevated in comparison with pigs of Group 7b (Figure 3.3). The first increase in temperatures (up to 40.8˚C) in Group 7a occurred on day 16 where 3/6 pigs were affected and they had grey, soft scouring for 5 days. On day 30, a second peak (temperatures up to 40.6˚C) affected all of the pigs in this group. A more significant fever peak (temperatures up to 42.0°C) occurred on day 74 with 4/6 pigs affected. There were no pigs from Group 7a or 7b that developed muscle wasting, scruffy coats and other signs of PMWS up to day 81 (Figure 3.11).

A **B**

Figure 3.11: Photographs of pigs from Group 7a. **A:** Prior to exposure to a PCV2-positive pig and faeces collected at Farm B and **B:** With no signs of wasting or PMWS disease 81 days post exposure to PCV2.

Group 7a pigs became infected with PCV2 during the study as demonstrated by PCR on tonsil tissue.

3.3.2 Relationship between clinical cases, age and breed

Fisher's exact test showed that there was a statistically significant difference between clinical cases and age categories in Group 1 to 4 ($p = 0.000$). Eleven pigs from Groups 1 and 3 fulfilled our clinical case definition, whereas there were no pigs from Groups 2 and 4 that showed typical signs of PMWS. There was no significant difference ($p = 0.135$) between clinical cases of PMWS in Auckland Island pigs and commercial pigs.

3.4 Discussion

This study was designed to determine whether it was possible to transmit postweaning multisystemic wasting syndrome (PMWS) from affected to susceptible pigs and reproduce clinical disease in pigs of different ages and serological status. The exclusion of many of the postulated co-factors was also a key consideration of the study design and determined whether disease expression requires an infectious cause other than porcine circovirus type 2 (PCV2).

The current study demonstrated that PMWS could be transmitted to clinically healthy pigs through direct contact with PMWS-affected pigs, in agreement with other oro-nasal experimental studies (Bolin et al. 2001, Okuda et al. 2003, Kristensen et al. 2004). Clinical signs were consistent with descriptions provided in previous reports including muscle wasting, dyspnoea, coughing, diarrhoea, and rough hair coat (Harding & Clark 1997, Harding et al. 1998, Sorden 2000). Typical histopathological lesions of PMWS were found and PCV2 was detected within those lesions by *in situ* hybridisation (Sorden 2000) (Chapter 4, section 4.3.3, page 103).

Daily rectal temperatures of pigs in Group 1 rose above 41˚C within the first 2 to 3 days after exposure to the PMWS-affected pigs, followed by another peak as disease developed and the pigs began to waste and die. Five of these naïve pigs died unusually rapidly (within 22 days) after developing typical signs of PMWS (100% case fatality), whereas a sixth died of a bleeding accident at day 5 and showed no evidence of the disease. The herd from the source farm of Group 1 remained free of the disease, however, when pigs from that herd of the same age were exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd, they became infected with PCV2 but did not develop any disease with no mortality occurring. This indicates that PMWS is transmissible to susceptible pigs by direct exposure and that PCV2 alone is not sufficient to produce clinical signs consistent with PMWS.

Four-week-old pigs of Group 3 (PCV2-positive, PMWS-negative on day 0) showed mildly elevated rectal temperatures within the first three weeks, but then 3/9 pigs of the group developed typical signs of PMWS and died before completion of the study. These findings suggest that despite prior exposure to PCV2, susceptible pigs develop clinical signs of disease after direct exposure to PMWS-affected pigs but do not deteriorate as fast as naïve animals.

The pigs from the on-site negative control group (Group 5) became ill during the second half of the study period. Onset of infection was noticed as an elevation of individual rectal temperatures >41˚C with a biphasic curve similar to that noted in Group 1. The pyrexia failed to respond to antibiotic therapy, consistent with reports from other PMWS infections (Segalés et al. 1997, Harding et al. 1998, Madec et al. 2000, Segalés et al. 2004a). Wasting and rough hair coats appeared during the subsequent 20 days but

no mortality occurred before the conclusion of the study. These observations suggest that PMWS was transmitted to the group via an inadvertent, indirect route. Despite careful biosecurity measures, potential routes may have been mechanical or personnel vectors, rodent vector or short distance (2m) airborne spread. It was necessary to keep these pigs adjacent to the exposed pigs because of biosecurity requirements for the study with the possibility of indirect transmission being recognised from the beginning of the study. Comparative group of pigs (Group 6) which were held 'off-site' at their source farm were evaluated and found to have no evidence of PMWS. This indicates that PMWS infection is also transmissible via indirect routes of exposure and confirms the contagious nature of the disease.

New Zealand is currently free of porcine reproductive and respiratory syndrome virus (PRRSV) and testing confirmed that pigs from this study were free of this agent. Porcine parvovirus (PPV) was not transmitted to Group 1 or Group 3 pigs during the study excluding this pathogen as a causal agent for PMWS (Chapter 4, section 4.3.6, page 111). Groups 1, 2 and 7a were of an archaic genotype, yet results showed that exposure of these pigs to PMWS-affected pigs at four weeks of age produced fulminant PMWS. However, exposure to PCV2-contaminated faeces and a PCV2-positive PMWS-negative pig produced PCV2 infection but no disease. Pigs were kept in traditional semi-outdoor housing using commercial management practices without vaccine use. There was no evidence of any differences in immunosuppression between groups which developed disease and those that did not. Therefore, it appears that disease expression was determined by exposure to PMWS-affected pigs and was affected by age. Prior exposure of pigs to PCV2 reduced the prevalence and speed of development of the disease but did not determine whether or not it occurred. The susceptibility of young (4-week-old) pigs to PMWS is consistent with the observations of Bailey et al. (2001) of normal architecture of the mucosal immune system being reached by about 6 weeks of age, with absolute numbers of $CD8^+$ T-cells continuing to increase. The logical consequence of this is that pigs older than 6-weeks-of-age are better able to resist viral challenge in comparison to younger pigs. However, in the present study Group 5 pigs were approximately 8 weeks old before they developed fever in response to presumptive exposure to a PMWS causing agent. Consequently, the period of susceptibility to PMWS occurs from 4 to 8 weeks.

There was limited availability of pigs from Farm A hence the low number of animals per group in Groups 1, 2 and 7a. However, our findings clearly demonstrate that PCV2 infection alone is not sufficient to produce PMWS. Mixing PCV2-negative (PMWS-negative) with PCV2-positive (PMWS-negative) pigs did not result in clinical signs consistent with PMWS within the exposure period of 81 days.

3.5 Conclusions

We conclude that an infectious agent other than PCV2 is a necessary cause for the occurrence of PMWS and hypothesise that an un-identified aetiological agent exists. Further research is required to identify this agent and to determine its possible routes of transmission.

Chapter 4

Pathological and microbiological findings of a natural transmission study on postweaning multisystemic wasting syndrome in pigs

4.1 Introduction

Over the last decade, postweaning multisystemic wasting syndrome (PMWS) has been identified in many countries worldwide including the USA, UK, Asia and most of Europe (Allan & Ellis 2000, Segalés & Domingo 2002). It is considered to be an important disease of pigs with a significant economic impact on affected farms, through a loss in productivity caused by increased mortality rates and a reduction in daily weight gain of growing pigs.

The first cases of PMWS in weaners were identified in high health pig herds in Saskatchewan, western Canada in 1991, with further evidence reported in 1996 (Harding & Clark 1997, Ellis et al. 1998). These authors suggested naming the new disease 'postweaning multisystemic wasting syndrome' due to the typical clinical signs observed in affected pigs. PMWS affects recently weaned pigs, usually in the age range of 5 to 12 weeks (Harding & Clark 1997, Allan & Ellis 2000) and is clinically characterised by progressive weight loss, with or without respiratory signs, diarrhoea, pallor, and jaundice (Harding & Clark 1997, Allan et al. 1999a, Allan & Ellis 2000). Morbidity can range from 10% to 50% of pigs in the at-risk age group on affected farms with a case fatality rate of up to 80% (Segalés & Domingo 2002).

The most frequent gross pathological findings seen in PMWS-affected pigs are poor body condition, non-collapsed lungs with associated bacterial bronchopneumonia, and lymphadenopathy (Harding & Clark 1997, Rosell et al. 1999, Segalés & Domingo 2002, Segalés et al. 2004b). Characteristic histological lesions attributable to PMWS are typically found in lymphoid tissues (tonsils, lymph nodes, spleen, Peyer's patches and thymus), although inflammatory infiltrates associated with PMWS have also been observed in other organs (Harding & Clark 1997, Rosell et al. 1999, Darwich et al.

2004). A variable extent of lymphocyte depletion with loss of follicular structure is found in almost all PMWS-affected pigs (Segalés & Domingo 2002). Infiltration of large mononuclear, binucleate or multinucleated histiocytes (also referred to as giant cells, multinucleated giant cells or heterokaryon) can also be observed. Another prominent feature of PMWS is the presence of intensely basophilic to amphophilic, intracytoplasmic or intranuclear inclusion bodies in histiocytic cells of lymphoid tissues, and, more sporadically, in epithelial cells of other organs (kidney, lung, blood vessel, pancreas, liver, and intestine) (Morozov et al. 1998, Rosell et al. 1999, McNeilly et al. 1999, Allan & Ellis 2000, Rosell et al. 2000, Sirinarumitr et al. 2000).

Research has been conducted worldwide in an effort to identify the likely aetiological agent(s) of PMWS and to elucidate its pathogenesis. It is recognised that the immune system plays an important role in the development of the disease and distinctive lymphoid lesions. At present, cells of the monocyte/macrophage lineage are considered as the main target cells for porcine circovirus type 2 (PCV2) (Ellis et al. 1998, Rosell et al. 1999, Chianini et al. 2003, Gilpin et al. 2003, Chang et al. 2006b, Hamberg et al. 2007), which is the most frequently suggested causal agent for PMWS.

To provide further evidence for the aetiology and pathogenesis of PMWS, we conducted a study to determine if clinical PMWS could be transmitted from affected to non-affected pigs. The main transmission routes of PMWS are assumed to be direct contact with affected pigs and vectors such as feed, equipment and clothing. The primary aim of this study was to produce clinical PMWS in healthy pigs of a susceptible age. A secondary aim was to collect ante mortem and post mortem samples from affected pigs in order to complete a detailed microbiological investigation of potential causal agents of PMWS. This was completed, in part, by the exclusion of known significant porcine pathogens in the New Zealand pig population. This chapter outlines the main pathological and microbiological findings (including clinical pathology).

4.2 Materials and methods

4.2.1 Case definition of PMWS

PMWS-affected study animals were sourced from two properties fulfilling the below criteria and that had previously been confirmed to have PMWS. Individual pigs were confirmed as infected based on clinical signs of wasting with and without dyspnoea. The study animals confirmed to be free of PMWS were sourced from properties with no history of PMWS infection. These animals did not fulfil the below criteria and showed no clinical signs consistent with PMWS.

The following case definition of PMWS-affected farms was applied to source farms for this study (Stone 2004). A PMWS-positive diagnosis at the farm-level required fulfilment of the following criteria:

(1) Current or historical evidence of non-responsive wasting[7] in 6 to 12-week-old pigs with a high case fatality rate; combined with

(2) Acute phase mortality rate in the target age group of approximately 15% or greater, but at least twice the pre-acute mortality; or, elevated mortality rate in the post acute phase.

The criteria to diagnose PMWS at the animal level consisted of:

(1) Clinical signs of wasting with or without dyspnoea or icterus;

(2) Histopathological lesions in lymphoid tissues such as depletion of lymphoid[8] cells and

  (2.1) presence of infiltrative (eventually multinucleated) histiocytes[9] in the cortex[10] or,

[7] Wasting was assessed on the loss or lack of fat and/or muscle covering of the animal.

[8] Lymphoid tissues contain aggregates or follicles of lymphocytes. Lymphoid depletion is marked by a progressive loss of lymphocytes in these follicular structures.

[9] Histiocytes (mononuclear or multinucleated) are tissue macrophages. Their function is to phagocyte material of foreign origin (e.g. viruses, bacteria, fungi) and to stimulate lymphocytes and other immune cells to respond to the pathogen. Over time mononuclear histiocytes can fuse to multinucleated histiocytes (Alastair Johnstone, personal communication).

[10] The cortex of a lymph node consists of follicles, trabeculae, fine stromal elements, and lymph sinuses (Banks 1986).

(2.2) evidence of lympho-histiocytic inflammatory and degenerative epithelial changes in non-lymphoid organs (e.g. liver, lung, kidney) but definitively,

(2.3) evidence of botryoid inclusion bodies within lesions.

4.2.2 Experimental design

Origin of animals

Study animals originated from four different farms (Farm A to D) (refer to Chapter 3, section 3.2.2, page 47 for details).

PMWS-free study animals

The study animals diagnosed as free of PMWS were sourced from two properties (Farm A and B) with no history of PMWS infection. Pigs free of PCV2 were obtained from Farm A, a herd comprised of pigs of an archaic genotype (Auckland Island pigs). This herd is free of the most common and exotic pig pathogens, including PCV2, porcine parvovirus (PPV), Aujeszky's disease (AujD) and porcine reproductive and respiratory syndrome virus (PRRSV). Pigs obtained from Farm B, a high health status, commercial New Zealand piggery, were known to be endemically infected with PCV2, but free of other pathogens (PRRSV, PPV, and AujD). The PMWS-free study animals did not fulfil the animal-level criteria listed above and did not show clinical signs consistent with PMWS and were therefore deemed free of the disease.

PMWS-affected study animals

PMWS-affected study animals were sourced from two properties (Farm C and D) which had previously been confirmed to have PMWS and fulfilled the above criteria, with recent and historical post weaning mortality rates regularly in excess of 20%. The study pigs were diagnosed as PMWS-positive based on clinical signs of wasting with or without dyspnoea.

Study design

Study animals were separated into eight groups (Groups 1 to 7b, Table 4.1).

Table 4.1: Summary of experimental groups of a natural transmission study on PMWS outlining origin, number of pigs per group, age (weeks) and PCV2 and PMWS status, at the commencement of the experiment.

Experimental group	Farm of origin	N	Age (weeks)	PCV2 status	PMWS status	Study housing
1	A[1]	6	4	–	–	Outdoor shelter[2]
2	A	3	13	–	–	Outdoor shelter
3	B[3]	10	4	+	–	Outdoor shelter
4	B	10	12	+	–	Outdoor shelter
5	B	10	4	+	–	Outdoor shelter
6	B	6	4	+	–	Commercial pig farm
7a	A	6	4	–	–	Indoor housing[4]
7b	B	6	4	+	–	Indoor housing
Exposure	C[5] D[5]	4 12	8	+ +	+ +	Outdoor shelter

[1] Private research herd, archaic genotype, fully-enclosed housing.

[2] Purpose-built outdoor research facility at Massey University, Palmerston North, New Zealand, located on a grazing paddock previously used for ruminants only. Shelter was made of plywood and straw bales with separate pens for Groups 1 to 5.

[3] Commercial piggery, modern genotype, fully-enclosed housing.

[4] A separate room on a Massey University research farm without any other pigs.

[5] Commercial piggeries, composite genetic mix, outdoor and indoor housing.

– = negative

+ = positive

The exposure group (PMWS-affected pigs) consisted of 16 weaners with typical clinical signs of wasting from Farm C and D serving as a direct source of PMWS to healthy pigs via direct transmission. Pigs of the exposure group were mixed with healthy pigs of Groups 1 to 4 for up to 56 days, with each group housed in separate pens. The PMWS-affected weaners were initially stratified by farm of origin and randomly allocated to groups, ensuring that pigs from each PMWS-affected farm were represented per group. These exposure pigs were then rotated between Group 1 to 4 after the first week in an attempt to minimise any variation in shedding between PMWS-affected pigs within groups. Group 5 was not directly exposed to PMWS-affected pigs but was housed 2 metres adjacent to Groups 1 to 4 in a separate pen, serving as an on-site negative control group. Group 6 remained on the source farm (Farm B) for the duration of the exposure period and served as an off-site control group.

Group 7a and 7b comprised of six pigs from each of Farms A and B, which were held in the same pen for a period of 81 days. These pigs were exposed to a 12-week-old pig from Farm B and to faeces collected from 12-week-old pigs from Farm B. Both groups acted as internal study comparisons for Groups 1 and 3.

Animals of Groups 1 and 7a, which were at the at-risk age for PMWS (4 weeks), were PCV2-negative on day 0 of the study and were assumed to become infected with both PMWS and PCV2, respectively, during the study period. Pigs of Groups 3 and 7b, which were at the same at-risk age (4 weeks), were PCV2-positive on day 0 of the study but it was expected that only Group 3 would become infected with PMWS after exposure to PMWS-affected pigs.

Two unforeseen deaths occurred within the first week of the study which were not related to PMWS. One pig (ID 22) had to be euthanased on day 3 after developing acute meningitis, leaving 9 animals in Group 3, and one pig (ID 6) died of a blood sampling accident on day 5, leaving 5 animals in Group 1. Both pigs were excluded from any further investigation.

Data collection

All study animals, with the exception of the Group 6 pigs, were observed twice daily and rectal temperatures and respiratory and heart rates recorded once daily. Rectal temperatures and heart rates were measured during the morning feeding. Respiratory rates were recorded while animals were resting in the early afternoon. Pigs were assessed and given a body condition score based on the amount of fat and/or muscle covering, after first signs of weight loss became apparent, with scores recorded at three day intervals. All healthy study animals had a body condition score of 3.5 on day 0 of the study. A body condition score rating system of 1 (poor) to 5 (fat), according to the New Zealand Animal Welfare Code 2005 for pigs (National Animal Welfare Advisory Committee 2005) (Appendix I) was utilised. A pig assessed to have a body condition score of <3 was diagnosed as wasting.

Pigs with an elevated temperature (≥40˚C) for at least two consecutive days prior to their first signs of weight loss, with or without development of scruffy hair coats, were defined as clinical cases of PMWS.

Clinical outcomes

The occurrence of clinical signs indicative of PMWS is outlined in Chapter 3, section 3.3.1, page 56. Table 4.2 illustrates the groups showing clinical evidence of PMWS during the study period (Groups 1, 3 and 5). Case fatalities were observed in Group 1 and 3. There was no evidence of PMWS in any of the other groups.

Table 4.2: Summary of clinical PMWS cases and case fatalities, stratified by experimental group, in a natural transmission study of PMWS. Groups 1 to 4 were exposed to PMWS-affected pigs, while Groups 7a and 7b had exposure to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd. Groups 5 and 6 served as on-site and off-site study controls, respectively.

Experimental group	Clinical PMWS cases/number of animals in group	Characteristics of case development	Number of case fatalities	Study day of case fatality occurrence
1[a]	5/5	Fast and fulminant	5/5	18, 21, 22
2[b]	0/3	-	0/3	-
3[c]	6/9	Slow and more varied	3/6	21, 33, 44
4[d]	0/10	-	0/10	-
5[e]	7/10	Moderate and uniform	0/7	-
6[f]	0/6	-	0/6	-
7a[g]	0/6	-	0/6	-
7b[h]	0/6	-	0/6	-

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-positive, PMWS-negative, 4-week-old, off-site control group.
[g] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[h] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

4.2.3 Necropsy and histopathology

Pigs were euthanased prior to the planned study termination day if they showed rapidly deteriorating general conditions, moderate to severe dyspnoea, or serious reasons not associated with PMWS, to ensure a humane endpoint and suitable timing of pathological examination. Overdose of sodium pentobarbital by intravenous injection was used for euthanasia. At end of the study, the remaining pigs within Groups 1 to 5 were also euthanased by this method, whereas pigs from Groups 6, 7a and 7b were euthanased by a captive bolt pistol followed by immediate exsanguination. All 73 study pigs were necropsied at the Institute of Veterinary, Animal and Biological Sciences at Massey University (Palmerston North, New Zealand). Samples of the following tissues were collected at post mortem: Skin, muscle, bone marrow, brain, tonsil, heart, lung, liver, pancreas, spleen, kidney, adrenal gland, bladder, stomach, ileum, ovary, testicle, and lymph nodes including the submandibular, mesenteric, and superficial inguinal lymph nodes. The tissues were fixed by immersion in neutral buffered 10% formalin, embedded in paraffin wax, sectioned at 4µm, and stained with haematoxylin and eosin (H&E) for microscopic analysis (Gill et al. 1974). Sections from selected tissues were also stained for acid-fast organisms (Ziehl-Neelsen method) (Culling et al. 1985) and leptospires (Warthin-Starry method) (Young 1969).

Sections of lymphoid organs (lymph nodes, tonsil, spleen, Peyer's patches, BALT[11] of lung) were assessed by an experienced pathologist for depletion of lymphoid cells, loss of lymphoid follicle structure[12], presence of infiltrative mononuclear and multinucleated histiocytes, and evidence of botryoid inclusion bodies within lesions. A subjective scoring system for the severity of histopathological lesions, ranging from 0 to 4 (0, normal; 1, minimal; 2, mild; 3, moderate; 4, marked), was determined by assessment against the histological characteristics of tissues of confirmed PMWS-negative pigs. The remaining, non-lymphoid organs were assessed for the presence of any pathological alterations. The histopathologist was blinded to the exposure status of study animals.

[11] BALT is the bronchus associated lymphoid tissue of the lung.

[12] Any alteration of the normal lymphoid cell distribution appropriate to the site under consideration, i.e. whether lymph node, spleen, and mucosa associated lymphoid tissues (MALT).

4.2.4 *In situ* hybridisation

Selected tissue sections of lymph node (mandibular, superficial inguinal or mesenteric lymph node), Peyer's patches, kidney, liver, lung and spleen from each study animal were tested for PCV2 nucleic acid using the *in situ* hybridisation (ISH) technique. Tissue sections were cut to 8μm from paraffin-embedded blocks and mounted on to Superfrost Plus glass slides (Biolab Scientific). The ISH testing was then completed at the Veterinary Diagnostic Laboratory of Iowa State University (Ames, Iowa, USA), according to a previously described method of Rosell et al. (1999). A scoring system for absence (0) and low to abundant (1 to 3) amount of PCV2 nucleic acid detected was used.

4.2.5 Bacteriology

To investigate the concurrent presence of pulmonary bacterial infections, only reference cases of experimental groups with indications of a pulmonary infection were sampled at post mortem. These indications included gross evidence of consolidated lobules or visible mucopurulent exudates in the airways. Lungs were cultured aerobically for a spectrum of pneumonic bacteria using routine culture media such as MacConkey agar, 5% Sheep blood agar, Colistin Naladixin Acid blood agar (CNA), and selective *Mycoplasma* media.

4.2.6 Haematology

Whole blood samples were collected from all pigs of Groups 1 to 5 and Groups 7a and 7b according to the sampling schedule as outlined in Table 4.3. Animals of Group 6 were not blood sampled during the study.

Table 4.3: Sampling days for collection of whole blood of experimental groups during a natural transmission study on PMWS.

Experimental group	Sampling days
1[a]	0, 8, 11
2[b]	0, 8, 11, 29
3[c]	0, 8, 11, 29
4[d]	0, 8, 11, 29
5[e]	0, 8, 11, 29
7a[f] and 7b[g]	0, 5, 8, 11, 15, 22, 29, 36, 43, 50, 57, 64, 71, 81

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Blood was collected from the jugular vein into specified vacutainer tubes as required for whole blood (Ethylenediamine Tetraacetic Acid additive (EDTA)) and processed for haemograms and blood smears at a commercial laboratory at Massey University (New Zealand Veterinary Pathology). Routine complete blood counts and leukocyte differentiation were performed on each sample using an automated blood cell analyzer (Advia 120, Bayer). The analyses included a count of white blood cells (WBC) and red blood cells (RBC), haemoglobin concentration (HGB), haematocrit value (HCT) and haematocrit value indices (mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC)), and platelet count (PLT).

4.2.7 Serology

To investigate the concurrent presence of major porcine viral pathogens, serum samples collected at post mortem and on study day 0 were tested for antibodies against PCV2, AujD, PRRSV, PPV, bovine viral diarrhoea (BVD) and hepatitis E virus (HEV). Serum was harvested from blood samples collected from the jugular vein using vacutainer tubes without any additives. Serum samples were frozen at -84˚C (MDF-U50V, Sanyo) until analysis.

Testing for PCV2 antibodies

Serum samples of all pigs in Group 4, 7a and 7b on day 0, and in Group 1, 3, 4, 5, 7a, and 7b on the termination day, were tested for the presence of PCV2 antibodies. Serum was also collected from 10 randomly selected weaners (4-weeks-old) and 10 growers (12-weeks-old) from Farm B to act as control groups. PCV2 serology was performed at the Elizabeth Macarthur Agricultural Institute (Menangle, NSW, Australia), using the Synbiotics SERELISA® PCV2 Ab Mono Blocking test. Test results were measured as optical densities (OD) and expressed in sample ratios[13] or percentage inhibition values (PIs). PIs of ≥80%, 79% to 60%, or <60% were considered test-positive, inconclusive, or test-negative, respectively (Finlaison et al. 2007). These reference values were determined via internal test evaluation prior to testing, with the objective of achieving a high negative predictive value[14].

Testing for AujD, PRRSV, PPV, BVD, and HEV

Serum samples of pigs in Group 1 on day 0, and pigs in Group 1 and 3 on termination day, were also analysed for the presence of viral antibodies against AujD, PRRSV, PPV and BVD. Diagnostic tests were performed at the MAF's Investigation and Diagnostic Centre (Wallaceville, New Zealand), using the commercial ELISA kits HerdChek* PRV gB Antibody Test Kit (IDEXX Laboratories Inc. 2003), HerdChek* PRRS 2XR Antibody Test Kit (IDEXX Laboratories Inc. 2006), SVANOVIR® PPV-Ab (Svanova Biotech AB 2004), and HerdChek* Bovine Viral Diarrhea Virus Antigen Test Kit/Serum Plus (IDEXX Laboratories Inc. 2007) for AujD, PRRSV, PPV, and BVD, respectively. The same reference values were applied as provided by the test manufacturers.

Serum samples of pigs in Group 1 and 2 on day 0, and pigs in Group 1, 2, 3, 4, 5, 7a, and 7b on termination day were also analysed for antibodies against HEV. Serum samples from the randomly selected weaners (n = 10) and growers (n = 10) from Farm B were also tested for HEV antibodies as control groups. Serology was performed at

[13] Sample ratio = OD sample / OD N, which means: Sample ratio = Average of the sample optical densities (OD sample) if the test is performed with duplicate samples, divided by the average of the negative control optical densities (OD N) (Synbiotics Europe SAS 2005).

[14] High confidence that a sero-negative tested animal is truly sero-negative.

Living Cell Technologies Ltd (Auckland, New Zealand), using a commercial HEV-antibody ELISA kit (Cat# WE7396, WanTai Biological Pharmacy Enterprise Co. Ltd, Beijing, China) according to the manufacturer's instructions.

4.2.8 Polymerase chain reaction

To eliminate common New Zealand porcine pathogens and identify potential causal agents involved in the pathogenesis of PMWS, faecal samples, tonsil tissues and peripheral blood mononuclear cells (PBMCs), collected from experimental groups on day 0 and at post mortem, were tested for the presence of various porcine deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses using nested polymerase chain reaction (PCR) assays (Table 4.4).

Table 4.4: Tissue samples of experimental groups and references of the virus-specific primers used in nested PCR, stratified by DNA and RNA viruses.

			Experimental groups tested		
Virus type	Pathogen	Tissue	Day 0	PM	Reference
RNA	BCV	Faeces	1	1, 3	(Gelinas et al. 2001)
	EMCV	Faeces	1, 3	1, 3	(Vanderhallen & Koenen 1998) and Garkavenko (not published)
	HEV	Faeces	1, 2, 3	1, 2, 3, 4, 5, 7a, 7b	(Erker et al. 1999)
	NLCV	Faeces	1, 3	1, 3	(van der Poel et al. 2000)
	PEC	Faeces	1, 3	1, 3	(Guo et al. 1999)
	PEV CPE I	Faeces	1, 2, 3	1, 2, 3, 4, 5, 7a, 7b	(Zell et al. 2000)
	PEV CPE II	Faeces	1, 3	1, 3, 7a, 7b	(Zell et al. 2000)
	PEV CPE III	Faeces	1, 2, 3	1, 2, 3, 4, 5, 7a, 7b	(Zell et al. 2000)
	PHEV	Faeces	1	1, 3	(Sasseville et al. 2002)
	PRRSV	Faeces	1	1, 3	(Guarino et al. 1999)
	Reovirus	Faeces	1, 3	1, 3	(Seliger et al. 1992)
	Rotavirus	Faeces	1, 3	1, 3	(Zhang et al. 1998)
DNA	PCMV	PBMC	1, 3	1, 3	(Hamel et al. 1999)
	PCV1	PBMC	1, 3	1, 3	(Kim & Chae 2003)
	PCV2	Faeces	1, 2, 3	1, 2, 3, 4, 5, 7a, 7b	(Larochelle et al. 1999a) and Garkavenko (not published)
		Tonsil		3, 5, 7a, 7b	(Larochelle et al. 1999a) and Garkavenko (not published)
	PLHV	PBMC	1, 3	1, 3	Garkavenko (not published)
	PPV	PBMC	1, 3	1, 3	(Soares et al. 2003)

BCV – Bovine coronavirus
EMCV – Encephalomyocarditis virus
HEV – Hepatitis E virus
NLCV – Norwalk-like calicivirus
PCMV – Porcine cytomegalovirus
PCV1/PCV2 – Porcine circovirus type 1/type 2
PEC – Porcine enteric calicivirus
PEV CPE I to III – Porcine enterovirus, cytopathogen effect group I to III
PHEV – Porcine hemagglutinating encephalomyelitis virus
PLHV – Porcine lymphotropic herpesvirus
PPV – Porcine parvovirus
PRRSV – Porcine reproductive and respiratory syndrome virus

Samples from day 0 were tested in series if the result from the post mortem sample was positive to any pathogen listed, to ascertain whether the infection was pre-existing or acquired during the experimental exposure to PMWS-affected pigs. A small number of randomly selected samples from day 0 were tested if the results from the post mortem samples of an experimental group were all negative to any pathogen listed. PBMCs were separated from blood samples collected using vacutainer tubes containing acid citrate dextrose (ACD) and were stored at -84°C until analysed. PCR testing was performed at Living Cell Technologies Ltd (Auckland, New Zealand). Details on isolations of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) from samples and PCR assays are given in Appendix III.

In order to act as a control group, faecal samples of randomly selected weaners (n = 10) and growers (n = 10) from Farm B which were tested serologically, were also tested by PCR for the presence of PCV2 and porcine enterovirus (PEV).

4.2.9 Data management and analysis

Data management

Descriptive statistics were calculated for each group and the frequency of macroscopic and microscopic findings, serology, and PCR results are presented. Haematological data that were normally distributed were summarised using the number of observations, mean, and 95% confidence interval, while non-normally distributed data were summarised as minimum, 25th percentile, median, 75th percentile and maximum.

Some collected data were transformed to simplify the interpretation and comparison of findings between the experimental groups. Histopathology scores (0 to 4) of characteristic lesions in lymphoid tissues were combined to create a dichotomous outcome variable of lesion presence (histopathology scores of 1 to 4) or absence (histopathology score of 0). Loss of body condition (wasting) was used as the primary clinical indication for the presence of PMWS. To assess whether presence or absence of characteristic lymphoid lesions at post mortem is typical for clinically PMWS-affected pigs, body condition scores (1 to 5) at termination day were combined to create a dichotomous outcome variable of body condition score category for wasting pigs (0)

(body condition scores <3) or thriving pigs (1) (body condition scores ≥3). ISH scores (0 to 3) were categorised into presence (scores of 1 to 3) and absence (score of 0). Ratios of neutrophils to lymphocytes were calculated as an indicator of lymphocytopenia in peripheral blood after both haematological parameters were log_{10} transformed in an attempt to fit normal distribution.

Statistical analysis

Fisher's exact test was used to evaluate the association between the loss of body condition and presence or absence of characteristic lesions in lymphoid tissues. Fisher's exact test was also used to evaluate the association between selected tissues and number of pigs with presence of PCV2 nucleic acid within tissue lesions, as determined by ISH.

On single blood sampling days, differences in the ratio of neutrophils to lympocytes between experimental groups were investigated using an analysis of variance (ANOVA). If the F-test statistic on an individual bleeding day was significant, the difference between each pair of experimental groups was evaluated using the Bonferroni multiple comparison test.

SPSS 13.0 for Windows and SAS® 9.1.2. (SAS Institute Inc., USA, 2004) were used for statistical analysis, with statistical significance at $p < 0.05$.

4.3 Results

4.3.1 Macroscopic findings

All macroscopic findings at necropsy and the frequency of lesions per experimental group are summarised in Table 4.5.

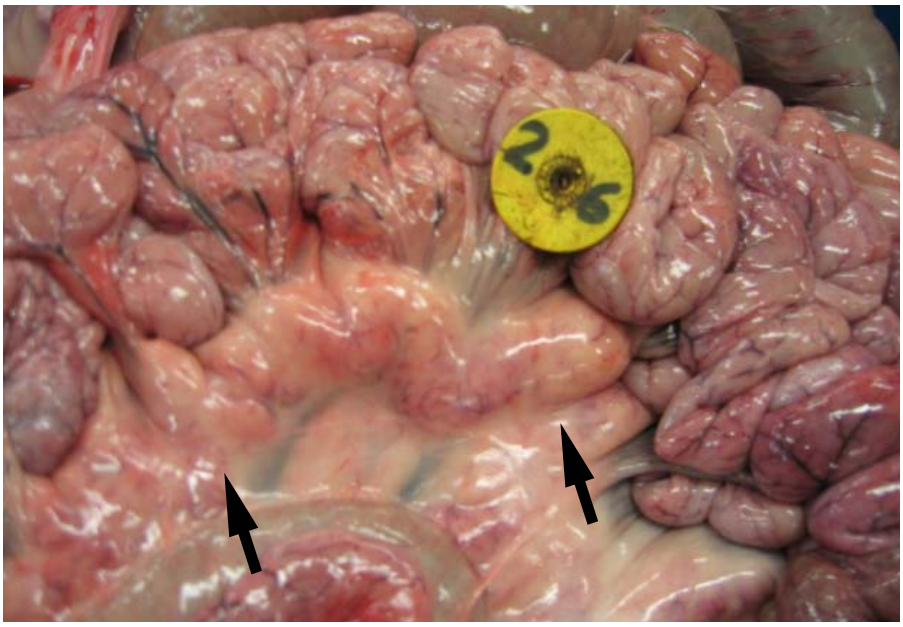
Table 4.5: Summary of macroscopic findings in a natural transmission study on PMWS. Data are presented as frequency per experimental group of pigs (number of affected/number of examined animals).

Tissue	Finding/lesion	Group 1[a] (*n* = 5)	Group 2[b] (*n* = 3)	Group 3[c] (*n* = 9)	Group 4[d] (*n* = 10)	Group 5[e] (*n* = 10)	Group 6[f] (*n* = 6)	Group 7a[g] (*n* = 6)	Group 7b[h] (*n* = 6)
Body condition	Muscle wasting	5/5	-	4/9	-	-	-	-	-
Lymph nodes[i]	Enlargement	4/5	-	1/9	2/10	2/10	-	1/6	1/6
Lung	Consolidation	1/5	2/3	2/9	2/10	-	-	-	-
	Congestion	2/5	-	1/9	1/10	-	-	-	-
	Non-collapsed	1/5	1/3	2/9	-	-	-	-	-
	Sub-acute pneumonia	-	-	-	4/10	4/10	-	-	-
Heart	Pericarditis	1/5	-	1/9	-	1/10	-	-	-
	Epicarditis	-	-	-	-	-	-	1/6	-
Thorax	Pleuritis	1/5	-	-	-	1/10	-	-	-
Abdominal cavity	Peritonitis	2/5	1/3	1/9	1/10	2/10	-	-	-
Intestine	Colitis	-	-	-	-	-	-	-	-
	Intussusception	-	-	1/9	-	-	-	-	-
Stomach	Ulcer	-	-	-	2/10	-	-	-	-
	Signs of gastritis	2/5	-	-	2/10	-	-	-	-
	Infarct	-	-	1/9	-	-	-	-	-
Kidney	White surface spots	-	-	1/9	-	-	-	-	-
	Renal cyst	-	-	-	-	2/10	-	-	-
Joints	Septic polyarthritis	-	-	-	-	-	-	-	-

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs; [b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs; [c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs; [d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs; [e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group; [f] PCV2-positive, PMWS-negative, 4-week-old, off-site control group; [g] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces; [h] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces; [i] including superficial inguinal lymph node, mandibular lymph node and mesenteric lymph node; '-' indicates there was no lesion found.

A variety of gross lesions were observed among experimental groups. The changes found in animals with clinical signs of disease were not pathognomonic but suggestive for PMWS. Poor body condition, enlargement of one or more lymph nodes (Figure 4.1, A), and a diffuse non-collapsing lung with areas of consolidation (Figure 4.1, B) were most frequently found in groups with clinical signs of disease (Group 1 and 3), but were occasionally observed in individual pigs from other groups.

A



B

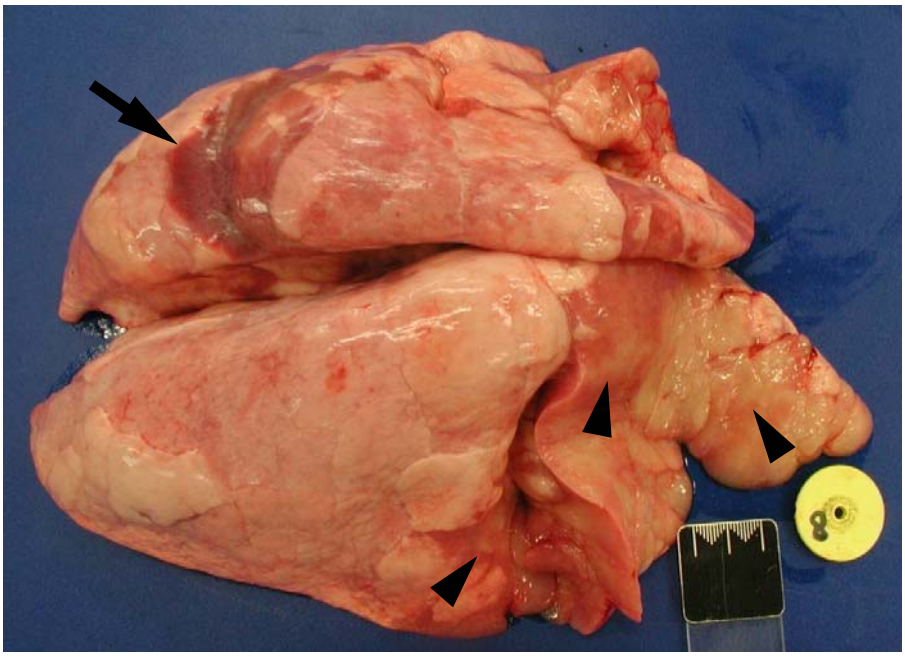


Figure 4.1: Photographs of **A:** enlarged mesenteric lymph nodes (*arrows*) from a pig of Group 5 with wasting symptoms and **B:** Non-collapsed lungs from a study animal within Group 2 which presented clinical signs of severe dyspnoea. There is an irregular, patchy distribution of consolidated lobules in the caudal lobes (*arrow*) and extensive consolidation of the middle and anterior lobes (*arrow heads*).

While sub-acute pneumonia of anterioventral distribution was confined to Group 4 and 5; pericarditis, peritonitis, and stomach lesions also occurred infrequently in other groups. No macroscopic alterations were seen in the spleen, tonsil, pancreas, adrenal gland, urinary bladder, testis/ovary, skin, brain or bone marrow in any study pig. No

lesions were found in any pigs in Group 6 (off-site control group). Histopathological investigation was required because gross lesions at post mortem observed in animals with clinical signs of disease were not sufficient to diagnose PMWS with confidence.

4.3.2 Microscopic findings

Histopathological lesions distinctive of PMWS were observed in experimental groups where clinical signs of PMWS were present in animals (Groups 1, 3, and 5) but also found to a lesser extent, in other groups. Microscopic lesions attributable to PMWS were largely confined to lymphoid tissues (including lymph nodes, spleen, tonsil, and ileal Peyer's patches) and parenchymal organs such as lung, liver, kidney, stomach, and intestine.

Lymphocellular depletion affecting lymphoid follicles or lymphocytic aggregates was detected in almost all experimental groups, but varied in degree between the groups, depending on their PCV2 status at the beginning of the experiment and their exposure to PMWS during the experiment. Loss of follicular structure was mainly observed in those groups with clinical occurrence of PMWS or exposure to PCV2. Progressive multifocal to diffuse infiltration of large mononuclear and multinucleated histiocytes was seen repeatedly in all experimental groups, but were particularly numerous in the acute stage of PMWS-affected pigs. Basophilic, botryoid intracytoplasmic inclusion bodies in histiocytic cells were frequently present in the clinically, most severely, affected pigs (Group 1) but were very rarely found among the other experimental groups.

Inflammatory infiltrates typically associated with PMWS were also observed in other non-lymphoid tissues. Interstitial pneumonia and/or bronchopneumonia, non-suppurative and granulomatous interstitial nephritis, non-suppurative gastritis and non-suppurative hepatitis including hydropic changes were the most commonly reported lesions. The degree of lesion severity varied between experimental groups according to their exposure status. Microscopic findings in tissues and the frequency of lesions observed in each experimental group are summarised in Table 4.6. A detailed description of the main histologic lesions within each experimental group follows.

Table 4.6: Summary of microscopic findings in examined tissues in a natural transmission study on PMWS. Data are presented as frequency per experimental group of pigs (number of affected/number of examined tissues). Skin and bone marrow tissue were not examined in any group.

Tissue	Lesion	Group 1[a] (*n* = 5)	Group 2[b] (*n* = 3)	Group 3[c] (*n* = 9)	Group 4[d] (*n* = 10)	Group 5[e] (*n* = 10)	Group 6[f] (*n* = 6)	Group 7a[g] (*n* = 6)	Group 7b[h] (*n* = 6)
Lymph nodes[i]	Mature lymphocyte depletion	5/5	2/3	9/9	7/10	10/10	3/6	6/6	5/6
	Loss of follicular definition	5/5	-	3/9	1/10	8/10	-	5/6	2/6
	Histiocyte infiltration	5/5	1/3	3/9	1/10	10/10	1/6	6/6	3/6
	Multinucleated histiocytes	3/5	1/3	1/9	1/10	10/10	1/6	6/6	2/6
	Circovirus inclusions	5/5	-	-	-	1/10	-	-	2/6
Spleen	Mature lymphocyte depletion	5/5	2/3	7/9	4/9	8/9	3/6	6/6	3/6
	Loss of PALS[j] definition	5/5	-	2/9	-	5/9	-	1/6	-
	Histiocyte infiltration	5/5	-	2/9	-	8/9	-	3/6	1/6
	Multinucleated histiocytes	2/5	-	1/9	-	8/9	-	2/6	-
	Circovirus inclusions	3/5	-	-	-	-	-	-	-
Tonsil	Mature lymphocyte depletion	3/3	2/3	5/9	3/10	10/10	1/5	3/5	2/6
	Loss of lymphocytic aggregates definition	3/3	-	1/9	-	7/10	-	1/5	-
	Histiocyte infiltration	3/3	1/3	2/9	-	10/10	1/5	4/5	1/6
	Multinucleated histiocytes	2/3	1/3	1/9	1/10	10/10	1/5	4/5	1/6
	Circovirus inclusions	2/3	-	-	-	-	-	-	-
Lung - peribronchial lymphoid tissue	Mature lymphocyte depletion	2/2	-	3/8	1/10	9/9	-	2/5	1/6
	Loss of BALT[k] definition	-	-	-	-	3/9	-	2/5	1/6
	Histiocyte infiltration	2/2	-	3/8	-	9/9	-	2/5	1/6
	Multinucleated histiocytes	1/2	-	1/8	-	8/9	-	1/5	1/6
	Circovirus inclusions	1/2	-	-	-	-	-	-	1/6
Lung	Bronchopneumonia (pleuropneumonia, necrotising bronchopneumonia)	-	2/3	3/9	4/10	-	-	-	1/6
	Interstitial or broncho-interstitial pneumonia	4/5	-	3/9	4/10	9/9	-	4/5	4/6
Intestine - Peyer's patches	Mature lymphocyte depletion	5/5	1/3	7/8	6/10	10/10	1/5	4/6	3/6
	Loss of lymphocytic aggregates definition	3/5	-	1/8	-	4/10	-	2/6	-
	Histiocyte infiltration	4/5	1/3	2/8	2/10	10/10	-	4/6	-
	Multinucleated histiocytes	2/5	1/3	1/8	1/10	10/10	-	4/6	1/6
	Circovirus inclusions	4/5	-	-	-	-	-	-	-
Intestine	Enteritis, non-suppurative	-	1/3	1/9	6/10	2/10	-	-	-
	Enteritis, suppurative	-	-	-	-	2/10	-	-	-
	Enteritis, necrohaemorrhagic	-	-	-	-	-	-	-	-

Table 4.6. continued

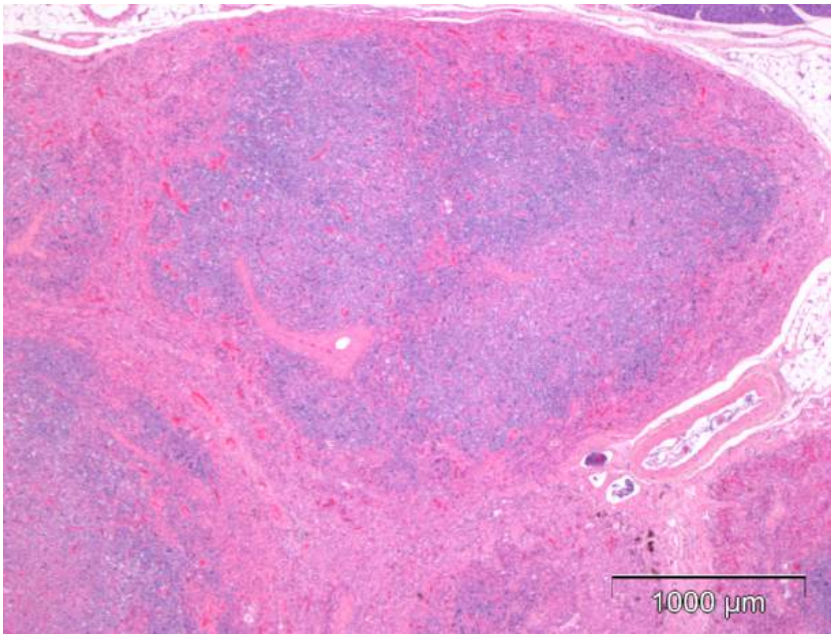
Tissue	Lesion	Group 1 (*n* = 5)	Group 2 (*n* = 3)	Group 3 (*n* = 9)	Group 4 (*n* = 10)	Group 5 (*n* = 10)	Group 6 (*n* = 6)	Group 7a (*n* = 6)	Group 7b (*n* = 6)
Intestine	Duodenitis, fibrinonecrotic	2/5	-	-	-	-	-	-	-
	Ileitis	4/5	-	-	1/10	3/10	-	1/6	-
	Colitis, non-suppurative	1/5	-	-	1/10	-	-	-	-
	Colitis, suppurative	-	-	-	-	-	-	1/6	-
	Colitis, granulomatous	-	-	-	-	1/10	-	-	-
Stomach	Gastritis, non-suppurative	1/5	1/3	2/7	7/10	4/10	-	4/6	2/6
	Gastritis, granulomatous	1/5	-	2/7	-	3/10	-	-	-
	Gastritis, granulomatous ulcerative	1/5	-	-	3/10	-	-	-	-
	Gastric infarct	3/5	-	-	-	-	-	-	-
Kidney	Nephritis, non-suppurative or granulomatous interstitial	-	2/3	4/9	3/10	7/9	-	5/6	1/6
Liver	Hepatitis, non-suppurative	-	1/3	4/9	2/10	4/10	-	6/6	1/6
	Focal hepatic necrosis	-	-	-	-	-	-	1/6	-
	Apoptosis	2/5	-	1/9	-	-	-	-	-
	Hydropic degeneration	-	2/3	7/9	5/10	10/10	-	5/6	6/6
Heart	Myocarditis, non-suppurative interstitial	-	-	1/9	-	1/10	-	-	-
	Epicarditis, fibrinous	-	-	2/9	-	-	-	-	-
Brain	Meningitis, non-suppurative	-	-	-	-	5/10	ne	ne	ne
	Meningitis, suppurative	-	-	1/9	-	-	ne	ne	ne
Adrenal gland	Focal non-suppurative inflammation	-	-	-	4/10	1/5	ne	-	-
Urinary bladder	Cystitis, lymphocytic	-	-	1/6	1/10	-	ne	-	-
Pancreas		ne	-	-	-	-	ne	ne	-
Testis/ovary		-	-	-	-	-	ne	ne	-

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs; [b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs; [c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs; [d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs; [e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group; [f] PCV2-positive, PMWS-negative, 4-week-old, off-site control group; [g] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces; [h] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces; [i] including superficial inguinal lymph node, mandibular lymph node and mesenteric lymph node; [j] PALS – periarteriolar lymphocyte sheath; [k] BALT – bronchus associated lymphoid tissue; '-' indicates there was no lesion found; ne – not examined.

Group 1 (at day 0: PCV2-negative, PMWS-negative, 4-weeks-old, directly exposed to PMWS-affected pigs)

Overall, pigs of Group 1 (n = 5) demonstrated the most severe histological lesions consistent with those seen in cases of PMWS. In lymph nodes, where the follicular oblation was not complete (Figure 4.2), infiltration of histiocytes (mononuclear and multinucleated) was a prominent feature (Figure 4.3).

A



B

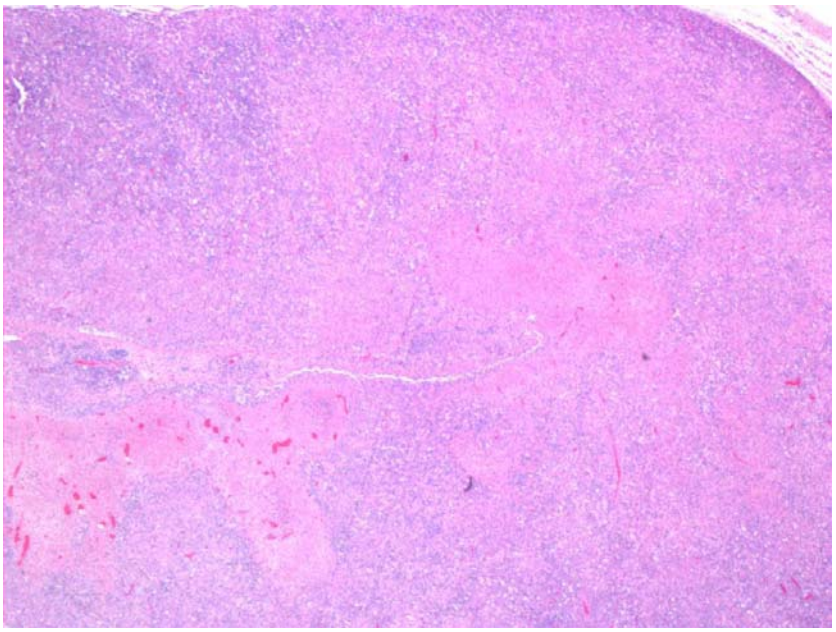


Figure 4.2: Microscopic slides of **s**ections of lymph nodes from pigs of Group 1 with typical clinical signs of PMWS, illustrating the significant between-animal variability of lesions. **A:** Still recognisable follicular architecture and **B:** Complete loss of follicular structure (same magnification). (Haematoxylin and eosin).

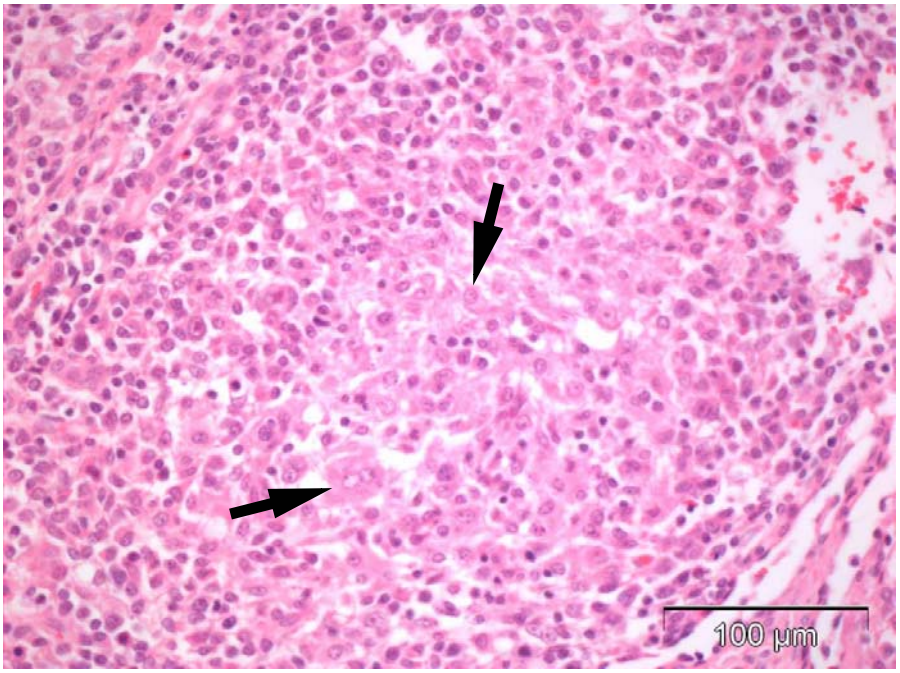


Figure 4.3: Microscopic slide of a follicular centre of a lymph node from a PMWS-affected pig of Group 1 with infiltration of large mononuclear and multinucleated histiocytes (*arrows*). (Haematoxylin and eosin).

Botryoid intracytoplasmic inclusion bodies were seen in large numbers, particularly within histiocytes (Figure 4.4).

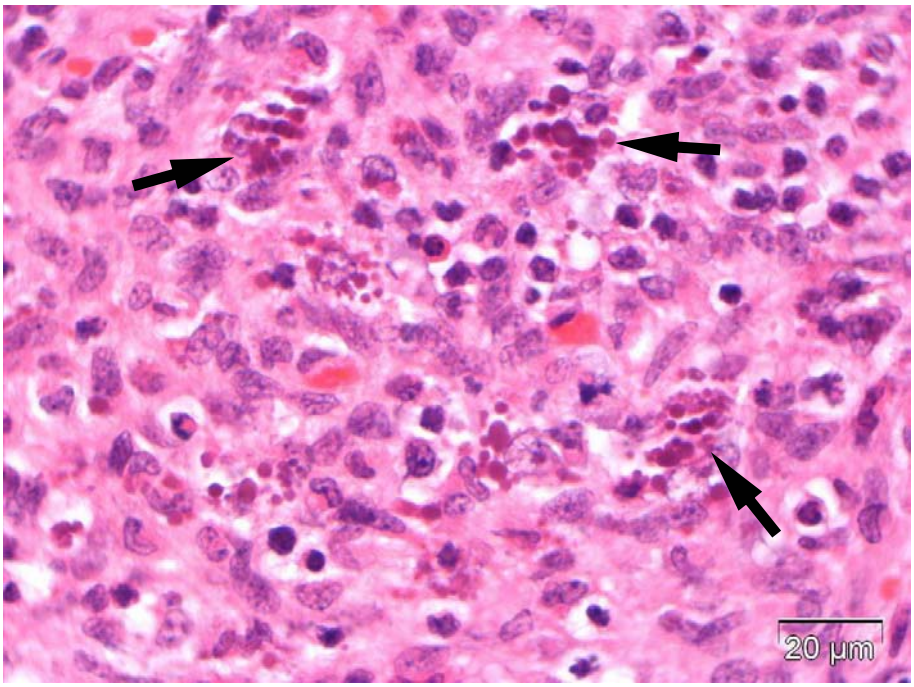


Figure 4.4: Microscopic slide of a lymph node from a PMWS-affected pig in Group 1. Large numbers of botryoid amphophilic intracytoplasmic inclusion bodies (*arrows*) are present within histiocytes in an area of severely disrupted lymph node cortex. (Haematoxylin and eosin).

Lymphoid depletion, loss of follicular definition in the periarteriolar lymphocyte sheaths (PALS), and infiltration of histiocytic cells were evident in the spleen of all Group 1 pigs. Tonsil sections and Peyer's patches showed effacing of lymphocytic aggregates with infiltration of histiocytic cells. The tonsils of 2/5 animals were not examined.

Broncho-interstitial pneumonia was evident in 4/5 pigs. The peribronchial lymphoid tissue was assessed for the evidence of inflammatory reaction as a primary effect of PMWS, which is characterised by the presence of lymphoid and macrophage cells, and degenerative epithelial changes. Marked lymphocyte depletion combined with infiltration of histiocytes was prominent in two cases (Figure 4.5).

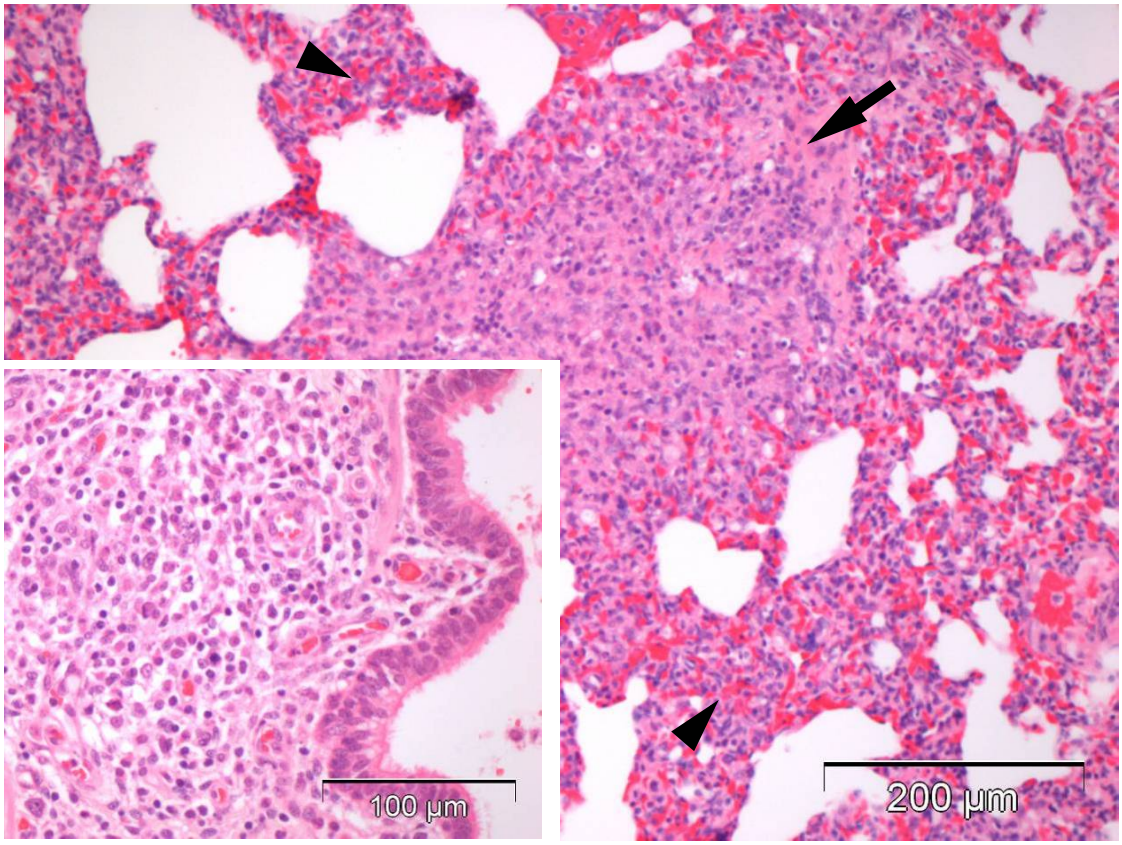


Figure 4.5: Microscopic slides of a lung section from a pig of Group 1, affected by PMWS. Broncho-interstitial pneumonia with thickening of alveolar (*arrow heads*) and interlobular septa (*arrow*). The inset shows histiocyte infiltration in peribronchial lymphoid tissue. (Haematoxylin and eosin).

The other two animals had severe secondary bacterial bronchopneumonia and consequential suppurative and necrotising changes. It was impossible in these cases, therefore, to assess the inflammatory reaction as a primary effect of PMWS.

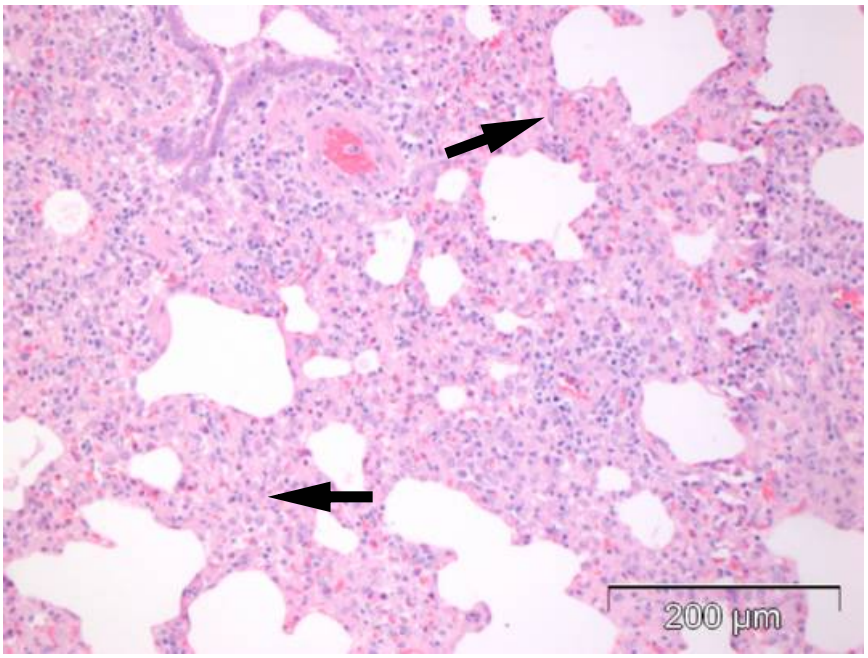
Group 2 (at day 0: PCV2-negative, PMWS-negative, 13-weeks-old, directly exposed to PMWS-affected pigs)

In contrast to Group 1, the pigs from Group 2 (n = 3) showed only minor microscopic lesions. Mild to moderate lymphoid depletion was evident in the lymph nodes, spleen and tonsils of 2/3 pigs (ID 7 and 9), but only in the Peyer's patches of the remaining pig (ID 8). This pig also developed a chronic/subacute suppurative bronchopneumonia with hyperplastic lymphoid aggregates (Figure 4.1, B). A mild subacute to chronic form of bronchopneumonia was also observed in pig ID 9. No viral inclusion bodies were observed in any lymphoid tissue in this experimental group. Other histological changes seen were focal, non-suppurative chronic interstitial nephritis (ID 8 and 9) and hydropic degeneration in the liver (ID 7 and 9).

Group 3 (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old, directly exposed to PMWS-affected pigs)

There was a more varied development of clinical disease in Group 3 (n = 9) compared to Group 1, and the degree and frequency of lesions characteristic of PMWS within this group were consistent with this. In the lymph nodes, spleen, and Peyer's patches, minimal to moderate lymphocyte depletion was evident in almost all animals, whereas minor loss of follicular structure and infiltration of mononuclear and multinucleated histiocytes occurred in only a few cases. There were no botryoid inclusion bodies seen in any lymphoid tissues. A third (3/9) of the pigs in this group (ID 12, 25, and 27) suffered from severe granulomatous interstitial pneumonia (Figure 4.6); with another third developed a mild form of chronic bronchopneumonia (ID 13, 21 and 24).

A



B

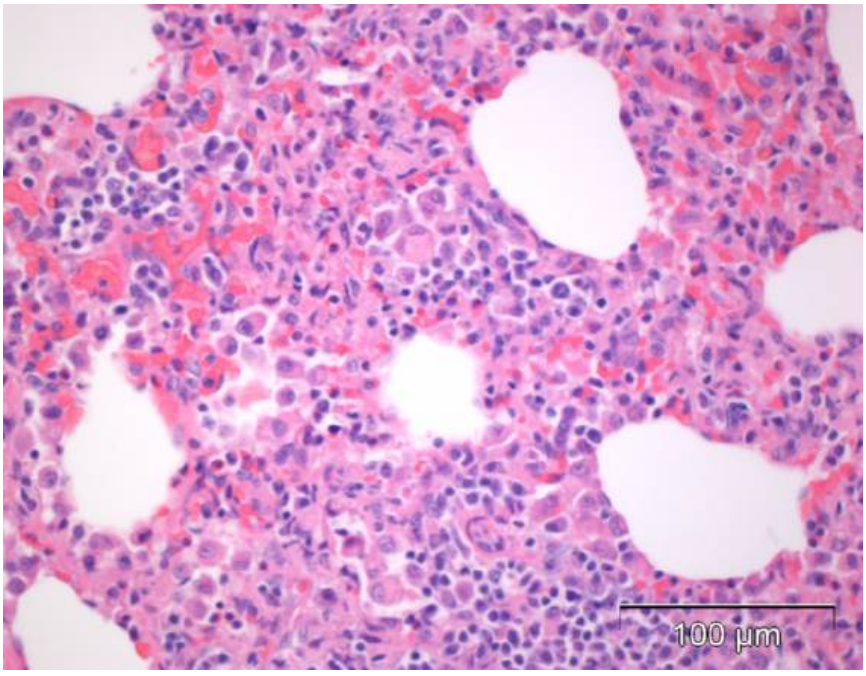


Figure 4.6: Microscopic slides of a section of lung from a pig in Group 3 with severe granulomatous interstitial pneumonia. **A:** Thickening of alveolar septa (*arrows*) and **B:** Tissue infiltration of macrophages and other mononuclear leukocytes. (Haematoxylin and eosin).

There was evidence of inflammatory changes affecting a wider range of organs. Focal non-suppurative chronic interstitial nephritis was evident in 3/9 pigs (ID 13, 24 and 25) and severe multifocal to diffuse granulomatous nephritis was found in 1/9 pig (ID 12). Mild focal non-suppurative hepatitis with moderate to severe diffuse hydropic

degeneration was detected in the livers of 4/9 (ID 12, 13, 24 and 25) and 7/9 (ID 12, 13, 18, 21, 24, 25 and 28) pigs, respectively (Figure 4.7).

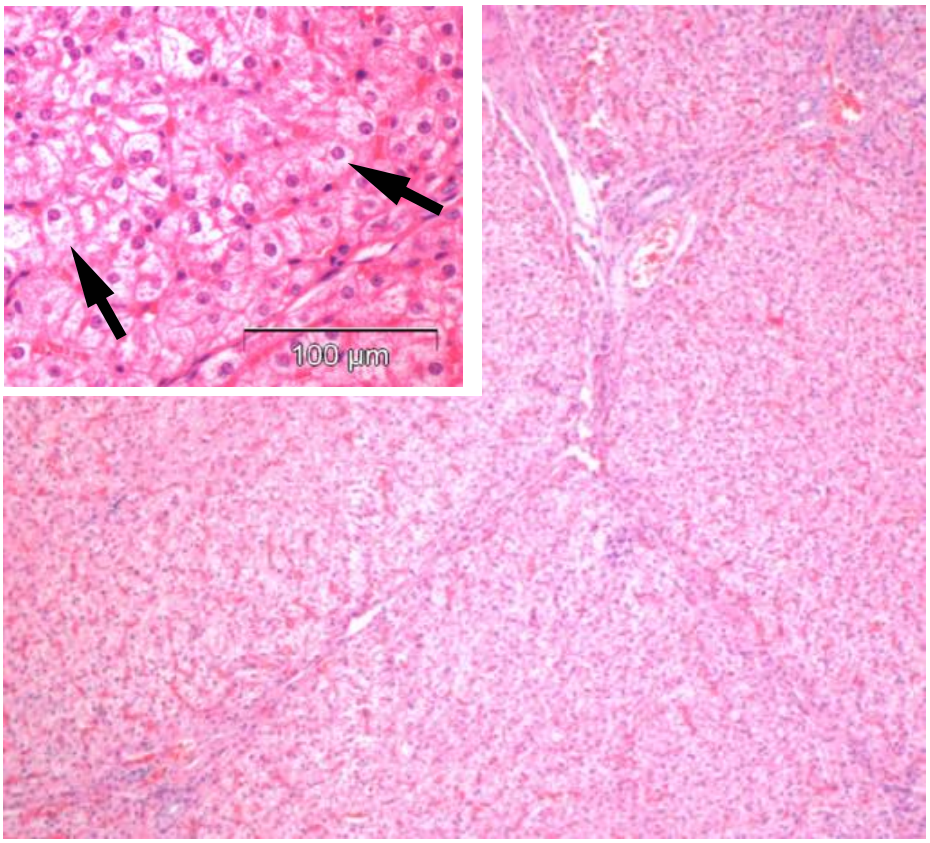


Figure 4.7: Microscopic slides of a section of liver tissue from a pig of Group 3 with severe diffuse hydropic degeneration. The inset shows the vacuolation within the hepatocytes (*arrows*). (Haematoxylin and eosin).

Group 4 (at day 0: PCV2-positive, PMWS-negative, 12-weeks-old, directly exposed to PMWS-affected pigs)

Histological lesions typical of PMWS were present in some tissues despite a lack of clinical evidence suggesting the successful transmission of PMWS to pigs in Group 4 (*n* = 10). Although mild lymphocyte depletion was detected in lymph nodes and Peyer's patches, only minimal to mild loss of follicular structure, and infrequent infiltration of mononuclear and multinucleated histiocytes were observed (ID 35, 39 and 40). Minor lymphocyte depletion was detected in the spleen (ID 31, 35, 36 and 40) and tonsil (ID 31, 34 and 35). Lymphoid lesions within the peribronchial tissue were rare and minimal

with lymphoid depletion detected in 1/10 pigs (ID 34). Severe diffuse suppurative bronchopneumonia with hyperplastic peribronchial lymphoid tissue was evident in 4/10 pigs (ID 36, 37, 38 and 39), while another 4/10 pigs (ID 33, 34, 35 and 40) developed mild broncho-interstitial pneumonia.

Scattered mild non-suppurative nephritis was evident in 1/10 pigs (ID 32) and two other pigs (ID 35 and 40) had mild chronic non-suppurative interstitial nephritis. Focal mild non-suppurative changes were observed in the liver of 2/10 pigs (ID 32 and 38), while diffuse, mild vacuolar degeneration of hepatocytes was present in 5/10 pigs (ID 31, 33, 36, 39 and 40).

Group 5 (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old, on-site control group)

The earliest clinical signs indicative of PMWS (wasting, poor hair coat) in Group 5 (n = 10) appeared by day 35. Observed lesions, at the microscopic level, were considered more uniform across the group in comparison to pigs of other experimental groups. Moderate to severe lymphocyte depletion was observed in lymphoid tissues (lymph nodes, spleen, tonsil and Peyer's patches) of all pigs. Loss of follicular structure and infiltration of mononuclear and multinucleated histiocytes were relatively less severe in relation to other groups, but occurred in all examined lymphoid tissues. Intracytoplasmic inclusion bodies were detected only within the lymph nodes of 1/10 pigs (ID 16).

Interstitial pneumonia was detected in 9/9 pigs and varied in severity from very mild to severe. Mild to marked lymphocyte depletion in the BALT with moderate to severe infiltration of histiocytes was identified. Infiltration of multinucleated histiocytes, however, was minimal and intra-alveolar macrophages were only occasionally detected in 1/10 pigs (ID 17).

The majority of the group showed kidney lesions consisting of diffuse mild interstitial nephritis (1/10 pigs, ID 11) or non-suppurative interstitial nephritis which was either mildly (4/10 pigs, ID 17, 23, 26, 29), or moderately (2/10 pigs, ID 16 and 19), focally extensive (Figure 4.8).

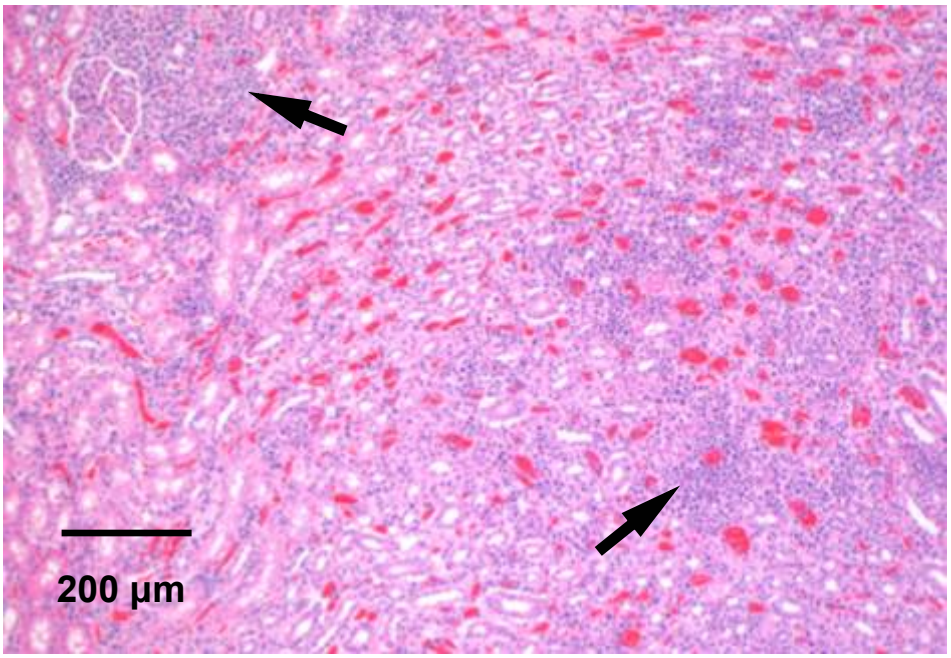


Figure 4.8: Microscopic slide of a section of kidney tissue from a pig of Group 5 with focally extensive non-suppurative interstitial nephritis. Note the lymphocyte infiltration (*arrows*) in the interstitial tissue. (Haematoxylin and eosin).

Changes in the liver of 4/10 pigs (ID 16, 17, 26, and 30) consisted of mild, focal non-suppurative inflammation with mild to severe diffuse hydropic vacuolation in hepatocytes, particularly in periportal zones. Mild, focal non-suppurative meningitis was noted in 5/10 pigs (ID 16, 17, 19, 26, and 29).

Group 6 (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old, off-site control group)

Animals from Group 6 (n = 6) demonstrated no clinical signs suggestive of PMWS and only minimal histological lesions were identified. Minimal lymphocyte depletion in the lymph nodes (Figure 4.9) and spleen developed in 3/6 pigs. An infiltration of histiocytes and a few multinucleated histiocytes in lymph node tissue was shown in 1/6 pigs (Figure 4.9, inset), while identical lesions were also found in its tonsillar tissue. No other obvious abnormalities, on the microscopic level, were identified in the examination of the remaining tissues of Group 6 pigs.

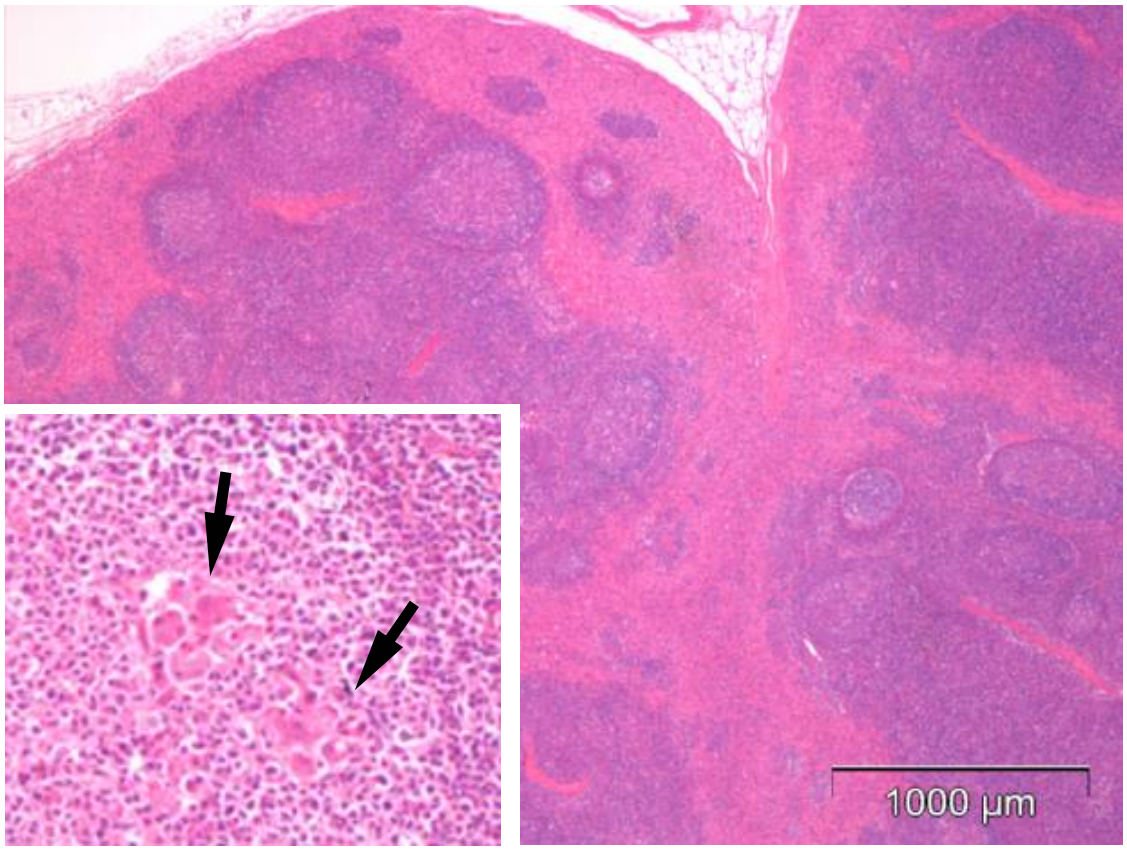


Figure 4.9: Microscopic slides of representative lymph node changes of Group 6 control pigs with minimal lymphocyte depletion. Inset: In one pig, there were folliculocentric aggregations of multinucleated histiocytes (*arrows*). (Haematoxylin and eosin).

Group 7a (at day 0: PCV2-negative, PMWS-negative, 4-weeks-old, directly exposed to PCV2-positive pig and faeces) and Group 7b (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old, directly exposed to PCV2-positive pig and faeces)

No pigs in either Group 7a (n = 6), or of Group 7b (n = 6), demonstrated clinical signs characteristic of PMWS.

Group 7a

Minimal to marked lymphocyte depletion occurred in the lymph nodes of 6/6 pigs from Group 7a, combined with a minor loss of follicular structure (similar to Group 6, Figure 4.9). The infiltration of histiocytes was moderate and the amount of multinucleated histiocytes present varied from minimal to numerous. In the spleen, minimal

lymphocyte depletion was detected with minimal to moderate invasion of histiocytic cells in 3/6 pigs (ID 41, 42, and 46), with the infiltration of multinucleated histiocytes considered minimal in 2/6 of these pigs (ID 41 and 42). Minor lymphocyte depletion with minimal infiltration of histiocytes was identified in the tonsillar tissue of 3/5 pigs (ID 42, 43, and 46), although histiocytic infiltration was abundant in 1/5 pigs (ID 42). Lesions in Peyer's patches, identified in 2/6 pigs (ID 42 and 46), consisted of mild to moderate lymphocyte depletion, with mild loss of follicular structure. These lesions also had minimal to marked histiocyte infiltration and multinucleated histiocytes. No intracytoplasmic inclusion bodies were observed in any examined lymphoid tissue.

Histological changes within the peribronchial lymphoid tissue of the lung were minimal and detected in 2/5 pigs (ID 43 and 44). Both animals had mild lymphocyte depletion with minor loss of follicular structure and infiltration of a few histiocytes. The majority (4/5) of the group, however, developed very mild diffuse interstitial pneumonia including thickening of the alveolar septa.

Minor focal lesions in kidney tissue consistent with non-suppurative interstitial nephritis were found in 5/6 pigs. Scattered focal to multifocal, mild non-suppurative hepatitis was also observed in every pig in the group and diffuse moderate hydropic degeneration of hepatic cells was very prominent.

Group 7b

In contrast to animals of Group 7a, Group 7b developed only infrequent and mild lesions similar to those seen in Group 6. Minimal to mild lymphocyte depletion was the main lesion affecting lymphoid tissues of pigs in this group. Minor infiltration of histiocytes occurred in the lymph nodes, spleen (ID 95), and tonsil (ID 90) of two pigs only, whereas minimal numbers of multinucleated histiocytes were also detected in Peyers' patches (ID 95) and tonsil (ID 90). Intracytoplasmic inclusion bodies were seen very rarely and in low numbers (ID 90 and 91).

Lymphocyte depletion, loss of follicular structure, infiltration of mononuclear and multinucleated histiocytes, and mild incursion of inclusion bodies were identified in the peribronchial lymphoid tissue of 1/6 pigs (ID 90). A relatively small number of inclusion bodies were also detected. Mild diffuse lesions indicating non-suppurative

interstitial pneumonia occurred in 4/6 pigs (ID 90, 91, 92, and 93). One pig (ID 94) also showed signs of a mild, resolving bronchopneumonia.

Minimal focal, interstitial, non-suppurative inflammation was detected in the kidneys of 1/6 pigs (ID 93). Although mild to moderate diffuse hydropic degeneration of hepatocytes was noted in every pig in Group 7b, mild focal non-suppurative hepatitis was seen in 1/6 pigs (ID 93).

Evaluation of histological lesions in lymphoid tissues and body condition score

Associations between body condition score (0/1) and the presence of lesions in various lymphoid tissues characteristic of PMWS are summarised in Table 4.7.

Table 4.7: Associations between body condition score categories 0 and 1 and the presence of microscopic lesions in various lymphoid tissues of pigs involved in a natural transmission study of PMWS.

			Body condition score category					
			0		1			
Lesions	Tissue	Presence	n	%	n	%	Total	P[1]
Lymphocyte depletion	Superficial inguinal lymph node	no	0	0.0%	4	100.0%	4	0.040
		yes	19	59.4%	13	40.6%	32	
	Mandibular lymph node	no	0	0.0%	6	100.0%	6	0.006
		yes	19	63.3%	11	36.7%	30	
	Mesenteric lymph node[2]	no	1	14.3%	6	85.7%	7	0.041
		yes	17	60.7%	11	39.3%	28	
	Spleen[2]	no	4	40.0%	6	60.0%	10	0.454
		yes	15	60.0%	10	40.0%	25	
	Tonsil[3]	no	3	25.0%	9	75.0%	12	0.071
		yes	14	63.6%	8	36.4%	22	
	Peyer's patches	no	1	12.5%	7	87.5%	8	0.016
		yes	18	64.3%	10	35.7%	28	
	Peribronchial lymphoid tissue[4]	no	4	22.2%	14	77.8%	18	0.001
		yes	12	80.0%	3	20.0%	15	
Loss of follicular definition	Superficial inguinal lymph node	no	7	35.0%	13	65.0%	20	0.023
		yes	12	75.0%	4	25.0%	16	
	Mandibular lymph node	no	7	31.8%	15	68.2%	22	0.002
		yes	12	85.7%	2	14.3%	14	
	Mesenteric lymph node	no	8	34.8%	15	65.2%	23	0.006
		yes	11	84.6%	2	15.4%	13	
	Spleen[2]	no	9	37.5%	15	62.5%	24	0.004
		yes	10	90.9%	1	9.1%	11	
	Tonsil[3]	no	8	34.8%	15	65.2%	23	0.026
		yes	9	81.8%	2	18.2%	11	
	Peyer's patches	no	12	42.9%	16	57.1%	28	0.044
		yes	7	87.5%	1	12.5%	8	
	Peribronchial lymphoid tissue[5]	no	12	41.4%	17	58.6%	29	0.092
		yes	3	100.0%	0	0.0%	3	
Histiocyte infiltration	Superficial inguinal lymph node	no	5	29.4%	12	70.6%	17	0.018
		yes	14	73.7%	5	26.3%	19	
	Mandibular lymph node	no	2	14.3%	12	85.7%	14	< 0.001
		yes	17	77.3%	5	22.7%	22	
	Mesenteric lymph node	no	5	29.4%	12	70.6%	17	0.018
		14	4	44.4%	5	55.6%	9	
	Spleen[2]	no	7	33.3%	14	66.7%	21	0.005
		yes	12	85.7%	2	14.3%	14	

Table 4.7. continued

			Body condition score category					
			0		1			
Lesion	Tissue	Presence	n	%	n	%	Total	P[1]
Histiocyte infiltration	Tonsil[3]	no	5	27.8%	13	72.2%	18	0.015
		yes	12	75.0%	4	25.0%	16	
	Peyer's patches	no	6	35.3%	11	64.7%	17	0.093
		yes	13	68.4%	6	31.6%	19	
	Peribronchial[4] lymphoid tissue	no	4	21.1%	15	78.9%	19	< 0.001
		yes	12	85.7%	2	14.3%	14	
Multinucleated histiocytes	Superficial inguinal lymph node	no	8	40.0%	12	60.0%	20	0.107
		yes	11	68.8%	5	31.3%	16	
	Mandibular lymph node	no	6	33.3%	12	66.7%	18	0.044
		yes	13	72.2%	5	27.8%	18	
	Mesenteric lymph node	no	9	42.9%	12	57.1%	21	0.192
		yes	10	66.7%	5	33.3%	15	
	Spleen[2]	no	10	41.7%	14	58.3%	24	0.035
		yes	9	81.8%	2	18.2%	11	
	Tonsil[3]	no	7	36.8%	12	63.2%	19	0.166
		yes	10	66.7%	5	33.3%	15	
	Peyer's patches	no	9	42.9%	12	57.1%	21	0.192
		yes	10	66.7%	5	33.3%	15	
	Peribronchial[4] lymphoid tissue	no	8	34.8%	15	65.2%	23	0.026
		yes	8	80.0%	2	20.0%	10	
Circovirus inclusions	Superficial inguinal lymph node	no	12	41.4%	17	58.6%	29	0.008
		yes	7	100.0%	0	0.0%	7	
	Mandibular lymph node	no	13	43.3%	17	56.7%	30	0.020
		yes	6	100.0%	0	0.0%	6	
	Mesenteric lymph node	no	13	43.3%	17	56.7%	30	0.020
		yes	6	100.0%	0	0.0%	6	
	Spleen[2]	no	16	50.0%	16	50.0%	32	0.234
		yes	3	100.0%	0	0.0%	3	
	Tonsil[3]	no	15	46.9%	17	53.1%	32	0.485
		yes	2	100.0%	0	0.0%	2	
	Peyer's patches	no	15	46.9%	17	53.1%	32	0.106
		yes	4	100.0%	0	0.0%	4	
	Peribronchial[4] lymphoid tissue	no	15	46.9%	17	53.1%	32	0.485
		yes	1	100.0%	0	0.0%	1	

[1] Fisher's exact test; [2] one missing value; [3] two missing values; [4] three missing values; [5] four missing values.

Lymphocyte depletion was significantly ($p < 0.05$) associated with a body condition score of <3 in all organs examined, except the spleen and tonsil. Loss of follicular structure, except for peribronchial tissue, and infiltration of histiocytes, excluding Peyer's patches, were significantly related ($p < 0.05$) to loss of body condition in all tissues, whereas infiltration of multinucleated histiocytes was considered significant in the mandibular lymph nodes, spleen, and peribronchial lymphoid tissues only. Significant associations between circovirus inclusion bodies and a body condition score <3, however, were limited to three lymph node tissues.

4.3.3 Detection of PCV2 by the *in situ* hybridisation method

The *in situ* hybridisation (ISH) method was applied to detect PCV2 nucleic acid in selected sections of lymph nodes, Peyer's patches, kidney, liver, lung and spleen from each study animal. PCV2 presence within tissue lesions was indicated by a dark purple intracellular staining as illustrated in Figure 4.10.

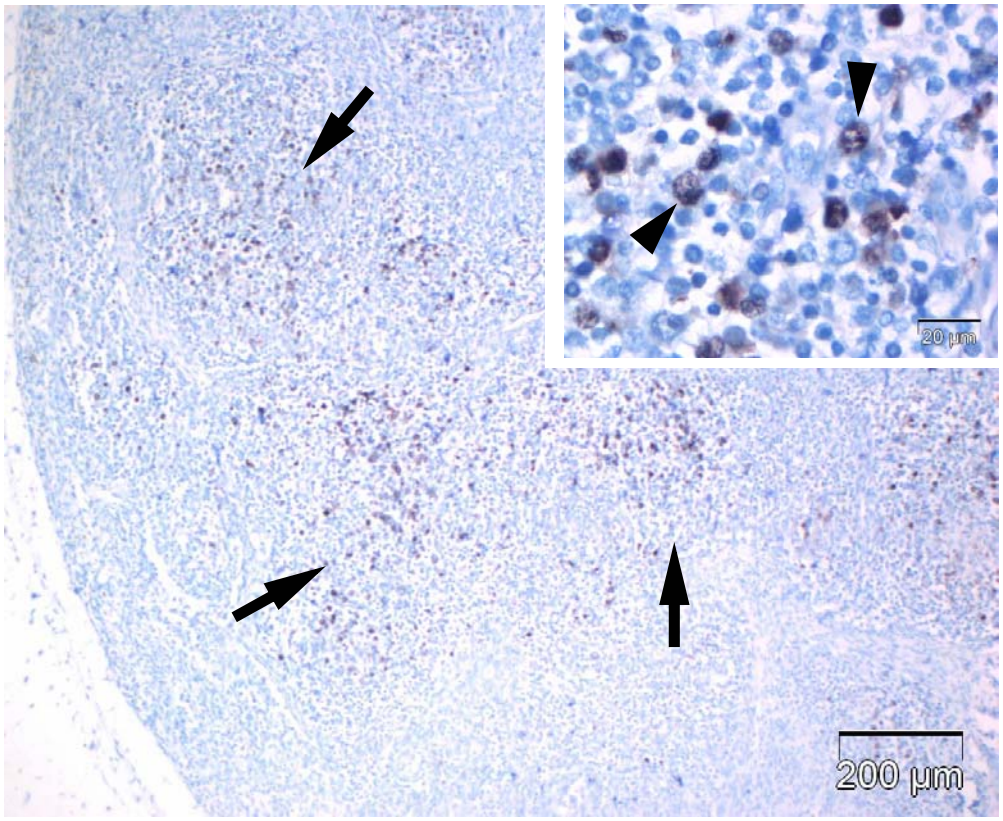


Figure 4.10: Microscopic slide of a lymph node section of a Group 1 animal clinically affected by PMWS with detection of PCV2 nucleic acid (low amount) by the *in situ* hybridisation technique. Note the clustered dark purple granular staining (*arrows*) within areas of poorly defined follicular structure. The inset depicts the intracytoplasmic staining of PCV2 at a higher magnification (*arrow heads*).

The numbers of animals clinically affected with PMWS and/or positive to the ISH method, stratified by experimental group, are shown in Table 4.8.

Table 4.8: Frequency of study animals affected with clinical signs typical of PMWS and/or positive to *in situ* hybridisation (ISH) technique (detecting PCV2 DNA), stratified by experimental group.

Experimental group		Clinically affected	ISH positive	Clinically affected **and** ISH positive
Group 1[a]	(n = 5)	5	4	4
Group 2[b]	(n = 3)	0	0	0
Group 3[c]	(n = 9)	6	1	0
Group 4[d]	(n = 10)	0	0	0
Group 5[e]	(n = 10)	7	4	2
Group 6[f]	(n = 6)	0	4	0
Group 7a[g]	(n = 6)	0	6	0
Group 7b[h]	(n = 6)	0	1	0

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-positive, PMWS-negative, 4-week-old, off-site control group.
[g] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[h] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Positive tests for ISH were recorded in 4/5 and 2/7 clinically affected pigs of Groups 1 and 5, respectively, while other clinical cases of Groups 1, 3, and 5 were negative. Control animals of Group 6, 7a and 7b did not demonstrate any clinical signs consistent with PMWS, however, some animals were tested ISH positive.

Table 4.9 summarises the presence of PCV2 nucleic acid, as identified via the ISH technique, in selected tissues from study animals. Among all groups, PCV2 was most common in sections of lymph nodes, Peyer's patches, and splenic tissue ($p < 0.001$). All lung tissue tested was negative on ISH. PCV2 DNA was detected in 4/55 pigs (ID 5, 30, 11, one pig of control Group 6). There was no difference observed in the amount of PCV2 between positively stained tissues of the same animal. Detailed ISH results of each experimental group, stratified by the amount of PCV2 nucleic acid detected within lesions of selected tissues, are given in Appendix IV.

Table 4.9: Results of *in situ* hybridisation, testing for the presence of PCV2, stratified by tissue type and experimental group of pigs in a transmission study of PMWS.

			PCV2 present		
Tissue	Group	Total number	Number	Percent (%)	P[a]
Lymph node	1[b]	5	4	80	< 0.001
	2[c]	3	0	0	
	3[d]	9	1	11	
	4[e]	10	0	0	
	5[f]	10	3	30	
	6[g]	6	4	67	
	7a[h]	6	4	67	
	7b[i]	6	0	0	
Peyer's patches	1	5	2	40	< 0.001
	2	3	0	0	
	3	9	0	0	
	4	10	0	0	
	5	10	1	10	
	6	6	1	17	
	7a	6	3	50	
	7b	6	1	17	
Spleen	1	5	1	20	< 0.001
	2	3	0	0	
	3	9	0	0	
	4	10	0	0	
	5	10	0	0	
	6	6	2	33	
	7a	6	2	33	
	7b	6	0	0	
Liver	1	5	1	20	0.091
	2	3	0	0	
	3	9	0	0	
	4	10	0	0	
	5	10	0	0	
	6	6	0	0	
	7a	6	0	0	
	7b	6	0	0	
Kidney	1	5	0	0	0.164
	2	3	0	0	
	3	9	1	11	
	4	10	0	0	
	5	10	0	0	
	6	6	0	0	
	7a	6	0	0	
	7b	6	0	0	

[a] Fisher's exact test; [b] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs; [c] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs; [d] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs; [e] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs; [f] PCV2-positive, PMWS-negative, 4-week-old, on-site control group; [g] PCV2-positive, PMWS-negative, 4-week-old, off-site control group; [h] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces; [i] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

4.3.4 Bacteriology

Cultures completed on swabs collected from lung tissue of study animals with gross lesions suggestive of microbial infection were negative under aerobic conditions (ID 8, 19, 35, and 37). Two pigs (ID 8 and 27) were positive for *Staphylococcus aureus*, however, after enrichment culture. Bacteria morphologically consistent with *Mycobacterium spp*. and spirochaetes were not detected on histological examination of sections after staining with Ziehl-Neelsen and Warthin-Starry silver stains, respectively.

4.3.5 Haematology

A clear relationship between factors measured in the complete blood counts and apparent clinical signs and moderate to severe histopathological lesions, characteristic of PMWS, was not observed during the first 29 days post-exposure. Details of complete blood counts are given in Appendices V to VIII. Variations in the percentages and absolute values of differential leukocytes between the groups were observed during the 29 days post-exposure, predominantly affecting neutrophils and lymphocytes. Histograms illustrating the percentage and absolute values of differential leukocytes among groups are shown in Appendices IX to XIII and Appendices XIV to XVIII, respectively. Detailed data on absolute values and percentages of differential leukocytes of the experimental groups are also provided in Appendices XIX to XXVI.

Group 1 (at day 0: PCV2-negative, PMWS-negative, 4-weeks-old, directly exposed to PMWS-affected pigs, *n* = 5)

As all pigs from Group 1 died before the fourth blood sampling day (day 29), haematological changes were followed over the first 11 days post-exposure only in this group.

Clear signs of lymphocyte depletion were visible via histopathology and leukocyte differentiation of peripheral blood cells also revealed a relatively low count of lymphocytes to day 11 post-exposure. The percentage of neutrophils, however, was consistently higher than the percentage of lymphocytes and showed a statistically significant inverse neutrophil to lymphocyte ratio on bleeding days 0 and 8 (Figure 4.11, A and B). The relative number of monocytes almost doubled within 11 days.

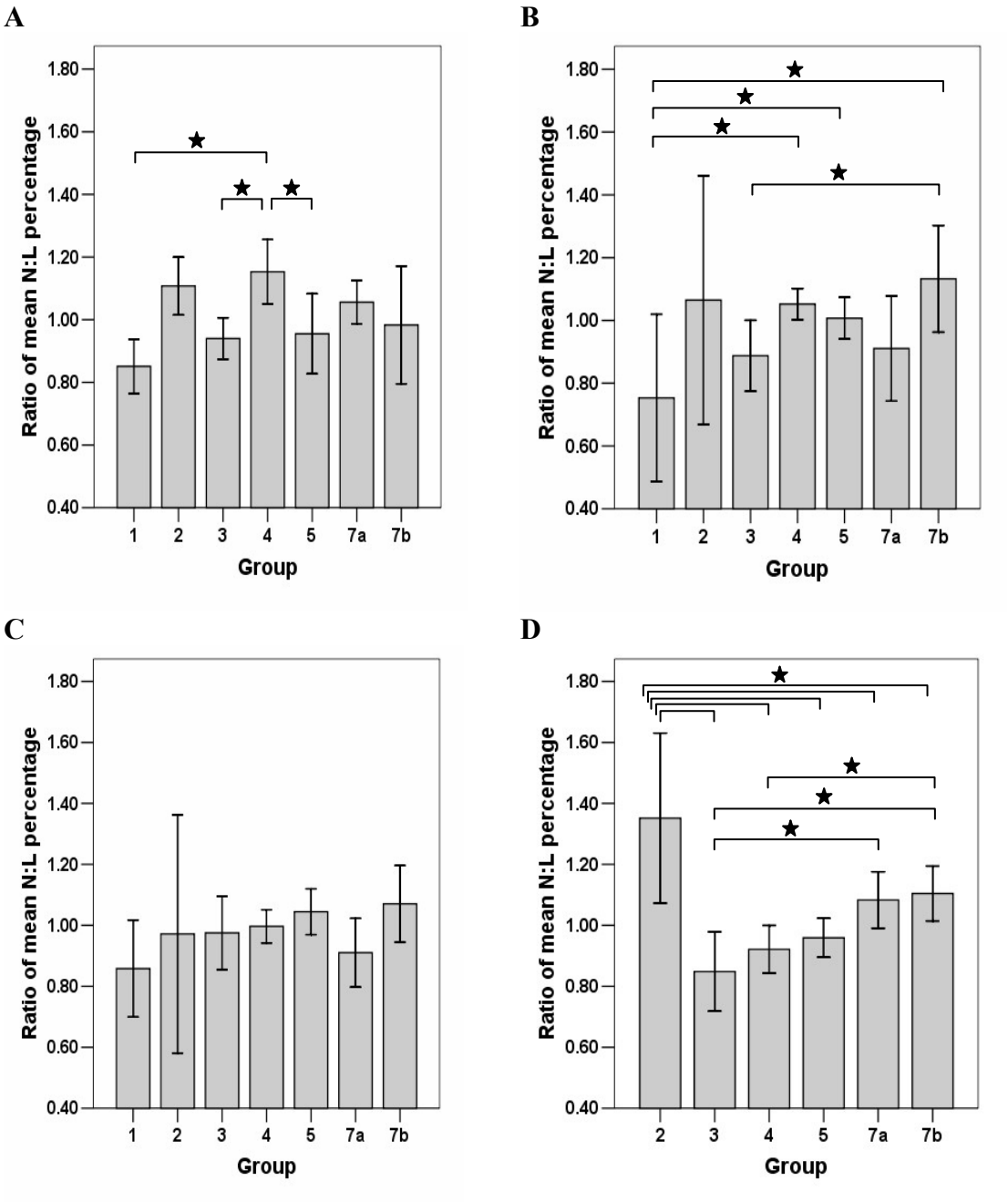


Figure 4.11: Ratios of mean neutrophils to lymphocytes (N:L) percentage in blood samples of experimental groups of pigs in a natural transmission study on PMWS, stratified by sampling day (**A**: 0, **B**: 8, **C**: 11 and **D**: 29 days post exposure). At day 0: Group 1 (PCV2-negative, PMWS-negative, 4-week-old), Group 2 (PCV2-negative, PMWS-negative, 13-week-old), Group 3 (PCV2-positive, PMWS-negative, 4-week-old), Group 4 (PCV2-positive, PMWS-negative, 12-week-old), Group 5 (PCV2-positive, PMWS-negative, 4-week-old), Group 7a (PCV2-negative, PMWS-negative, 4-week-old), Group 7b (PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. ★ indicates significant difference between groups ($p < 0.05$). Error bars represent 95% confidence interval.

Figure 4.11 shows the mean neutrophil to lymphocyte ratios by days post-exposure, stratified by experimental group. There were significant differences in mean neutrophil to lymphocyte ratios between the experimental groups on day 0 ($p = 0.002$), day 8 ($p < 0.001$) and day 29 ($p < 0.001$). The difference between groups on day 11 was marginally significant ($p = 0.051$).

Group 2 (at day 0: PCV2-negative, PMWS-negative, 13-weeks-old, directly exposed to PMWS-affected pigs, *n* = 3)

Neutrophil, lymphocyte and eosinophil counts dropped considerably from 0 to 8 days post-exposure, but recovered to normal values by day 29. This group displayed a normal neutrophil to lymphocyte ratio, except on day 11 where the percentage value of lymphocytes was lower than that of neutrophils (Figure 4.11).

Group 3 (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old, directly exposed to PMWS-affected pigs, *n* = 9)

Differentiation of leukocytes showed a slightly different trend from that seen in Groups 1 and 2. Despite fluctuations in the percentage of the main leukocytes, the percentage of neutrophils remained higher than lymphocytes and resulted in an inverse neutrophil to lymphocyte ratio on all four blood sampling days, similar to Group 1 (Figure 4.11).

Group 4 (at day 0: PCV2-positive, PMWS-negative, 12-weeks-old, directly exposed to PMWS-affected pigs, *n* = 10)

The neutrophil percentages of Group 4 doubled over time, while lymphocyte counts remained relatively steady, causing inverse neutrophil to lymphocyte ratios from day 11 onwards (Figure 4.11).

Group 5 (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old, on-site control group, *n* = 10)

No clinical signs considered typical of disease transmission were obvious from the haemogram in Group 5 animals to day 29 post-exposure. White blood cell counts increased slowly from day 0 to 29 due to the continuous rise of neutrophils, while the

lymphocyte count remained steady after doubling within the first 8 days. Inverse neutrophil to lymphocyte ratios, however, were observed on days 0 and 29 (Figure 4.11).

Group 7a (at day 0: PCV2-negative, PMWS-negative, 4-weeks-old, directly exposed to PCV2-positive pig and faeces, *n* = 6) and Group 7b (at day 0: PCV2-positive, PMWS-negative, 4-weeks-old, directly exposed to PCV2-positive pig and faeces, *n* = 6)

Leukocyte differentiation revealed a peak of neutrophil counts in Group 7a on day 8, while steady numbers of neutrophils were observed in Group 7b. Lymphocyte counts of Group 7a increased constantly to a peak on day 29, whereas Group 7b had a constantly high lymphocyte count after day 0. These findings were reflected in inverse neutrophil to lymphocyte ratios on days 8 and 11 for Group 7a, and on day 0 for Group 7b (Figure 4.11).

No significant changes were observed in the complete blood counts of blood samples collected in Groups 7a and 7b weekly until day 81. Details are provided in Appendices XXVII to XXIX. A histogram illustrating the haematological parameters of Group 7a and 7b by sampling day is given in Appendix XXX.

Leukocyte differentiations did not show significant changes in either group, with the exception of day 81, where both groups experienced a large decrease in lymphocytes, monocytes, eosinophils (Appendix XXXI), and basophils (Appendix XXXII). Group 7b also demonstrated a significant decrease in neutrophils on the day of termination. In both groups, the ratio of neutrophils to lymphocytes was normal after day 29 until the end of study (Figure 4.12). Group 7a demonstrated an inverse ratio on day 81 due to a significant increase in percentage of neutrophils.

Figure 4.12 depicts the mean neutrophil to lymphocyte ratios for experimental Groups 7a and 7b on weekly blood sampling days until day 81. The differences in mean neutrophil to lymphocyte ratios between both groups were statistically significant ($p < 0.05$) on days 8, 11, 15, 57, and 81 but not significantly different on the remaining sampling days.

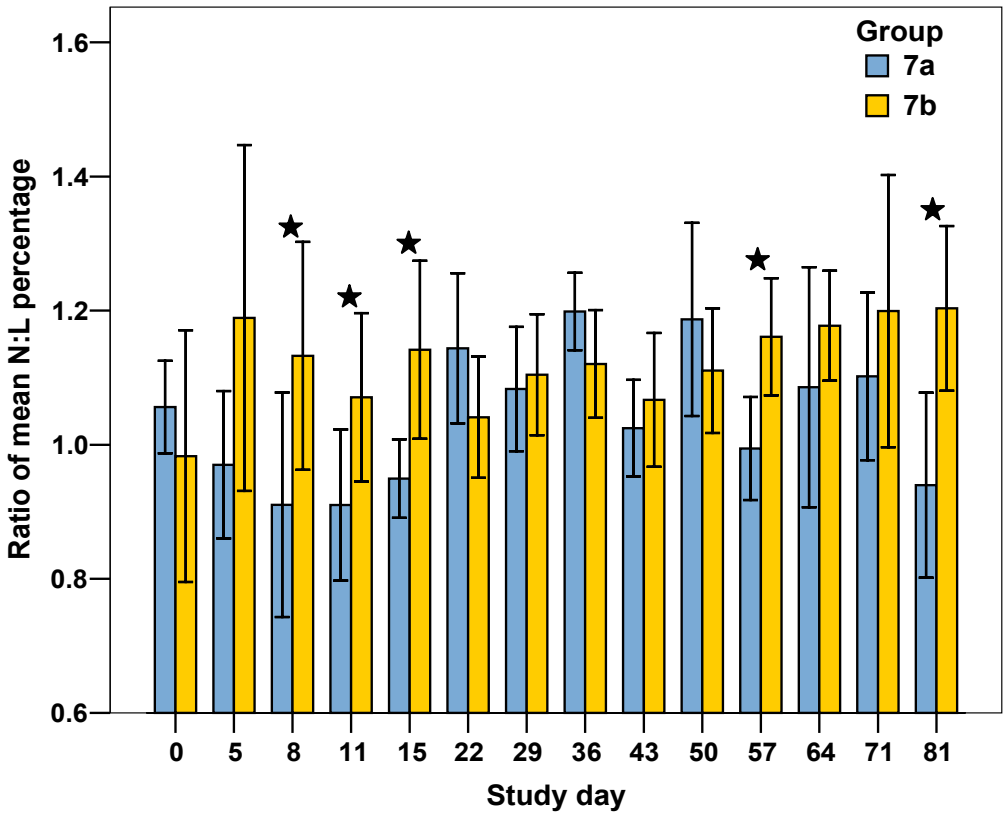


Figure 4.12: Ratios of mean neutrophils to lymphocytes (N:L) percentage in blood samples during a natural transmission study on PMWS. At day 0: Group 7a (PCV2-negative, PMWS-negative, 4-week-old), Group 7b (PCV2-positive, PMWS-negative, 4-week-old). Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. ★ indicates significant difference between groups ($p < 0.05$). Error bars represent 95% confidence interval.

4.3.6 Serology

Serology test results for detection of antibodies against PCV2, PPV, AujD, PRRSV, and BVD are shown in Figure 4.13 and Table 4.10.

Antibodies against PCV2

Increased levels of serum antibodies against PCV2 were measured in Groups 1, 4 and 7a, by a commercial PCV2 ELISA test, after exposure to PMWS-affected pigs and PCV2-contaminated faeces and a PCV2-positive 12-week-old pig from Farm B (Figure 4.13).

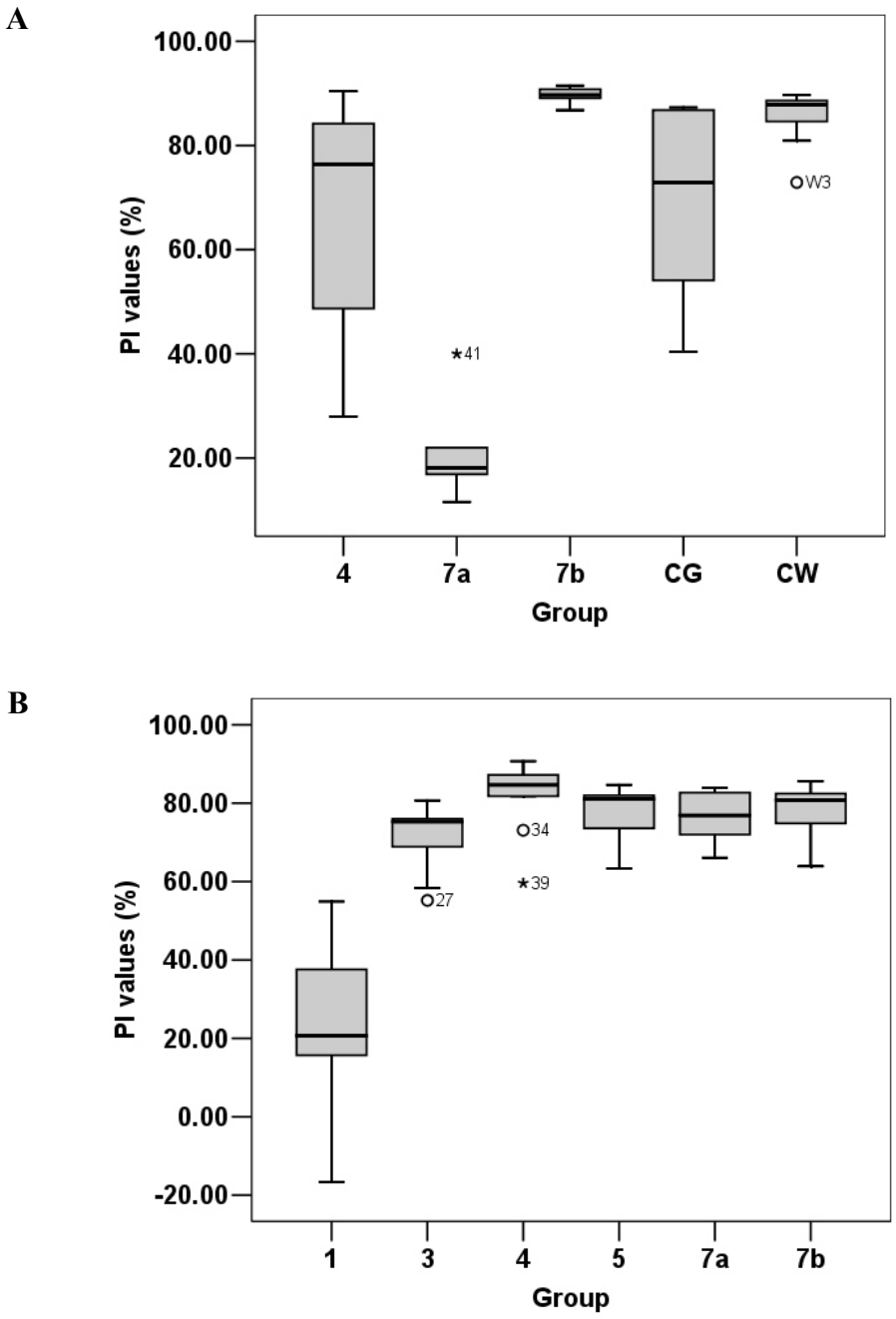


Figure 4.13: Boxplots of percentage inhibition (PI) results from a commercial PCV2 ELISA test assessing PCV2 antibodies in pig sera from a natural transmission study on PMWS. **A**: PI values on day 0 of the study. On day 0: Group 1 (PCV2-negative, PMWS-positive, 4-week-old), Group 3 (PCV2-positive, PMWS-negative, 4-week-old), Group 4 (PCV2-positive, PMWS-negative, 12-week-old), Group 5 (PCV2-positive, PMWS-negative, 4-week-old), Group 7a (PCV2-negative, PMWS-negative, 4-week-old), Group 7b (PCV2-positive, PMWS-negative, 4-week-old), CG (PCV2-positive, PMWS-negative, 12-week-old), CW (PCV2-positive, PMWS-negative, 4-week-old). Groups 1, 3, and 4 were directly exposed to PMWS-affected pigs for 56 days. Group 5 was not directly exposed and served as on-site control. Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. CG and CW acted as off-site controls, without exposure to PMWS. **B**: PI values at post mortem. Horizontal bars represent the median value and box length is the interquartile range (IQR); outliers (○) are values between 1.5 to 3x IQR and extreme values (⋆) lie beyond 3x IQR. Numbers represent pig identification numbers.

Pigs of Group 1 and 7a originating from a PCV2-negative farm (Farm A), seroconverted after exposure to PMWS-affected pigs or PCV2-contaminated faeces and a PCV2-positive pig. Animals of Group 1 did not reach the test-positive PI values (80%) at post mortem, but a slight antibody increase was evident. Group 7a was PCV2-negative at the beginning of the study but seroconverted to PCV2 after 81 days of exposure to the virus.

Pigs from Group 3, 4, 5 and 7b originated from Farm B (PCV2-positive), but PCV2 antibody levels of Group 4 and 7b differed at day 0. After exposure to PCV2 and PMWS-affected pigs PI values of both groups were positive. Test results of Group 3 were mainly inconclusive at post mortem after exposure to PMWS, whereas Group 5, the on-site control group, showed a greater proportion of test-positive values.

Antibodies against PPV, AujD, PRRSV, BVD, and HEV

Based on serology test results, there was no indication of infection with PPV, AujD, PRRSV or BVD in Group 1 and 3 (clinically diseased pigs) (Table 4.10). Several pigs from each of the seven groups, however, had antibodies against HEV at post mortem.

Table 4.10: Frequency (number of test positive/number of tested animals per group) of viral antibodies detected in porcine sera in a natural transmission study of PMWS at day 0 and post mortem (PM). Numbers in brackets indicate inconclusive test results.

Experimental group	N	PCV2 Day 0	PCV2 PM	PPV PM	AujD PM	PRRSV PM	HEV PM	BVD PM
1[a]	5	ne	0/5	0/5	0/5	0/5	1/5	0/5
2[b]	3	ne	ne	ne	ne	ne	3/3	ne
3[c]	9	ne	1/9 (5)	0/9	0/9	0/9	8/9 (1)	0/9
4[d]	10	3/10 (4)	8/10 (1)	ne	ne	ne	10/10	ne
5[e]	10	ne	6/10 (4)	ne	ne	ne	7/10	ne
7a[f]	6	0/6	2/6 (4)	ne	ne	ne	2/6 (1)	ne
7b[g]	6	6/6	5/6 (1)	ne	ne	ne	2/6	ne

ne – not examined
PCV2 – Porcine circovirus type 2
PPV – Porcine parvovirus
AujD – Aujeszky's disease
PRRSV – Porcine reproductive and respiratory syndrome virus
HEV – Porcine hepatitis E virus
BVD – Bovine viral diarrhoea
[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

4.3.7 PCR analysis for viral pathogens

The results of PCR testing for the presence of porcine viral pathogens in experimental groups are summarised for DNA viruses in Table 4.11 and for RNA viruses in Table 4.12.

Table 4.11: Frequency (number of detected/number of tested animals per experimental group) of DNA viruses detected in faeces, tonsil tissue, and peripheral blood mononuclear cells (PBMC) of pigs in a natural transmission study on PMWS on day 0 and at post mortem (PM) using polymerase chain reaction (PCR) method.

		PCV2 Faeces		PCV2 tonsil issue	PCV1 PBMC		PPV PBMC		PLHV PBMC		PCMV PBMC	
Experimental group	N	Day 0	PM	PM	Day 0	PM	Day 0	PM	Day 0	PM	Day 0	PM
1[a]	5	0/5	4/5	ne	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
2[b]	3	0/3	1/3	ne	ne	ne	ne	ne	ne	ne	ne	ne
3[c]	9	0/9	2/9	9/9	0/9	0/9	0/9	0/9	1/9	1/9	4/9	4/9
4[d]	10	ne	0/10	ne	ne	ne	ne	ne	ne	ne	ne	ne
5[e]	10	ne	0/10	10/10	ne	ne	ne	ne	ne	ne	ne	ne
7a[f]	6	ne	0/6	6/6	ne	ne	ne	ne	ne	ne	ne	ne
7b[g]	6	ne	0/6	4/6	ne	ne	ne	ne	ne	ne	ne	ne

ne – not examined
PCV2 – Porcine circovirus type 2
PCV1 – Porcine circovirus type 1
PPV – Porcine parvovirus
PLHV – Porcine lymphotropic herpesvirus
PCMV – Porcine cytomegalovirus
[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Table 4.12: Frequency (number of detected/number of tested animals per experimental group) of RNA viruses detected in faeces of pigs in a natural transmission study on PMWS on day 0 and at post mortem (PM) using reverse transcription-polymerase chain reaction (RT-PCR) method.

	PRRSV		HEV		PEV CPE I		PEV CPE II		PEV CPE III		PEC		Rotavirus		Norwalk-like calicivirus	
Group	Day 0	PM	Day 0	PM	Day 0	PM	Day 0	PM	Day 0	PM	Day 0	PM	Day 0	PM	Day 0	PM
1[a] ($n = 5$)	0/5	0/5	0/5	5/5	0/5	5/5	0/5	1/5	0/5	5/5	0/5	0/5	0/5	2/5	0/5	4/5
2[b] ($n = 3$)	ne	ne	0/3	0/3	0/3	3/3	ne	ne	0/3	1/3	ne	ne	ne	ne	ne	ne
3[c] ($n = 9$)	ne	0/9	0/10	3/9	3/4	2/9	0/1	0/9	3/6	8/9	1/9	1/9	0/1	0/9	0/5	3/9
4[d] ($n = 10$)	ne	ne	ne	1/10	ne	0/10	ne	ne	ne	2/10	ne	ne	ne	ne	ne	ne
5[e] ($n = 10$)	ne	ne	ne	10/10	ne	10/10	ne	ne	ne	10/10	ne	ne	ne	ne	ne	ne
7a[f] ($n = 6$)	ne	ne	ne	0/6	ne	0/6	ne	0/6	ne	0/6	ne	ne	ne	ne	ne	ne
7b[g] ($n = 6$)	ne	ne	ne	0/6	ne	0/6	ne	0/6	ne	0/6	ne	ne	ne	ne	ne	ne

ne – not examined
PRRSV – Porcine reproductive and respiratory syndrome virus
HEV – Porcine hepatitis E virus
PEV CPE I to III – Porcine enterovirus with cytopathogen effect groups I to III
PEC – Porcine enteric calicivirus
[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

PCV2 and porcine circovirus type 1 (PCV1) antigen

PCV2 antigen was detected in almost every pig tested, either in faeces or tonsillar tissue, independent of health status at post mortem. PCV1, however, was not present in study groups with clinical cases of PMWS (Groups 1 and 3).

PPV, PRRSV, HEV and PEV CPE I to III antigen

There was no PPV or PRRSV infection detected in Groups 1 and 3 at the end of the study period according to PCR testing and confirmed the serological results of these groups at post mortem. Pigs in these and other experimental groups had, however, acquired other viral infections during the course of the study. HEV and PEV CPE I and III antigens, including Norwalk-like calicivirus in Group 1, were detected at post mortem in Groups 1 and 5 in clinically diseased pigs. Pigs in Group 2 were infected with PEV CPE I at post mortem, with the exception of one case which was infected with PEV CPE III. Pigs in Group 3 were predominantly infected by PEV CPE III, including a few pigs infected prior to day 0 of the study. Other RNA viruses such as porcine enteric calicivirus (PEC) and rotavirus were detected by PCR testing in individual pigs of Groups 1 and 3. Groups 1 and 3 were test-negative on day 0 and at post mortem for the following RNA viruses: reovirus, porcine hemagglutinating encephalomyelitis virus (PHEV), encephalomyocarditis virus (EMCV), and bovine coronavirus (BCV).

PCR results of faecal samples from randomly selected weaners and growers of Farm B demonstrated that the herd was endemically infected with PEV CPE III, PCV2 and HEV (Table 4.13).

Table 4.13: Herd profile of viral pathogens tested on a commercial pig farm from which healthy, PCV2-positive, 4 and 12-week-old pigs were sourced for a natural transmission study on PMWS. The frequency (number of detected/number of tested animals) of positive samples is stratified by age group. Numbers in brackets indicate inconclusive test results.

Age group	N	PCV2 PCR	PEV CPE I RT-PCR	PEV CPE II RT-PCR	PEV CPE III RT-PCR	PCV2 ELISA	HEV ELISA
4 weeks old	10	0/10	0/10	0/10	4/10	9/10 (1)	8/10
12 weeks old	10	1/10	0/10	0/10	2/10	3/10 (3)	0/10

PCV2 – Porcine circovirus type 2
PEV CPE I to III – Porcine enterovirus with cytopathogen effect groups I to III
HEV – Porcine hepatitis E virus
PCR – Polymerase chain reaction
RT-PCR – Reverse transcriptase polymerase chain reaction
ELISA – Enzyme-linked immunosorbent assay

4.4 Discussion

The primary aim of this study was to produce clinical signs of postweaning multisystemic wasting syndrome (PMWS) in healthy pigs of a susceptible age. This allowed the collection of ante mortem and post mortem samples from confirmed PMWS cases for detailed microbiological investigation to determine the potential causal agent(s) of PMWS. The results of the sample testing support the hypothesis that a novel infectious agent exists, in addition to, or other than, porcine circovirus type 2 (PCV2), and is likely to be a necessary cause for the occurrence of PMWS.

Macroscopic findings

Macroscopic lesions characteristic for PMWS as previously reported by others (Harding & Clark 1997, Rosell et al. 1999, Segalés & Domingo 2002, Segalés et al. 2004b) were found in this study. Muscle wasting, lymph node enlargement, pulmonary consolidation and non-collapsed lungs were commonly observed in pigs of Group 1 and 3, whereas other gross lesions typically associated with PMWS were also detected in other experimental groups, albeit at a lower incidence. This is consistent with observations made by Harding & Clark (1997) where macroscopic lesions typically associated with PMWS are not always present in all affected pigs. The low incidence could be attributed to the small number of study animals per experimental group.

Microscopic findings in lymphoid tissues

Characteristic histological lesions associated with PMWS were found in lymphoid tissues including the tonsils, lymph nodes, spleen and Peyer's patches. These lesions typically consisted of lymphocyte depletion with loss of follicular structure, large infiltration of histiocytic cells and/or multinucleated giant cells and intracytoplasmic inclusion bodies (Harding & Clark 1997, Rosell et al. 1999, Allan & Ellis 2000). A variable degree of lymphocellular depletion in lymphoid tissues was frequently observed in most study groups (although minimal and rare in control Group 6), whereas moderate to severe loss of follicular structure was predominantly seen in groups with acute cases of PMWS (Group 1 and 5).

Pigs from Group 1 developed fulminant clinical signs typical of PMWS and died within three weeks after exposure to PMWS-affected pigs. Their lymphoid lesions consisted of severely marked lymphocyte depletion and a total loss of follicular structure, concurrent with a prominent infiltration of large mononuclear and multinucleated histiocytes in follicular centres. When pigs from the same herd of the same age (Group 7a) were exposed only to a PCV2-positive pig and faeces collected from PCV2-positive, PMWS-negative pigs, they developed similar lesions in lymphoid tissues but of reduced severity. These pigs became infected with PCV2 but did not develop clinical signs consistent with PMWS. This suggests that lesions observed in lymphoid tissues of Group 7a were most likely caused by PCV2, which is currently the most recognised causal agent of PMWS.

There was a more varied development of clinical disease in Group 3 compared to Group 1, and the degree and frequency of microscopic lesions characteristic of PMWS in this group reflected this. Group 3 pigs were of the same age as Group 1 pigs, but originated from a PMWS-free herd, endemically infected with PCV2. After direct exposure to PMWS-affected pigs, most animals demonstrated minimal to moderate lymphocyte depletion in lymphoid tissues, while only a few cases had minor loss of follicular structure with minimal to moderate infiltration of mononuclear and multinucleated histiocytes. Considering the severity of lesions in Group 1, this observation suggests that pre-exposure to PCV2 may ease the fulminant impact and disease expression of PMWS in weaned pigs, resulting in a relatively mild infection. Another explanation

may be that mild lymphoid lesions detected at post mortem in Group 3 animals were in the process of resolving (convalescence or late stage of disease) and do not represent the maximal extent of damage typically seen in the acute stage of PMWS (Quintana et al. 2001, Opriessnig et al. 2006b). The difference may also be related to individual variation in pig's immunological response to a PMWS/PCV2-infection.

Pigs from the on-site control group (Group 5), did not have direct exposure to PMWS-affected pigs and did not demonstrate clinical signs indicative of PMWS until day 34 of the study. It was assumed that some pigs from this study group would suffer more severe clinical signs if the study continued. Lymphoid lesions within this group were more consistent in comparison to other experimental groups. Moderate to severe lymphocyte depletion was observed in each individual pig, with moderate loss of follicular structure and minimal to moderate infiltration of mononuclear and multinucleated histiocytes. An increased degree of lymphocyte depletion and loss of follicular structure in comparison to Group 3 suggested that Group 5 was in an acute phase of PMWS when the study finished. These findings are in agreement with that of Quintana et al. (2001) and Opriessnig et al. (2006b), suggesting that histological lesions observed in PMWS-affected pigs change during the course of disease.

Histopathological investigation of Groups 6 and 7b, which were the off-site control group and comparison group for Group 3, respectively, revealed similar findings. Pigs of these groups were healthy with prior exposure to PCV2 and rare and very mild changes in lymphoid tissues were evident. Minimal to mild lymphocyte depletion was the predominant lesion, whereas infiltration of mononuclear and multinucleated histicytes was rarely observed. Neither of these study groups were in contact with PMWS-affected pigs nor developed clinical signs of PMWS, therefore, it can be assumed that minor lymphocyte depletion or minimal granulomatous inflammation is caused by other infectious or non-infectious factors, for example PCV2 or physical distress. Krakowka et al. (2005) classified pigs with minor lymphocyte depletion as subclinically PCV2-infected animals, however, Darwich et al. (2002) reported that even PCV2-negative pigs can show a slight lymphocyte depletion.

Statistically significant associations between the observed lymphoid lesions and muscle wasting were found for all pigs in this study, considering wasting the most common and

characteristic clinical sign of PMWS. Our findings have highlighted that loss of follicular structure and histiocytic infiltration in lymphoid tissues are the most frequent and significant lesions associated with wasting, followed by lymphocyte depletion. This supports the histological observations that pigs with obvious signs of wasting were more likely to show histopathological lesions distinctive of PMWS, which were not dominant in healthy PCV2-infected control animals.

Intracytoplasmic inclusion bodies

Intracytoplasmic botryoid inclusion bodies are associated with the presence of PCV2 DNA and are one of the accepted diagnostic criteria for PMWS (Stone 2004). These inclusion bodies were frequently present in histiocytes of clinically severe PMWS cases in pigs of Group 1, but occasionally found in single animals of experimental Group 5 and 7b, although pigs of the latter group did not demonstrate clinical signs consistent with PMWS. A number of pigs from Groups 3 and 5 with clinical signs of PMWS, did not show inclusion bodies at the histopathological level. Krakowka et al. (2005) reported that depending on the severity of disease, inclusion bodies are not always found on microscopic tissue examination accounting for this finding. Intracytoplasmic inclusion bodies, therefore, are neither very specific nor pathognomonic for PMWS.

Microscopic findings in non-lymphoid tissues

Mild to severe interstitial pneumonia is the most common lesion found in lungs of PMWS-affected pigs (Rosell et al. 1999, Allan & Ellis 2000, Quintana et al. 2001). Interstitial (broncho-) pneumonia was also observed in our experimental groups, with varying lesion severity. Pigs in the acute stage of PMWS (Group 1 and 5) developed moderate to severe interstitial pneumonia, some with concurrent bacterial infection, while pigs of Group 7a and 7b had only minor tissue changes associated with interstitial pneumonia. Bacterial co-infections in PCV2-infected lungs were evident in a recent *in vitro* study on porcine alveolar macrophages (Chang et al. 2006a). PCV2 alone is able to impair the function of alveolar macrophages significantly by temporarily inhibiting phagocytosis and persistently affecting their microbicidal capability. Consequently, lung tissue is more susceptible to secondary pathogens, due to weakening of an important alveolar defence mechanism. In addition, increased cytokine and chemokine

production may intensify respiratory distress and exacerbate interstitial pneumonia through an increased inflammatory reaction (Chang et al. 2006a).

Similar to pathological findings of previous studies on PMWS (Harding & Clark 1997, Rosell et al. 1999, Quintana et al. 2001, Chianini et al. 2003), non-suppurative and granulomatous interstitial nephritis was also detected in most of our experimental groups, including Groups 7a and 7b. Although observed lesions were of a relatively minor severity, there was no specific pattern of changes distinguishable between PMWS-affected groups and PCV2-infected groups. Consequently, it can be argued that these changes are not pathognomonic of PMWS, as PCV2-infected groups were also affected.

In all experimental groups, with the exception of control Group 6, microscopic changes were observed in the liver. The two main lesions detected in hepatic tissue were focal mild non-suppurative parenchymal inflammation and diffuse mild to severe vacuolar hydropic degeneration of hepatocytes. There was no significant difference in the degree of lesions apparent between PMWS-affected and PCV2-infected groups. These results support the microscopic findings of Rosell et al. (2000) and Quintana et al. (2001), who found lymphohistiocytic inflammatory lesions in liver tissue of numerous pigs with naturally acquired PMWS. Comparable hepatic lesions were also observed in a study with inoculated hepatitis E virus (HEV), PCV2-negative pigs (Halbur et al. 2001). Findings suggested that rare lymphohistiocytic infiltrates in hepatic sinusoids can be considered as physiological background changes in pigs, whereas mild diffuse inflammation and vacuolar degeneration of hepatic cells are effects of HEV-infection. Porcine HEV appears to be present in the pig population worldwide (Meng et al. 1999, van der Poel et al. 2001, Banks et al. 2004), including an estimated seroprevalence of up to 90% in the New Zealand pig population (Garkavenko et al. 2001). Although our study pigs were infected with HEV, we cannot clarify whether the observed inflammatory lesions were due to infection with this pathogen or due to physiological changes. HEV-infection in PMWS-affected pigs was further investigated in a recent study by Martin et al. (2007). These authors found an association between HEV infection and a relatively mild hepatitis, independent of the pig's PMWS status, and concluded that PCV2 is responsible for severe inflammatory lesions in liver tissue. However, the mild nature of the lesions and the similar hepatic lesions between both

PMWS-affected and PCV2-positive pigs in our study provides evidence against this pathogen acting as the sole causal agent of these lesions. In summary, it seems that HEV and PCV2 may both play an independent role in the development of hepatitis lesions found in young pigs.

In situ hybridisation findings

When applying Sorden's criteria to diagnose PMWS (Sorden 2000), the detection of PCV2 within typical histological lesions in lymphoid tissues is required. Frequently used techniques to determine PCV2 DNA or antigen presence within lesions are *in situ* hybridisation (ISH), immunohistochemistry (IHC) and indirect immunofluorescence (IIF) (Segalés & Domingo 2002, Chae 2004). ISH was applied in the present study to detect PCV2 DNA in tissue sections of target samples. While PCV2 DNA was detected in 19/55 pigs tested, with 4/5 and 2/7 clinically affected pigs of Group 1 and 5 tested positive with ISH, respectively. The remaining clinical cases of Groups 1, 3, and 5 were ISH-negative. A possible explanation for the low number of clinical cases positive by ISH, may be that lesions were in the process of resolving and PCV2 nucleic acids were therefore removed from areas of inflammation (Opriessnig et al. 2006b). A number of pigs within groups lacking clinical signs consistent with PMWS (Groups 6, 7a, and 7b), however, were tested ISH positive. This confirms findings of previous studies where a high virus load of PCV2 was consistently found in lesions of pigs suffering from PMWS but PCV2 was also detected in lymphoid tissues of clinically normal pigs (Larochelle et al. 1999b, Allan & Ellis 2000, Calsamiglia et al. 2002, Segalés & Domingo 2002). These findings suggest that the detection of PCV2 within lymphoid tissues of clinically affected pigs cannot act as a diagnostic criterion for PMWS with a high level of specificity.

Haematological findings

Histological and haematological findings of previous research have indicated that an immune system dysfunction in PMWS-affected pigs, modulated by PCV2, may be responsible for the development of the clinical signs of PMWS (Segalés et al. 2000, Darwich et al. 2004). Lymphocyte depletion and histiocytic infiltration in lymphoid tissues was one of the most characteristic microscopic lesions and are reflected in an

absolute lymphopenia and monocytosis of naturally PMWS-affected pigs (Segalés et al. 2000, Segalés et al. 2001, Darwich et al. 2003). In the present study, histopathological findings at post mortem could not be compared with haematological findings as the last blood sampling day was on day 29 of the 56 study days. Nonetheless, attempts were made to find patterns between haematology results and development of clinical signs characteristic of PMWS during the first half of the study. No clear differences were observed in parameters of the complete haemogram between the experimental groups with and without apparent clinical signs. Changes in the differential leukocytes, however, were noticed despite the limited number of study animals in some groups. Inverse neutrophil to lymphocyte ratios, as a result of an increased number of neutrophils and decreased amount of lymphocytes, were predominantly observed in groups with apparent clinical signs of PMWS.

After exposure to PMWS-affected pigs, no other experimental group in this study showed fulminant clinical signs consistent with PMWS at the same severity as Group 1. Early signs of absolute lymphopenia in peripheral blood cells occurred after the first fever peak on day 6. Although, no haematology results were available at post mortem, marked lymphocyte depletion in lymphoid tissues indicated a severe lymphopenia at the time of death. The rising number of blood monocytes suggested a proliferation of the monocyte/macrophage lineage cells, which correlated with the increased infiltration of macrophages, as observed in lymphoid tissues of PMWS diseased pigs (Rosell et al. 1999, Chianini et al. 2003, Segalés et al. 2004a). The findings of Group 1 confirm observations of a previous study by Nielsen et al. (2003). These authors, however, proposed PCV2 infection as the cause of lymphopenia and leukopenia in experimentally PCV2 infected and immunostimulated specific pathogen free (SPF) piglets.

When pigs from the same source herd, of the same age (4-week-old) as Group 1 were exposed to PCV2 only, they became infected with PCV2 but did not develop any clinical signs typical of PMWS, and none died. These clinical observations correlated with the haematological findings of Group 7a. There were no indications of leukopenia, lymphopenia or monocytosis which is typically associated with PMWS.

Group 3 pigs were 4 weeks old, and originated from a PCV2-positive, PMWS-negative herd. They showed mildly elevated rectal temperatures within the first three weeks and

3/8 (37.5%) pigs developed typical signs of PMWS after the last blood sampling day on study day 29. At post mortem, this group showed a more variable degree and frequency of lesions characteristic of PMWS than that seen in Group 1. As discussed previously, it is possible that lymphoid lesions were in the process of convalescence and may have been of greater severity if examined earlier in the study. Haematological alterations observed during the first half of the study would support this assumption, because differential leukocyte counts indicated lymphopenia, neutrophilia and monocytosis, corresponding to haematological changes as described in previous studies in naturally PMWS-affected pigs (Segalés et al. 2000, Darwich et al. 2004, Sipos et al. 2004). When comparing Groups 1 and 7a with the findings of Group 3, it appears that development of lymphopenia, neutrophilia and monocytosis consistent with PMWS was determined by age and exposure to PMWS-affected pigs, with prior exposure to PCV2 reducing the prevalence and speed of disease development.

The evaluation of the neutrophil to lymphocyte ratios revealed there is a statistically significant difference between the experimental groups on each blood sampling day, with the exception of study day 11. There was no known physiological explanation for this phenomenon and it may be that additional factors, such as a limited number of animals and animals of different breeds and herds of origin have influenced the statistical outcome and resulted in a marginal significance compared to other days.

Serological findings

ELISA tests confirmed that seroconversion to PCV2 was present in naïve study animals due to direct transmission from infected animals. Pigs originating from Farm A were clearly sero-negative to PCV2 prior the exposure to PMWS-affected animals or PCV2 pathogen. Group 7a developed a very strong immune response to PCV2 after 81 days of exposure to a PCV2-positive, PMWS-negative pig, whereas Group 1 pigs were still sero-negative at post mortem after exposure to PMWS-affected pigs. It can be assumed that the weak immune reaction observed in Group 1 was a the result of a rapidly weakening immune system, which was unable to respond to the pathogen rather than a lack of sufficient time to produce PCV2 antibodies prior to death.

Pigs originating from Farm B had serum antibodies against PCV2 on day 0 of the transmission study as tested by ELISA, although the concentrations varied considerably between age groups. These findings indicate that at study day 0, weaners of 4 weeks-of-age still had high levels of maternal antibodies against PCV2, whereas 12-week-old grower pigs had lost their passively acquired antibodies to some extent during the growing period (Rodriguez-Arrioja et al. 2002, Larochelle et al. 2003, Sibila et al. 2004). The grower pigs seroconverted strongly to PCV2 after exposure to PMWS-diseased pigs for 56 days, pointing to a robust, active immune response induced through PCV2-infection.

Although only animals of Group 7b were tested for PCV2 antibodies at study day 0, it can be assumed that pigs of Groups 3 and 5 had similarly high levels of antibodies against PCV2 because they were the same age and originated from the same herd. Animals in Group 5, however, showed a more uniform development of clinical disease in comparison to the variation evident in Group 3. Both groups had histopathological lesions consistent with PMWS and relative high levels of PCV2 antibodies at post mortem, but Group 5 was infected via an inadvertent, indirect route later in the study at approximately 8 weeks-of-age. A possible explanation for the observed difference in the development of PMWS could be the level of protective PCV2 antibodies at the time of infection (Ostanello et al. 2005). Our findings from Groups 3 and 5 also suggest that passively acquired antibodies to PCV2 may not be protective against the development of clinical symptoms in weaners, similarly to findings by Hassing et al. (2004). In contrast, observations made under field and experimental conditions in previous studies (Rodriguez-Arrioja et al. 2002, Allan et al. 2002a, Sibila et al. 2004, Opriessnig et al. 2004b, McKeown et al. 2005, Opriessnig et al. 2006a, Calsamiglia et al. 2007, Rose et al. 2007) suggested that passive antibodies may be protective. In summary, our data support the common view that PCV2 is involved in the pathogenesis of PMWS, but do not support the hypothesis that PCV2 is the primary causal agent of PMWS.

Our study strongly indicates that PCV2 alone is not sufficient for expression of PMWS, pointing to the existence of a novel infectious agent (Lawton et al. 2004a, Lawton et al. 2004b) or exposure to a spectrum of known pathogens or non-infectious factors that modify the expression of PCV2-infection to produce clinical disease. Several experimental studies have investigated the relationship between concurrent infection

with PCV2 and either, porcine reproductive and respiratory syndrome virus (PRRSV), or porcine parvovirus (PPV) (Kennedy et al. 2000, Ellis et al. 2000, Harms et al. 2001, Opriessnig et al. 2004a, Hasslung et al. 2005) and have shown that enhanced PCV2 replication and an increased severity of histopathological lesions are associated with PCV2-infection in dually infected pigs (Allan et al. 2000a, Rovira et al. 2002). This evidence strongly suggests that the clinical outcome of dual infection with PCV2 and either agent is likely to be more severe, than with either agent acting alone. Similar findings have been reported for numerous other concurrent infections with porcine pathogens in field observations (Rodriguez-Arrioja et al. 1999, Pallarés et al. 2002, Ellis et al. 2004, Wellenberg et al. 2004). Evidence of a strong association between putative co-infections and outbreaks of PMWS was not, however, demonstrated in field studies by Larochelle et al. (2003) and Madec et al. (2000).

Polymerase chain reaction findings

PRRSV, Aujeszky's disease virus and swine influenza virus are putatively linked co-infections for PMWS (Allan & Ellis 2000, Ellis et al. 2000), and are absent from New Zealand, whereas other potential co-pathogens such as PPV, are far more widespread in New Zealand than the prevalence of clinical PMWS would explain. In the present study, polymerase chain reaction (PCR) results and serology by ELISA on terminal serum samples confirmed the absence of these infectious porcine pathogens in Group 1, 3 and 5 in which PMWS was successfully transmitted from affected pigs. To identify potential infectious porcine pathogens responsible for the development of PMWS, we tested post mortem samples for a large selection of porcine viral pathogens. Results of our molecular investigation strongly indicated that HEV, porcine enterovirus (PEV) cytopathogenic effect (CPE) I and III, including Norwalk-like calicivirus in Group 1, were present in Group 1 and 5, while PEV CPE III was predominantly present in Group 3. After comparison with the 'viral profile' of the source farm (Farm B), PEV CPE I appeared to be the pathogen most strongly associated with PMWS development. PEV has been previously identified in tissues of PMWS-affected pigs (Pogranichniy et al. 2002, Horlen et al. 2007) but to the author's knowledge, this is the first study to propose the possible association between PEV and PMWS. Additional research is currently underway to identify novel agents that may have been transmitted from PMWS-diseased pigs in this study.

4.5 Conclusions

The findings of our study suggest that PCV2 alone is not sufficient to produce clinical PMWS in pigs, and provide support for the hypothesis that another, aetiological pathogen is involved in the development of the disease. Additional research is currently underway to identify novel agents. Preliminary findings of our molecular investigation are most indicative of PEV CPE I as a causally associated infectious pathogen for PMWS.

Chapter 5

Genetic characterisation of porcine circovirus type 2 isolates from a natural transmission study on postweaning multisystemic wasting syndrome in pigs

5.1 Introduction

Porcine circovirus (PCV) was first identified by German researchers in 1974 as a persistent contaminant of porcine-kidney 15 (PK-15) cell cultures (Tischer et al. 1974). It was described as a picornavirus-like particle which is a small spherical virus morphologically similar to picornaviruses. A few years later, Tischer et al. (1982) published the morphological details of the virus and proposed to name it porcine circovirus due to its circular genome structure.

The pathogenicity and epidemiology of PCV have been investigated and it has been concluded that it is non-pathogenic in pigs (Tischer et al. 1986). However, genetic analyses have revealed antigenic and genomic differences between PCV of PK-15 cell cultures and PCV viruses from field cases of PMWS, which strongly indicates that two distinct genotypes exist (Allan et al. 1998b, Meehan et al. 1998, Hamel et al. 1998, Morozov et al. 1998, Allan et al. 1999b). As a result of this, PCV of PK-15 cell line has been designated as type 1 (PCV1), and PCV strains of the virus associated with field cases of PMWS have been designated as type 2 (PCV2) (Allan et al. 1998b, Meehan et al. 1998). Both PCV1 and PCV2 belong to the family *Circoviridae*, genus *Circovirus*. PCV is a non-enveloped isometric particle of 17 ± 1.3nm diameter and is comprised of a covalently closed circular single-stranded DNA genome of 1.76 kilobases[15] (Tischer et al. 1982). The genome possesses several open reading frames (ORF), which encode different viral proteins. The two main genes are ORF1 and ORF2 and represent about 93% of the viral genome (Morozov et al. 1998, Mankertz et al. 2000, de Boisséson et al. 2004). ORF1 is a replicase gene which encodes the replication protein required for genome replication (Mankertz et al. 1998), whereas ORF2 is a capsid gene expressing

[15] One kilobase equals to 1,000 base pairs or nucleotides.

the major structural protein, and the antigenic structure of the virus (Nawagitgul et al. 2000).

PCV2 is the currently, most recognised aetiological agent associated with PMWS. Several experimental studies have failed to reproduce clinical disease similar to that experienced under field conditions by inoculating piglets with PCV2 alone (Krakowka et al. 2000, Magar et al. 2000a, Fenaux et al. 2002). Moreover, genetic comparison of PCV2 isolates originating from PMWS-affected and non-affected farms were insufficient to explain PMWS causation (Grierson et al. 2004b, de Boisséson et al. 2004). However, observations of a few studies have associated changes in the ORF2 gene with a difference in virulence (Fenaux et al. 2004, McKeown et al. 2006), suggesting that nucleotide changes in ORF2 may determine the host immune response and clinical appearance of PMWS. A recent study by Opriessnig et al. (2006c) provided the first experimental evidence of a difference in virulence between PCV2 isolates from cases with and without PCV2-associated lesions.

The main objectives of this chapter were: (1) to genetically characterise PCV2 isolates originating from a natural transmission study of PMWS and other PMWS-affected pigs in New Zealand by molecular methods, and; (2) to evaluate the genetic differences among the PCV2 isolates.

5.2 Materials and methods

5.2.1 Samples

At necropsy various tissue samples were collected from each pig in the PMWS transmission study (Chapter 3, page 43) and stored at -84°C (MDF-U50V, Sanyo). Samples were chosen that represented both PMWS-affected and non-affected experimental groups of pigs for PCV2 sequencing. Refer to Chapter 3 (section 3.2, page 47) for details of groups, animals and their history. A summary of selected tissue samples is given in Table 5.1. Additional tissue samples originating from PMWS-affected farms on the South Island of New Zealand (NZ) during 2006 and 2007 were also sequenced for comparison.

Table 5.1: Summary of selected tissues for complete sequencing of the PCV2 genome. Tissues were collected from pigs with typical clinical signs of PMWS in a natural transmission study of PMWS (North Island, New Zealand) and from animals originating from PMWS-affected pig farms (South Island, New Zealand).

Study group	Age at post mortem (weeks)	Clinical signs of PMWS present in study group or on farm	Sample ID	Source of PCV2 in New Zealand	Tissue	Histopathological findings
Exposure group	10	Chronic wasting, scruffy hair coats, pale skin	53, 62	PMWS-affected farms (Farm C and D), North Island, early 2005	Faeces	Not completed
1[a]	7	High fever (>41°C), unusually rapid wasting	3	Transmitted from pigs of exposure group in early 2005	Mandibular lymph node	Moderate lymphocyte depletion, marked loss of cortico-medullary definition and histiocyte infiltration, mild infiltration of multinucleated histiocytes, moderate circovirus-associated inclusion bodies
5[b]	12	High fever (>41°C), early signs of wasting, scruffy hair coats	17	Transmitted from pigs of exposure group in early 2005	Mandibular lymph node	Moderate lymphocyte depletion, moderate infiltration of histiocytes, marked infiltration of multinucleated histiocytes
6[c]	12	None	6	Commercial pig farm (Farm B), North Island, mid 2005	Mandibular lymph node	Minimal lymphocyte depletion, minimal infiltration of histiocytes, mild infiltration of multinucleated histiocytes
7a[d]	15	None	42	Transmitted from pigs of commercial pig farm (Farm B) in late 2005	Mandibular lymph node	Minimal lymphocyte depletion, marked infiltration of histiocytes, mild infiltration of multinucleated histiocytes
-	8	Fever, conjunctivitis cyanotic ear tips, wasting, intermittent diarrhoea	E	Canterbury Region, South Island, early 2006	Pooled sample of lymphoid tissues	Minimal lymphocyte depletion, marked infiltration of histiocytes, mild infiltration of multinucleated histiocytes
-	9	Acutely infected, wasting, diarrhoea	PIC1, PIC2	South Island, early 2007	Mesenteric lymph node	Not examined
-	8	Acutely infected, wasting, diarrhoea	CB1	South Island, early 2007	Mesenteric lymph node	Not examined
-	9	Acutely infected, wasting, diarrhoea	NP8	South Island, early 2007	Mesenteric lymph node	Not examined

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs; [b] PCV2-positive, PMWS-negative, 4-week-old, on-site control group; [c] PCV2-positive, PMWS-negative, 4-week-old, off-site control group; [d] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

5.2.2 Isolation of DNA from tissues

Deoxyribonucleic acid (DNA) was extracted from lymphoid tissue (DNeasy blood & tissue kit, Qiagen) and extracted from faecal samples (Generation DNA Purification System, Capture Column Kit, Gentra, USA), according to the manufacturer's protocols. Extracted DNA samples were stored at -20°C.

5.2.3 PCR amplification of complete PCV2 genome

The DNA of each sample was amplified by polymerase chain reaction (PCR) (GeneAmp®, PCR System 9700, Applied Biosystems), using two previously published PCV2-specific pairs of PCR primers[16] (Fenaux et al. 2000). The primer pairs, CV1-CV2 and CV3-CV4, produced two overlapping DNA fragments of 989bp and 1,092bp (base pairs or nucleotides), respectively, and represented the complete PCV2 genome (Figure 5.1). The final 20µl PCR reaction contained 1x PCR buffer (10x PCR buffer, Invitrogen), 1.5mM of $MgCl_2$ (Invitrogen), 1pmol of each primer, 0.1mM of deoxyribonucleotide triphosphates (dNTPs) (Global Science), 1U of Taq DNA polymerase (Invitrogen) and 2 µl of extracted DNA. The PCR included an initial enzyme-activation step at 94°C for 2 minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 61°C for 30 seconds and extension at 72°C for 15 seconds. A final elongation was not used. $MgCl_2$ concentration was optimised by titration.

[16] Primers are synthetic single strands of DNA of various lengths designed to bind to specific complementary target sites in a DNA template (Riley 2004).

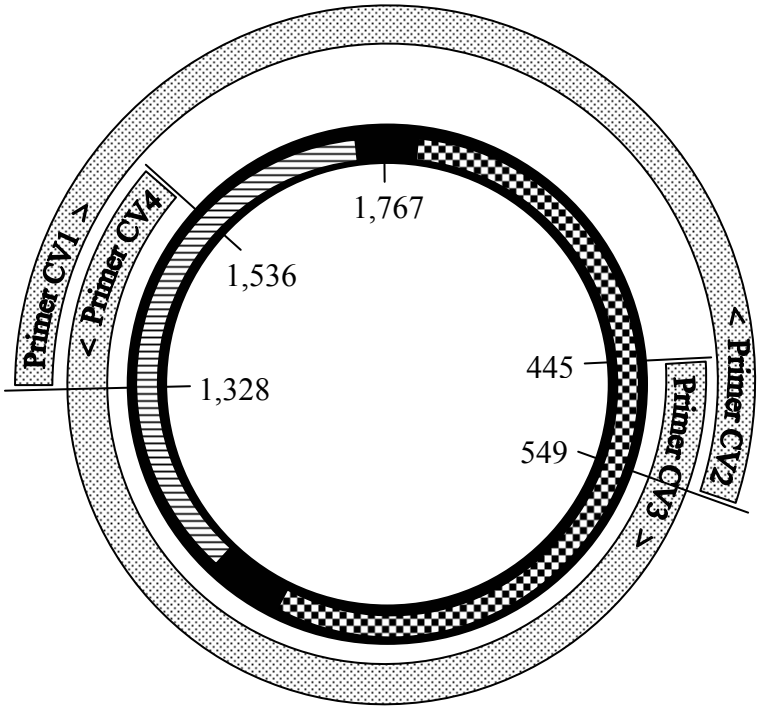


Figure 5.1: Schematic outline of the complete circular genome of PCV2 (inner circle with 1,767 nucleotides) with its capsid protein region (hatched part on inner circle, ORF2 gene) and replication protein region (chequered part on inner circle, ORF1 gene). For full-length sequencing of PCV2 two sets of primers (CV1 to CV4) were used to generate two overlapping segments.

5.2.4 Sequencing, sequence alignments and cluster analysis

The PCR products were loaded onto a TAE agarose gel containing 2% agarose, acetic acid and Tris-EDTA buffer and stained with ethidium bromide. PCR products were then purified using the QIAquick PCR purification kit (Qiagen), according to the manufacturer's protocol, and sequenced at the Allan Wilson Centre at Massey University (Palmerston North, New Zealand), using a capillary genetic analyzer (ABI3730 Genetic Analyzer, Applied Biosystems Inc.). Sequences were analysed and edited with the programme MT, Navigator PPC (version 1.0.2b3).

Eight PCV2 sequences published in the nucleotide database (NCBI 2007) were selected for analysis to identify areas of genomic differences that may be associated with the clinical appearance of PMWS (Table 5.2).

Table 5.2: Identification of PCV2 isolates retrieved from the GenBank database (NCBI 2007).

Country of origin	GenBank accession number	Genome length (nucleotides)	Disease association	Year detected	Reference
Australia	AY754018.1	1,768	none	2000	(Muhling et al. 2006)
Canada	AF055392.1	1,768	PMWS	1998	(Meehan et al. 1998)
China	AY556474	1,768	PMWS	2004	(NCBI 2007)
France 1	AF055393.1	1,767	PMWS	1998	(Meehan et al. 1998)
France 2	AF201311	1,767	PMWS	1999	(Mankertz et al. 2000)
USA 1	AF264042	1,768	PMWS	1998	(Fenaux et al. 2000)
USA 2	AF055391.1	1,768	PMWS	1998	(Meehan et al. 1998)
USA 3	DQ397521.1	1,768	Respiratory disease, no PMWS diagnosis	2003	(Opriessnig et al. 2006c)

Sequences were selected based on their completeness, geographical origin, clinical history of the isolates, and citations in previous publications. The eight sequences were downloaded and linearised at the origin of replication, which is the designated starting point of numbering the circular PCV2 genome (Hamel et al. 1998) (refer Figure 5.1). PCV2 isolate France 2 (AF201311) was taken as a reference sequence to align all isolates analysed in this study. MEGA (version 3.1, (Kumar et al. 2004)) was used to align nucleotide sequences and complete amino acid alignments. Incomplete PCV2 amplicons (ID 3, 53, and 62) were excluded from further analysis. A cluster analysis was performed on the aligned genomes and an unrooted phylogenetic dendrogram was constructed, based on the number of nucleotide differences using the Neighbor-Joining method. Bootstrap values[17] were determined on 1,000 replicates. Complete nucleotide genomes and amino acid sequences of the capsid protein (ORF2) were compared, pairwise, for detection of homology using AlignX of Vector NTI Advance 10.3.0.

[17] The bootstrap test is one of the most commonly used tests of reliability of an inferred tree. Bootstrapping measures how consistently the data support the given taxon bipartitions of the inferred tree in comparison to the original tree after nucleotides have been randomly chosen and replaced. Bootstrap numbers represent confidence values in the chosen analysis. Bootstrap values close to 100% mean uniform support. In general, bootstrap values of >95% for a given interior branch indicate to a 'correct' topology at that branch (Anonymous 2007a).

5.3 Results

5.3.1 Electrophoretic band pattern

Figure 5.2 shows a gel electrophoresis of the amplicons generated by the PCR assay, which were generated for each selected tissue sample. The amplification products were of an expected size (approximately 989bp and 1,092bp for primer pairs CV1-CV2 and CV3-CV4, respectively). Other significant bands or artefacts were not observed.

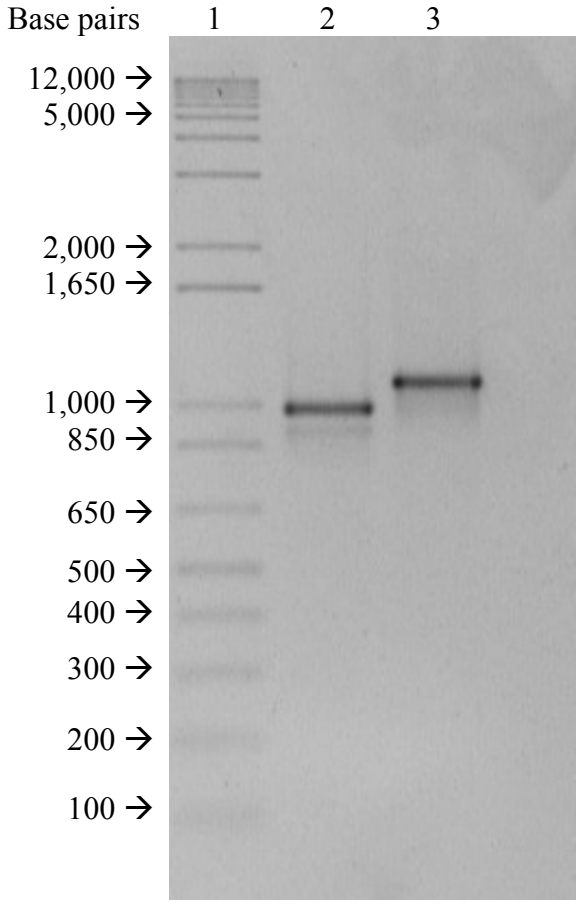


Figure 5.2: Gel electrophoresis of amplified fragments of PCV2 DNA detected in one sample (ID 17). Lane 1: 1Kb Plus DNA ladder (Invitrogen) with different size of base pair fragments indicated by arrows on the left margin. Lane 2: PCR amplification products of primer pairs CV1-CV2 and Lane 3: CV3-CV4.

5.3.2 Alignments and phenetic analyses of PCV2 isolates

The New Zealand PCV2 isolates sequenced in this study were aligned and compared for nucleotide differences. PCV2 amplicons of three samples (ID 3, 53, and 62) were incomplete with 45, 851, and 989 missing nucleotides, respectively. Various changes of the PCR assay and the concentration of extracted DNA repeatedly failed to amplify complete fragments. Genetic divergence was apparent between PCV2 isolates from clinically healthy and PMWS-affected pigs with 62 nucleotide differences, where 43/62 of genomic changes were within the ORF2 region. Details on the nucleotide sequence alignment are given in Appendix XXXIII.

Eight other PCV2 genome sequences from the NCBI database were also included in this study. Percentage values of identity between all analysed sequences are listed in Table 5.3. The nucleotide identity among all PCV2 isolates associated with clinical symptoms of PMWS was at least 95% and within NZ isolates alone was 100%, whereas a higher degree of similarity (>95%) was observed between PCV2 from PMWS-affected and non-affected pigs. Homology of the amino acid sequences of the capsid protein ranged from 90% to 100% in pigs affected with PMWS, and >89% between PCV2 isolates from PMWS-affected and non-affected pigs.

Table 5.3: Pairwise comparison of complete PCV2 genomes and amino acid sequences of the capsid protein (ORF2). PCV2 isolates of PMWS cases are shown in bold.

		Sequence identity (%)[a]															
Isolate	Nucleotides	**17**	42	6	**China**	Australia	**USA 2**	**Canada**	USA 3	**USA 1**	**PIC2**	**PIC1**	**E**	**France 1**	**France 2**	**CB1**	**NP8**
17	1,767	–	96	96	95	96	97	97	99	99	100	100	100	100	100	100	100
42	1,767	94	–	100	98	97	97	97	99	99	96	96	96	97	99	96	96
6	1,767	94	100	–	98	97	97	97	99	99	96	96	96	97	99	96	96
China	1,768	90	94	94	–	96	97	97	98	99	95	95	95	96	98	95	95
Australia	1,768	90	93	93	90	–	96	96	97	97	96	96	96	96	97	96	96
USA 2	1,768	93	94	94	94	92	–	99	99	100	97	97	97	96	97	97	97
Canada	1,768	93	94	94	94	93	97	–	100	100	97	97	97	96	97	97	97
USA 3	1,768	92	93	93	93	92	96	97	–	99	99	99	99	97	96	99	99
USA 1	1,768	93	95	95	94	92	99	97	96	–	99	99	99	97	96	99	99
PIC2	1,766	99	94	94	90	90	93	93	92	93	–	100	100	100	100	100	100
PIC1	1,767	100	94	94	90	90	93	93	92	93	99	–	100	100	100	100	100
E	1,766	99	94	94	90	90	93	93	92	93	99	100	–	100	100	100	100
France 1	1,767	99	95	95	90	90	94	94	93	94	98	99	99	–	100	100	100
France 2	1,767	98	94	94	90	90	94	93	92	94	98	98	99	100	–	100	100
CB1	1,767	99	94	94	90	90	93	93	92	93	99	100	100	99	98	–	100
NP8	1,766	100	94	94	90	90	93	93	92	93	99	100	100	99	99	100	–

[a] Values listed present percentage identity of complete nucleotide sequences (upper right) and amino acid sequence homologies of the capsid gene (ORF2) (lower left).

Figure 5.3 presents the heterogeneity in aligned amino acid sequences of ORF2 between all PCV2 isolates included in this study. Repeatable motifs of amino acid changes could not be associated with the presence of PMWS.

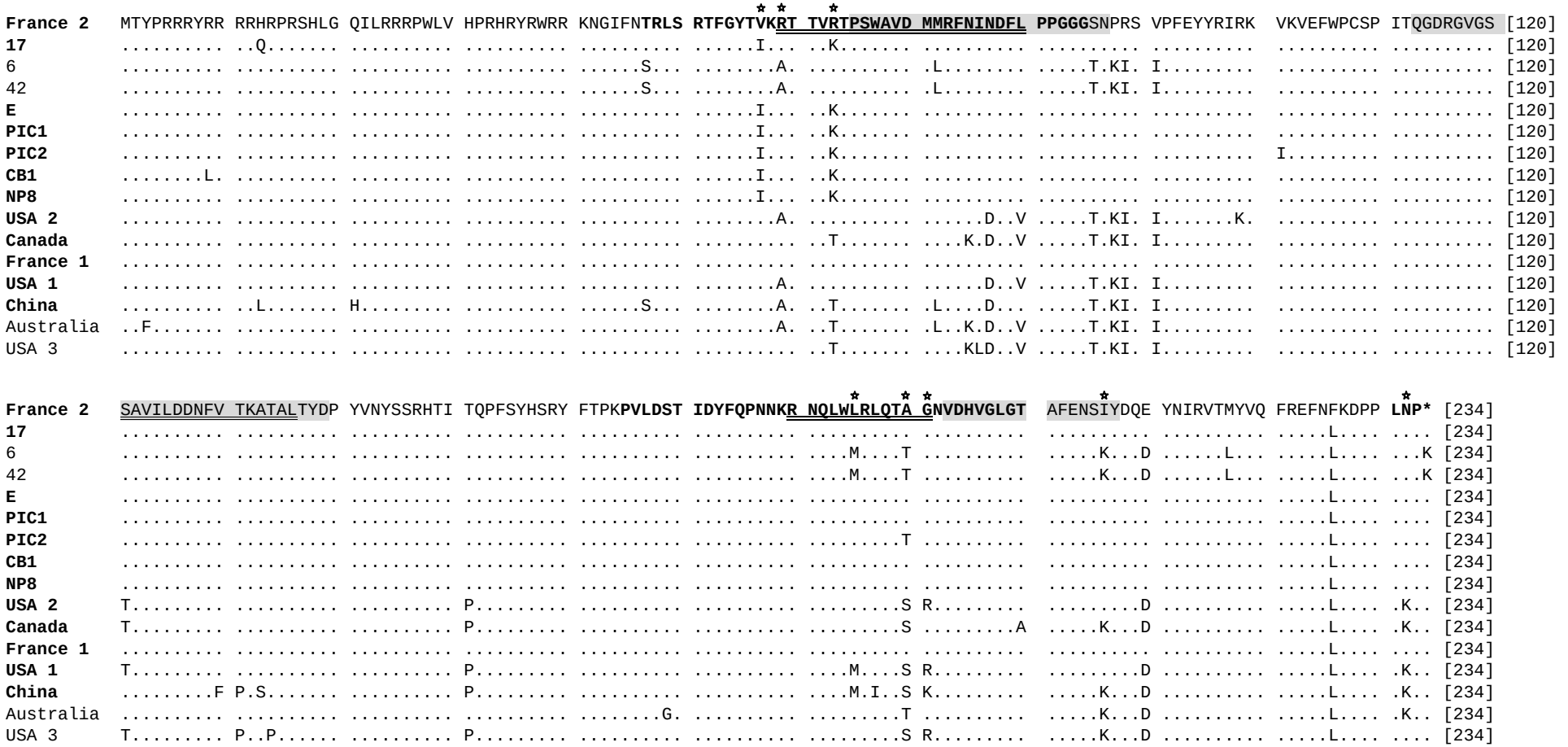


Figure 5.3: Comparative amino acid alignment of the viral capsid protein (encoded by ORF2 gene) of 16 PCV2 isolates with PMWS cases shown in bold. 'France 2' is used as reference isolate and only differences are shown for the other isolates. Immunoreactive domains published previously are indicated as follows: highlighted for Mahé et al. (2000); double underlined for Larochelle et al. (2002); in bold for Lekcharoensuk et al. (2004); and ✯ for mutations reported by de Boisséson et al. (2004).

Figure 5.4 depicts the cluster analysis of complete PCV2 genomes in an unrooted dendrogram. All NZ isolates associated with PMWS appeared in the same cluster together. French isolates (n = 2) were in relative proximity to PCV2 isolates from healthy NZ pigs but clearly separated from the Australian strain. Some clustering effect was observed in association with cases of PMWS.

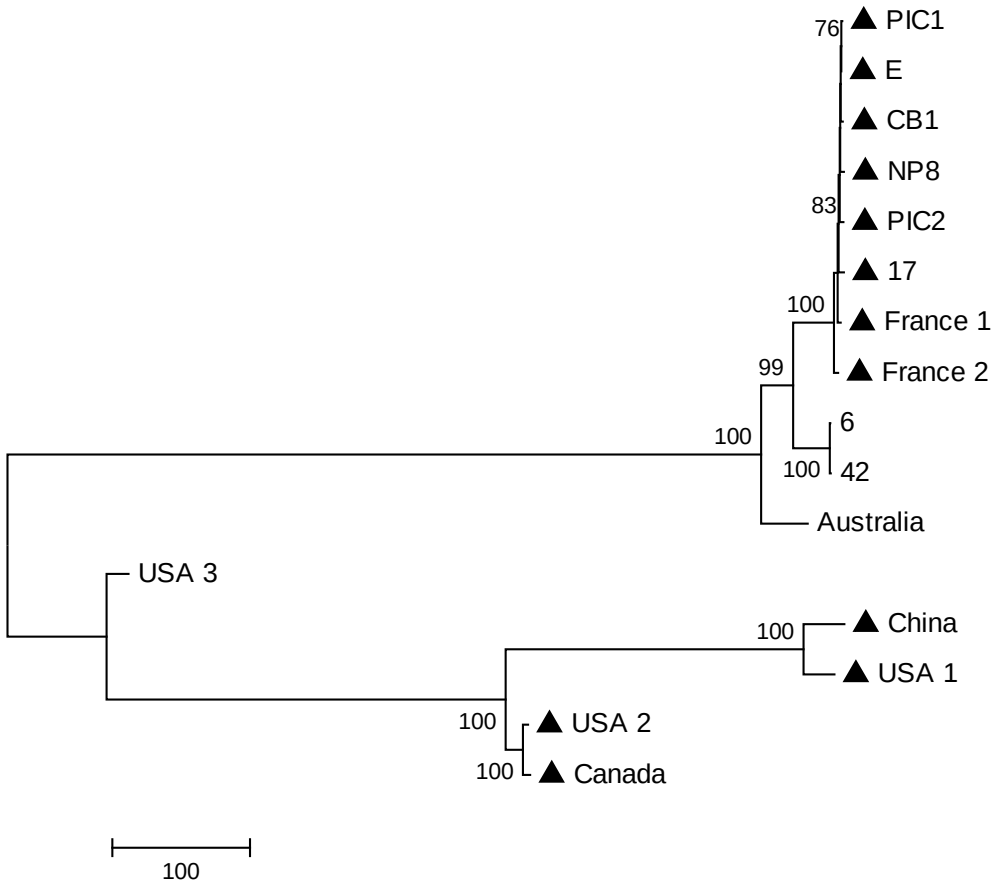


Figure 5.4: Cluster analysis of 16 full-length PCV2 genomes selected from pigs from a transmission study on PMWS, acutely infected pigs from commercial farms in New Zealand and comparable isolates available from GenBank with ▲ = PCV2 genomes of PMWS cases. A consensus phylogram of 1,000 Neighbour-Joining trees was generated based on the number of nucleotides differences of aligned PCV2 genomes. The numbers refer to bootstrap values with significance above 70% and branch lengths are proportional to the number of nucleotide differences, represented by the scale bar.

5.4 Discussion

This molecular study was conducted to genetically characterise porcine circovirus type 2 (PCV2) isolates from healthy pigs and those affected with postweaning multisystemic wasting syndrome (PMWS) from our transmission trial, in an attempt to evaluate whether the variants differed genetically in virulence.

Our study showed that PCV2 isolates associated with PMWS in New Zealand shared 100% nucleotide identity with each other, and showed at least 95% sequence identity with PCV2 variants from PMWS-affected pigs originating from other countries. Similarly, high identity values have been reported from other phylogenetic studies (Meehan et al. 1998, Mankertz et al. 2000, Fenaux et al. 2000, Choi et al. 2002, Larochelle et al. 2002, Muhling et al. 2006), indicating a relatively stable PCV2 genome overall with minor genetic variation among isolates of different geographic origin. In this study, there was a greater degree of similarity (>95%) observed between PCV2 from PMWS-affected and non-affected pigs, insinuating that there is little identifiable correlation between strains and health status, which supports previous findings of case-control studies (Pogranichniy et al. 2002, Grierson et al. 2004b, de Boisséson et al. 2004). These observations suggest that differences in PCV2 nucleotide sequences are not sufficient to explain the aetiology of PMWS compared to sub-clinical PCV2-infections.

It has been suggested that multiple PCV2 genotypes can circulate simultaneously in individual PMWS-affected pigs under field conditions, and by chance the less virulent genotype is isolated for sequencing (Opriessnig et al. 2006c). If this suggestion has merit, studies reporting genomic data from PMWS-affected pigs may encounter misclassification issues. This would also apply to PCV2 isolates submitted to the nucleotide database of NCBI. Our cluster analysis indicates that there was an association between nucleotide differences and PMWS, however, we cannot draw valid inference due to the small sample size of PCV2 variants. An increased sample of PCV2 isolates from healthy and PMWS-affected pigs from New Zealand and increased numbers of PCV2 strains from other countries would be required to support our findings.

The capsid gene (ORF2) of PCV2 is more heterogenic when compared to the complete genome and shows reduced sequence identity with ORF2 genes of other PCV2 isolates. In our study, the capsid protein shared as little as 90% amino acid homology with other published PCV2 isolates, irrespective of their association with PMWS. These findings are in agreement with previously reported variability values (Fenaux et al. 2000, Mankertz et al. 2000, Larochelle et al. 2002, Pogranichniy et al. 2002). Because of greater amino acid alterations observed in the capsid protein, Mankertz et al. (2000) speculated that the modified protein might be associated with altered pathogenicity of PCV2 leading to a different tropism and changed virus-host interaction. Indeed, three regions of higher amino acid heterogeneity on ORF2 were identified in the study of Larochelle et al. (2002), and the first two corresponded with antigenic domains determined by peptide scanning analysis in an earlier study by Mahé et al. (2000). However, no repeatable amino acid motifs for these two regions could be associated with PCV2 isolates originating from PMWS-affected or healthy pigs (Larochelle et al. 2002). Isolates analysed in our study showed a greater heterogeneity in immunoreactive regions but failed to identify an amino acid mutation specific to a pathogenic state, such as in the findings of de Boisséson et al. (2004).

While several molecular studies have failed to identify specific mutations in PCV2 isolates associated with the occurrence of PMWS, it is possible that more than one change in amino acid sequence is necessary to determine the virulence of PCV2. The virulence of viruses is generally regulated by multiple genes and an influence from other genes cannot be excluded (Larochelle et al. 2002). The first evidence of differences in virulence between PCV2 isolates was published in 2006 (Opriessnig et al. 2006c). Two PCV2 isolates from cases with and without PCV2-associated lesions were compared experimentally and genetically after inoculation of seven-week-old specific pathogen free (SPF) pigs. Isolates differed significantly in their antibody profiles, the amount of virus in serum and in lymphoid tissues, along with the severity of macroscopic and microscopic lesions. This study failed to reproduce clinical signs consistent with PMWS despite using a virus isolate cloned from a pig with confirmed PMWS.

Much research is currently focused on determining the molecular basis of PCV2 virulence and identifying mutations, but it is important to remember that many

experimental PCV2 inoculation studies have failed to reproduce clinical signs typical of PMWS (Krakowka et al. 2000, Magar et al. 2000a, Fenaux et al. 2002). PCV2 vaccines which have been commercially available in some countries since last year are formulated with inactivated PCV2 isolates (Circovac®, Merial Inc.), PCV1-2 chimera products (Suvaxyn® PCV2 One Dose, Fort Dodge) or PCV2 antigens expressed in an inactivated Baculovirus (Porcilis PCV®, Intervet) and appear to have a protective effect in the field with reductions in mortality and clinical signs typical of PMWS (Charreyre et al. 2006a, Auvigne et al. 2006, Joisel et al. 2006). Further research is needed to expand our understanding of the importance of specific molecular differences between PCV2 isolates, their pathogenicity and effect on disease expression, and subsequently on the epidemiology of PMWS.

5.5 Conclusions

Genetic differences were found between isolates from healthy and PMWS-affected pigs as determined by the phenetic analysis. There were no significant molecular changes detected between ORF2 genes of PCV2 isolates from healthy and PMWS-affected pigs, which would identify a mutation specific for a pathogenic state. Multiple genes might determine viral virulence but disease expression probably requires another pathogen and is not caused by a virulent 'subtype' of PCV2.

Chapter 6

General discussion

This thesis reports on our investigation into the aetiology and natural transmission of postweaning multisystemic wasting syndrome (PMWS), a wasting disease primarily affecting weaned pigs. Since its first detection in Canada in 1991 (Harding & Clark 1997), PMWS has spread into many countries worldwide (Allan & Ellis 2000, Segalés & Domingo 2002). Significant production losses occur through reduced daily weight gain, increased morbidity and mortality rates, resulting in vast economic impacts on pig producion systems. Multiple hypotheses have been formulated in an effort to explain the likely causation of PMWS, predominantly focusing on porcine circovirus type 2 (PCV2) as the only aetiological pathogen. New Zealand's first cases of PMWS were identified in September 2003 (Lawton et al. 2004b). Following the disease outbreak, investigations have indicated that the spread of disease in New Zealand is similar to that of a propagating epidemic, suggesting the introduction of a novel pathogenic agent for PMWS.

An experimental transmission study was conducted in a purpose-built outdoor facility at Massey University (Palmerston North, New Zealand), to investigate the aetiology of PMWS. We demonstrated that clinical signs consistent with PMWS can easily be reproduced by the exposure of healthy pigs to clinically PMWS-affected pigs (Chapter 3). The study provides evidence that PMWS is a transmissible disease and that a pathogen distinct from PCV2 (but perhaps concomitant with PCV2) is likely involved in the aetiology of the disease. The study has resulted in the collection of a range of biological samples throughout the course of PMWS that are being used to continue work on the detection of novel pathogens.

Prior to our study, uncertainty about the disease induction and modes of PMWS transmission were evident despite comprehensive experimental research. The design of our experimental study (Chapter 3) determined that PMWS transmission can occur through direct contact between clinically healthy and PMWS-affected pigs. These pigs

were housed in the same pens and the first typical clinical signs of PMWS developed in exposed pigs after one week. These observations confirm the findings of previous experimental transmission studies (Kristensen et al. 2004, Kristensen et al. 2006). Our trial has also demonstrated that indirect transmission of PMWS is possible when unexposed pigs, kept as on-site controls adjacent to the PMWS exposed pigs, became infected during the study via an inadvertent route. Careful biosecurity measures were maintained but transmission may have been through mechanical, personnel or rodent vectors, or through short distance airborne spread. The potential route of PMWS transmission through birds was suggested by Mackinnon (2000) after the disease was introduced into the UK in 1999. This is especially relevant to outdoor production farms and may have contributed to the recent PMWS outbreak on the South Island of New Zealand (Neumann et al. 2007). However, birds were thought to be an unlikely vector in our study as all direct contact was prevented through installation of a bird-proof wire mesh around the study facility. Further studies are required to identify vectors and potential routes of indirect transmission of PMWS.

PMWS predominantly affects weaned pigs and was observed in New Zealand in pigs of 4 to 12 weeks of age (Stone 2004, Rawdon et al. 2004, Loth & Stone 2005, Neumann et al. 2007). In our study, 4 and 12-week-old healthy pigs were exposed to PMWS causing agent(s) for 56 days to define the susceptible age of infection (Chapter 3). Pigs older than 12 weeks were not susceptible to the development of clinical signs of PMWS, whereas 4-week-old pigs were susceptible, showing clinical signs one week after exposure. In conclusion, PMWS is a highly age-dependent disease with pigs of younger age (4 to 8-weeks-old) being at a higher risk of developing clinical symptoms of PMWS than those that are older than 12 weeks.

In our study, pigs developed clinical signs typical of PMWS (Chapter 3) and had macroscopic and microscopic changes characteristic for PMWS (Chapter 4). Although disease expression and pathological lesions can vary considerably among affected pigs, our findings were very similar to reports from other PMWS-affected countries (Daft et al. 1996, Harding & Clark 1997, Segalés et al. 1997, LeCann et al. 1997, Kennedy et al. 1998, Onuki et al. 1999, Drew et al. 2004). Therefore, it would be logical to assume that: (1) the disease conditions in New Zealand do not differ markedly from those overseas; and (2) PMWS is probably caused by the same pathogen(s). Porcine

reproductive and respiratory syndrome virus (PRRSV), Aujeszky's disease virus and porcine parvovirus (PPV) have been proposed as necessary contributors to PMWS causation (Pogranichniy et al. 2002, Ellis et al. 2003, Wellenberg et al. 2004), but have been ruled-out in this study through negative results in serological and molecular tests (Chapter 4). Therefore, the hypothesis of a novel infectious agent for PMWS remains valid and is supported by epidemiological evidence that PMWS occurs as a propagating epidemic, with characteristics consistent with those produced by a novel infectious agent (Morris et al. 2002, Lawton et al. 2004a, Vigre et al. 2005).

Porcine circovirus (PCV) infection has long been associated with PMWS and the virus has been implicated as the causative agent for the disease (Ellis et al. 1998, Allan et al. 1998a), although an exclusive causal relationship has not been confirmed. The view that PMWS is caused by PCV2 with disease expression modified by the presence of viral co-infections (Rodriguez-Arrioja et al. 1999, Kennedy et al. 2000, Harms et al. 2001, Pallarés et al. 2002, Ellis et al. 2004, Hasslung et al. 2005), immunostimulation (Krakowka et al. 2001, Kyriakis et al. 2002), genetics (López-Soria et al. 2004, Opriessnig et al. 2006a) or management effects (Allan et al. 2003) is difficult to reconcile with epidemiological evidence that: (1) PMWS has occurred in countries where PCV2 has long been endemic (Horner 1991, Larochelle et al. 1999a, Magar et al. 2000b); and (2) PCV2 infection is widespread and frequently found on PMWS unaffected farms (Larochelle et al. 1999a, Rodriguez-Arrioja et al. 2000, Pogranichniy et al. 2002). PCV2 as the causal agent of PMWS, with the involvement of proposed co-factors of infectious or non-infectious nature have largely been excluded by the findings of our transmission study (Chapters 3 and 4). Further support for this is provided by the fact that PMWS could not be reproduced in susceptible pigs through the exposure of PCV2 alone (Chapter 3). It is justified, therefore, to assume that an infectious novel pathogen for PMWS exists. Extensive laboratory investigations are ongoing to identify this agent and additional clinical research is planned to gain further insight on the aetiology of PMWS.

Despite the lack of evidence that identifies significant differences between PCV2 strains on PMWS-affected and un-affected farms (Grierson et al. 2004b, de Boisséson et al. 2004), a recent hypothesis attempts to explain the association between PCV2 infection and prevalence of PMWS, by the suggestion that PCV2 strains differ genetically in

virulence (Opriessnig et al. 2006c). Genetic differences observed in PCV2 isolates from healthy and PMWS-affected study animals (Chapter 5) were not sufficient to support this hypothesis. Moreover, the genomic homology of >95% between PCV2 from PMWS-affected and un-affected pigs does not support that there is a correlation between strains and health status. Although multiple genes might determine viral virulence, it appears most likely that disease expression requires another pathogen and is not caused by a virulent 'subtype' of PCV2.

Case definitions are important for emerging diseases in order to successfully identify cases. The first formal case definition of PMWS, known as 'Sorden's criteria', was published in 2000 (Sorden 2000), and is the most commonly used case definition for PMWS. However, not all recognised PMWS cases fulfil the case definition, for example histopathological lesions, defined as characteristic for PMWS, can also be found in healthy pigs without clinical symptoms of PMWS (Chapter 4), suggesting that not all of the proposed criteria are pathognomonic for PMWS. Another issue with the case definition for PMWS is that some of the required histopathological lesions (including the presence of PCV2 DNA within those lesions) may be in the process of resolving and, as a result, obvious clinical cases of PMWS are not confirmed in histopathology (Chapter 4). This indicates that case definitions need to be revised over time to prevent misdiagnosis of cases and, consequently, sustain the spread of disease. It is difficult to develop useful, accurate case definitions for PMWS, especially given the uncertainty about the aetiology of the disease. The identification of the causal agent(s) of PMWS will, therefore, allow greater diagnostic accuracy in the future.

The objectives of this transmission study were: (1) to produce clinical PMWS in healthy pigs of a susceptible age and serological status; and (2) to collect ante mortem and post mortem samples from affected pigs to complete a detailed microbiological investigation of potential causal agents of PMWS. The limited availability of PCV2-negative pigs for our transmission study explains the low number of study animals in some experimental groups (Chapter 3) and it would be justified, therefore, to argue that our results are not statistically robust due to a small sample size. Nevertheless, our study could reproduce clinical signs of PMWS in susceptible pigs and demonstrated that PCV2 infection alone is not sufficient to produce the disease. The pathological investigation (including clinical pathology) in our study (Chapter 4), was predominantly focused on the

diagnosis of PMWS cases to confirm our clinical observations made during the course of disease. To investigate the process of pathogenesis of PMWS and the development of histopathological changes in PMWS-affected pigs, a different study design would be required with larger sample sizes and consecutive euthanasia of study animals at weekly intervals.

This study has provided considerable information for pig producers and the pig industry at a national and international level. Findings of our investigations suggest that, avoiding direct contact with clinically PMWS-affected pigs, and the maintainance of high biosecurity measures on un-affected pig farms would likely prevent disease introduction through personnel, feed and equipment vectors. Sound health management systems on pig production and rearing sites should be instituted to reduce the risk of PMWS, and other common pig diseases as the susceptible age range of pigs found in our study was 4 to 8-weeks-old. As PMWS is easily transmitted via pig-to-pig contact, we recommend separating pigs immediately after the first signs of disease occur (elevated rectal temperatures (>40°C), refusal to feed), to prevent further spread of the infectious agent(s) within the herd. Furthermore, certain identification of the aetiological agent(s) of PMWS may greatly benefit the development of a live-animal diagnostic test, aid in the development of a specific PMWS vaccine and resolve the current issue of inaccurate case definitions for PMWS and their interpretation. PCV2 vaccines, which have been commercially available in some countries since last year, are formulated with inactivated PCV2 isolates (Circovac®, Merial Inc.), PCV1-2 chimera products (Suvaxyn® PCV2 One Dose, Fort Dodge) or PCV2 antigens expressed in an inactivated Baculovirus (Porcilis PCV®, Intervet). The vaccines appear to have a protective effect with reductions in mortality and clinical signs typical of PMWS according to preliminary observations in the field (Charreyre et al. 2006a, Auvigne et al. 2006, Joisel et al. 2006). This supports the involvement of PCV2 in the aetiology of PMWS, however, it does not rule out the existence of other causal pathogens. The future will show whether these PCV2 vaccines perform adequately in the field as cases of vaccine failure have not yet had time to be reported.

Despite the limited availability of pigs from Farm A (archaic genotype) for use in this study our findings show that PMWS primarily affects pigs of 4 to 8-weeks-of-age and support the likely involvement of a novel infectious agent(s). The disease is

transmissible through direct contact to clinically PMWS-affected pigs and may also be transmitted indirectly via mechanical and personnel vectors. Further research is required to explore the possibilities of bird vectors and short distance airborne spread. Genetic differences between PCV2 isolates from healthy and PMWS-affected pigs are not sufficient to explain the prevalence of PMWS, therefore, it is most unlikely that the disease expression is caused by a virulent 'subtype' of PCV2. Laboratory investigations are ongoing to identify a novel agent involved in PMWS. This work will clarify the aetiology of this complex disease and will assist in the development of a live-animal diagnostic test. In addition to this, useful control measures could be developed to restrain the spread of PMWS at the national and international level and reduce the vast economic loss to the pig industry worldwide.

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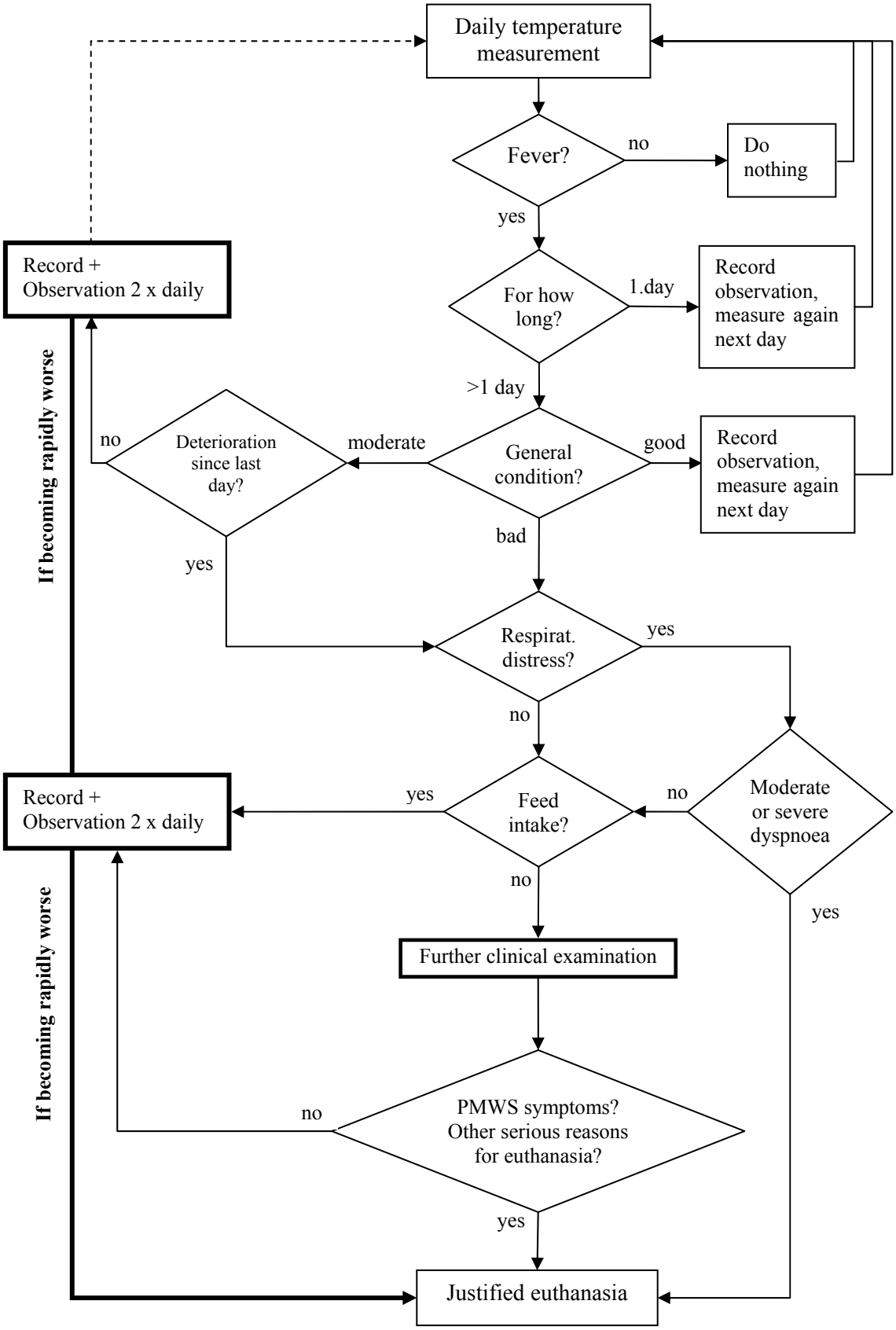
Appendices

Appendix I: Condition scoring of grower and finisher pigs.

	Numerical Score	Pelvic Bones	Loin	Vertebrae	Ribs
	**1**	Pelvic bones very prominent.	Loin very narrow. Sharp edges on transverse spinal process. Flank very hollow.	Prominent and sharp throughout the length of the backbone.	Individual ribs very prominent.
	**2**	Pelvic bones obvious but some slight cover.	Loin narrow. Only very slight cover to edge of transverse spinal process. Flank rather hollow.	Prominent.	Rib cage less apparent. Difficult to see individual ribs.
	**3**	Pelvic bones covered.	Edge of transverse spinal processes covered and rounded.	Visible over the shoulder. Some cover further back.	Covered but can be felt.
	**4**	Pelvic bones only felt with firm pressure.	Edge of transverse spinal processes felt only with firm pressure.	Felt only with firm pressure.	Rib cage not visible. Quite difficult to feel any ribs.
	**5**	Pelvic bones impossible to feel.	Impossible to feel bones. Flank full and rounded.	Impossible to feel vertebrae.	Difficult to feel ribs.

Appendix II: Procedure of defining humane endpoint - early termination by euthanasia.



Appendix III: Isolation of RNA and DNA.

RNA isolation and reverse transcription-PCR assay (RT-PCR)

Faecal samples (30 mg) were dissolved with TRIzol reagent (GIBCO-BRL, Gaithersburg, MD, USA) according to the manufacturer's protocol. Complementary deoxyribonucleic acid (cDNA) was generated using 2 U M-MVL reverse transcriptase (Life Technologies, BRL) as per the manufacturer's directions and RNA reverse transcribed in the presence of random primers at 42°C for 1 h.

DNA isolation

Viral DNA from faeces, tonsil tissues and PBMC was extracted using Generation® DNA Purification System, Capture Column Kit (Gentra 2005) according to the manufacturer's protocol.

PCR assay for amplification of DNA and RNA isolates

All amplifications of DNA/RNA were performed using an automated thermocycler (582 BR003646, Bio-Rad icycler). Different sets of virus specific primers (Table 3.3) were used to amplify 100ng of DNA/ RNA in a PCR reaction mixture with the final concentration of 1.5 mmol/L $MgCl_2$, 0.2 mmol/L (each) deoxyribonucleotide triphosphate (dNTP), 1.00 μmol/L of each primer, and 2.5 U of Taq DNA polymerase (Eppendorf) per 50 μl.

Appendix IV: *In situ* hybridisation (ISH) results of different experimental groups in a transmission study on PMWS. Numbers (%) of pigs are stratified by amount of PCV2 nucleic acid detected within lesions of selected tissues.

		Score				
Tissue	Group	absent	low	moderate	abundant	Total
Lymph node	1	1 (20)	2 (40)	1 (20)	1 (20)	5 (100)
	2	3 (100)	0 (0)	0 (0)	0 (0)	3 (100)
	3	8 (89)	1 (11)	0 (0)	0 (0)	9 (100)
	4	10 (100)	0 (0)	0 (0)	0 (0)	10 (100)
	5	7 (70)	1 (10)	2 (20)	0 (0)	10 (100)
	6	2 (33)	3 (50)	1 (17)	0 (0)	6 (100)
	7a	2 (33)	4 (67)	0 (0)	0 (0)	6 (100)
	7b	6 (100)	0 (0)	0 (0)	0 (0)	6 (100)
Peyer's patches	1	3 (60)	1 (20)	0 (0)	1 (20)	5 (100)
	2	3 (100)	0 (0)	0 (0)	0 (0)	3 (100)
	3	9 (100)	0 (0)	0 (0)	0 (0)	9 (100)
	4	10 (100)	0 (0)	0 (0)	0 (0)	10 (100)
	5	9 (90)	1 (10)	0 (0)	0 (0)	10 (100)
	6	5 (83)	1 (17)	0 (0)	0 (0)	6 (100)
	7a	3 (50)	3 (50)	0 (0)	0 (0)	6 (100)
	7b	5 (83)	1 (17)	0 (0)	0 (0)	6 (100)
Spleen	1	4 (80)	0 (0)	0 (0)	1 (20)	5 (100)
	2	3 (100)	0 (0)	0 (0)	0 (0)	3 (100)
	3	9 (100)	0 (0)	0 (0)	0 (0)	9 (100)
	4	10 (100)	0 (0)	0 (0)	0 (0)	10 (100)
	5	10 (100)	0 (0)	0 (0)	0 (0)	10 (100)
	6	4 (67)	1 (17)	1 (17)	0 (0)	6 (100)
	7a	4 (67)	2 (33)	0 (0)	0 (0)	6 (100)
	7b	6 (100)	0 (0)	0 (0)	0 (0)	6 (100)
Liver	1	4 (80)	0 (0)	0 (0)	1 (20)	5 (100)
	2	3 (100)	0 (0)	0 (0)	0 (0)	3 (100)
	3	9 (100)	0 (0)	0 (0)	0 (0)	9 (100)
	4	10 (100)	0 (0)	0 (0)	0 (0)	10 (100)
	5	10 (100)	0 (0)	0 (0)	0 (0)	10 (100)
	6	6 (100)	0 (0)	0 (0)	0 (0)	6 (100)
	7a	6 (100)	0 (0)	0 (0)	0 (0)	6 (100)
	7b	6 (100)	0 (0)	0 (0)	0 (0)	6 (100)
Kidney	1	0 (0)	0 (0)	0 (0)	0 (0)	5 (100)
	2	0 (0)	0 (0)	0 (0)	0 (0)	3 (100)
	3	0 (0)	1 (10)	0 (0)	0 (0)	9 (100)
	4	0 (0)	0 (0)	0 (0)	0 (0)	10 (100)
	5	0 (0)	0 (0)	0 (0)	0 (0)	10 (100)
	6	0 (0)	0 (0)	0 (0)	0 (0)	6 (100)
	7a	0 (0)	0 (0)	0 (0)	0 (0)	6 (100)
	7b	0 (0)	0 (0)	0 (0)	0 (0)	6 (100)

Appendix V: Mean and (95% confidence interval) for parameters of complete blood count on day 0 of natural transmission study on PMWS by experimental group.

Experimental group	N	Parameter						
		White blood cell count ($x10^9$/l)	Red blood cell count ($x10^{12}$/l)	Haemoglobin concentration (g/l)	Haematocrit value (l/l)	MCV (fl)	MCHC (g/l)	Platelet count ($x10^9$/l)
1[a]	6	12.95 (9.10 - 16.80)	6.34 (5.82 - 6.87)	121.2 (109.47 - 132.93)	0.360 (0.326 - 0.394)	56.74 (54.815 - 58.665)	336.8 (330.38 - 343.22)	292.4 (161.59 - 423.21)
2[b]	3	12.66 (7.69 - 17.64)	7.93 (7.55 - 8.31)	142.0 (135.43 - 148.57)	0.413 (0.385 - 0.442)	52.00 (48.341 - 55.659)	343.7 (337.93 - 349.40)	265.0 (81.22 - 448.78)
3[c]	10	12.76 (10.65 - 14.88)	6.55 (6.26 - 6.84)	119.1 (109.73 - 128.47)	0.359 (0.334 - 0.384)	54.61 (51.684 - 57.536)	333.5 (321.27 - 345.73)	173.6 (124.45 - 222.75)
4[d]	10	16.67 (14.34 - 18.99)	6.33 (5.94 - 6.71)	116.6 (112.45 - 120.75)	0.344 (0.330 - 0.358)	54.46 (52.566 - 56.354)	338.9 (331.53 - 346.27)	138.4 (94.22 - 182.58)
5[e]	10	14.21 (7.09 - 21.33)	6.00 (5.39 - 6.61)	116.9 (107.56 - 126.24)	0.329 (0.292 - 0.366)	55.12 (52.671 - 57.569)	357.9 (327.29 - 388.51)	396.9 (67.71 - 726.09)
7a[f]	6	9.41 (8.58 - 10.24)	7.03 (6.71 - 7.36)	141.8 (133.04 - 150.62)	0.410 (0.380 - 0.440	58.65 (54.865 - 62.435)	344.8 (341.49 - 348.18)	375.0 (15.71 - 734.29)
7b[g]	6	12.90 (10.44 - 15.36)	6.38 (6.03 - 6.72)	98.7 (86.82 - 110.51)	0.305 (0.276 - 0.334)	47.43 (44.485 - 50.381)	324.7 (313.10 - 336.23)	504.3 (161.80 - 846.86)

MCV – Mean corpuscular volume

MCHC – Mean corpuscular haemoglobin concentration

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.

[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.

[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.

[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.

[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.

[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix VI: Mean and (95% confidence interval) for parameters of complete blood count on day 8 of natural transmission study on PMWS by experimental group.

		Parameter						
Experimental group	N	White blood cell count ($x10^9$/l)	Red blood cell count ($x10^{12}$/l)	Haemoglobin concentration (g/l)	Haematocrit value (l/l)	MCV (fl)	MCHC (g/l)	Platelet count ($x10^9$/l)
1[a]	5	18.78 (0.00- 38.00)	6.11 (5.52 - 6.77)	111.8 (97.50 - 126.00)	0.330 (0.285 - 0.383)	53.88 (51.739 - 56.011)	339.5 (336.45 - 342.55)	261.3 (0.00 - 534.65)
2[b]	3	8.77 (3.15 - 14.40)	6.97 (5.64 - 8.30)	119.3 (104.16 - 134.51)	0.340 (0.297 - 0.383)	48.70 (45.414 - 51.986)	352.3 (333.52 - 371.14)	304.0 (0.00 - 968.20)
3[c]	9	18.71 (12.56 - 24.86)	6.13 (5.73 - 6.53)	106.1 (101.20 - 111.02)	0.321 (0.305 - 0.337)	52.23 (49.925 - 54.542)	332.9 (325.66 - 340.11)	329.4 (257.30 - 401.59)
4[d]	10	20.19 (17.39 – 23.00)	6.26 (5.91 - 6.61)	110.1 (106.62 - 113.58)	0.332 (0.319 - 0.345)	53.10 (51.084 - 55.116)	332.2 (327.12 - 337.28)	288.6 (197.83 - 379.37)
5[e]	10	18.65 (14.95 - 22.36)	5.74 (5.29 - 6.19)	101.3 (93.25 - 109.35)	0.303 (0.281 - 0.325)	53.19 (51.378 - 55.002)	332.6 (315.06 - 350.14)	357.8 (258.07 - 457.53)
7a[f]	6	14.97 (10.62 - 19.33)	7.21 (6.89 - 7.54)	138.8 (131.34 - 146.33)	0.387 (0.366 - 0.407)	53.35 (50.608 - 56.092)	361.5 (354.94 - 368.06)	402.0 (146.86 - 657.14)
7b[g]	6	17.88 (14.32 - 21.45)	6.89 (6.43 - 7.35)	103.0 (92.09 - 113.91)	0.303 (0.276 - 0.330)	43.95 (40.975 - 46.925)	339.8 (331.35 - 348.32)	517.3 (335.73 - 698.93)

MCV – Mean corpuscular volume

MCHC – Mean corpuscular haemoglobin concentration

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.

[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.

[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.

[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.

[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.

[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix VII: Mean and (95% confidence interval) for parameters of complete blood count on day 11 of natural transmission study on PMWS by experimental group.

		Parameter						
Experimental group	N	White blood cell count ($x10^9$/l)	Red blood cell count ($x10^{12}$/l)	Haemoglobin concentration (g/l)	Haematocrit value (l/l)	MCV (fl)	MCHC (g/l)	Platelet count ($x10^9$/l)
1[a]	5	11.37 (8.63 - 14.11)	6.14 (5.43 - 6.86)	110.0 (95.36 - 124.64)	0.314 (0.276 - 0.352)	51.30 (50.165 - 52.435)	348.0 (341.26 - 354.74)	466.6 (390.65 - 542.55)
2[b]	3	11.62 (9.45 - 13.80)	7.37 (6.74 - 8.01)	124.0 (105.25 - 142.75)	0.350 (0.300 - 0.399)	47.97 (44.861 - 51.073)	351.0 (346.70 - 355.30)	460.0 (322.16 - 597.84)
3[c]	9	20.46 (17.77 - 23.15)	5.91 (5.43 - 6.40)	99.4 (93.74 - 105.15)	0.297 (0.280 - 0.313)	50.52 (48.256 - 52.788)	334.3 (325.66 - 343.00)	303.8 (199.24 - 408.31)
4[d]	10	19.99 (18.34 - 21.65)	6.01 (5.70 - 6.31)	104.6 (100.87 - 108.33)	0.305 (0.292 - 0.318)	50.99 (49.276 - 52.704)	341.9 (337.03 - 346.77)	466.4 (363.32 - 569.48)
5[e]	10	19.20 (16.35 - 22.04)	5.91 (5.52 - 6.31)	101.9 (97.05 - 106.75)	0.309 (0.290 - 0.328)	52.48 (50.842 - 54.120)	330.0 (313.62 - 346.38)	398.6 (282.00 - 515.20)
7a[f]	6	13.24 (11.64 - 14.83)	6.76 (6.37 - 7.14)	129.7 (122.50 - 136.84)	0.347 (0.332 - 0.361)	51.82 (48.838 - 54.795)	371.0 (364.33 - 377.67)	612.2 (206.90 - 1017.43)
7b[g]	6	17.80 (15.97 - 19.63)	6.75 (6.30 - 7.19)	99.8 (90.94 - 108.72)	0.293 (0.274 - 0.313)	43.65 (40.298 - 47.002)	339.5 (329.37 - 349.63)	521.5 (395.71 - 647.29)

MCV – Mean corpuscular volume
MCHC – Mean corpuscular haemoglobin concentration
[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix VIII: Mean and (95% confidence interval) for parameters of complete blood count on day 29 of natural transmission study on PMWS by experimental group.

		Parameter						
Experimental group	N	White blood cell count ($x10^9$/l)	Red blood cell count ($x10^{12}$/l)	Haemoglobin concentration (g/l)	Haematocrit value (l/l)	MCV (fl)	MCHC (g/l)	Platelet count ($x10^9$/l)
2[a]	3	18.44 (7.79 - 29.10)	7.75 (7.27 - 8.23)	131.0 (116.10 - 145.90)	0.400 (0.375 - 0.425)	51.53 (48.741 - 54.325)	328.3 (314.21 - 342.46)	418.3 (218.30 - 618.37)
3[b]	8	30.93 (18.73 - 43.14)	5.84 (5.34 - 6.34)	101.1 (93.70 - 108.55)	0.333 (0.308 - 0.357)	57.19 (53.887 - 60.488)	303.3 (294.39 - 312.11)	245.1 (178.35 - 311.90)
4[c]	10	23.68 (20.76 - 26.60)	5.82 (5.50 - 6.13)	105.1 (100.41 - 109.79)	0.336 (0.323 - 0.349)	58.03 (56.372 - 59.688)	311.8 (307.76 - 315.84)	308.6 (202.36 - 414.84)
5[d]	10	19.09 (16.91 - 21.27)	5.67 (5.37 - 5.96)	102.5 (95.62 - 109.38)	0.334 (0.311 - 0.357)	58.90 (57.105 - 60.695)	306.4 (299.73 - 313.07)	261.1 (160.05 - 362.15)
7a[e]	6	17.76 (14.39 - 21.13)	5.67 (5.34 – 6.00)	98.3 (92.91 - 103.75)	0.272 (0.254 - 0.290)	47.85 (45.072 - 50.628)	363.8 (347.83 - 379.84)	616.0 (299.15 - 932.85)
7b[f]	6	15.65 (13.82 - 17.47)	6.32 (5.94 - 6.70)	93.7 (88.33 - 99.00)	0.275 (0.264 - 0.286)	43.73 (41.741 - 45.725)	339.0 (328.46 - 349.54)	681.0 (585.21 - 776.79)

MCV – Mean corpuscular volume

MCHC – Mean corpuscular haemoglobin concentration

[a] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.

[b] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.

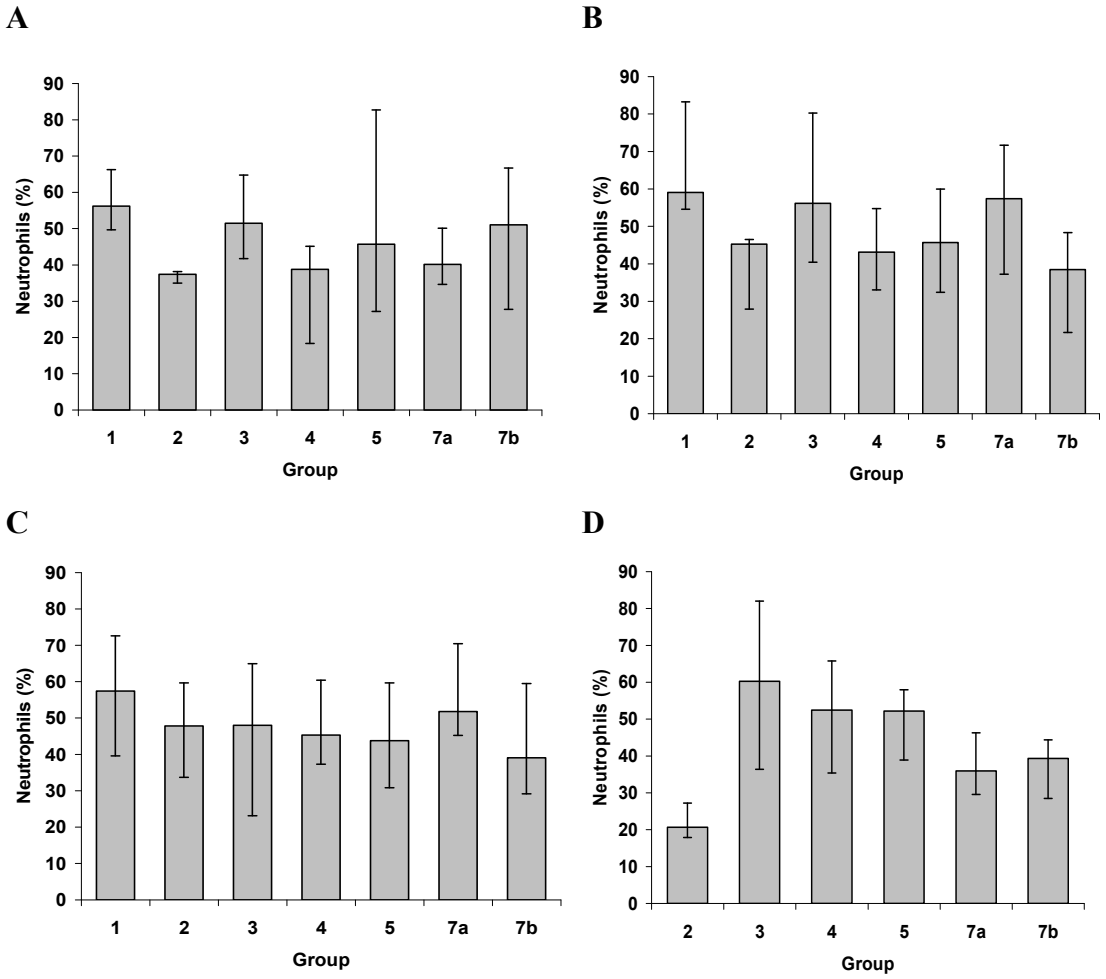
[c] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.

[d] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.

[e] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

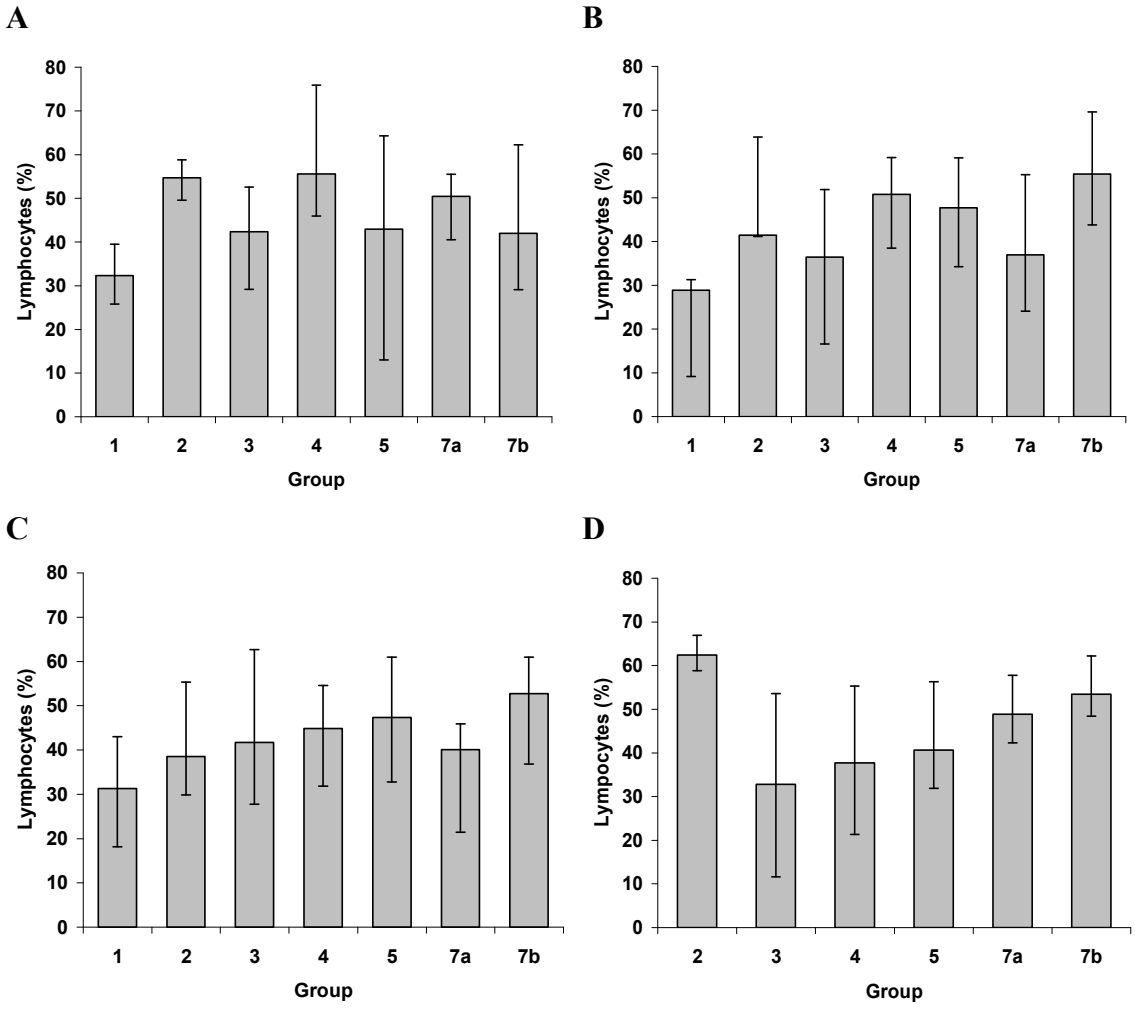
[f] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix IX: Histograms of the median within-group percentage (%) of blood neutrophils.



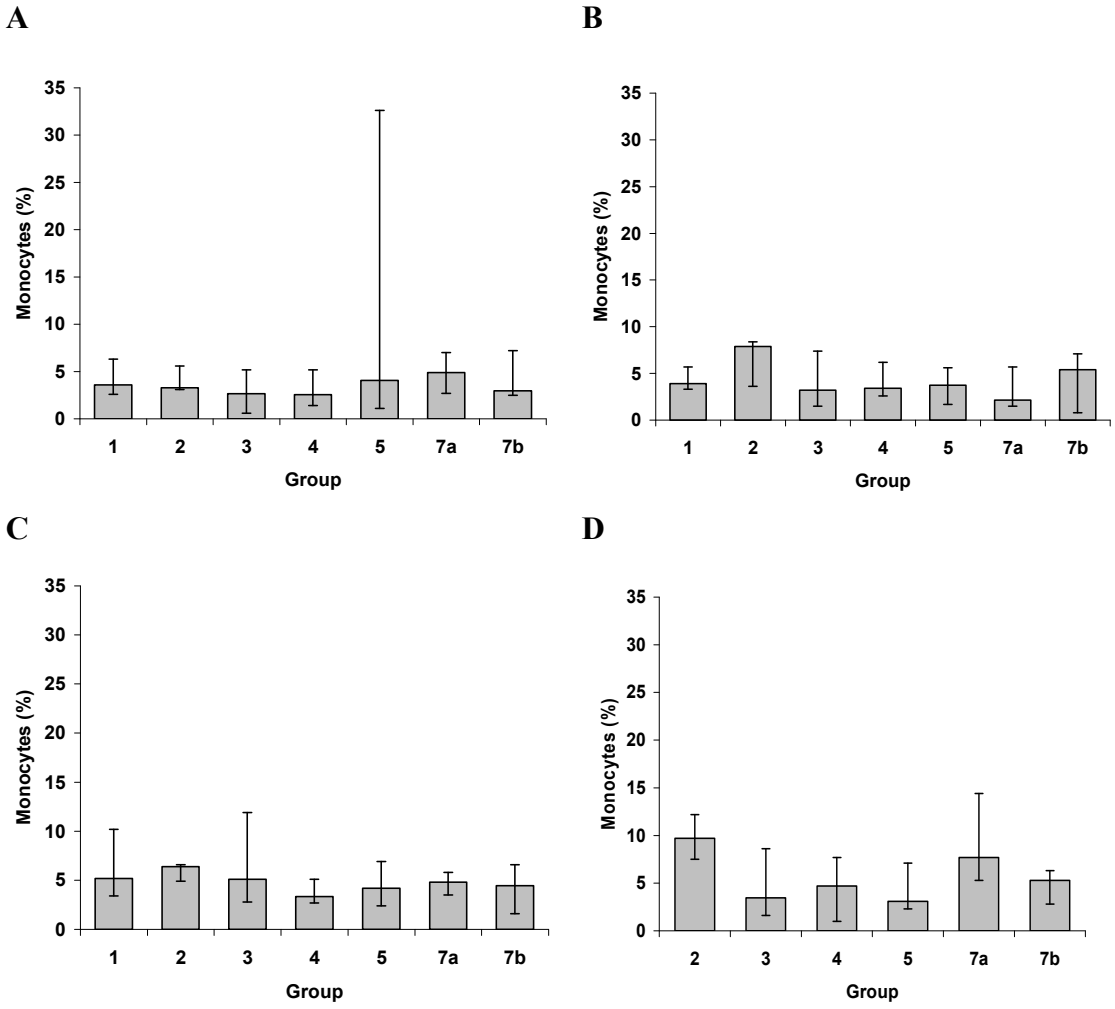
Histograms of the median within-group percentage (%) of blood neutrophils measured in pigs during a natural transmission study on PMWS, stratified by sampling day post-exposure. **A, B, C,** and **D** illustrate results at day 0, 8, 11, and 29 post-exposure, respectively. At day 0: Group 1 (n = 5, PCV2-negative, PMWS-negative, 4-week-old), Group 2 (n = 3, PCV2-negative, PMWS-negative, 13-week-old), Group 3 (n = 9, PCV2-positive, PMWS-negative, 4-week-old), Group 4 (n = 10, PCV2-positive, PMWS-negative, 12-week-old), Group 5 (n = 10, PCV2-positive, PMWS-negative, 4-week-old), Group 7a (n = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (n = 6, PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Error bars represent range (minimum to maximum values).

Appendix X: Histograms of the median within-group percentage (%) of blood lymphocytes.



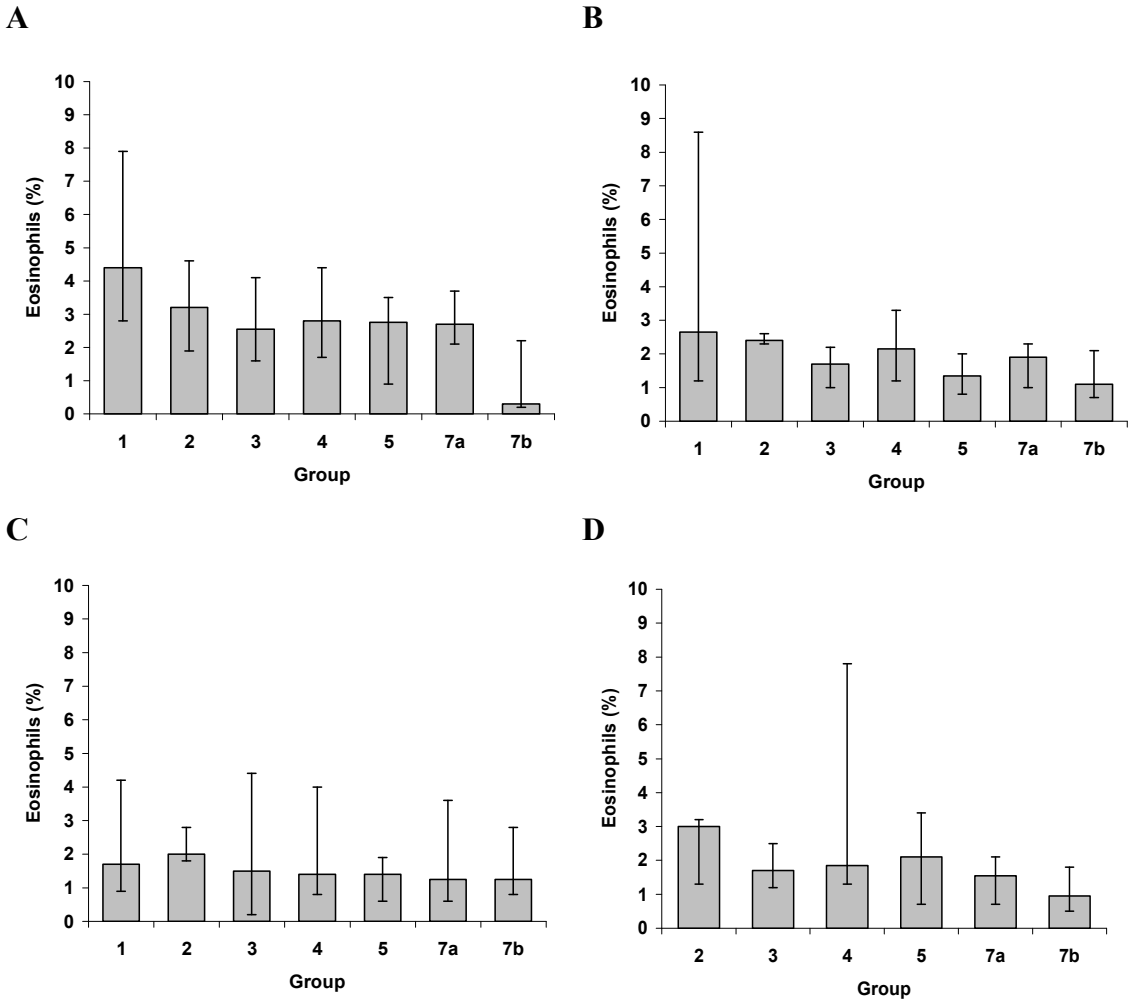
Histograms of the median within-group percentage (%) of blood lymphocytes measured in pigs during a natural transmission study on PMWS, stratified by sampling day post-exposure. **A, B, C,** and **D** illustrate results at day 0, 8, 11, and 29 post-exposure, respectively. At day 0: Group 1 (n = 5, PCV2-negative, PMWS-negative, 4-week-old), Group 2 (n = 3, PCV2-negative, PMWS-negative, 13-week-old), Group 3 (n = 9, PCV2-positive, PMWS-negative, 4-week-old), Group 4 (n = 10, PCV2-positive, PMWS-negative, 12-week-old), Group 5 (n = 10, PCV2-positive, PMWS-negative, 4-week-old), Group 7a (n = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (n = 6, PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Error bars represent range (minimum to maximum values).

Appendix XI: Histograms of the median within-group percentage (%) of blood monocytes.



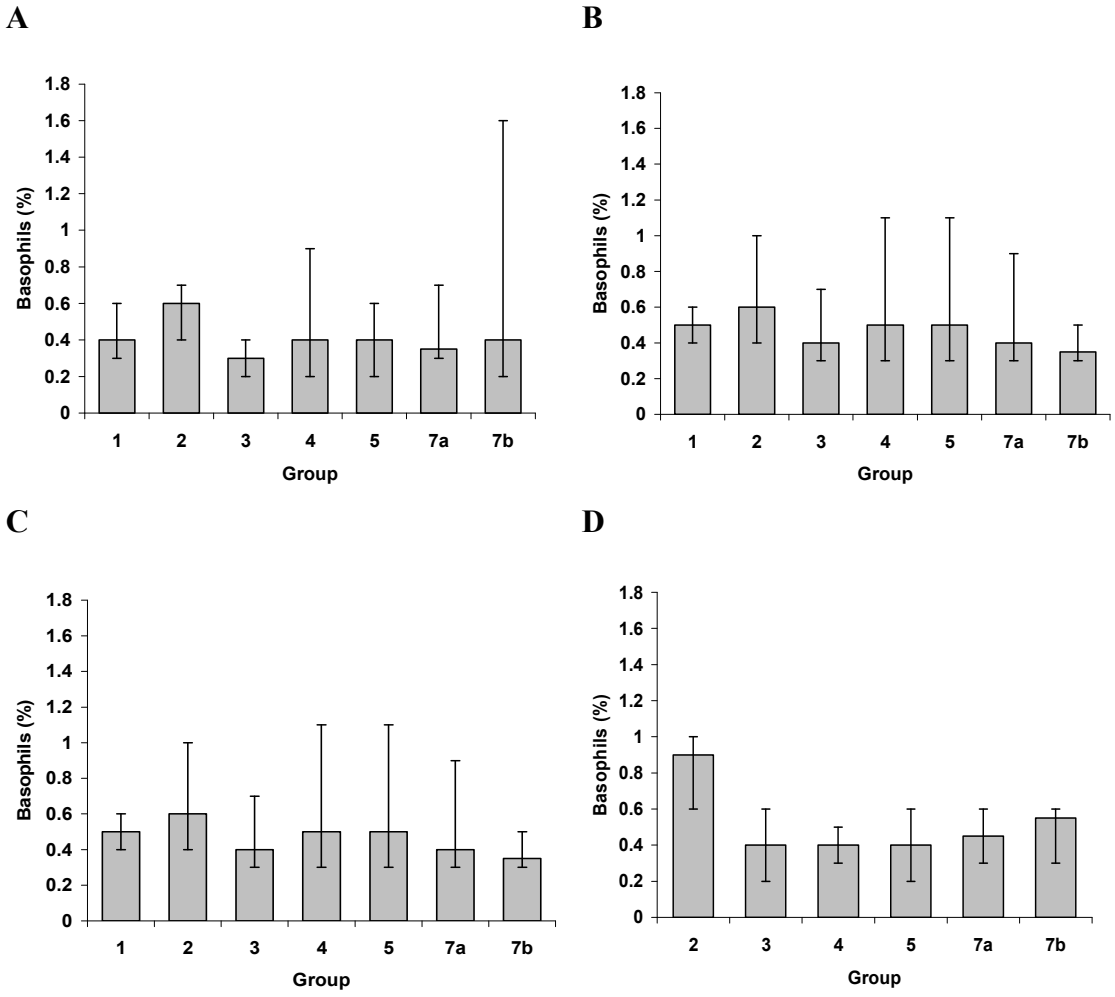
Histograms of the median within-group percentage (%) of blood monocytes measured in pigs during a natural transmission study on PMWS, stratified by sampling day post-exposure. **A, B, C,** and **D** illustrate results at day 0, 8, 11, and 29 post-exposure, respectively. At day 0: Group 1 (n = 5, PCV2-negative, PMWS-negative, 4-week-old), Group 2 (n = 3, PCV2-negative, PMWS-negative, 13-week-old), Group 3 (n = 9, PCV2-positive, PMWS-negative, 4-week-old), Group 4 (n = 10, PCV2-positive, PMWS-negative, 12-week-old), Group 5 (n = 10, PCV2-positive, PMWS-negative, 4-week-old), Group 7a (n = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (n = 6, PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Error bars represent range (minimum to maximum values).

Appendix XII: Histograms of the median within-group percentage (%) of blood eosinophils.



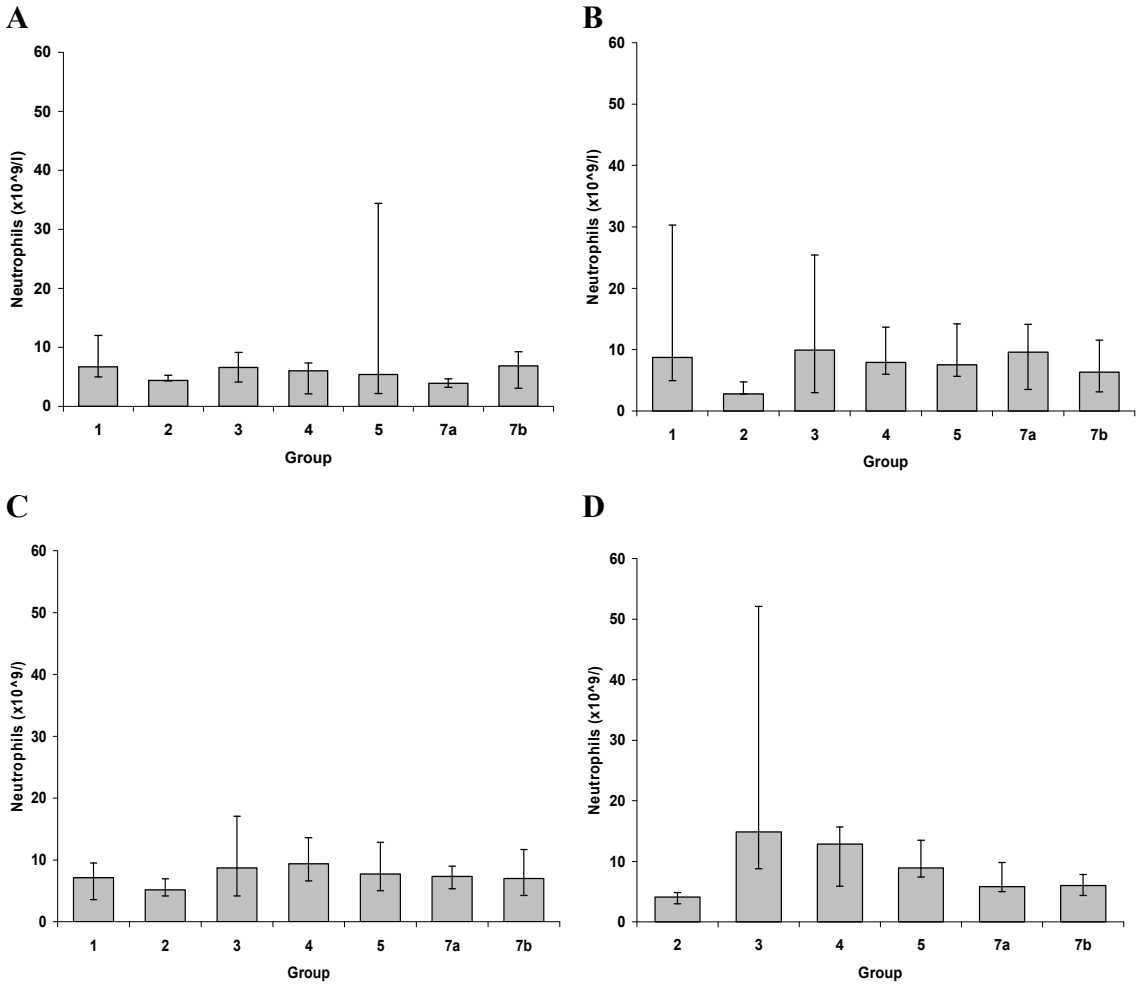
Histograms of the median within-group percentage (%) of blood eosinophils measured in pigs during a natural transmission study on PMWS, stratified by sampling day post-exposure. **A, B, C,** and **D** illustrate results at day 0, 8, 11, and 29 post-exposure, respectively. At day 0: Group 1 (n = 5, PCV2-negative, PMWS-negative, 4-week-old), Group 2 (n = 3, PCV2-negative, PMWS-negative, 13-week-old), Group 3 (n = 9, PCV2-positive, PMWS-negative, 4-week-old), Group 4 (n = 10, PCV2-positive, PMWS-negative, 12-week-old), Group 5 (n = 10, PCV2-positive, PMWS-negative, 4-week-old), Group 7a (n = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (n = 6, PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Error bars represent range (minimum to maximum values).

Appendix XIII: Histograms of the median within-group percentage (%) of blood basophils.



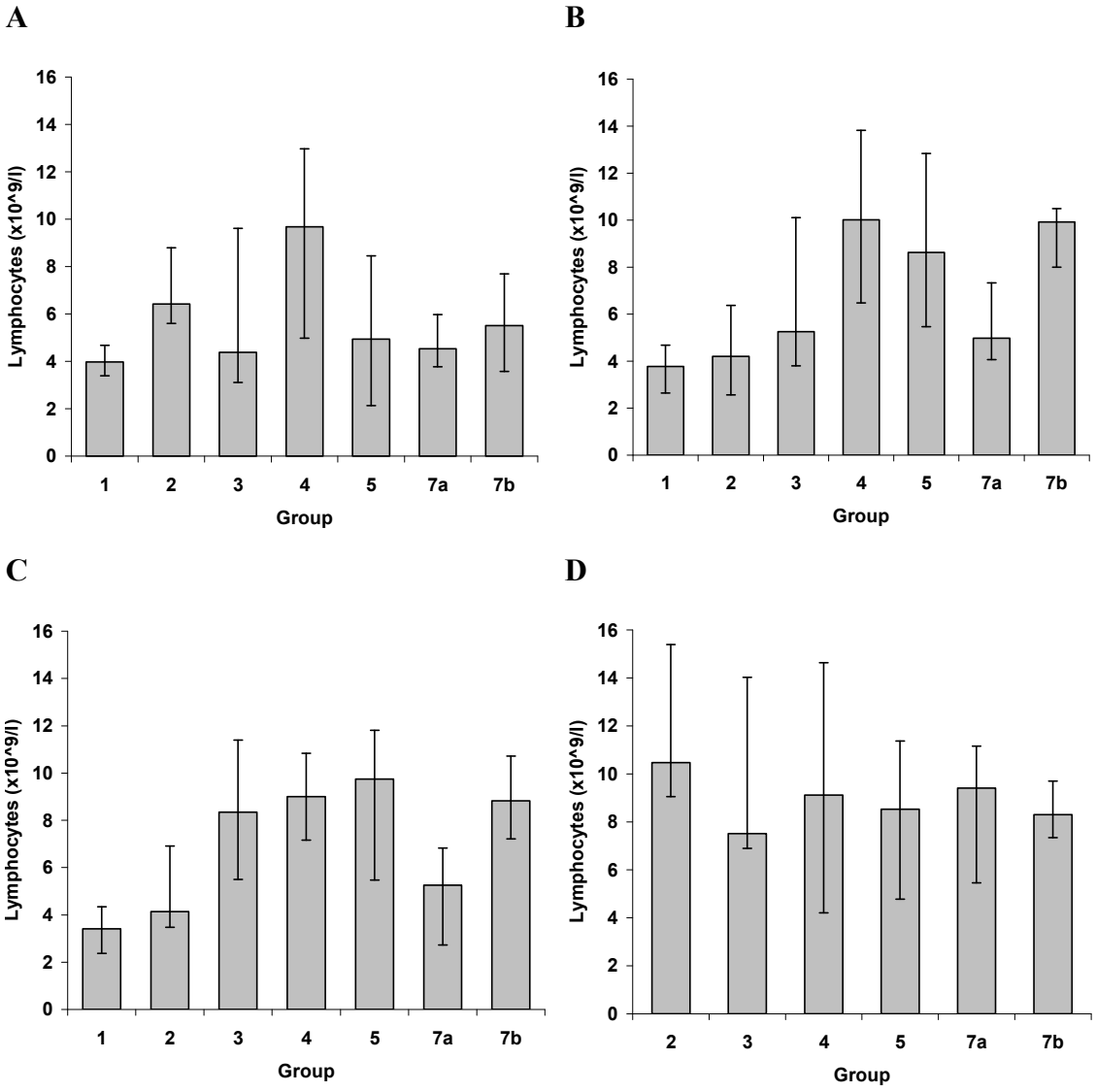
Histograms of the median within-group percentage (%) of blood basophils measured in pigs during a natural transmission study on PMWS, stratified by sampling day post-exposure. **A, B, C,** and **D** illustrate results at day 0, 8, 11, and 29 post-exposure, respectively. At day 0: Group 1 (n = 5, PCV2-negative, PMWS-negative, 4-week-old), Group 2 (n = 3, PCV2-negative, PMWS-negative, 13-week-old), Group 3 (n = 9, PCV2-positive, PMWS-negative, 4-week-old), Group 4 (n = 10, PCV2-positive, PMWS-negative, 12-week-old), Group 5 (n = 10, PCV2-positive, PMWS-negative, 4-week-old), Group 7a (n = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (n = 6, PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Error bars represent range (minimum to maximum values).

Appendix XIV: Histograms of the median within-group values of blood neutrophil counts (x10^9/l).



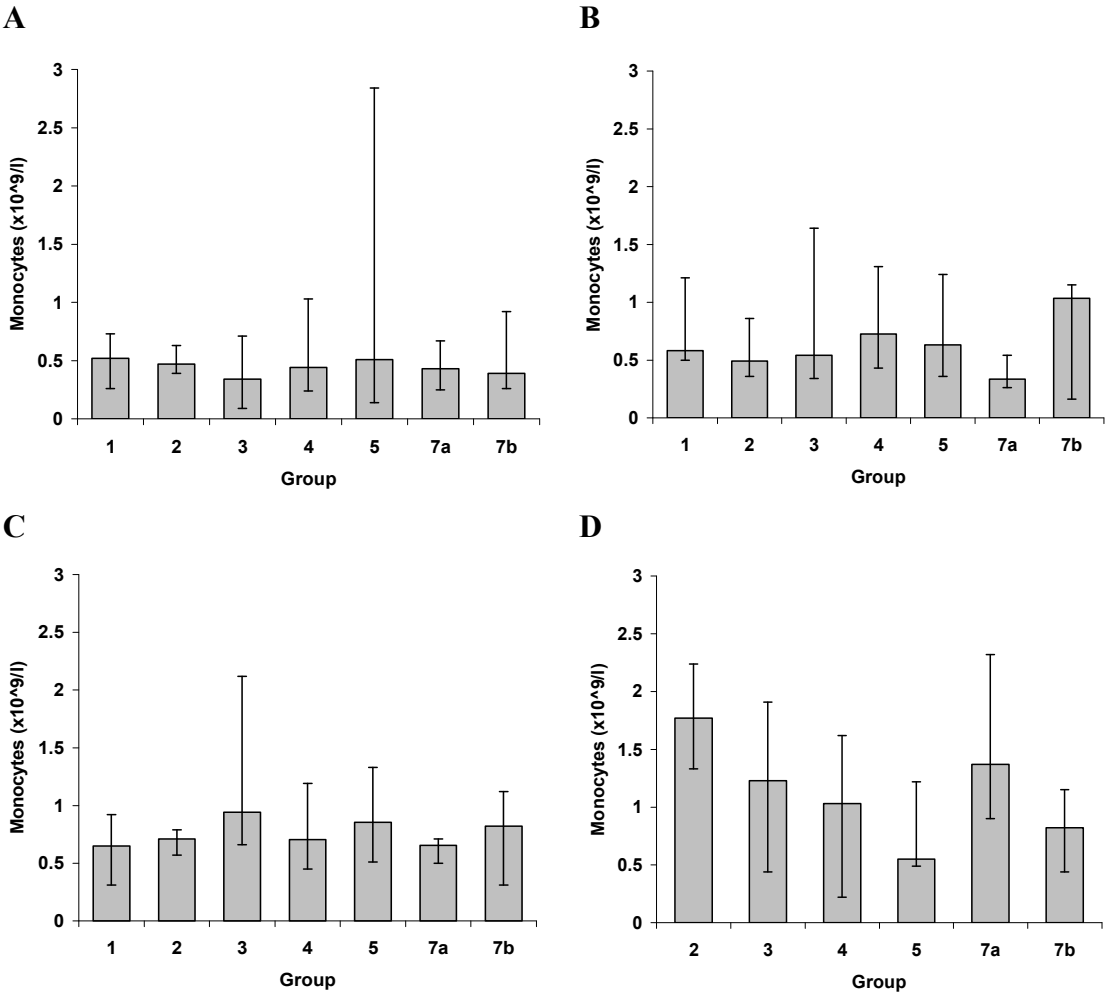
Histograms of the median within-group values of blood neutrophil counts (x10^9/l) measured in pigs during a natural transmission study on PMWS stratified by sampling day post-exposure. **A, B, C,** and **D** illustrate results at day 0, 8, 11, and 29 post-exposure, respectively. At day 0: Group 1 (*n* = 5, PCV2-negative, PMWS-negative, 4-week-old), Group 2 (*n* = 3, PCV2-negative, PMWS-negative, 13-week-old), Group 3 (*n* = 9, PCV2-positive, PMWS-negative, 4-week-old), Group 4 (*n* = 10, PCV2-positive, PMWS-negative, 12-week-old), Group 5 (*n* = 10, PCV2-positive, PMWS-negative, 4-week-old), Group 7a (*n* = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (*n* = 6, PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Error bars represent range (minimum to maximum values).

Appendix XV: Histograms of the median within-group values of blood lymphocyte counts (x10^9/l).



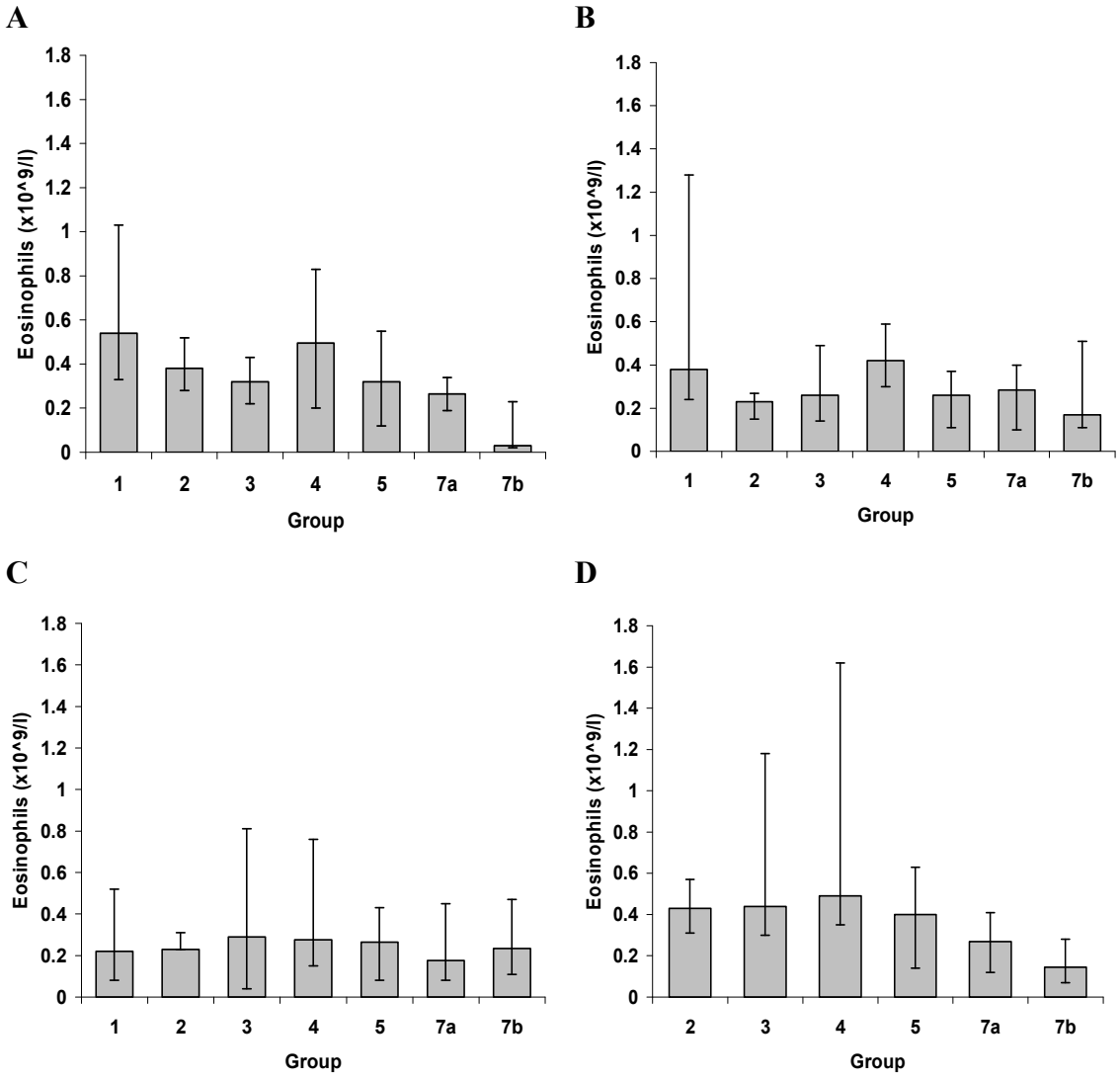
Histograms of the median within-group values of blood lymphocyte counts (x10^9/l) measured in pigs during a natural transmission study on PMWS stratified by sampling day post-exposure. **A, B, C,** and **D** illustrate results at day 0, 8, 11, and 29 post-exposure, respectively. At day 0: Group 1 (*n* = 5, PCV2-negative, PMWS-negative, 4-week-old), Group 2 (*n* = 3, PCV2-negative, PMWS-negative, 13-week-old), Group 3 (*n* = 9, PCV2-positive, PMWS-negative, 4-week-old), Group 4 (*n* = 10, PCV2-positive, PMWS-negative, 12-week-old), Group 5 (*n* = 10, PCV2-positive, PMWS-negative, 4-week-old), Group 7a (*n* = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (*n* = 6, PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Error bars represent range (minimum to maximum values).

Appendix XVI: Histograms of the median within-group values of blood monocyte counts (x10^9/l).



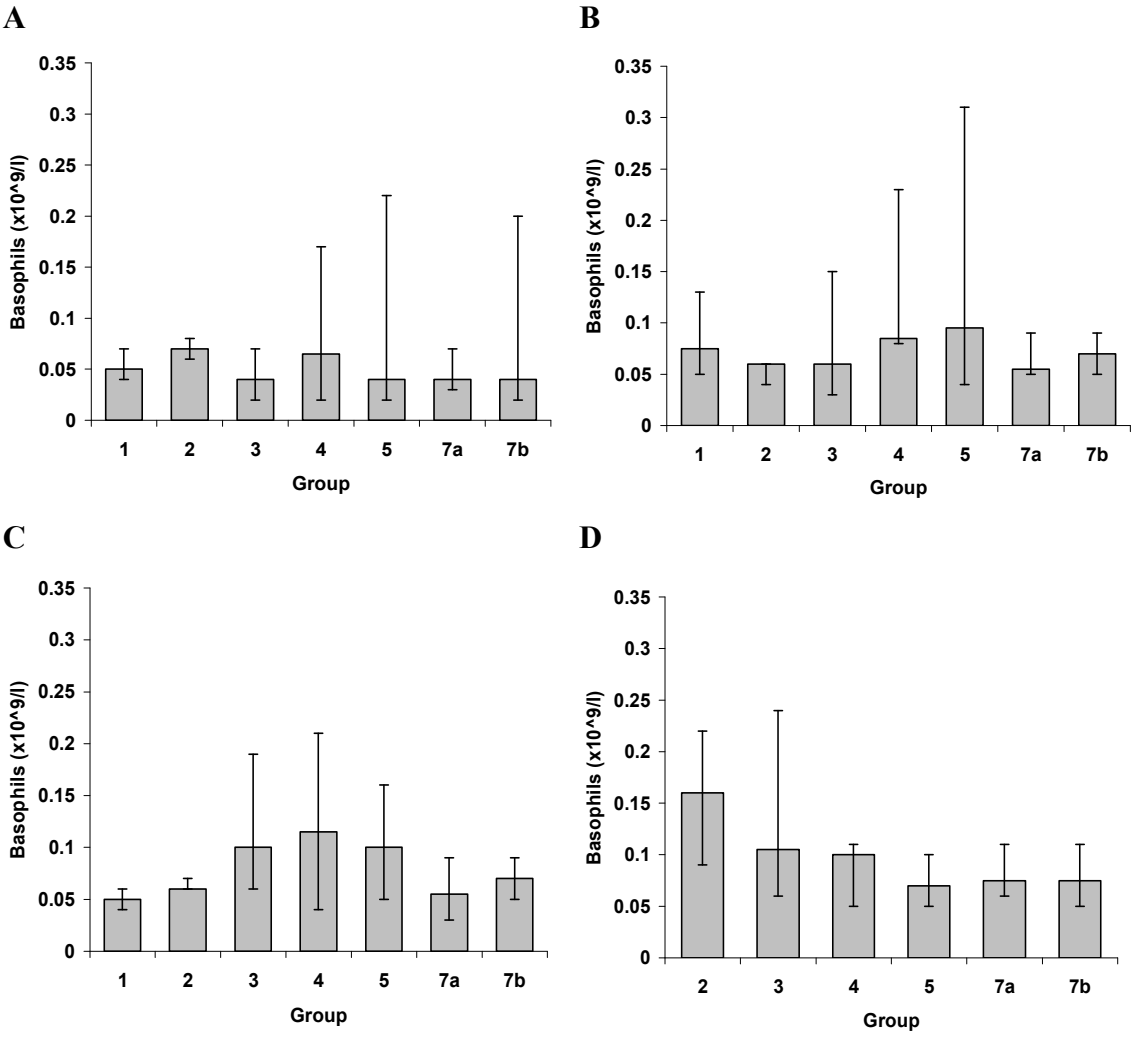
Histograms of the median within-group values of blood monocyte counts (x10^9/l) measured in pigs during a natural transmission study on PMWS stratified by sampling day post-exposure. **A, B, C,** and **D** illustrate results at day 0, 8, 11, and 29 post-exposure, respectively. At day 0: Group 1 (*n* = 5, PCV2-negative, PMWS-negative, 4-week-old), Group 2 (*n* = 3, PCV2-negative, PMWS-negative, 13-week-old), Group 3 (*n* = 9, PCV2-positive, PMWS-negative, 4-week-old), Group 4 (*n* = 10, PCV2-positive, PMWS-negative, 12-week-old), Group 5 (*n* = 10, PCV2-positive, PMWS-negative, 4-week-old), Group 7a (*n* = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (*n* = 6, PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Error bars represent range (minimum to maximum values).

Appendix XVII: Histograms of the median within-group values of blood eosinophil counts (x10^9/l).



Histograms of the median within-group values of blood eosinophil counts (x10^9/l) measured in pigs during a natural transmission study on PMWS stratified by sampling day post-exposure. **A, B, C,** and **D** illustrate results at day 0, 8, 11, and 29 post-exposure, respectively. At day 0: Group 1 (*n*=5, PCV2-negative, PMWS-negative, 4-week-old), Group 2 (*n* = 3, PCV2-negative, PMWS-negative, 13-week-old), Group 3 (*n* = 9, PCV2-positive, PMWS-negative, 4-week-old), Group 4 (*n* = 10, PCV2-positive, PMWS-negative, 12-week-old), Group 5 (*n* = 10, PCV2-positive, PMWS-negative, 4-week-old), Group 7a (*n* = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (*n* = 6, PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Error bars represent range (minimum to maximum values).

Appendix XVIII: Histograms of the median within-group values of blood basophil counts (x10^9/l).



Histograms of the median within-group values of blood basophil counts (x10^9/l) measured in pigs during a natural transmission study on PMWS stratified by sampling day post-exposure. **A, B, C,** and **D** illustrate results at day 0, 8, 11, and 29 post-exposure, respectively. At day 0: Group 1 (*n* = 5, PCV2-negative, PMWS-negative, 4-week-old), Group 2 (*n* = 3, PCV2-negative, PMWS-negative, 13-week-old), Group 3 (*n* = 9, PCV2-positive, PMWS-negative, 4-week-old), Group 4 (*n* = 10, PCV2-positive, PMWS-negative, 12-week-old), Group 5 (*n* = 10, PCV2-positive, PMWS-negative, 4-week-old), Group 7a (*n* = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (*n* = 6, PCV2-positive, PMWS-negative, 4-week-old). Experimental Groups 1 to 4 were directly exposed to PMWS-affected pigs for 56 days, with the exception of Group 1 animals which died before day 29 post-exposure. Group 5 served as on-site control group. Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Error bars represent range (minimum to maximum values).

Appendix XIX: Minimum, maximum and quartiles for absolute values of total leukocytes on study day 0 of a natural transmission study on PMWS in cell type and by experimental group.

Leukocyte	Experimental group	N	Minimum	Percentile			Maximum
				25th	50th	75th	
Neutrophils (x10^9/l)	1[a]	6	4.99	6.47	6.69	8.16	12.04
	2[b]	3	4.32	4.32	4.40	5.23	5.23
	3[c]	10	4.11	5.55	6.59	7.62	9.15
	4[d]	10	2.11	5.11	6.03	7.11	7.35
	5[e]	10	2.16	3.58	5.40	6.74	34.40
	7a[f]	6	3.22	3.28	3.91	4.05	4.67
	7b[g]	6	3.05	4.38	6.86	8.21	9.26
Lymphocytes (x10^9/l)	1	6	3.39	3.73	3.97	4.27	4.68
	2	3	5.60	5.60	6.43	8.79	8.79
	3	10	3.11	3.80	4.39	7.01	9.61
	4	10	4.98	8.31	9.68	10.80	12.97
	5	10	2.13	3.80	4.94	6.17	8.45
	7a	6	3.78	4.27	4.53	5.17	5.98
	7b	6	3.58	4.08	5.51	7.20	7.69
Monocytes (x10^9/l)	1	6	0.26	0.41	0.52	0.65	0.73
	2	3	0.39	0.39	0.47	0.63	0.63
	3	10	0.09	0.19	0.34	0.56	0.71
	4	10	0.24	0.29	0.44	0.68	1.03
	5	10	0.14	0.44	0.51	0.52	2.84
	7a	6	0.25	0.34	0.43	0.51	0.67
	7b	6	0.26	0.32	0.39	0.79	0.92
Eosinophils (x10^9/l)	1	6	0.33	0.51	0.54	0.68	1.03
	2	3	0.28	0.28	0.38	0.52	0.52
	3	10	0.22	0.28	0.32	0.39	0.43
	4	10	0.20	0.40	0.49	0.57	0.83
	5	10	0.12	0.16	0.32	0.39	0.55
	7a	6	0.19	0.20	0.26	0.31	0.34
	7b	6	0.02	0.03	0.03	0.12	0.23
Basophils (x10^9/l)	1	6	0.04	0.04	0.05	0.07	0.07
	2	3	0.06	0.06	0.07	0.08	0.08
	3	10	0.02	0.03	0.04	0.05	0.07
	4	10	0.02	0.05	0.06	0.08	0.17
	5	10	0.02	0.03	0.04	0.06	0.22
	7a	6	0.03	0.03	0.04	0.04	0.07
	7b	6	0.02	0.03	0.04	0.09	0.20

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix XX: Minimum, maximum and quartiles for absolute values of total leukocytes on study day 8 of a natural transmission study on PMWS in cell type and by experimental group.

Leukocyte	Experimental group	N	Minimum	Percentile			Maximum
				25th	50th	75th	
Neutrophils (x10^9/l)	1[a]	5	4.96	6.57	8.72	19.78	30.30
	2[b]	3	2.79	2.79	2.79	4.74	4.74
	3[c]	9	3.00	6.84	9.94	11.23	25.42
	4[d]	10	6.01	6.81	7.90	9.06	13.66
	5[e]	10	5.65	6.58	7.53	9.75	14.22
	7a[f]	6	3.49	4.02	9.57	11.92	14.12
	7b[g]	6	3.12	4.79	6.32	9.46	11.55
Lymphocytes (x10^9/l)	1	5	2.64	3.00	3.78	4.44	4.68
	2	3	2.56	2.56	4.20	6.37	6.37
	3	9	3.80	4.36	5.26	7.95	10.11
	4	10	6.48	9.61	10.02	11.18	13.83
	5	10	5.47	6.15	8.63	10.44	12.84
	7a	6	4.07	4.74	4.97	5.75	7.34
	7b	6	8.00	9.54	9.91	10.46	10.50
Monocytes (x10^9/l)	1	5	0.50	0.52	0.58	0.91	1.21
	2	3	0.36	0.36	0.49	0.86	0.86
	3	9	0.34	0.47	0.54	0.74	1.64
	4	10	0.43	0.52	0.72	0.88	1.31
	5	10	0.36	0.44	0.63	0.76	1.24
	7a	6	0.26	0.26	0.33	0.48	0.54
	7b	6	0.16	0.39	1.03	1.11	1.15
Eosinophils (x10^9/l)	1	5	0.24	0.28	0.38	0.86	1.28
	2	3	0.15	0.15	0.23	0.27	0.27
	3	9	0.14	0.19	0.26	0.37	0.49
	4	10	0.30	0.34	0.42	0.48	0.59
	5	10	0.11	0.17	0.26	0.33	0.37
	7a	6	0.10	0.16	0.28	0.38	0.40
	7b	6	0.11	0.14	0.17	0.31	0.51
Basophils (x10^9/l)	1	5	0.05	0.05	0.07	0.11	0.13
	2	3	0.04	0.04	0.06	0.06	0.06
	3	9	0.03	0.05	0.06	0.11	0.15
	4	10	0.08	0.08	0.08	0.13	0.23
	5	10	0.04	0.07	0.09	0.13	0.31
	7a	6	0.05	0.05	0.05	0.06	0.09
	7b	6	0.05	0.06	0.07	0.08	0.09

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix XXI: Minimum, maximum and quartiles for absolute values of total leukocytes on study day 11 of a natural transmission study on PMWS in cell type and by experimental group.

Leukocyte	Experimental group	N	Minimum	Percentile			Maximum
				25th	50th	75th	
Neutrophils ($x10^9$/l)	1[a]	5	3.57	3.80	7.14	7.78	9.51
	2[b]	3	4.20	4.20	5.14	6.95	6.95
	3[c]	9	4.20	7.78	8.69	11.55	17.04
	4[d]	10	6.61	7.71	9.40	10.71	13.60
	5[e]	10	5.02	6.43	7.73	9.97	12.83
	7a[f]	6	5.34	5.46	7.34	8.13	8.99
	7b[g]	6	4.28	6.09	6.99	8.52	11.67
Lymphocytes ($x10^9$/l)	1	5	2.37	2.81	3.41	3.87	4.34
	2	3	3.47	3.47	4.14	6.91	6.91
	3	9	5.50	7.30	8.35	9.11	11.40
	4	10	7.16	8.32	8.99	9.60	10.83
	5	10	5.47	7.45	9.74	10.96	11.81
	7a	6	2.73	4.40	5.26	5.43	6.83
	7b	6	7.22	8.46	8.82	10.21	10.72
Monocytes ($x10^9$/l)	1	5	0.31	0.48	0.65	0.70	0.92
	2	3	0.57	0.57	0.71	0.79	0.79
	3	9	0.66	0.84	0.94	1.75	2.12
	4	10	0.45	0.57	0.70	0.79	1.19
	5	10	0.51	0.63	0.85	1.03	1.33
	7a	6	0.50	0.51	0.65	0.69	0.71
	7b	6	0.31	0.67	0.82	0.96	1.12
Eosinophils ($x10^9$/l)	1	5	0.08	0.08	0.22	0.22	0.52
	2	3	0.23	0.23	0.23	0.31	0.31
	3	9	0.04	0.17	0.29	0.45	0.81
	4	10	0.15	0.25	0.27	0.44	0.76
	5	10	0.08	0.15	0.26	0.31	0.43
	7a	6	0.08	0.10	0.17	0.21	0.45
	7b	6	0.11	0.16	0.23	0.29	0.47
Basophils ($x10^9$/l)	1	5	0.04	0.04	0.05	0.06	0.06
	2	3	0.06	0.06	0.06	0.07	0.07
	3	9	0.06	0.07	0.10	0.11	0.19
	4	10	0.04	0.08	0.11	0.15	0.21
	5	10	0.05	0.08	0.10	0.16	0.16
	7a	6	0.03	0.04	0.05	0.07	0.09
	7b	6	0.05	0.06	0.07	0.08	0.09

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix XXII: Minimum, maximum and quartiles for absolute values of total leukocytes on study day 29 of a natural transmission study on PMWS in cell type and by experimental group.

Leukocyte	Experimental group	N	Minimum	Percentile			Maximum
				25th	50th	75th	
Neutrophils (x10^9/l)	2[a]	3	3.00	3.00	4.11	4.85	4.85
	3[b]	8	8.77	11.05	14.84	22.43	52.09
	4[c]	10	5.92	11.39	12.85	13.78	15.66
	5[d]	10	7.40	7.86	8.90	10.64	13.49
	7a[e]	6	5.03	5.49	5.81	7.83	9.81
	7b[f]	6	4.35	4.64	6.00	6.97	7.85
Lymphocytes (x10^9/l)	2	3	9.05	9.05	10.48	15.39	15.39
	3	8	6.89	7.04	7.51	11.11	14.02
	4	10	4.21	7.77	9.11	10.18	14.63
	5	10	4.78	6.16	8.53	9.39	11.37
	7a	6	5.46	7.52	9.41	9.95	11.16
	7b	6	7.34	7.59	8.30	9.49	9.70
Monocytes (x10^9/l)	2	3	1.33	1.33	1.77	2.24	2.24
	3	8	0.44	0.57	1.23	1.69	1.91
	4	10	0.22	0.80	1.03	1.51	1.62
	5	10	0.49	0.52	0.55	0.89	1.22
	7a	6	0.90	0.95	1.37	1.86	2.32
	7b	6	0.44	0.44	0.82	1.04	1.15
Eosinophils (x10^9/l)	2	3	0.31	0.31	0.43	0.57	0.57
	3	8	0.30	0.36	0.44	0.54	1.18
	4	10	0.35	0.38	0.49	0.75	1.62
	5	10	0.14	0.30	0.40	0.48	0.63
	7a	6	0.12	0.15	0.27	0.34	0.41
	7b	7	0.07	0.12	0.14	0.26	0.28
Basophils (x10^9/l)	2	3	0.09	0.09	0.16	0.22	0.22
	3	8	0.06	0.09	0.10	0.11	0.24
	4	10	0.05	0.08	0.10	0.11	0.11
	5	10	0.05	0.06	0.07	0.08	0.10
	7a	6	0.06	0.06	0.07	0.08	0.11
	7b	7	0.05	0.06	0.07	0.09	0.11

[a] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[e] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[f] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix XXIII: Minimum, maximum and quartiles for percentages of total leukocytes on study day 0 of a natural transmission study on PMWS in cell type and by experimental group.

Leukocyte	Experimental group	N	Minimum	Percentile			Maximum
				25th	50th	75th	
Neutrophils (%)	1[a]	6	49.7	56.0	56.2	62.5	66.3
	2[b]	3	35.0	35.0	37.4	38.2	38.2
	3[c]	10	41.7	44.3	51.4	60.0	64.8
	4[d]	10	18.4	30.3	38.8	40.1	45.1
	5[e]	10	27.2	40.6	45.8	56.8	82.7
	7a[f]	6	34.6	37.6	40.2	42.7	50.1
	7b[g]	6	27.8	35.2	51.1	65.4	66.7
Lymphocytes (%)	1	6	25.8	25.9	32.3	35.9	39.5
	2	3	49.6	49.6	54.7	58.8	58.8
	3	10	29.2	31.3	42.4	48.6	52.6
	4	10	45.9	52.9	55.6	60.7	75.9
	5	10	13.0	31.1	42.9	51.4	64.3
	7a	6	40.5	47.2	50.5	55.5	55.5
	7b	6	29.1	29.6	42.0	57.8	62.2
Monocytes (%)	1	6	2.6	3.2	3.6	4.4	6.3
	2	3	3.1	3.1	3.3	5.6	5.6
	3	10	0.6	1.8	2.7	4.7	5.2
	4	10	1.4	2.3	2.6	4.2	5.2
	5	10	1.1	2.6	4.1	4.4	32.6
	7a	6	2.7	3.1	4.9	5.5	7.0
	7b	6	2.5	2.6	2.9	5.4	7.2
Eosinophils (%)	1	6	2.8	3.0	4.4	6.8	7.9
	2	3	1.9	1.9	3.2	4.6	4.6
	3	10	1.6	2.0	2.5	3.3	4.1
	4	10	1.7	2.2	2.8	3.6	4.4
	5	10	0.9	1.3	2.7	3.1	3.5
	7a	6	2.1	2.2	2.7	3.3	3.7
	7b	6	0.2	0.2	0.3	0.7	2.2
Basophils (%)	1	6	0.3	0.4	0.4	0.4	0.6
	2	3	0.4	0.4	0.6	0.7	0.7
	3	10	0.2	0.3	0.3	0.4	0.4
	4	10	0.2	0.3	0.4	0.5	0.9
	5	10	0.2	0.3	0.4	0.5	0.6
	7a	6	0.3	0.3	0.35	0.4	0.7
	7b	6	0.2	0.2	0.4	0.5	1.6

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix XXIV: Minimum, maximum and quartiles for percentages of total leukocytes on study day 8 of a natural transmission study on PMWS in cell type and by experimental group.

Leukocyte	Experimental group	N	Minimum	Percentile			Maximum
				25th	50th	75th	
Neutrophils (%)	1[a]	5	54.6	55.4	59.1	72.7	83.3
	2[b]	3	27.9	27.9	45.3	46.5	46.5
	3[c]	9	40.5	44.8	56.2	66.9	80.3
	4[d]	10	33.1	36.3	43.2	44.0	54.8
	5[e]	10	32.4	39.9	45.7	51.8	60.0
	7a[f]	6	37.2	38.6	57.4	70.5	71.7
	7b[g]	6	21.7	28.4	38.5	48.4	48.4
Lymphocytes (%)	1	5	9.2	18.6	28.9	30.5	31.3
	2	3	41.2	41.2	41.5	63.9	63.9
	3	9	16.6	25.2	36.5	45.5	51.9
	4	10	38.5	48.8	50.8	52.5	59.2
	5	10	34.3	40.5	47.7	53.4	59.1
	7a	6	24.1	24.1	36.9	51.7	55.3
	7b	6	43.8	47.2	55.4	62.7	69.6
Monocytes (%)	1	5	3.3	3.5	3.9	4.9	5.7
	2	3	3.6	3.6	7.9	8.4	8.4
	3	9	1.5	2.8	3.2	5.7	7.4
	4	10	2.6	2.8	3.4	4.2	6.2
	5	10	1.7	2.5	3.7	4.4	5.6
	7a	6	1.5	1.8	2.1	3.0	5.7
	7b	6	0.8	2.5	5.4	6.8	7.1
Eosinophils (%)	1	5	1.2	1.4	2.6	6.1	8.6
	2	3	2.3	2.3	2.4	2.6	2.6
	3	9	1.0	1.2	1.7	1.9	2.2
	4	10	1.2	1.9	2.15	2.4	3.3
	5	10	0.8	1.0	1.35	1.7	2.0
	7a	6	1.0	1.2	1.9	2.3	2.3
	7b	6	0.7	0.8	1.1	1.9	2.1
Basophils (%)	1	5	0.4	0.4	0.5	0.6	0.6
	2	3	0.4	0.4	0.6	1.0	1.0
	3	9	0.3	0.3	0.4	0.4	0.7
	4	10	0.3	0.4	0.5	0.6	1.1
	5	10	0.3	0.4	0.5	0.7	1.1
	7a	6	0.3	0.3	0.4	0.5	0.9
	7b	6	0.3	0.3	0.3	0.5	0.5

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix XXV: Minimum, maximum and quartiles for percentages of total leukocytes on study day 11 of a natural transmission study on PMWS in cell type and by experimental group.

Leukocyte	Experimental group	N	Minimum	Percentile			Maximum
				25th	50th	75th	
Neutrophils (%)	1[a]	5	39.6	42.4	57.4	58.3	72.6
	2[b]	3	33.7	33.7	47.8	59.7	59.7
	3[c]	9	23.1	42.7	48.0	55.5	65.0
	4[d]	10	37.3	41.2	45.3	49.7	60.4
	5[e]	10	30.8	34.2	43.8	48.6	59.7
	7a[f]	6	45.2	46.1	51.8	56.5	70.4
	7b[g]	6	29.2	33.3	39.1	46.2	59.5
Lymphocytes (%)	1	5	18.1	27.4	31.3	32.5	43.0
	2	3	29.8	29.8	38.5	55.3	55.3
	3	9	27.8	34.1	41.7	45.8	62.7
	4	10	31.8	41.4	44.9	51.5	54.6
	5	10	32.8	44.1	47.4	55.8	61.0
	7a	6	21.4	34.1	40.1	44.5	45.9
	7b	6	36.8	45.9	52.8	58.5	61.0
Monocytes (%)	1	5	3.4	3.6	5.2	5.4	10.2
	2	3	4.9	4.9	6.4	6.6	6.6
	3	9	2.8	3.8	5.1	6.7	11.9
	4	10	2.7	2.9	3.4	4.1	5.1
	5	10	2.4	3.6	4.2	6.4	6.9
	7a	6	3.5	4.0	4.8	5.5	5.8
	7b	6	1.6	3.9	4.5	6.1	6.6
Eosinophils (%)	1	5	0.9	0.9	1.7	1.7	4.2
	2	3	1.8	1.8	2.0	2.8	2.8
	3	9	0.2	1.0	1.5	1.9	4.4
	4	10	0.8	1.3	1.4	2.0	4.0
	5	10	0.6	0.8	1.4	1.6	1.9
	7a	6	0.6	0.9	1.2	1.6	3.6
	7b	6	0.8	0.8	1.2	1.6	2.8
Basophils (%)	1	5	0.3	0.3	0.5	0.6	0.7
	2	3	0.5	0.5	0.5	0.5	0.5
	3	9	0.3	0.4	0.4	0.6	1.0
	4	10	0.2	0.4	0.6	0.8	1.0
	5	10	0.3	0.4	0.6	0.7	0.8
	7a	6	0.2	0.3	0.4	0.6	0.7
	7b	6	0.3	0.4	0.4	0.4	0.5

[a] PCV2-negative, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[e] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[f] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[g] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix XXVI: Minimum, maximum and quartiles for percentages of total leukocytes on study day 29 of a natural transmission study on PMWS in cell type and by experimental group.

Leukocyte	Experimental group	N	Minimum	Percentile			Maximum
				25th	50th	75th	
Neutrophils (%)	2[a]	3	17.9	17.9	20.7	27.2	27.2
	3[b]	8	36.4	52.9	60.3	65.7	82.0
	4[c]	10	35.4	46.5	52.5	59.3	65.8
	5[d]	10	38.9	41.7	52.2	56.4	58.0
	7a[e]	6	29.6	32.7	36.0	43.3	46.3
	7b[f]	6	28.5	32.9	39.4	43.1	44.4
Lymphocytes (%)	2	3	58.8	58.8	62.4	66.9	66.9
	3	8	11.6	27.8	32.8	37.8	53.6
	4	10	21.3	32.9	37.7	45.3	55.3
	5	10	31.9	35.1	40.6	49.2	56.3
	7a	6	42.3	43.9	48.9	55.0	57.8
	7b	6	48.4	48.8	53.4	59.2	62.2
Monocytes (%)	2	3	7.5	7.5	9.7	12.2	12.2
	3	8	1.6	1.8	3.5	7.4	8.6
	4	10	1.0	3.3	4.7	5.9	7.7
	5	10	2.3	2.7	3.1	4.0	7.1
	7a	6	5.3	5.3	7.7	10.2	14.4
	7b	6	2.8	3.5	5.3	6.3	6.3
Eosinophils (%)	2	3	1.3	1.3	3.0	3.2	3.2
	3	8	1.2	1.3	1.7	2.0	2.5
	4	10	1.3	1.7	1.8	3.4	7.8
	5	10	0.7	1.7	2.1	2.5	3.4
	7a	6	0.7	0.9	1.5	1.9	2.1
	7b	6	0.5	0.8	0.9	1.7	1.8
Basophils (%)	2	3	0.6	0.6	0.9	1.0	1.0
	3	8	0.2	0.3	0.4	0.5	0.6
	4	10	0.3	0.3	0.4	0.5	0.5
	5	10	0.2	0.3	0.4	0.4	0.6
	7a	6	0.3	0.4	0.4	0.6	0.6
	7b	6	0.3	0.4	0.5	0.6	0.6

[a] PCV2-negative, PMWS-negative, 13-week-old, directly exposed to PMWS-affected pigs.
[b] PCV2-positive, PMWS-negative, 4-week-old, directly exposed to PMWS-affected pigs.
[c] PCV2-positive, PMWS-negative, 12-week-old, directly exposed to PMWS-affected pigs.
[d] PCV2-positive, PMWS-negative, 4-week-old, on-site control group.
[e] PCV2-negative, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.
[f] PCV2-positive, PMWS-negative, 4-week-old, exposed to PCV2-positive pig and faeces.

Appendix XXVII: Mean and (95% confidence interval) for parameters of complete blood count in a natural transmission study on PMWS. At day 0: Group 7a (*n* = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (*n* = 6, PCV2-positive, PMWS-negative, 4-week-old). Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days.

		Study day						
Blood parameter	Group	0	5	8	11	15	22	29
WBC ($x10^9$/l) (white blood cell)	7a	9.41 (8.58-10.24)	12.12 (9.84-14.39)	14.97 (10.62-19.33)	13.24 (11.64-14.83)	16.23 (14.23-18.22)	20.23 (17.65-22.8)	17.76 (14.39-21.13)
	7b	12.90 (10.44-15.36)	15.49 (12.28-18.69)	17.88 (14.32-21.45)	17.80 (15.97-19.63)	19.02 (16.58-21.46)	16.88 (15.26-18.51)	15.65 (13.82-17.47)
RBC ($x10^{12}$/l) (red blood cell count)	7a	7.03 (6.71-7.36)	7.28 (6.88-7.67)	7.21 (6.89-7.54)	6.76 (6.37-7.14)	6.74 (6.47-7)	6.14 (5.82-6.45)	5.67 (5.34-6.00)
	7b	6.38 (6.03-6.72)	6.60 (6.32-6.87)	6.89 (6.43-7.35)	6.75 (6.30-7.19)	6.92 (6.65-7.19)	6.61 (6.26-6.96)	6.32 (5.94-6.70)
HGB (g/l) (haemoglobin concentration)	7a	141.8 (133.04-150.62)	143.2 (136.98-149.35)	138.8 (131.34-146.33)	129.7 (122.50-136.84)	125.0 (121.35-128.65)	109.8 (104.3-115.36)	98.3 (92.91-103.75)
	7b	98.7 (86.82-110.51)	99.0 (92.29-105.71)	103.0 (92.09-113.91)	99.8 (90.94-108.72)	107.0 (98.62-115.38)	98.3 (88.47-108.19)	93.7 (88.33-99.00)
HCT (l/l) (haematocrit value)	7a	0.410 (0.380-0.440)	0.392 (0.372-0.412)	0.387 (0.366-0.407)	0.347 (0.332-0.361)	0.338 (0.328-0.349)	0.300 (0.284-0.316)	0.272 (0.254-0.290)
	7b	0.305 (0.276-0.334)	0.302 (0.284-0.319)	0.303 (0.276-0.330)	0.293 (0.274-0.313)	0.308 (0.283-0.334)	0.288 (0.264-0.312)	0.275 (0.264-0.286)
MCV (fl) (mean corpuscular volume)	7a	58.65 (54.865-62.435)	53.95 (51.512-56.388)	53.35 (50.608-56.092)	51.82 (48.838-54.795)	50.22 (48.188-52.245)	49.17 (47.196-51.137)	47.85 (45.072-50.628)
	7b	47.43 (44.485-50.381)	45.43 (43.44-47.427)	43.95 (40.975-46.925)	43.65 (40.298-47.002)	44.40 (41.968-46.832)	43.87 (41.612-46.121)	43.73 (41.741-45.725)
MCHC (g/l) (mean corpuscular haemoglobin concentration)	7a	344.8 (341.49-348.18)	365.7 (360.85-370.49)	361.5 (354.94-368.06)	371.0 (364.33-377.67)	370.3 (366.95-373.72)	364.5 (361.47-367.53)	363.8 (347.83-379.84)
	7b	324.7 (313.10-336.23)	330.2 (322.03-338.3)	339.8 (331.35-348.32)	339.5 (329.37-349.63)	348.2 (336.56-359.78)	338.5 (327.5-349.5)	339.0 (328.46-349.54)
PLT ($x10^9$/l) (platelet count)	7a	375.0 (15.71-734.29)	466.2 (267.66-664.67)	402.0 (146.86-657.14)	612.2 (206.90-1017.43)	770.5 (684.96-856.04)	389.0 (218.43-559.57)	616.0 (299.15-932.85)
	7b	504.3 (161.80-846.86)	462.7 (329.84-595.5)	517.3 (335.73-698.93)	521.5 (395.71-647.29)	537.0 (438.69-635.31)	497.7 (437.51-557.82)	681.0 (585.21-776.79)

Table XXVII continued

Blood parameter	Group	Study day						
		36	43	50	57	64	71	81
WBC ($x10^9$/l) (white blood cell)	7a	14.27 (12.75-15.8)	16.87 (14.93-18.81)	13.51 (11.01-16)	16.87 (14.42-19.32)	19.17 (17.15-21.2)	14.31 (11.69-16.92)	13.68 (10.71-16.64)
	7b	15.11 (14.57-15.65)	18.70 (15.41-21.99)	16.52 (15.55-17.49)	19.22 (16.15-22.29)	17.63 (14.7-20.55)	20.20 (15.75-24.65)	7.88 (4.65-11.12)
RBC ($x10^{12}$/l) (red blood cell count)	7a	5.81 (5.61-6)	6.30 (6.12-6.48)	7.11 (6.72-7.49)	6.39 (6.18-6.6)	6.20 (6.11-6.29)	6.37 (6.22-6.52)	6.18 (5.53-6.84)
	7b	6.33 (6.04-6.63)	6.45 (6.27-6.63)	6.78 (6.43-7.12)	6.59 (6.21-6.97)	6.35 (6.11-6.59)	6.05 (5.77-6.33)	6.38 (6.03-6.74)
HGB (g/l) (haemoglobin concentration)	7a	102.0 (99.37-104.63)	110.2 (105.05-115.29)	127.2 (119.53-134.8)	112.5 (108.43-116.57)	108.8 (106.49-111.18)	113.8 (111.04-116.62)	112.2 (103.02-121.31)
	7b	94.8 (90.93-98.73)	98.8 (94.21-103.46)	106.3 (103.19-109.48)	108.1 (100.93-115.36)	103.1 (98.31-107.97)	97.0 (91.04-102.96)	107.1 (100.85-113.44)
HCT (l/l) (haematocrit value)	7a	0.288 (0.282-0.294)	0.318 (0.305-0.332)	0.353 (0.331-0.375)	0.308 (0.295-0.322)	0.315 (0.307-0.323)	0.337 (0.33-0.343)	0.317 (0.288-0.345)
	7b	0.282 (0.272-0.291)	0.298 (0.284-0.313)	0.315 (0.308-0.322)	0.313 (0.293-0.333)	0.306 (0.295-0.317)	0.297 (0.283-0.312)	0.311 (0.287-0.336)
MCV (fl) (mean corpuscular volume)	7a	49.58 (47.91-51.257)	50.48 (49.046-51.92)	49.72 (48.39-51.044)	48.27 (47.145-49.389)	50.63 (49.15-52.117)	52.80 (51.674-53.926)	51.68 (50.106-53.261)
	7b	44.63 (42.53-46.737)	46.02 (43.925-48.108)	46.78 (44.69-48.877)	47.63 (45.263-49.994)	47.90 (45.194-50.606)	49.23 (47.5-50.957)	48.83 (46.225-51.433)
MCHC (g/l) (mean corpuscular haemoglobin concentration)	7a	354.5 (349.52-359.48)	346.7 (340.71-352.63)	360.5 (354.83-366.17)	365.2 (360.07-370.26)	346.7 (341.93-351.41)	338.3 (336.27-340.4)	352.5 (338-367)
	7b	335.8 (330.14-341.52)	333.0 (325.51-340.49)	335.8 (329.73-341.94)	345.6 (336.18-354.96)	340.1 (332.44-347.85)	325.9 (314.53-337.18)	344.4 (332.2-356.65)
PLT ($x10^9$/l) (platelet count)	7a	641.0 (593.82-688.18)	507.2 (311.03-703.31)	526.7 (321.4-731.94)	467.2 (210.14-724.19)	234.8 (99-370.67)	429.8 (182.89-676.78)	225.8 (55.51-396.16)
	7b	647.3 (615.29-679.37)	467.2 (335.26-599.07)	617.3 (569.5-665.17)	522.6 (416.02-629.12)	441.1 (367.67-514.62)	347.9 (273.46-422.25)	155.6 (47.59-263.56)

Appendix XXVIII: Minimum (Min), maximum (Max) and quartiles for absolute values of total leukocytes in pigs of a natural transmission study on PMWS by cell type. At day 0: Group 7a (n = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (n = 6, PCV2-positive, PMWS-negative, 4-week-old). Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days. Each haematological parameter was measured in SI units ($x10^9$/l).

		Group 7a					Group 7b				
			Percentile					Percentile			
Day	Leukocyte	Min	25th	50th	75th	Max	Min	25th	50th	75th	Max
0	Neutrophils	3.22	3.28	3.91	4.05	4.67	3.05	4.38	6.86	8.21	9.26
	Lymphocytes	3.78	4.27	4.53	5.17	5.98	3.58	4.08	5.51	7.20	7.69
	Monocytes	0.25	0.34	0.43	0.51	0.67	0.26	0.32	0.39	0.79	0.92
	Eosinophils	0.19	0.20	0.27	0.31	0.34	0.02	0.03	0.03	0.12	0.23
	Basophils	0.03	0.03	0.04	0.04	0.07	0.02	0.03	0.04	0.09	0.20
5	Neutrophils	3.54	4.84	6.02	7.05	7.55	2.84	3.66	4.18	5.35	12.42
	Lymphocytes	3.46	3.63	4.63	6.19	8.89	4.89	6.71	8.06	11.18	14.34
	Monocytes	0.27	0.38	0.44	0.45	0.74	0.19	0.54	0.58	0.65	1.02
	Eosinophils	0.08	0.18	0.32	0.33	0.49	0.13	0.14	0.20	0.35	0.40
	Basophils	0.03	0.04	0.09	0.12	0.32	0.05	0.06	0.08	0.12	0.12
8	Neutrophils	3.49	4.02	9.57	11.92	14.12	3.12	4.79	6.32	9.46	11.55
	Lymphocytes	4.07	4.74	4.97	5.75	7.34	8.00	9.54	9.92	10.46	10.50
	Monocytes	0.26	0.26	0.34	0.48	0.54	0.16	0.39	1.04	1.11	1.15
	Eosinophils	0.10	0.16	0.29	0.38	0.40	0.11	0.14	0.17	0.31	0.51
	Basophils	0.05	0.05	0.06	0.06	0.09	0.05	0.06	0.07	0.08	0.09
11	Neutrophils	5.34	5.46	7.35	8.13	8.99	4.28	6.09	6.99	8.52	11.67
	Lymphocytes	2.73	4.40	5.26	5.43	6.83	7.22	8.46	8.82	10.21	10.72
	Monocytes	0.50	0.51	0.66	0.69	0.71	0.31	0.67	0.82	0.96	1.12
	Eosinophils	0.08	0.10	0.18	0.21	0.45	0.11	0.16	0.24	0.29	0.47
	Basophils	0.03	0.04	0.06	0.07	0.09	0.05	0.06	0.07	0.08	0.09
15	Neutrophils	6.64	6.67	7.64	8.75	11.20	5.41	6.00	6.69	6.88	7.91
	Lymphocytes	5.02	6.26	6.49	7.34	7.80	8.12	8.75	9.90	11.42	17.32
	Monocytes	0.39	0.74	0.83	1.08	1.08	0.45	0.77	1.03	1.10	1.16
	Eosinophils	0.04	0.10	0.13	0.19	0.28	0.09	0.11	0.24	0.36	0.39
	Basophils	0.04	0.07	0.08	0.09	0.22	0.04	0.05	0.07	0.11	0.45
22	Neutrophils	4.85	5.85	6.66	7.13	8.92	4.79	6.5	7.17	8.36	9.15
	Lymphocytes	7.54	9.61	10.01	13.48	14.88	5.89	7.67	8.25	9.31	10.02
	Monocytes	0.84	1.32	1.73	2.49	2.97	0.75	0.77	0.81	0.89	1.18
	Eosinophils	0.14	0.16	0.29	0.46	0.58	0.07	0.18	0.28	0.55	0.75
	Basophils	0.07	0.11	0.12	0.14	0.20	0.05	0.05	0.07	0.08	0.10
29	Neutrophils	5.03	5.49	5.82	7.83	9.81	4.35	4.64	6.00	6.97	7.85
	Lymphocytes	5.46	7.52	9.41	9.95	11.16	7.34	7.59	8.30	9.49	9.70
	Monocytes	0.90	0.95	1.37	1.86	2.32	0.44	0.44	0.82	1.04	1.15
	Eosinophils	0.12	0.15	0.27	0.34	0.41	0.07	0.12	0.15	0.26	0.28
	Basophils	0.06	0.06	0.08	0.08	0.11	0.05	0.06	0.08	0.09	0.11

Table XXVIII continued

		Group 7a					Group 7b				
			Percentile					Percentile			
Day	Leukocyte	Min	25th	50th	75th	Max	Min	25th	50th	75th	Max
36	Neutrophils	3.58	3.70	4.20	4.89	4.98	4.46	4.94	5.37	6.32	6.46
	Lymphocytes	6.33	7.26	8.54	9.70	9.81	6.93	7.36	8.64	8.77	9.78
	Monocytes	0.88	1.04	1.30	1.54	1.54	0.51	0.54	0.80	0.91	1.13
	Eosinophils	0.05	0.06	0.09	0.13	0.20	0.10	0.10	0.11	0.13	0.36
	Basophils	0.05	0.07	0.08	0.08	0.09	0.06	0.06	0.07	0.09	0.09
43	Neutrophils	5.22	5.48	7.33	8.70	9.83	6.77	6.88	6.94	7.49	8.93
	Lymphocytes	6.82	6.83	8.00	8.20	8.74	6.83	7.58	8.30	10.31	16.32
	Monocytes	0.43	0.96	1.17	1.34	1.59	0.51	0.66	0.99	1.49	1.51
	Eosinophils	0.07	0.09	0.36	0.48	0.59	0.12	0.15	0.33	0.39	0.48
	Basophils	0.05	0.06	0.07	0.08	0.09	0.04	0.06	0.07	0.20	0.56
50	Neutrophils	2.35	2.83	3.85	5.63	7.91	4.36	5.23	6.18	7.42	7.42
	Lymphocytes	5.15	6.34	7.81	9.25	9.53	7.02	7.82	9.47	9.83	9.95
	Monocytes	0.24	0.67	1.00	1.17	1.17	0.32	0.56	0.70	0.88	1.16
	Eosinophils	0.06	0.06	0.12	0.20	0.49	0.09	0.14	0.19	0.37	0.42
	Basophils	0.07	0.07	0.09	0.10	0.10	0.07	0.08	0.09	0.10	0.10
57	Neutrophils	4.93	6.51	7.66	8.96	9.76	4.96	5.04	6.42	7.27	8.15
	Lymphocytes	5.53	5.67	7.21	9.20	9.58	7.21	8.56	9.70	14.52	15.28
	Monocytes	0.85	0.91	1.03	1.37	1.91	0.51	0.97	1.12	1.29	1.30
	Eosinophils	0.16	0.16	0.33	0.40	0.46	0.10	0.15	0.19	0.56	0.94
	Basophils	0.04	0.05	0.08	0.08	0.09	0.07	0.11	0.12	0.32	0.46
64	Neutrophils	3.88	5.34	6.46	7.12	7.85	3.68	5.09	5.65	6.20	6.59
	Lymphocytes	4.65	5.82	8.20	10.55	12.24	8.10	8.17	9.49	10.29	16.73
	Monocytes	1.14	1.79	2.06	4.10	11.23	0.76	0.81	1.02	1.52	2.38
	Eosinophils	0.15	0.15	0.33	0.42	0.78	0.09	0.10	0.22	0.72	0.94
	Basophils	0.08	0.09	0.12	0.18	0.19	0.06	0.06	0.10	0.17	0.19
71	Neutrophils	3.54	3.79	5.68	5.98	7.10	4.32	5.16	6.19	7.57	7.85
	Lymphocytes	4.76	5.10	7.61	9.65	10.94	8.37	8.60	9.96	11.47	25.87
	Monocytes	0.48	0.69	0.91	1.09	1.35	0.80	0.89	1.06	1.41	2.17
	Eosinophils	0.12	0.17	0.23	0.39	0.40	0.08	0.18	0.25	0.66	0.84
	Basophils	0.03	0.05	0.07	0.10	0.10	0.09	0.10	0.24	0.32	0.34
81	Neutrophils	2.45	6.53	7.94	9.12	11.79	0.62	1.18	2.79	4.20	4.33
	Lymphocytes	3.94	4.17	5.48	5.71	7.77	1.98	2.15	4.13	7.59	8.47
	Monocytes	0.14	0.20	0.26	0.46	0.60	0.11	0.11	0.24	0.46	0.58
	Eosinophils	0.04	0.06	0.09	0.12	0.21	0.01	0.03	0.04	0.05	0.15
	Basophils	0.02	0.04	0.05	0.07	0.07	0.03	0.03	0.04	0.05	0.07

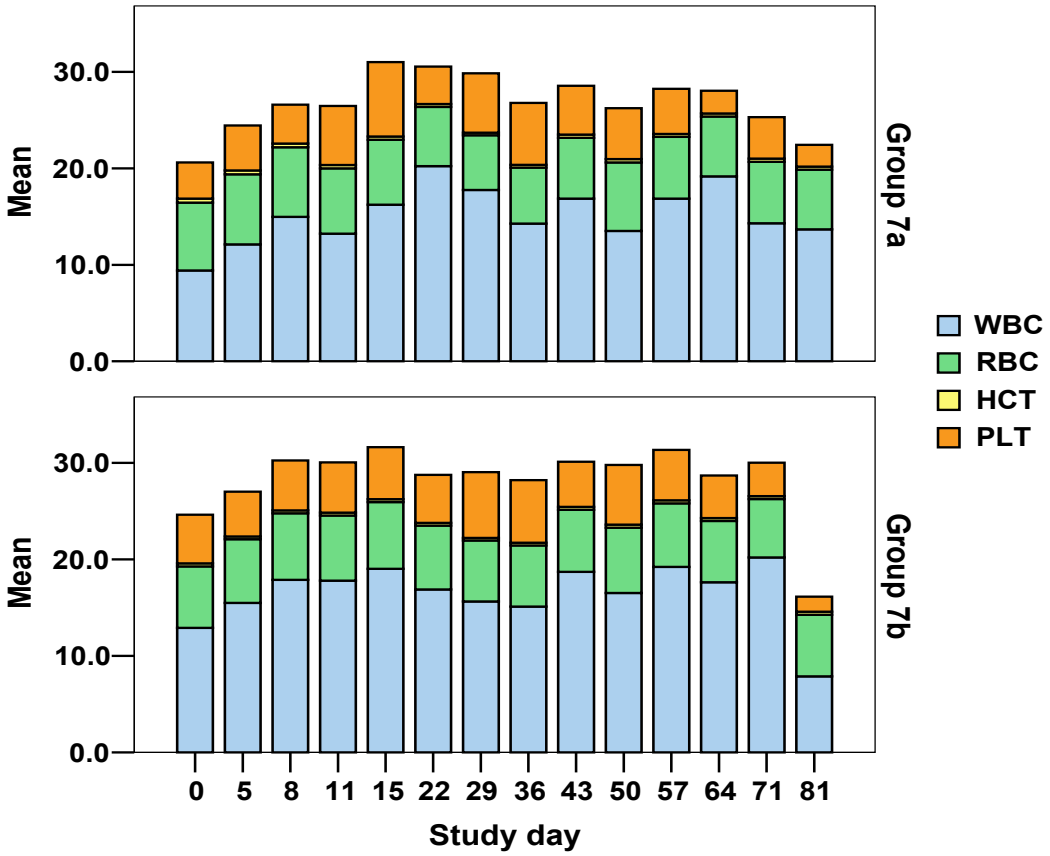
Appendix XXIX: Minimum (Min), maximum (Max) and quartiles for percentage of total leukocytes in pigs of a natural transmission study on PMWS by cell type. At day 0: Group 7a (*n* = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (*n* = 6, PCV2-positive, PMWS-negative, 4-week-old). Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd for 81 days.

		Group 7a					Group 7b				
			Percentile					Percentile			
Day	Leukocyte	Min	25th	50th	75th	Max	Min	25th	50th	75th	Max
0	Neutrophils	34.60	37.60	40.15	42.70	50.10	27.80	35.20	51.05	65.40	66.70
	Lymphocytes	40.50	47.20	50.45	55.50	55.50	29.10	29.60	42.00	57.80	62.20
	Monocytes	2.70	3.10	4.90	5.50	7.00	2.50	2.60	2.95	5.40	7.20
	Eosinophils	2.10	2.20	2.70	3.30	3.70	0.20	0.20	0.30	0.70	2.20
	Basophils	0.30	0.30	0.35	0.40	0.70	0.20	0.20	0.40	0.50	1.60
5	Neutrophils	35.80	38.20	52.70	54.70	57.40	14.90	28.00	32.65	37.30	62.90
	Lymphocytes	33.00	35.80	40.40	50.00	55.70	34.00	49.90	59.80	64.60	75.30
	Monocytes	3.00	3.10	3.35	4.60	4.90	1.00	3.60	4.40	5.40	5.50
	Eosinophils	0.50	1.10	3.15	3.60	3.80	0.90	1.00	1.10	2.20	3.60
	Basophils	0.30	0.30	0.75	1.30	2.00	0.20	0.60	0.60	0.60	0.70
8	Neutrophils	37.20	38.60	57.45	70.50	71.70	21.70	28.40	38.50	48.40	48.40
	Lymphocytes	24.10	24.10	36.95	51.70	55.30	43.80	47.20	55.45	62.70	69.60
	Monocytes	1.50	1.80	2.15	3.00	5.70	0.80	2.50	5.40	6.80	7.10
	Eosinophils	1.00	1.20	1.90	2.30	2.30	0.70	0.80	1.10	1.90	2.10
	Basophils	0.30	0.30	0.40	0.50	0.90	0.30	0.30	0.35	0.50	0.50
11	Neutrophils	45.20	46.10	51.80	56.50	70.40	29.20	33.30	39.10	46.20	59.50
	Lymphocytes	21.40	34.10	40.05	44.50	45.90	36.80	45.90	52.75	58.50	61.00
	Monocytes	3.50	4.00	4.80	5.50	5.80	1.60	3.90	4.45	6.10	6.60
	Eosinophils	0.60	0.90	1.25	1.60	3.60	0.80	0.80	1.25	1.60	2.80
	Basophils	0.20	0.30	0.40	0.60	0.70	0.30	0.40	0.40	0.40	0.50
15	Neutrophils	45.80	45.80	47.05	56.20	56.40	21.60	34.00	36.50	40.00	45.20
	Lymphocytes	32.80	37.30	41.75	44.40	46.40	46.50	51.50	55.60	60.30	69.00
	Monocytes	2.90	4.40	5.50	5.80	6.00	2.40	4.40	5.05	6.10	6.60
	Eosinophils	0.30	0.70	0.80	1.00	1.60	0.50	0.60	1.10	2.00	2.20
	Basophils	0.30	0.40	0.50	0.50	1.50	0.30	0.30	0.35	0.60	1.80
22	Neutrophils	25.50	27.60	31.70	41.20	43.30	35.30	38.50	41.10	44.50	53.60
	Lymphocytes	45.70	47.60	53.85	55.20	64.80	34.50	46.00	51.00	55.20	56.50
	Monocytes	5.10	6.10	8.40	12.20	14.20	4.10	4.40	4.85	6.20	6.60
	Eosinophils	0.70	0.80	1.50	2.40	2.80	0.50	1.00	1.65	2.90	4.40
	Basophils	0.40	0.50	0.60	0.80	0.80	0.20	0.30	0.45	0.50	0.50
29	Neutrophils	29.60	32.70	36.00	43.30	46.30	28.50	32.90	39.35	43.10	44.40
	Lymphocytes	42.30	43.90	48.85	55.00	57.80	48.40	48.80	53.45	59.20	62.20
	Monocytes	5.30	5.30	7.70	10.20	14.40	2.80	3.50	5.30	6.30	6.30
	Eosinophils	0.70	0.90	1.55	1.90	2.10	0.50	0.80	0.95	1.70	1.80
	Basophils	0.30	0.40	0.45	0.60	0.60	0.30	0.40	0.55	0.60	0.60

Table XXIX continued

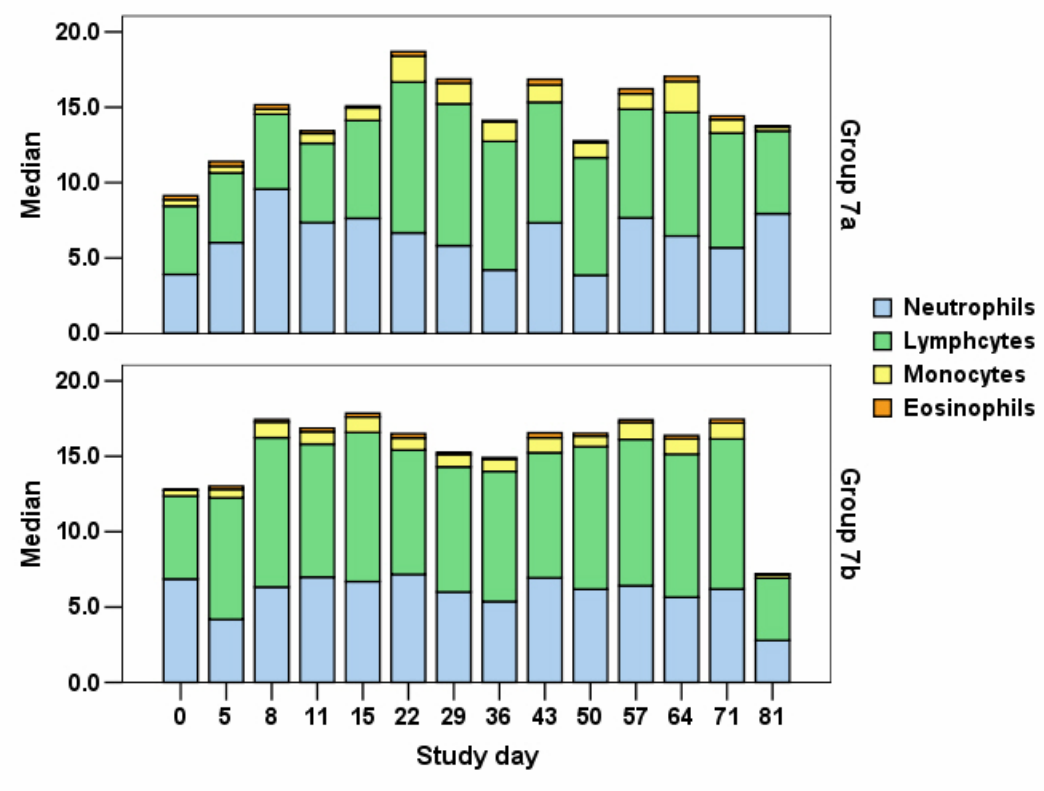
		Group 7a					Group 7b				
			Percentile					Percentile			
Day	Leukocyte	Min	25th	50th	75th	Max	Min	25th	50th	75th	Max
36	Neutrophils	24.50	29.80	30.25	31.80	33.10	30.50	31.30	35.05	42.50	44.00
	Lymphocytes	51.90	57.30	59.15	59.70	63.00	47.20	49.40	56.40	60.50	61.50
	Monocytes	5.40	8.40	8.70	10.50	12.60	3.30	3.80	5.20	6.10	7.30
	Eosinophils	0.40	0.50	0.70	0.80	1.20	0.60	0.60	0.75	0.90	2.50
	Basophils	0.40	0.50	0.55	0.60	0.60	0.40	0.40	0.50	0.60	0.60
43	Neutrophils	34.20	39.00	43.55	46.00	50.60	28.40	35.90	41.30	46.40	48.60
	Lymphocytes	40.20	43.30	44.90	51.10	54.60	43.30	46.80	48.10	53.80	61.90
	Monocytes	3.20	5.90	6.55	7.80	8.60	3.50	3.60	5.45	6.00	8.40
	Eosinophils	0.50	0.50	2.00	2.50	3.90	0.80	0.80	1.50	2.40	2.70
	Basophils	0.30	0.40	0.40	0.50	0.50	0.30	0.30	0.40	1.10	2.10
50	Neutrophils	22.60	26.90	29.15	33.90	48.50	28.00	32.00	37.75	40.10	46.80
	Lymphocytes	38.80	57.00	60.15	63.80	64.40	44.30	50.60	54.75	56.80	62.80
	Monocytes	2.70	6.10	6.65	7.00	8.20	1.70	3.60	4.10	5.60	7.50
	Eosinophils	0.40	0.60	0.80	2.30	3.00	0.60	0.80	1.10	2.40	2.60
	Basophils	0.50	0.60	0.60	0.70	0.70	0.40	0.50	0.60	0.60	0.60
57	Neutrophils	40.10	40.40	43.35	47.40	54.40	23.90	29.90	32.20	37.70	41.10
	Lymphocytes	32.10	40.30	45.30	48.60	51.10	49.10	50.30	59.60	62.00	65.50
	Monocytes	4.60	5.00	7.30	7.80	10.60	3.00	4.80	5.10	8.00	8.10
	Eosinophils	0.80	0.90	1.90	2.80	3.30	0.60	0.80	1.10	3.20	5.50
	Basophils	0.30	0.40	0.40	0.40	0.50	0.50	0.50	0.70	1.50	2.00
64	Neutrophils	18.70	23.00	34.90	41.10	47.60	24.50	25.10	32.80	37.00	39.50
	Lymphocytes	20.00	35.30	46.65	54.50	58.80	53.30	53.80	55.10	63.40	63.70
	Monocytes	6.60	10.20	11.50	19.70	48.30	4.70	5.00	6.70	9.10	10.10
	Eosinophils	0.70	0.80	1.70	2.30	4.70	0.40	0.50	1.40	4.20	5.40
	Basophils	0.50	0.50	0.60	0.80	1.10	0.40	0.40	0.60	0.70	1.00
71	Neutrophils	27.00	31.50	36.30	45.10	48.90	13.10	33.00	34.60	37.70	39.80
	Lymphocytes	41.00	42.70	53.50	60.50	64.60	52.40	53.50	53.80	55.20	78.20
	Monocytes	4.80	5.80	5.95	6.10	8.60	4.30	5.10	5.80	6.60	7.20
	Eosinophils	0.60	1.60	1.95	2.30	2.40	0.20	0.90	1.20	4.20	5.40
	Basophils	0.30	0.40	0.50	0.60	0.60	0.50	0.60	0.70	1.60	1.90
81	Neutrophils	34.60	51.00	52.10	59.60	73.00	21.00	23.20	34.10	39.00	43.00
	Lymphocytes	24.40	37.00	42.55	44.00	58.90	50.90	54.30	61.60	70.40	72.60
	Monocytes	0.90	1.40	2.75	3.40	3.40	2.20	2.90	3.80	3.90	4.60
	Eosinophils	0.20	0.40	0.75	0.90	1.60	0.30	0.30	0.40	1.30	1.90
	Basophils	0.10	0.30	0.35	0.50	0.60	0.30	0.40	0.40	1.30	1.70

Appendix XXX: Histograms of the mean values of haematological parameters.



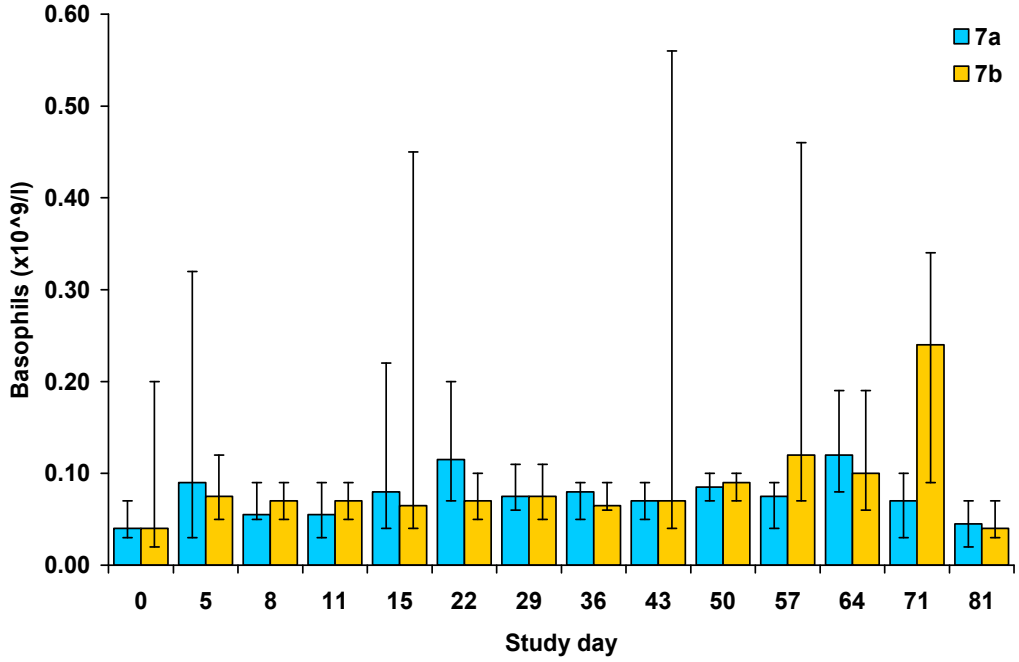
Histograms of the mean values of haematological parameters in two experimental groups of pigs during a natural transmission study on PMWS from day 0 to day 81 post-exposure. At day 0: Group 7a (*n* = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (*n* = 6, PCV2-positive, PMWS-negative, 4-week-old). Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd. White blood cells (WBC) ($x10^9$/l); red blood cells (RBC) ($x10^{12}$/l); haematocrit (HCT) (l/l); platelets (PLT) ($x10^{11}$/l).

Appendix XXXI: Histograms of the median values of differential leukocytes.



Histograms of the median values of differential leukocytes in two experimental groups of pigs during a natural transmission study on PMWS from day 0 to day 81 post-exposure. At day 0: Group 7a (n = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (n = 6, PCV2-positive, PMWS-negative, 4-week-old). Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd.

Appendix XXXII: Histograms of the median values of basophil counts.



Histograms of the median values of basophil counts ($x10^9$/l) of pigs during a natural transmission study on PMWS from day 0 to day 81 post-exposure. At day 0: Group 7a (n = 6, PCV2-negative, PMWS-negative, 4-week-old), Group 7b (n = 6, PCV2-positive, PMWS-negative, 4-week-old). Both Group 7a and 7b were directly exposed to PCV2-contaminated faeces and a PCV2-positive pig from a PMWS-free herd. Error bars represent range (minimum to maximum values).

Appendix XXXIII: Nucleotide sequence alignment of PCV2 genomes isolated from a transmission study on PMWS and other New Zealand pigs acutely infected with PMWS. PCV2 isolates of PMWS cases are shown in bold. The ORF2 gene is highlighted and sequences for primers CV1 to CV4 are in bold. The PCV2 isolate 'France 2' is used as reference and only nucleotide differences are indicated for the other isolates. Not amplified segments of PCV2 isolates are indicated by hyphens.

```
France 2  ACCAGCGCAC TTCGGCAGCG GCAGCACCTC GGCAGCACCT CAGCAGCAAC ATGCCCAGCA AGAAGAATGG AAGAAGCGGA CCCCAACCCC ATAAAAGGTG  [ 100]
53        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 100]
62        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 100]
3         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 100]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 100]
6         .......... .......... .......... .......... .......... .......... .......... .......... ........A. ..........  [ 100]
42        .......... .......... .......... .......... .......... .......... .......... .......... ........A. ..........  [ 100]
E         .......... .......... .......... .......... .......... .....T.... .......... .......... .......... ..........  [ 100]
PIC1      .......... .......... .......... .......... .......... .....T.... .......... .......... .......... ..........  [ 100]
PIC2      .......... .......... .......... .......... .......... .....T.... .......... .......... .......... ..........  [ 100]
CB1       .......... .......... .......... .......... .......... .....T.... .......... .......... .......... ..........  [ 100]
NP8       .......... .......... .......... .......... .......... .....T.... .......... .......... .......... ..........  [ 100]

France 2  GGTGTTCACT CTGAATAATC CTTCCGAAGA CGAGCGCAAG AAAATACGGG ATCTTCCAAT ATCCCTATTT GATTATTTTA TTGTTGGCGA GGAGGGTAAT  [ 200]
53        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 200]
62        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 200]
3         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 200]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 200]
6         .........G .......... .......... .......... .......... .G..C..... C......... .......... .......... ..........  [ 200]
42        .........G .......... .......... .......... .......... .G..C..... C......... .......... .......... ..........  [ 200]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 200]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 200]
PIC2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 200]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 200]
NP8       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 200]

France 2  GAGGAAGGAC GAACACCTCA CCTCCAGGGG TTCGCTAATT TTGTGAAGAA GCAGACTTTT AATAAAGTGA AGTGGTATTT GGGTGCCCGC TGCCACATCG  [ 300]
53        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 300]
62        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 300]
3         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 300]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 300]
6         .......... .......... .......... .......... .......... ...A...... .......... .......... T......... ..........  [ 300]
42        .......... .......... .......... ..T....... .......... ...A...... .......... .......... T......... ..........  [ 300]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 300]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 300]
PIC2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 300]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 300]
NP8       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 300]
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France 2  AGAAAGCGAA AGGAACAGAT CAGCAGAATA AAGAATACTG CAGTAAAGAA GGCAACTTAC TGATGGAGTG TGGAGCTCCT AGATCTCAGG GACAACGGAG  [ 400]
53        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 400]
62        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 400]
3         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 400]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 400]
6         .......... ......T... .......... .......T.. .......... .......... .T.....A.. .......... ........A. ..........  [ 400]
42        .......... ......T... .......... .......T.. .......... .......... .T.....A.. .......... ........A. ..........  [ 400]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 400]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 400]
PIC2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 400]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 400]
NP8       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 400]

                                                                                  Primer CV3 -->
France 2  TGACCTGTCT ACTGCTGTGA GTACCTTGTT GGAGAGCGGG AGTCTGGTGA CCGTTGCAGA GCAGCACCCT GTAACGTTTG TCAGAAATTT CCGCGGGCTG  [ 500]
53        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 500]
62        .......... .......... .......... .......... ....------ ---------- ---------- ---------- ---------- ----------  [ 500]
3         .......... .......... .......... .......... ...------- ---------- ---------- ---------- --------.. ..........  [ 500]
17        .......... .......... .......... .......... .......... .......... ....T..... .......... .......... ..........  [ 500]
6         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 500]
42        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 500]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 500]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 500]
PIC2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 500]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 500]
NP8       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 500]

                                                                        <-- Primer CV2
France 2  GCTGAACTTT TGAAAGTGAG CGGGAAAATG CAGAAGCGTG ATTGGAAGAC TAATGTACAC GTCATTGTGG GGCCACCTGG GTGTGGTAAA AGCAAATGGG  [ 600]
53        .......... .....----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
62        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 600]
3         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 600]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 600]
6         T......... .......... .......... .......... .......... C......... .......... .......... .......... ..........  [ 600]
42        T......... .......... .......... .......... .......... C......... .......... .......... .......... ..........  [ 600]
E         .....-.... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 600]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 600]
PIC2      .......... ....-..... .......... .......... .......... .......... .......... .......... .......... ..........  [ 600]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 600]
NP8       .....-.... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 600]
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France 2  CTGCTAATTT TGCAGACCCG GAAACCACAT ACTGGAAACC ACCTAGAAAC AAGTGGTGGG ATGGTTACCA TGGTGAAGAA GTGGTTGTTA TTGATGACTT  [ 700]
53        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 700]
62        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 700]
3         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]
6         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]
42        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]
PIC2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]
NP8       .....G.... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]

France 2  TTATGGCTGG CTGCCCTGGG ATGATCTACT GAGACTGTGT GATCGATATC CATTGACTGT AGAGACTAAA GGTGGAACTG TACCTTTTTT GGCCCGCAGT  [ 800]
53        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 800]
62        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 800]
3         .......... T......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 800]
17        .......... T......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 800]
6         .......... .....G.... .......... .......... .......... .......... .......... .......... .......... ..........  [ 800]
42        .......... .....G.... .......... .......... .......... .......... .......... .......... .......... ..........  [ 800]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 800]
PIC1      .......... .......... .......... ......A... .......... .......... .......... .......... .......... ..........  [ 800]
PIC2      ......T... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 800]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 800]
NP8       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 800]

France 2  ATTCTGATTA CCAGCAATCA GACCCCGTTG GAATGGTACT CCTCAACTGC TGTCCCAGCT GTAGAAGCTC TTTATCGGAG GATTACTTCC TTGGTATTTT  [ 900]
53        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 900]
62        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 900]
3         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
6         .......... .......... .......... .......... .......... .......... .......... .C........ .......... ..........  [ 900]
42        .......... .......... .......... .......... .......... .......... .......... .C........ .......... ..........  [ 900]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
PIC2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
NP8       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
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France 2  GGAAGAATGC TACAGAACAA TCCACGGAGG AAGGGGGCCA GTTCGTCACC CTTTCCCCCC CATGCCCTGA ATTTCCATAT GAAATAAATT ACTGAGTCTT  [1000]
53        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [1000]
62        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [1000]
3         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1000]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1000]
6         .......... .........G .......... .......... .......... .......... .......... .......... .......... ..........  [1000]
42        .......... .........G .......... .......... .......... .......... .......... .......... .......... ..........  [1000]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1000]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1000]
PIC2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1000]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1000]
NP8       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1000]

France 2  TTTTATCACT TCGTAATGGT TTTTATTATT CATTAAGGGT TAAGTGGGGG GTCTTTAAAA TTAAATTCTC TGAATTGTAC ATACATGGTT ACACGGATAT  [1100]
53        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [1100]
62        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [1100]
3         .......... .......... .......... .......... .......... ........G. .......... .......... .......... ..........  [1100]
17        .......... .......... .......... .......... .......... ........G. .......... .......... .......... ..........  [1100]
6         .......... .......... .......... ....T..... .......... ........G. .......... .......... .....G.... ..........  [1100]
42        .......... .......... .......... ....T..... .......... ........G. .......... .......... .....G.... ..........  [1100]
E         .......... .......... .......... .......... .......... ........G. .......... .......... .......... ..........  [1100]
PIC1      .......... .......... .......... .......... .......... ........G. .......... .......... .......... ..........  [1100]
PIC2      .......... .......... .......... .......... .......... ........G. .......... .......... .......... ..........  [1100]
CB1       .......... .......... .......... .......... .......... ........G. .......... .......... .......... ..........  [1100]
NP8       .......... .......... .......... .......... .......... ........G. .......... .......... .......... ..........  [1100]

France 2  TGTATTCCTG GTCGTATATA CTGTTTTCGA ACGCAGTGCC GAGGCCTACG TGGTCTACAT TTCCAGCAGT TTGTAGTCTC AGCCACAGCT GGTTTCTTTT  [1200]
53        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [1200]
62        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [1200]
3         .......... .......... .......... .......... .......... .......... .......... .......... .......... .A........  [1200]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... .A........  [1200]
6         ....G..... .......T.. .......... .......... .......... .......... ......TG.. C..A...... .T........ .A..C.....  [1200]
42        ....G..... .......T.. .......... .......... .......... .......... ......TG.. C..A...... .T........ .A..C.....  [1200]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... .A........  [1200]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... .A........  [1200]
PIC2      .......... .......... .......... .......... .......... .......... ......T... .......... .......... .A........  [1200]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... .A........  [1200]
NP8       .......... .......... .......... .......... .......... .......... .......... .......... .......... .A........  [1200]
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France 2  GTTGTTTGGT TGGAAGTAAT CAATAGTGGA ATCTAGGACA GGTTTGGGGG TAAAGTACCG GGAGTGGTAG GAGAAGGGCT GGGTTATGGT ATGGCGGGAG [1300]
53        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- [1300]
62        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- [1300]
3         .......... .......... .......... .......... .......... .......G.. .........T .......... .......... .......... [1300]
17        .......... .......... .......... .......... .......... .......G.. .........T .......... .......... .......... [1300]
6         ...A...... .......... ....G..... ...A...... .......... .......... .......... .......... .......... .......... [1300]
42        ...A...... .......... ....G..... ...A...... .......... .......... .......... .......... .......... .......... [1300]
E         .......... .......... .......... .......... .......... .......G.. .......... .......... .......... .......... [1300]
PIC1      .......... .......... .......... .......... .......... .......G.. .......... .......... .......... .......... [1300]
PIC2      .......... .......... .......... .......... .......... .......G.. .......... .......... .......... .......... [1300]
CB1       .......... .......... .......... .......... .......... .......G.. .......... .......... .......... .......... [1300]
NP8       .......... .......... .......... .......... .......... .......G.. .......... .......... .......... .......... [1300]

                                        Primer CV1 -->
France 2  GAGTAGTTTA CATAGGGGTC ATAGGTGAGG GCTGTGGCCT TTGTTACAAA GTTATCATCT AAAATAACAG CACTGGAGCC CACTCCCCTG TCACCCTGGG [1400]
53        ---------- ---------- ---------- ---------- ---------- ---------- ------.... .......... .......... .......... [1400]
62        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- [1400]
3         .......... .......... .......... .......... .......... .......... .G........ .......... .......... .......... [1400]
17        .......... .......... .......... .......... .......... .......... .G........ .......... .......... .......... [1400]
6         .......... .G........ ......T... .......... .A........ .......... .G........ .......T.. A......... .......... [1400]
42        .......... .G........ ......T... .......... .A........ .......... .G........ .......T.. A......... .......... [1400]
E         .......... .......... .......... .......... .......... .......... .G........ .......... .......... .......... [1400]
PIC1      .......... .......... .......... .......... .......... .......... .G........ .......... .......... .......... [1400]
PIC2      .......... .......... .......... .......... .......... .......... .G........ .......... .......... .......... [1400]
CB1       .......... .......... .......... .......... .......... .......... .G........ .......... .......... .......... [1400]
NP8       .......... .......... .......... .......... .......... .......... .G........ .......... .......... .......... [1400]

France 2  TGATCGGGGA GCAGGGCCAG AATTCAACCT TAACCTTTCT TATTCTGTAG TATTCAAAGG GCACAGAGCG GGGGTTTGAG CCCCCTCCTG GGGGAAGAAA [1500]
53        .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1500]
62        ---------- ---------- ---------- ---....... .......... ...-...-.. .........G. ......-... .......... .......... [1500]
3         .......... .......... .......... .......... .......... .......... ...-...... .......... .......... .......... [1500]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1500]
6         ....T..... .......... .......... .......... .......... .......... .T.T....AT TTT...G.TC ........C. .......... [1500]
42        ....T..... .......... .......... .......... .......... .......... .T.T....AT TTT...G.TC ........C. .......... [1500]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1500]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1500]
PIC2      .......... .......... .......... ...T...... .......... .......... .......... .......... .......... .......... [1500]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1500]
NP8       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1500]
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                                    <-- Primer CV4
France 2  GTCATTAATA TTGAATCTCA TCATGTCCAC CGCCCAGGAG GGCGTTCTGA CTGTGGTTCG CTTGACAGTA TATCCGAAGG TGCGGGAGAG GCGGGTGTTG [1600]
53        .......... .......... .......... .......... ......T... .......... .....T.... .......... .......... .......... [1600]
62        .......... .......... .......... .......... ......T... .......... .....T.... .......... .......... .......... [1600]
3         .......... .......... .......... .......... ......T... .......... .....T.... .......... .......... .......... [1600]
17        .......... .......... .......... .......... ......T... .......... .....T.... .......... .......... .......... [1600]
6         ...G...... ..A....... G......... .......... .......... .......AGC .......... .......... .......... ....C..... [1600]
42        ...G...... ..A....... G......... .......... .......... .......AGC .......... .......... .......... ....C..... [1600]
E         .......... .......... .......... .......... ......T... .......... .....T.... .......... .......... .......... [1600]
PIC1      .......... .......... .......... .......... ......T... .......... .....T.... .......... .......... .......... [1600]
PIC2      .......... .......... .......... .......... ......T... .......... .....T.... .......... .......... .......... [1600]
CB1       .......... .......... .......... .......... ......T... .......... .....T.... .....A.... .......... .......... [1600]
NP8       .......... .......... .......... .......... ......T.C. .......... T....T.... .......... .......... .......... [1600]

France 2  AAGATGCCAT TTTTCCTTCT CCAGCGGTAA CGGTGGCGGG GGTGGACGAG CCAGGGGCGG CGGCGGAGGA TCTGGCCAAG ATGGCTGCGG GGGCGGTGTC [1700]
53        .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1700]
62        .......... .......... .......... .......... .......... .......... .......... .......... .......... .....T.... [1700]
3         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1700]
17        .......... .......... .......... .......... .......... .......... .......... .......... .......... .....T.... [1700]
6         ..A....... .......... .......... .......... .......... .......... .......... .T........ .......... .......... [1700]
42        ..A....... .......... .......... .......... .......... .......... .......... .T........ .......... .......... [1700]
E         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1700]
PIC1      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1700]
PIC2      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1700]
CB1       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1700]
NP8       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1700]

France 2  TTCTTCTTCG GTAACGCCTC CTTGGATACG TCATATCTGA AAACGAAAGA AGTGCGCTGT AAGTATT [1767]
53        .......C.. .......... .......... .....C.... .......... .......... ....... [1767]
62        .......C.. .......... .......... .......... .......... .......... ....... [1767]
3         .......C.. .......... .......... .......... .......... .......... ....... [1767]
17        .......C.. .......... .......... .......... .......... .......... ....... [1767]
6         .......C.. .......... .......... .....G.... .......... .......... ....... [1767]
42        .......C.. .......... .......... .....G.... .......... .......... ....... [1767]
E         .......C.. .......... .......... .......... .......... .......... ....... [1767]
PIC1      .......C.. .......... .......... .......... .......... .......... ....... [1767]
PIC2      .......C.. .......... .......... .......... .......... .......... ....... [1767]
CB1       .......CA. .......... .......... .......... .......... .......... ....... [1767]
NP8       .......C.. .......... .......... .......... .......... .......... ....... [1767]
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