Detection and Analysis of Porcine Circovirus Type 2 in the Irish Pig Population

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Masters of Science is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Table of Contents

Abstract

Postweaning Multisystemic Wasting Syndrome (PMWS) is a recently emerged multifactorial clinical disease of swine that affects nursery and growing pigs and PCV2 is believed to be the main aetiological agent of the disease. PMWS has emerged in Ireland as a clinical entity in pig herds so there was therefore a need to determine the extent of PCV2 infection and associated disease in the pig population of Ireland.

The prevalence of PCV2 in Ireland was determined by testing a percentage of porcine sera for antibodies to PCV2 using the IPMA method; this method is based on PCV-free PK15 cell cultures infected with PCV2 virus. Ten serum samples (where available) from fifty randomly selected herds (426 sera samples) were tested, of the fifty herds only four were negative to PCV2. However these four herds were all hobby herds (less than 12 pigs). In most cases of the ten samples tested per herd eight to ten of the sera samples were positive for PCV2 antibodies indicating that there is widespread seroconversion to PCV2 in Ireland.

To determine the extent and emergence of the disease a study on suspect herds was performed. This study commenced on the 1st January 2004 and was completed on the 1st January 2005. This study involved working in collaboration with local pig veterinarians and regional veterinary laboratories so that any herd that developed any syndromes typical/similar to PMWS were reported and suspect herds tested for the presence of PCV2 antigen. Tissue samples (especially lymph nodes) were taken from necropsied pigs (approximately 4 per herd) and tested by using a combination of indirect fluorescent antibody staining of cryostat tissue sections,

immunohistochemsitry and histopathology. A total of 30 suspect herds in Republic of Ireland were tested over this twelve month period. 24 of these herds were found to be positive for PMWS and 6 herds negative. In Northern Ireland, 31 herds were tested and 14 of these herds were PMWS positive, based on PCV2 antigen levels.

PCV2 was isolated from diseased and non-diseased pigs in Ireland. The entire genome of PCV2 was amplified by PCR sequenced. Complete genomes of PCV2 were then aligned and a phylogentic analysis was performed. ROI and NI isolates were closely related to each other displaying 97-100% overall nucleotide homology. An amino acid (aa) alignment was performed on the two major Open Reading Frames, ORF1 and ORF2, as these encoded the major proteins, Replicase and Capsid, respectively. The majority of aa changes observed between PMWS positive and negative isolates occurred within ORF1.

A detailed longitudinal study was also carried out. A total of 4 herds were involved in the study, 2 herds from ROI which were reported as positive for the disease, one negative control herd from ROI and one positive herd from NI. The study involved monitoring approximately 5 litters from each of the 4 herds for a total of 10 weeks from birth in order to elucidate any co-factors that may lead to PMWS. Serum samples were tested for PCV2 antibody titre and PPV antibody, tonsil and faeces samples were tested for the presence of other viruses such as enterovirus 1 and 2, reovirus and adenovirus and faecal swabs for bacterial organisms such as haemolytic *E. coli*, *Campylobacter* spp. and *Salmonellae* spp. From the study it could be concluded that there was no distinguishable coinfection of PCV2 with any of the infectious organisms mentioned between positive and negative animals and herds.

Chapter 1: Literature Review

1.1 Introduction

In 1974, reseasrchers in Germany described a new, non-cytopathogenic, picornavirus-like contaminant in the porcine cell line, PK-15 (ATCC-CCL33). This name porcine circovirus (PCV) was proposed (Tischer *et al.*, 1982). Serological surveys for PCV antibodies revealed that the virus was widespread in the swine population. However, experimental infections of newborn to nine-month old conventional pigs with PCV did not result in clinical disease and the virus was regarded as non-pathogenic (Tischer *et al.*, 1986, Allan *et al.*, 1995).

In the late 1990's, an apparently novel PCV-like virus was first isolated from pigs with a wasting disease in Western Canada (Ellis *et al*, 1998). Shortly afterwards, almost identical viruses to this Canadian isolate were also recovered from diseased pigs in North America and Europe (Allan *et al.*, 1998b). These PCV virus isolates were shown to be antigenically and genetically distinct from the PCV contaminant of PK-15 cell cultures. It was proposed that the PCV isolates from diseased pigs should be designated porcine circovirus type 2 (PCV2) and the original PCV contaminant of PK-15 cell cultures designated porcine circovirus type 1 (PCV1) (Allan *et al*, 1999b).

PCV2 has since been associated with a number of disease syndromes in pigs and is now recognized at the casual agent of Postweaning Multisystemic Wasting Syndrome (PMWS), which is the subject of this study.

1.2 Circoviridae

PCV2 is a member of the family *Circoviridae,* a recently established family composed of small, non-enveloped, isometric DNA viruses with a circular single stranded DNA genome. This family is divided into two genera based on virion size and genomic organisation; *Circovirus* and *Gyrovirus* (Mankertz *et al.*, 2004). PCV2 is classified in the genus *Circovirus.* Other members of the genus circovirus include; porcine circovirus type 1 (PCV1); beak and feather disease virus; goose circovirus; canary circovirus; the human TT virus. (Kim and Chae, 2001; Todd *et al*, 2000; Cheung, 2004a). Circoviruses are characterised by a very compact genomic structure. In all viruses from the genus *Circovirus,* an intergenic region is flanked by two head to head arranged open reading frames. Circoviruses are the smallest known animal viruses, approximately 12 – 27nm in diameter, that replicate autonomously in mammalian cells. The DNA molecules are approximately 2,000 nucleotides in length, encoding two major ambisense open reading frames (Grierson, 2004b). Circoviruses appear to have an affinity for tissues and cells of the immune system, such as macrophages and epithelial cells and are highly resistant to inactivation by detergents and disinfectants (Segalés and Domingo, 2002; Chae, 2004).

1.2.1 Porcine Circovirus Type 1 (PCV1)

PCV1, first described in 1974, is a non-enveloped virus of 17 nm diameter containing SS circular DNA of only $0.58x10^6$ daltons. It has a buoyant density of 1.37 gm/cm^3 in cesium gradients, does not haemagglutinate erythocytes and is resistant to inactivation by exposure to pH 3.0, *chloroform* and heating at 56 or 70°C (Allan *et al.*,

1994a). It is probable that these properties are common to both PCV1 and PCV2. The virus multiplies in primary and permanent cell cultures of porcine origin where it causes a persistent infection (Tischer, 1986). PCV1 is non-pathogenic and infection is thought to be ubiquitous throughout the pig population (Walker *et al.*; 2000). The presence of PCV1 has not been associated with any recognised clinical signs or pathology and experimental infection of pigs (with this virus) has failed to produce clinical disease (Tischer, 1986; Meehan *et al.*, 1998; Allan and Ellis 2000a; Allan, 2002; Segalés and Domingo, 2002).

1.2.2 Porcine Circovirus Type 2 (PCV2)

Nucleotide sequence analysis of the circovirus isolates associated with PMWS, PCV2, revealed important differences compared to the previously known circovirus derived from PK-15, PCV1. The circular PCV2 genome contains 1767-68 nucleotides (Meehan *et al.*, 1998; Hamel *et al.*, 2000; Mankertz *et al.*, 2000) and genomic analysis of PCV2 virus from pigs around the world has shown that they belong to a phylogenetic cluster with a nucleotide sequence identity greater than 94% (Meehan *et al.*, 1998; Mankertz *et al.*, 2000; Larochelle *et al.*, 2002). Potentially, six ORFs larger than 200 nucleotides have been suggested for the PCV2 genome but proteins seem to be expressed only by ORF1 and ORF2. Little is known about the significance of the potential smaller ORFs. Little data exist on the biological and physio-chemical characteristics of PCV2. Exposure of PCV2 for 10 minutes at room temperature to a number of commercial disinfectants based on chlorhexidine, formaldehyde, iodine and alcohols leads to a 1.8 to 4.4 log $TCID_{50}$ reduction in virus titre (Royer *et al.*, 2001).

1.2.3 Comparison of PCV1 and PCV2

The distinct pathogenicity of PCV2 seems to be the most striking difference between the PCV1 and PCV2. PCV2 isolates have 80% nucleotide identity with PCV1 isolates. Both PCV1 and PCV2 contain two major open reading frames (ORF's) named ORF1 and ORF2, which encode the Rep (replicase) and Cap (capsid structural protein) proteins respectively. Sequence conservation between the *cap* and *rep* genes of PCV1 and PCV2 differs considerably (Cheung, 2004a). The origin of replication (Ori) and the *rep* gene, both involved in replication of the virus is conserved in 79.5 and 82% of the nucleotides, while the *cap* gene shows a higher sequence deviation between the two strains with 62%. Since the *cap* gene seems to be the only gene product substantially altered, it may contribute to the different pathogenicities of PCV1 and PCV2 (Segalés and Domingo, 2002; Mankertz *et al.*, 2004).

The potential formation of a palindromic structure as a result of interactions between the inverted repeats (palindrome) at the origin of DNA replication (Ori) and similarities among the putative proteins essential for virus replication indicate that PCV DNA may replicate via the rolling-circle replication stem-loop or via a recently proposed rolling-circle replication (RCR) "melting-pot" model (Cheung, 2004a; Cheung, 2004b). The Rep proteins of PCV contain the three conserved RCR motifs (RCR-I, II, III) and NTP-binding core homologous to the Rep proteins of other prokaryotic and eukaryotic RCR systems. Replication of PCV DNA requires two proteins (Rep and Rep') instead of just one multifunctional Rep protein. PCV1 and PCV2 each contain a nona-nucleotide that is flanked by a pair of palindromic sequences. It has been reported that the nona-

nucloetide and the palindrome are critical for PCV DNA replication (Cheung, 2004a; Cheung, 2004b).

1.3 Post-weaning Multisystemic Wasting Syndrome

PMWS is a recently described multi-factorial clinical entity, which affects nursery and growing pigs. The designation PMWS was coined to describe a clinical condition which is characterized by progressive loss of condition, associated with lesions in several organ systems. The disease was first identified in 1991 in Saskatchewan, Western Canada in a high health specific pathogen free (SPF) herd and was reported in 1996 (Clark, 1997; Chae, 2004) (Harding, 1996; Harding, 1997; Meehan *et al.*, 1998; Allan, 2002; Segalés and Domingo, 2002; Darwich *et al.*, 2004; Allan, 2005). This herd was free of the major enteric and respiratory pathogens that affect swine including porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), aujeszky's disease virus (ADV), and did not demonstrate mycoplasmal or actinobacillary pneumonia, atrophic rhinitis, salmonellosis, swine dysentery and transmissible gastroenteritis. The overall pathogen load was low with low incidence of medical treatment and low mortality in the herd.

The reasons for the emergence of epidemic PMWS in the 1990s are not understood; but since its occurrence the disease has resulted in major economical and welfare problems to pig farmers (Grierson *et al.*, 2004a). It has been estimated that PMWS costs around €600 million per year to the European Union (Armstrong, 2004;

Allan, 2005). Direct losses come from mortality in the nursery and fattening pigs, and from unthrifty pigs unable to reach market weight. Indirect losses come from increased use of antibiotics in an attempt to control concurrent bacterial infections, and changes in farm management practices in an attempt to reduce the impact of PMWS.

PMWS appears to be a multifactorial syndrome. However, it has been demonstrated that porcine circovirus type 2 (PCV2) plays a crucial role in the pathogenesis of PMWS in pigs and is now recognized as the significant aetiological agent of the disease (Segalés and Domingo, 2002). PCV2 nucleic acid and antigen has been demonstrated in abundance within the lesions of affected pigs and the subsequent isolation and characterisation of a PCV2 virus from diseased pigs was reported (Ellis *et al.*, 1998).

1.3.1 Coinfection with other agents

To demonstrate if porcine circovirus is the causative agent of PMWS many consistent and repeatable disease models have been developed using infectious (Allan et al, 1999a; Krakowka *et al.*, 2000; Allan *et al.*, 2003) and non-infectious (Krakowka *et al.*, 2001) co-factors (Allan, 2005). Tischer *et al* carried out the first of these experiments in 1986. Six minipigs at the age of 9 months with no antibodies to PCVs were intranasally infected with PCV1. However, the pigs showed no signs of disease, nor could any increase in body temperature be measured. However, all pigs were seen to seroconvert to PCV1. Post-mortem and histopathological examination gave no indication of a specific virus disease. The same experiment was then carried out on newborn pigs to determine if

the younger pigs would become ill more readily than the older pigs. Again no signs of disease developed in these pigs (Tischer, 1986). The presence of PCV2 antigen has been demonstrated in lesions of animals affected by PMWS (Ellis *et al.*, 1998). Balasch *et al* (1999) inoculated conventional pigs from high health status farms with tissue homogentates from pigs with PMWS intranasally. Pigs were necropsied 14 or 21 days post inoculation (dpi). PCV2 nucleic acid was found in close association with microscopic lesions showing that PCV2 is transmissible to pigs by the nasal route (Balasch *et al.*, 1999). In another study carried out by Magar *et al* (2000) lesions were seen to first occur in the lungs demonstrating the respiratory system as a route of entry. Alternatively, PCV2 may infect the nasopharynx and tonsils and spread to other sites in mononuclear cells via the blood or lymph (Magar *et al.*, 2000b). In the study carried out by Balasch *et al* (1999) severe clinical signs were not reproduced, although, some pigs showed comparatively mild signs such as diarrhoea, increased rectal temperatures and reduced average daily weight gain. Failure to reproduce PMWS clinically was also reported by Lecann *et al* (1997). Microscopic lesions, similar in type and location to those described in pigs with naturally occurring PMWS but not as intense, were reported and a strong relationship was observed between the lesions and the detection of PCV2 as is in natural cases of PMWS (Balasch *et al.*, 1999).

The failure of the above mentioned experimental models to reproduce clinical disease when pigs were infected with PCV2 alone led to further investigations. In an experiment designed by Ellis *et al;* (1999) to determine the involvement of the novel PCV2 virus isolate (PCV-Saskatoon-7) in PMWS, neonatal gnotobiotic piglets were

inoculated with tissue homogenates from low and high passage cell culture material. Gnotobiotic piglets were used in order to exclude any external environmental sources of infectious or toxic material. However, the inoculum, was subsequently shown to have been coinfected with porcine parvovirus (PPV). Evidence of PPV in lesions typical of PMWS in these inoculated piglets suggested this virus may also play a role in the pathogenesis of the field syndrome. PPV is endemic in swine populations and many weaned pigs may be infected with PPV. PPV has only rarely been associated with any disease in piglets other than foetal death. All pigs were infected intranasally, again suggesting that the disease can be transmitted by this route via the respiratory or digestive tracts. Clinical signs were not consistently observed in the gnotobiotes in spite of the presence of gross and histological lesions characteristic of PMWS. However, lesions observed in pigs coinfected with PCV2 and PPV in this study included lymphadenopathy of bronchial nodes with prominent giant cells, interstitial pneumonia and multifocal myositis involving smooth and cardiac muscle (Ellis, 1999). The absence of clinical signs in this case may have been due to the absence of environmental stressors such as management practices, exogenous microflora or pathogens that could be cofactors in the development of disease. In addition, the inocula had relatively low titres of PCV2 and PPV (Ellis, 1999). This accidental inoculation of the pigs with a combination of the two viruses led to further studies investigating the effects of the two viruses when inoculated intranasally either singly or in combination into colostrum deprived conventional pigs. In studies carried out by Kennedy *et al,* (2000), pigs inoculated with PCV2 alone developed mild to moderate histopathological changes characteristic of PMWS. Virus was isolated from tissues, indicating that lesions of PMWS can be induced by inoculating pigs with

PCV2 alone, thereby fulfilling Koch's postulates. In pigs inoculated with both PCV2 and PPV, severe clinical signs including jaundice and severe microscopical lesions characteristic of PMWS developed. Pigs infected with PPV alone, developed mild interstitial nephritis which was indistinguishable from that in those animals infected with PCV2 alone and were associated with the presence of PPV antigen in tubular epithelium cells. Concurrent infection with PPV increased the severity of lesions suggesting this and other agents may play an important role in modifying the clinical and pathological expression of PCV2 infection (Kennedy *et al.*, 2000).

Field studies were then necessary to determine if these viruses interact in naturally acquired PMWS. Affected tissues from field cases were examined by immunohistochemistry (IHC) and polymerase chain reaction (PCR) to determine if a synergistic effect of the two viruses in producing clinical disease existed. Both PPV and PCV2 are small, SS DNA viruses that have a small coding capacity and are therefore highly dependent on host cell functions. Little is known of the requirements for growth of PCV2 *in vivo,* only that it is dependent on the S-phase of cell-cycle. It has been hypothesised that replication of both PCV2 and PPV depends on cellular enzymes expressed during the S-phase. Therefore, these viruses may target the same cells *in vivo* and may interact in some way to synergistically enhance replication. The same physiological factors may enhance replication of both viruses and so the presence of both PPV and PCV2 in lesion sites could be related to disease. Another theory suggested by Ellis *et al* (2000) is that viral antigens in phagocytic cells in lesion sites may have been engulfed at extralesional sites and transported intracellularly into sites of tissue damage.

It has also been suggested that replication of both PCV2 and PPV may occur in rapidly replicating cells in sites of pre-existing tissue damage (Ellis *et al*, 2000). PCV2 and PPV have a predilection for lymphoid tissue. Therefore replication of these agents in synchrony could have profound immunomodulating consequences and predispose to debility and secondary infections (Allan et al, 1999a; Ellis *et al*, 2000).

In the study mentioned above carried out by Kennedy *et al*, (2000) both viruses were detected in typical lesions of PMWS. It has therefore being suggested that PPV may be one of several cofactors, including other infectious agents such as PRRSV, that act synergistically with PCV2 in the pathogenises of PMWS.

To determine the involvement of other pathogens in the development of PMWS, other experimental models were created, some of which involved the coinfection of pigs with PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV). PRRS is one of the most significant infectious diseases affecting the swine industry. Evidence from field surveys suggests that coinfections of pigs with these two viruses is common (Allan *et al*, 2000b) as PCV2 is ubiquitous in the pig population and PRRS is also very common in pigs. Preliminary studies have shown that both PCV2 and PRRSV were present in the livers of sows with PRRS-associated hepatitis and both PRRSV and PCV2 antigen or genome have been detected in tissues from the majority of PMWS cases (Harms *et al*, 2001). However, the detection of PCV2 and PRRSV in the same animals may only be co-incidence as these are two infectious agents with a high prevalence in commercial pig populations. Harms *et al*, (2001) infected 3- week old caesarean-derived,

colostrum-deprived (CD/CD) piglets with PCV2 alone, PRRSV alone and a combination of the two viruses. PCV2 alone was found to be pathogenic in CD/CD pigs and this corroborates the findings mentioned earlier. Coinfection with PCV2 and PRRSV induced severe clinical disease, high mortality and lesions consistent with PMWS (Harms *et al*, 2001). Allan *et al*, (2000b) also infected colostrum deprived pigs with PCV2 alone, PRRSV alone and a combination of the two. Neither clinical disease nor gross lesions were observed in pigs inoculated with PCV2 or PRRSV alone. Pigs inoculated with both viruses in this experiment did not exhibit clinical disease, but it is possible that disease and gross lesions would have occurred if the experiment was not terminated at 28 days PI. In the pigs inoculated with PRRSV alone or the combination of the two, PRRSV antigen was only sporadically detected in minimal amounts, indicating that PCV2 infection does not potentiate PRRSV replication. In contrast, in pigs inoculated with PCV2/PRRSV, PCV2 viral load and tissue distribution was enhanced when compared to pigs inoculated with PCV2 alone. Results of this study suggest that previously reported potentiation of PCV2 replication in pigs following coinfection with PPV is not unique and that coinfection of pigs with PCV2 and PRRSV also potentiates PCV2 replication. The commonality of target cell types for these viruses may play an important part in this synergism. The results possibly negate the proposed hypothesis that PCV2/PPV synergism may be related to the dependence for growth of both of these viruses on cellular enzymes expressed in the S phase of cell replication (Allan *et al*, 2000b).

Similar experimental infections were also carried out by Rovira *et al*, (2002) using conventional pigs. Prior to inoculation all pigs were sero-negative to PRRSV and

had low titres of antibodies to PCV2. In the dual inoculated pigs increased rectal temperature (>40.5°C), wasting, severe respiratory distress and intense paleness was observed and PMWS was reproduced. In the pigs inoculated with PRRSV alone, no respiratory clinical signs developed. However, the average daily weight gain was lower, wasting was observed and reduced feed conversion rates were detected. Finally, in pigs inoculated with PCV2 alone, the pigs became infected but no clinical signs developed. The amount of PCV2 nucleic acid detected was higher in PRRSV/PCV2 inoculated pigs than in PCV2 only inoculated pigs and was present in a wider range of tissues. This suggests that PRRSV infection promotes the replication of PCV2 (Rovira *et al.*, 2002). Because PPV and PRRSV may potentiate PCV2 infection, other infectious agents such as *Cryptosporidium parvum*, (Nunez *et al.*, 2003), and pulmonary aspergillosis (Segales *et al.*, 2003b) may have similar effects.

In all experimental models mentioned, it is possible that a number of other factors such as the age and source of the pigs, the environmental condition in which the pigs were held, the genetics of the pigs and the nature of the PCV2 inoculum used, may have played a role in the consistent experimental reproducibility of the disease.

1.3.2 Transmission of PCV2 Infection

The oronasal route (Balasch *et al.*, 1999) is considered the most likely route of PCV2 transmission as supported by experimental studies on PCV2 infection, which have mainly used the intranasal route of inoculation (Allan et al, 1999aa; Ellis *et al.*, 1999; Krakowka *et al.*, 2000; Krakowka *et al.*, 2001; Rovira *et al.*, 2002). Under field

conditions the majority of pigs seroconvert to PCV2 between 2 and 4 months of age indicating that horizontal transmission of PCV2 between pigs is very efficient. Horizontal transmission to susceptible pigs commingled with already infected pigs has been demonstrated under experimental conditions (Albina *et al.*, 2001; Bolin *et al.*, 2001; Allan, 2005). Cloned genomic DNAs of PCV2 have been shown to be infectious and capable of producing histological lesions consistent with PMWS, when injected intramuscularly, intraperitoneally or directly into the liver or lymph nodes of pigs (Fenaux *et al.*, 2002; Roca *et al.*, 2004; Allan *et al.*, 2005). Transplacental transmission of PCV2 has recently been demonstrated following intranasal infection of sows (Park *et al.*, 2005) indicating that vertical transmission of PCV2 is feasible. Recent data from Korea has shown PCV2 infection in about 13% of aborted foetuses and stillborn piglets (Kim J., 2004).

1.4 Epidemiology

Since it was first described PCV2-associated PMWS is now recognized in most pig producing countries (where pigs are both intensively and extensively produced) in Europe, North and South America and eastern Asia (Chae, 2004). In Europe PMWS was first diagnosed in France in 1996 and then it was subsequently diagnosed across Europe, Table 1.1. A similar situation was observed in Asia where the disease has been known since 1995 in Taiwan, 1996 in Japan, 1998 in Thailand and 2000 in South Korea. The disease was also seen to emerge in the North Island of New Zealand in September 2003 (Laval, 2004).

Year PMWS first Diagnosed	Country
1991	Canada
1995	Taiwan
1996	Japan
1996	France
1997	Spain
1997	Belgium
1997	The Netherlands
1998	Thailand
1998	Northern Ireland
1998	Germany
1999	England
1999	Italy
1999	Hungary
1999	Greece
1999	Czech Republic
2000	South Korea
2002	Croatia
2003	New Zealand

Table 1.1: Emergence of PMWS

It was originally believed that PCV2 was a 'new' virus of the *Circoviridae* family. However, recent testing of archived tissue samples has shown that infection with PCV2 in the pig population in many countries existed prior to emergence of clinical disease (Magar *et al.*, 2000a; Bielanski *et al.*, 2004). PCV2 has been detected in archived porcine tissues as far back as, 1969 in Belguim, 1973 in Northern Ireland, 1986 in Switzerland, Canada and Spain, 1989 in Japan and 1993 in Thailand (Staebler *et al.*). PCV2 is now considered a ubiquitous virus both in countries where PMWS has or has not been detected (Allan *et al*, 2000a; Segales *et al.*, 2004a). This would suggest that the host-virus relationship was formerly one of a stable sub-clinical persistent infection. Clinical disease associated with PCV2 infection is now widespread and nucleic acid and antigen

have been demonstrated in abundance in tissues of pigs with and without PMWS. The question remains what has caused this change in the symbiotic relationship between PCV2 and the pig population and what is now triggering (PCV2 to cause the) clinical disease in some pigs? Detection and genetic typing of PCV2 DNA in archived paraffin wax-embedded tissues was carried out to confirm the presence of PCV2 in the British pig population since at least 1970. Of the samples tested, 41% from the 1990's, 31% from the 1980's and 32% from the 1970's were found to be positive for PCV2 by PCR and IHC. PMWS as a disease only recently emerged and it was thought that maybe changes to the PCV2 genome could account for an apparent increase in virulence. However, recent experimental reproduction of PMWS using a Swedish isolate (when Sweeden was free of PMWS) provides strong evidence against varying virulence of PCV2 isolates being responsible. It has been hypothesised that the involvement of extraneous agents/factors, in combination with other viral agents, including various microbial agents and components of modern intensive farming practices and modern husbandry practices such as early weaning, or immunological or other stresses are contributory factors to the incidence of PMWS today. High genetic similarity between archival samples and contemporary isolates provide evidence against the involvement of a 'new' or modified virus in the aetiology of PMWS (Grierson, 2004b).

1.5 Pathogenesis

PMWS is now recognised as a disease of pigs where PCV2 infection is needed for expression of the clinical condition. However, it is also recognised that PCV2 infection, linked to other co-factors, is necessary for the consistent development of full clinical disease in pigs (Allan *et al*, 2005). Up to 20% of pigs that develop this disease may recover. In these animals clinical signs and lymphoid lesions may resemble those found in the initial stages of disease. However, most pigs infected with PCV2 will not develop clinical PMWS. When clinical PMWS is apparent, damage to the immune system is the main feature in affected pigs. Lymphocyte depletion of lymphoid tissues, changes in peripheral blood mononuclear cell (PBMC) sub-populations and altered cytokine expression patterns are common in both natural and experimental cases of PMWS in pigs (Clark, 1997; Rosell *et al.*, 1999; Darwich *et al.*, 2003a; Darwich *et al.*, 2003b; Nielsen *et al.*, 2003). Pigs with PMWS show different degrees of wasting, generalized lymphadenopathy, non-collapsed and tan-mottled lungs, sometimes with very marked interstitial oedema (Segales and Domingo, 2002). Less frequently, hepatic atrophy and kidney lesions may also be present. Thin-walled, fluid-filled intestines have also been found especially in the ileum and spiral colon (Krakowka S., 2003; Darwich L., 2004,). PCV2 can also induce hepatitis and liver failure is one of the causes of death in PMWS-affected pigs (Rosell *et al.*, 2000; Krakowka *et al.*, 2001).

The pathogeneses of naturally acquired PCV2 infection has not been fully characterised (Segales *et al,* 2004). Little is known about the growth of PCVs in their natural host or the tissues involved in PCV2 replication. As mentioned, it has been

speculated that viral transmission may occur vertically, by direct (oro-nasal) or sexual contact, since PCV2 has been detected in nasal cavaties (Sibila *et al*, 2001), semen (Hamel *et al*, 2000) and in foetuses between 32 and 114 days gestation (Ohlinger and Pesch, 2000). Still unknown is the identity of cells that support PCV2 replication. In naturally affected PMWS in pigs initial PCV2 replication probably takes place in macrophages and lymphoid tissues such as tonsil and regional lymph nodes or Peyer's patches if virus is swallowed and survives the low pH of the stomach (Royer *et al.*, 2001). After infection and replication in the resident mucosal macrophages and other antigen-presenting cells, PCV2 might be transported either intracellularly or free in lymph and/or blood. The normal traffic of PCV2 target cells to many tissues may contribute to the spread of viral infection to numerous organs (Rosell *et al.*, 1999). It appears that histiocytic infiltration is one of the initial events during PCV2 infection and coincides with macroscopic lymphadenopathy (Segales *et al.*, 2004b). However, it has also been noted that the large amount of PCV2 viral antigen found in macrophages and dentritic cells of diseased pigs appears to be the result of the accumulation of viral particles (Gilpin *et al.*, 2003; Vincent *et al.*, 2003) and not the result of active virus replication in these cells.

It has been shown that some virus-infected tissues are free of pathological changes but in general, changes can be found in almost all tissues in severely PMWS-affected pigs. Generally, the more severe the lymphoid lesions, the higher the number of PCV2 infected tissues. No direct correlation has been found between the severity of PMWS lymphoid lesions and the severity of lesions in other tissues such as lung, liver or

kidney. The pathogenesis of naturally occurring PCV2 infection in adult pigs is highly speculative. A suggested pathogenic scheme for PCV2 infection in nursery and fattening pigs is outlined below in figure 1.1 (Segales *et al.*, 2004a):

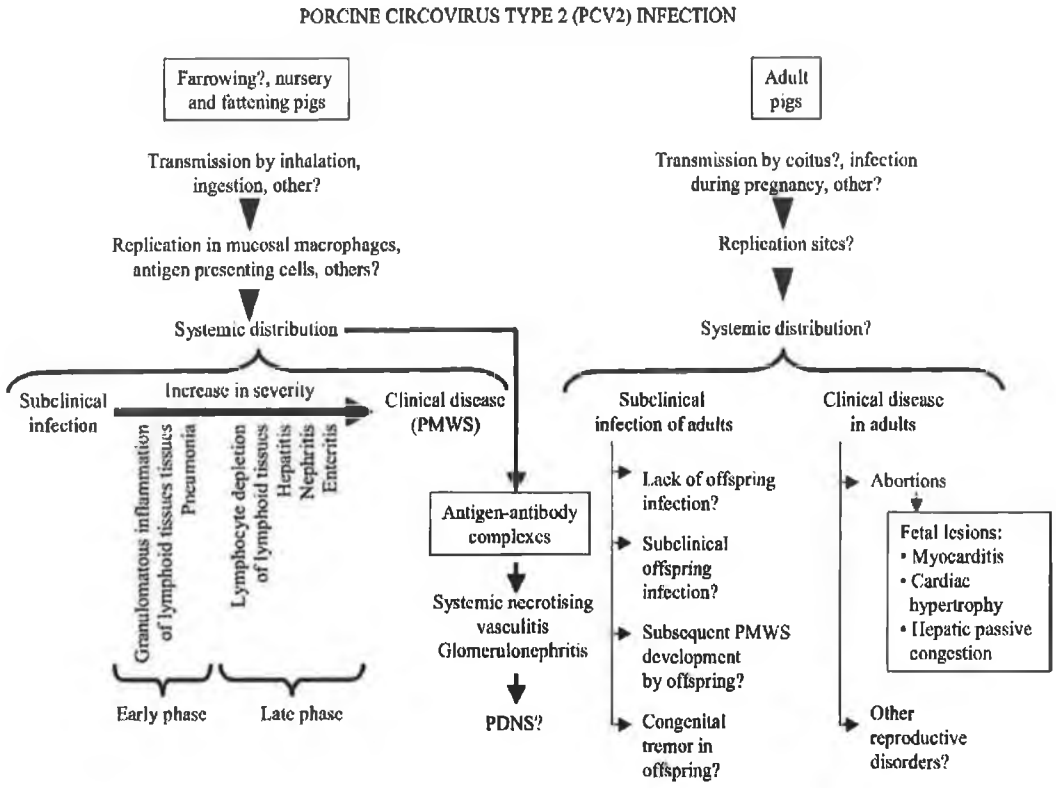

Figure 1.1: Suggested pathogenesis of natural PCV2 infection

It has been proposed that infection with PCV2 in particular conditions may be a cause of secondary immunodeficiency as a result of other organisms in pigs (Segales *et al.*, 2004b). Primary or secondary immunodeficiency increases the susceptibility of animals to infectious organisms of normally low pathogenicity or from an attenuated live vaccine. Immunodeficiency can manifest as recurrent illnesses that are usually difficult to control, failure to respond adequately to vaccination, unexplained neonatal illness and

death affecting more than one animal in a litter, and a variety of disease syndromes occurring concurrently in a herd. Some of these findings have been extensively described in PMWS outbreaks. The lack of antibiotic therapy response, the existence of a litter effect and the concurrence of other disease syndromes and well-known secondary pathogens may be features of immunosuppression in PMWS (Segales *et al.*, 2004a).

PCV2 may also infect the boar. PCV2 DNA has been detected in seminal fluid and non-sperm cell fractions of boar semen samples. Therefore, semen may be a significant vehicle for the transmission of PCV2. Production of high quality pathogen free semen is of particular importance to artificial insemination (AI) organizations within the pig industry (Chae, 2005).

1.6 Porcine Circovirus Disease (PCVD)

Since the identification of PCV2 and its subsequent association with PMWS, PCV2 has been increasingly isolated from pigs with other clinical signs. The terminology porcine circovirus diseases (PCVD) has recently been proposed to replace existing acronyms (Allan *et al.*, 2000d; Allan *et al.*, 2005). These clinical syndromes and diseases are divided into pre- and post-natal manifestations. In pre-natal disease PCV2 infection is linked to reproductive failures whereas the post-natal signs of the disease in Europe, Asia and North America are predominately PMWS, porcine respiratory disease complex (PRDC) and porcine nephropathy syndrome (PDNS) (Chae, 2004). PCV2 is also thought to play a major role in proliferative and necrotizing pneumonia (PNP),

congenital tremors, sow abortion and mortality syndrome (SAMS), granulomatous enteritis, exudative epidermitis and necrotizing lymphadenitis (O'Connor *et al.*, 2001; JungHyun *et al.*, 2004). PMWS, PDNS and reproductive disorders are the most important ones. However, the extent of the involvement of PCV2 in pig diseases other than PMWS is still not clear as this virus is ubiquitous.

1.6.1 Reproductive Disorders

There have been several reports of PCV2 associated reproductive failure under field conditions and experimental studies have demonstrated a deleterious effect on foetuses when PCV2 has been directly inoculated into them (Sanchez *et al.*, 2001b; Johnson *et al.*, 2002; Sanchez *et al.*, 2003; Pensaert *et al.*, 2004b; Sanchez *et al.*, 2004; Yoon *et al.*, 2004). PCV2 can infect the foetus and cause reproductive failure in the absence or presence of other well established reproductive pathogens. Consistent clinical signs on affected farms include elevated levels of abortion, stillbirths and foetal mummification. Reproductive failure is characterised by late-term abortion and delivery of stillborn near-term foetuses or premature piglets. Detection of PCV2 antigen and nucleic acid in stillborn piglets suggests that PCV2 can be present in large amounts in foetuses infected *in utero,* indicating that vertical infection may be an important means of viral transmission. This mode of transmission could be related not only to reproductive failure but also to the development of multisystemic disease in later life. Almost all descriptions of reproductive disorders associated with PCV2 are from North-America (O'Connor *et al.*, 2001), and very few cases have been reported in Europe (West *et al.*, 1999; O'Connor *et al.*, 2001).

Histopathologically there are no characteristic lesions in PCV2-infected aborted foetuses. Microscopic lung lesions have been reported in aborted foetuses in late gestation and stillborn piglets. When present, these lesions were mutifocal and mild to moderate in severity. Extensive areas of myocardial degeneration or necrosis, with oedema and mild fibrosis as well as a diffuse moderate infiltration of lymphocytes and macrophages, were observed (Segales *et al.*, 2004a; Chae, 2005). The primary PCV2 associated lesion in aborted or weak piglets appears to be myocarditis and PCV2 infection alone can produce severe lesions. It has been confirmed that PCV2 is associated with failure at all stages of gestation (Sanchez *et al.*, 2001a). The consequences of maternal infection at various stages of gestation may reflect the ability of the conceptus to support PCV2 replication rather than the effectiveness of the placental barrier (Chae, 2005; Ellis *et al.*, 2004; Harding, 2004; Kim J., 2004; Pensaert *et al.*, 2004a; Segales *et al.*, 2004a).

1.6.2 Porcine Dermatitis and Nephropathy Syndrome (PDNS)

PDNS is a relatively new and often fatal disease that primarily affects recently weaned and feeder pigs from 1.5 to 4 months of age. The syndrome was first recognised in the UK in 1993 (Smith et al., 1993). Since then it has been reported in several countries including Korea, Canada and the USA (Chae, 2005). PDNS is generally sporadic. The most common clinical signs are the development of round irregular shaped, red to purple skin lesions that coalesce to larger patches and plaques. Primary lesions in the skin and kidney are associated with a multisystemic vasculitis and skin lesions are usually first noted in the hindquarters, limbs and abdomen but may progress to

involve the thorax, flank or ears. Shortly after the appearance of the skin lesions, pigs may become pyrexic with rectal temperatures greater than 41°C. The kidneys become enlarged, pale and often exhibit petechial haemorrhages. These lesions are probably the result of immune disease complex. Renal and inguinal lymph nodes are usually enlarged and haemorrhagic. In fatal cases, cutaneous lesions result from severe necrotizing vasculitis affecting the dermis and subcutis, characterized by leukocytoclastic (the presence of neutrophils with nuclear fragments) inflammation involving capillaries, small and medium sized venules and arterioles, accompanied by epidermal necrosis and ulceration and dermal haemorrhage. Mildly affected animals may remain afebrile, bright and alert and most often recover. Severely affected animals may demonstrate lameness, anorexia or weight loss. Sudden death may occur but this is rare. There is increasing evidence to support a link between PDNS and PCV2. PCV2 antigen and/or nucleic acid have been found in tissues of pigs with PDNS. Many herds in both Europe and Korea have reported PMWS and PDNS outbreaks occurring simultaneously or in close chronology. However, there appears to be no direct relationship between PMWS and PDNS in affected herds. Pigs with PMWS do not progress to PDNS and vice versa. PCV2 nucleic acid has been demonstrated in more abundance in lymph nodes from pigs with PMWS than in those from pigs with PDNS. In contrast, PCV2 nucleic acid was in more abundant in the kidney from pigs with PDNS than in those with PMWS. It is possible that the difference in distribution of PCV2 between PMWS and PDNS could be due to different tissue tropism of different PCV2 strains. There is also evidence that the presence of other pathogens, such as *Pasteurella multocida* or combinations of pathogens

such as PCV2 and PRRSV may also induce PDNS (Chae, 2005; Ellis *et al.*, 2004; Harding, 2004)

1.7 Clinical and Post-Mortem Findings

PMWS primarily occurs in pigs between 25 and 120 days of age, with most cases occurring between 8 – 12 weeks. Outbreaks are now being increasingly reported in fattening pigs (Allan *et al.*, 2005). PMWS has been described in almost all types of farms, including farrow-to-finish and multi-site operations, and in sizes from 30 to 10,000 sow herds. Clinical expression of the disease is associated with the generation and accumulation of infectious virus in target tissues. Morbidity and mortality are variable, depending on the farm and on the batches of animals and the stage of the outbreak, co-infections and husbandry and management within affected areas; the usual rates are 4 – 30% and 70 – 80% respectively (Segalés and Domingo, 2002). Single pigs suffering from PMWS has also been observed sporadically on farms with very good production records and minimal postweaning mortality (Allan *et al.*, 2005).

There are six fundamental clinical signs of PMWS, which may be non-specific and variable and form the basis of a preliminary clinical diagnosis (Segalés and Domingo, 2002). The main clinical sign is wasting but it is usually seen concomitantly with other fundamental clinical signs such as, dyspnoea, enlarged lymph nodes (mainly inguinal superficial lymph nodes), diarrhoea, jaundice and pallor (Segalés and Domingo, 2002). Not all of the clinical signs may be present in a single pig; however, an affected

farm will present the majority over a period of time. Other clinical signs noted include coughing, pyrexia, icterus, gastric ulceration, meningitis while sudden death may arise due to secondary infections (Segalés and Domingo, 2002).

Although PMWS was first recognized in an SPF herd, additional infections or diseases are more commonly found in farms experiencing PMWS when compared to non-affected farms (Ellis *et al.*, 2004). It has been reported that pigs suffering from the disease may become immunosuppressed, resulting in an increased susceptibility to other pathogens including bacteria and less frequently viruses, mycoplasma and protozoa (Ellis *et al.*, 2000). Other viral and bacterial pathogens commonly found include, PRRSV, swine influenza virus (SIV), ADV, PPV *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, and *Mycoplasma hyopneumonia,* (Kim *et al* 2002). Diseases found with increased frequency include Glasser's disease, salmonellosis, streptococcal meningitis, postweaning colibacillosis, non-specific diarrhoea and hepatosis dietetica (Kim *et al* 2002). Whether or not the increased disease load found on PMWS-affected farms is a consequence of PMWS and the associated immune dysfunction has yet to be determined. It is probable that the final clinical outcome observed on farms affected with PMWS is the sum of the effects of the various concurrent diseases (Allan *et al.*, 2005).

It has been reported that, on some farms, most of the pigs developing PMWS correspond to a few litters, suggesting a potential "litter effect" (Madec *et al.*, 2000) and other reports have shown that castrated male pigs are more susceptible to PMWS than

females (Rodríguez-Arrioja *et al.*, 2002; Allan *et al.*, 2005). It has also been reported that pigs with lower birth and weaning weights and lighter pigs at the beginning of the fattening period, were more likely to develop PMWS (Allan *et al.*, 2005).

At necropsy, pigs suffering from PMWS present a variety of macroscopic and microscopic findings. Necropsy findings are mainly poor body condition, moderate pallor of the skin and various degrees of muscle wasting. Gross lesions in pigs with PMWS may be quite variable. The most striking lesions are: (i) non-collapsed lungs with interstitial oedema and enlargement of lymph nodes (mainly superficial inguinal, submandibular, mesenteric and mediastinal). However these lesions are not always present, (ii) mild to moderate atrophic discoloured livers (hepatic interlobular connective tissue may be prominent in severe cases). In the most advanced cases, pigs may have a flaccid liver, reduced in size, with marked loss of hepatocytes, increased amount of fibrous tissue at the lobular margins, and inflammatory infiltrates through all the hepatic tissue (Krakowka *et al.*, 2000; Rosell *et al.*, 2000); (iii) pale grossly enlarged kidneys may appear semi-translucent and waxy due to oedema and multifocal white foci may also be present on kidney cortices; (iv) other findings include bronchopneumonia which may be detectable due to bacterial infections; gastric ulceration of pars oesophaga (not directly related to effect of PCV2) of the stomach and fluid filled thin walled intestines especially the ileum and spiral colon; the spleen may be moderately enlarged, firm and non-congested on cut surface, and in the end stages of disease cachexia may develop (Segalés and Domingo, 2002). Although gross lesions are uncommon in the pancreas, there may be multifocal atrophy or regeneration in acinar epithelium that is associated with

interstitial lymphohistiocytic infiltration. Other organs such as gastric and intestinal mucosa, heart, adrenal glands, salivary glands and bone marrow may also show foci of lympho-histiocytic inflammatory infiltrates, although in a lesser intensity and smaller distribution than the main affected organs mentioned. Histologic lesions are characterized by the presence of syncytial multinucleated giant cells in lymph nodes, liver, spleen, tonsil, thymus and Peyers patches and grape-like circoviral intracytoplasmic inclusion bodies within the multisystemic lesions (Kim J *et al*, 2001). Usually intranuclear PCV2 antigen is found in affected tubular epithelial cells and scattered in mononuclear phagocytes (Allan and Ellis, 2000a).

The histopathological lymphoid lesions observed in PMWS affected farms are unique (Clark, 1997; Rosell *et al.*, 1999). Early microscopic lesions consist of infiltration of subcapsular sinuses by large histiocytic cells and giant multinucleate cells, and effacing of lymph follicles. Multinucleate giant cells may also appear in lymph follicles and in parafollicular zones. This is followed by depletion of lymphocytes in parafollicular zones, which vary in intensity from slight to intense (Clark, 1997; Rosell *et al.*, 1999). In the thymus, cortical atrophy is a prominent finding (Darwich *et al.*, 2003b). In a large number of cases it is possible to find cytoplasmic inclusions in histiocytes or dentritic cells. Inclusions are basophilic or amphophilic, rounded, and of very different sizes. Final stages of depletion show an empty lymphoid tissue, with a prominent network of stromal and accessory cells.

1.8 Diagnosis

From a clinical point of view the diagnosis of PMWS is difficult with clinical signs in each farm varying considerably. Diagnosis is generally based on clinical signs, post-mortem changes, histopathology and PCV2 antigen and/or nucleic acid detection. However, a pig or a group of pigs is considered to suffer from PMWS if they meet the following criteria: (i) clinical signs including growth retardation and wasting, frequently with dyspnoea and enlargement of lymph nodes, and occasionally jaundice, (ii) presence of characteristic histopathological lesions in lymphoid tissues (lymphocyte depletion together with granulomatous inflammation) and presence of PCV2 inclusion bodies in a proportion of infected pigs, and (iii) detection of PCV2 within the lesions in lymphoid tissues of affected pigs. In all PMWS cases, PCV2 is present in a variable amount in at least one tissue of the affected pig. However, in order to detect PCV2 and other concomitant infections a wide range of tissues including lymph nodes, tonsil, spleen, intestines, lung, liver and kidney should be tested (Allan, 2002a; Segalés and Domingo, 2002).

In order to establish the aetiological diagnosis of PMWS, diagnostic techniques are required that link the presence of virus with tissue lesions. Several methods have been developed to detect PCV2 in tissues and to correlate its detection with the presence of lesions. Histopathology, together with a technique to demonstrate PCV2 levels, has been the most important tool to establish a diagnosis of PMWS. The most powerful combination is one that allows the correlation between the severity of histopathological lesions in lymphoid tissues and the amount of PCV2 in the same tissues. A clinical

diagnosis may therefore be confirmed using a combination of immunohistochemistry (IHC) and/or indirect fluorescent antibody (IFA) (Racine *et al.*, 2004) or *in situ* hybridization (ISH) (McNeilly *et al.*, 1999; Rosell *et al.*, 1999; Choi and Chae, 2000; Kim and Chae, 2001) together with histopathology. Other techniques that may be used to detect PCV2 for diagnostic and research purposes include virus isolation (Allan *et al.*, 1998b), PCR (classical, multiplex or real-time), electron microscopy and DNA probes. Detection of PCV2 by virus isolation and PCR does not necessarily confirm a diagnosis of PMWS because PCV2 can be at a high concentration in healthy pigs. Information provided by IHC and ISH is more specific than that obtained by PCR as these methods provide cellular localisation and an indication of the level of expression of specific viral genes in tissue sections (JungHyun and ChanHee, 2004). Several serological techniques to detect antibodies to PCV2 have also been developed. These techniques are usually based on cell cultures infected with PCV2 and include indirect peroxidase monolayer assay (IPMA) (Mesu *et al.*, 2000). A commercial enzyme linked immunosorbent assay (ELISA) has recently become commercially available from Synbiotics. Two new ELISA kits have been manufactured, SERELISA® PCV2 Antibody (Ab) Mono Blocking for use on serum and faeces samples, this kit uses a single well blocking immunoenzymatic technique and SERELISA® Ag Capture for use on faeces samples this kit uses a single well sandwich immunoenzymatic technique for the detection of PCV2 antigen. Wells are sensitized with anti-PCV2 Ab, viral protein if present binds to the specific sites an anti-PCV2 preoxidase conjugate allows the revelation of the antigen, optical densities are recorded and used to determine the presence or absence of antigen as a function of threshold values.

When high concentrations of virus are detected in the presence of histological changes the animal is considered positive for PMWS. If slight lymphoid lesions together with low amounts of PCV2 are the dominating findings there are three possible interpretations: (i) the pigs are suffering from a subclinical PCV2 infection, (ii) they may be only at the initial stages of PMWS and the lesions and the amount of PCV2 could become more severe after this initial phase, (iii) the animals may be in a convalescent period after suffering from PMWS (Allan, 2002; Segalés and Domingo, 2002). Since sub-clinical PCV2 infection with viremia occurs on almost all farms detection of PCV2 antigen or nucleic acid in tissues from clinically healthy pigs, or in diseased pigs without clinical signs and gross lesions consistent with PMWS should be interpreted with caution (Rose *et al.*, 2003).

The case definition of PMWS does not exclude the concomitant presence of other diseases, and it implies that neither clinical signs nor gross lesions observed in suspected PMWS affected pigs are sufficient to diagnose the disease, although they are indicative. A herd case definition for PMWS should include the occurrence of a clinical process, characterised mainly by wasting and mortality, in excess of the expected and/or historical level for each farm, and the establishment of individual diagnoses of the disease in a number of pigs. However, the herd case definition for PMWS has not been formerly established yet (Segales *et al.*, 2003a; Allan *et al.*, 2005).

1.9 Possible Risk Factors for the Occurrence of PMWS

There is growing concern regarding PMWS and why this disease has gone from a sporadic, low incidence occurrence to an epidemic form. It has been hypothesized that husbandry and management practices may have contributed to this change. A profound change has been observed in pig production structure and techniques as a result of competitive economic pressures. The size of herds has dramatically increased with obvious changes in nutrition, housing, herd management, prophylaxes and general hygiene routines. However, this intensification of European pig production has brought with it a particular set of problems at the post-weaning stage of production. There are four golden rules set out to limit the spread of PMWS in farms. These are as follows: (i) Limit pig-pig contact; (ii) avoid stress; (iii) Good hygiene; and (iv) Good nutrition (Krakowka *et al.*, 2003).

Krakowka *et al* (2003) has postulated that the main risk factors associated with this disease are poor hygiene status in the post-weaning room, creep feed intake per piglet in the last week prior to weaning of less than 470g, draughts and poor air quality throughout the four weeks after weaning, poor weaning temperature and overcrowding. In addition, Krakowka *et al* (2003) has suggested that the water supply must be potable and within easy access, the number of piglets per pen should be less than 13, the number of litters of origin per pen should be less than 4 and to avoid commingled pigs of different age groups, in order to improve the severity of concurrent respiratory disorders and the overall farm disease level (Krakowka *et al.*, 2003).

Because PCV2 is ubiquitous, minimal efforts have been applied to control the infection. Effective control measures for PMWS have focused on the understanding, control and eradication of infectious/non-infectious factors/triggers on individual farms. Management measures, nutrition, the effect of concurrent viral infections, stimulation of the immune system, serum therapy, PCV2 vaccination and the infectious PCV2 status and the antibody titres to PCV2 of the sow at farrowing (Allan *et al.*, 2005; Marco, 2005) should all be monitored.

1.9.1 Management Practices

It has been suggested that several environmental factors might be necessary, in association with PCV2 infection, to lead to the clinical expression of PMWS. The implementation of the 20 Madec Principles, a list of zootechnical measures to lower the impact of the disease, has resulted in a reduction in post-weaning mortality (Madec, 2001). Madec's points are not new and they are based on the strict use of all-in-all-out management, reduction of stress, keeping stocking densities to a minimum, and good hygiene. Sometimes it is difficult to fulfill all 20 points on all farms so it has been suggested that at least 16 of the 20 points should be achieved in order to see a response (Allan *et al.*, 2005; Marco, 2005).

1.9.2 Nutrition

It has been reported that changes in the diet of PMWS-affected pigs has resulted in partial control of the disease (Donadeu *et al.*, 2004; Allan *et al.*, 2005). Such changes included an increase in nutrient density for young pigs diet and addition of commercial

feed additives with antioxidant effects, such as bioflavonoids, which are phytochemicals that act as antioxidants and have anti-inflammatory effects which may promote cardiovascular health (Allan *et al.*, 2005; Marco, 2005). Another recent study has shown that conjugated linoleic acid (CLA) modifies PCV2 experimental infection (Bassaganya-Riera *et al.*, 2003; Allan *et al.*, 2005). It has also been suggested that addition of vitamin E and/or selenium because of their antioxidant properties to feed might be beneficial on farms with PMWS (Allan *et al.*, 2005; Marco, 2005). Data from one farm in Denmark, however, showed no correlation between vitamin E levels in the blood of post weaning pigs and the outcome of PMWS (Baebko, 2004; Marco, 2005). However, there is not enough scientific information available to establish the full effect of nutrition on PMWS.

1.9.3 Effect of Concurrent Viral Infections

Viral co-infections have been used experimentally to reproduce PMWS as previously mentioned, and a wide range of infectious agents have been observed concomitant with PCV2 infection on PMWS affected farms (Segalés and Domingo, 2002). Therefore, the control of these coinfectious agents in the post weaning area should decrease the incidence of PMWS.

1.9.4 Stimulation of the Immune System

The development of clinical disease following immunostimulation in PCV2 experimentally infected pigs (Krakowka *et al.*, 2001; Allan *et al.*, 2005; Marco, 2005) has been supported by a number of on-farm studies where PCV2 infection and the use of certain commercially available vaccines or immunomodulators have acted as triggering

factors for PMWS (Allan *et al.*, 2001; Allan *et al.*, 2005; Marco, 2005) suggesting that immune activation may be an important triggering factor of PMWS and perhaps the pivotal event in the pathogenesis of this disease on some farms. However, from a practical point of view it is not feasible to exclude the use of vaccines from sanitary programmes. However, producers with PMWS affected herds could re-schedule the timing of vaccination as a potential plan to minimize the disease (Allan *et al.*, 2005).

1.10 Possible Treatments and Control Measures for PMWS-Affected Herds

1.10.1 Serum Therapy

Serum therapy has been shown to be useful in the control of PMWS (Marco, 2005). It involves the subcutaneous injection of suckling or nursery pigs with PCV2 hyperimmune sera from a commercial slaughterhouse (Ferreira, 2001; Waddilove, 2002; Allan *et al.*, 2005). However, this technique has given variable results and its use in some farms did not result in any significant effect (Allan *et al.*, 2005; Marco, 2005). Strict precautions must be applied when performing serum therapy. Blood collection must be from the same affected farm to reduce any risk of introducing other pathogens and collection and administration must be performed under strict conditions of hygiene. Serum kept frozen or serum from pigs that were already treated with serum when they were young leads to a reduction in its therapeutic properties (Allan *et al.*, 2005; Marco, 2005). The precise mechanism of action of serum therapy has not yet been elucidated

(Allan *et al.*, 2005). However, this technique has given variable results in different herds (Marco, 2005).

1.10.2 PCV2 Vaccination

Some experimental trials of vaccination have produced encouraging results. Vaccination with PCV2 proteins (ORF1 and ORF2) has shown a degree of protection. A more recent study using an inactivated vaccine in colostrum-deprived pigs challenged with PCV2 coinfection or previously immunostimulated, have shown a reduction in mortality (Marco, 2005).

An inactivated, ajuvanted PCV2 vaccine "Circovac" (MERIAL) for use in sows and gilts is now commercially available and in use under special license in some European countries and Canada. The efficacy of this vaccine in controlling PMWS under field conditions still remains to be elucidated (Allan *et al.*, 2005).

1.10.3 PCV2 status and Serological Titres to PCV2 of the Farrowing Sow

It has been reported that active PCV2 infection or low serological titres to PCV2 in sows at farrowing had a significant effect on the overall mortality of their offspring due to PMWS (Allan *et al.*, 2002). The protective effect of passive maternal immunity on PMWS development is supported by the fact that disease occurs once these titres have declined (Rodríguez-Arrioja *et al.*, 2002; Larochelle *et al.*, 2003; Sibila *et al.*, 2004). Therefore, measures that increase maternal immunity and decrease sow viremia at farrowing may diminish PMWS impact on piglet mortality (Allan *et al.*, 2005).

However, more recent studies in Denmark and the United Kingdom have shown that high levels of antibody to PCV2 in sows and gilts does not relate to any protection from PMWS in the piglets derived from these animals (Hassing *et al.*, 2004).

1.10.4 Genetics

Field observations from farmers have suggested that certain genetic lines of pigs, specifically in relation to boar lines, are more susceptible to PMWS. However, there is very little data to support this (Allan *et al.*, 2005; Marco, 2005). Recent experimental studies have shown that Landrace pigs were more susceptible to PMWS than Duroc and Large White Pigs (Opriessnig *et al.*, 2004). However, other studies have shown contradictory results with the use of the Pietran boar line; as the use of this genetic boar line did not seem to have any effect on the offspring in one study (Rose *et al.*, 2003). Another field study was carried out by Lopez-Soria *et al* (2004) to compare the effect of three different boar lines on the outcome of general and PCV disease-associated PMWS and their off spring. Lopez-Soria *et al.*, (2004) observed a significant effect on general postweaning and PMWS associated mortalities (Lopez-Soria *et al.*, 2004). These findings need to be expanded in an attempt to identify a possible combined epidemiologic, genetic and immunologic approach to the control of this disease (Darwich *et al.*, 2004).

There are many control measures that can be used with regard to PMWS. These can be combined successfully. However, there is no general protocol that will suit all

farms. Each individual farm will need to take a slightly different approach depending on their facilities, health status, genetics, etc.

1.11 PMWS in Ireland

1.11.1 PMWS in the Republic of Ireland

In 1998 an outbreak of ill-thrift and increase in mortality in pigs of five to six weeks of age, on an integrated 300-sow unit with a weaning age of approximately 26 days was investigated by Spillane *et al.*, (1998). The first signs noted included increase in mortality from 1.5 to 8% in the affected age group and respiratory disease in 20% of pigs characterised by an increase in coughing, sneezing and/or dyspnoea. Gross mortem examinations revealed lesions of pleurisy and pneumonia suggestive of *Mycoplasma hyopneumonia* and *Pasteurella multocida* infection. Histopathological examination of the liver from one affected pig revealed severe hepatic necrosis and histiocytic infiltration. Multifocal lesions of granulomatous inflammation were seen in the pancreas and kidney of this animal and there was severe lymphoid depletion and histiocytic infiltration of periarteriolar lymphoid sheaths in the spleen. Amphophilic intracytoplasmic inclusion bodies were seen in the infiltrated histiocytes. The histopathological lesions in this pig were similar to those of PMWS (Ellis *et al.*, 1998) and the results of immunoperoxidase staining (Allan *et al.*, 1998b) and the PCR technique indicated infection with PCV2. This was the first known report of PMWS and clinical PCV2 infection in the Republic of Ireland (Spillane *et al.*, 1998).

By the year 2000 PMWS had been diagnosed by Spillane in 7 herds since the original diagnoses in1998 and PCV2 was identified in tissues of affected pigs from each herd. There was no localised geographic distribution of affected pig herds (Spillane, 2000).

1.11.2 PMWS in Northern Ireland

In Northern Ireland an 11 week old pig was submitted for post-mortem examination in October 1997 from a herd with a history of sudden death; this was the first report of infection of pigs with PCV2 and associated PMWS-like lesions in pigs in NI and the British Isles (Kennedy *et al.*, 1998). Histopathological examination revealed mild lymphoid depletion and the presence of synctia in mesenteric lymph nodes of this animal. In November 1997 a 12 week old pig was submitted for PM examination from a herd in which there was evidence of ill-thrift in young pigs and respiratory disease in finishing pigs, this was the second reported case of PMWS in this country. Enteritis and moderate lesions of pneumonia were seen at post mortem and histopathological examination revealed severe mononuclear cell infiltration and formation of syncytia in the lamina propria of the small intestine. Moderate lymphoid depletion, histiocytic infiltration and syncytia were seen in the mesenteric lymph nodes. Lesions of bronchointerstitial pneumonia were also apparent. Immunohistochemical staining revealed that lymphoid lesions were associated with a large amount of PCV2 antigen (Kennedy *et al.*, 1998). The disease was not seen again until March 1999 when a diagnosis was confirmed on a different farm. This outbreak was associated at the time with the introduction of a new vaccination regimen for *Mycoplasma hyopneumonia*.

Following this sporadic outbreak the disease was not seen to emerge again until 2002 when a devastating outbreak with losses of up to 50% occurred on a farm in South Armagh. This disease outbreak was associated with importation of weaners from a PMWS affected farm in the ROI. From 2002 onwards, a total of 40 outbreaks of the disease have been recorded throughout NI, with losses ranging between 5 and 40% (Allan *et al.*, 2005).

To date no pattern of spread of this disease within NI or the ROI has been demonstrated nor has any linkage been demonstrated in NI between specific pig breeders and outbreaks of disease (Allan *et al.*, 2005).

1.12 Objectives for this Study

Since its occurrence PMWS has been reported in America, Europe, Asia and New Zealand. There was therefore a need to establish the current status of this viral disease on the island of Ireland. This project therefore was carried out as a collaborative project between Northern Ireland and Republic of Ireland. It was necessary to transfer techniques already used in Veterinary Sciences Division (VSD), NI to the Central Veterinary Research laboratory (CVRL), ROI so that an accurate diagnosis of PMWS could be achieved in the Republic of Ireland. An incidence study of suspect PMWS outbreaks was performed to determine the presence and emergence of PCV2 infection and associated disease in the pig population of Ireland. A serological survey was also carried out to determine the extent of PCV2 infection in Ireland. This was achieved by

testing a percentage of random serum samples spread throughout the country for antibodies to PCV2. To determine if different strains of the virus existed in Ireland it was necessary to isolate and type PCV2 strains from diseased and non-diseased pigs in Ireland. Detailed longitudinal studies on PCV2 associated diseased and non-diseased herds in Ireland were carried out in an attempt to elucidate any infectious/non-infectious "triggers" that may lead to PMWS.

Studies are on going on an all Ireland basis to try and identify any common factors or co-factors that could explain the epidemiology of the disease in Ireland.

Chapter 2: Materials and Methods

2.1 Immunoperoxidase Monolayer Assay (IPMA)

An immunoperoxidase monolayer assay (IPMA) for the determination of PCV2 antibody titres in sera was performed as previously described (Allan *et al.*, 2000c; Blanchard *et al.*, 2003). This technique is based on cell cultures infected with PCV2. PCV-free PK15 cells grown at 37°C in 10% CO_2 in minimal essential medium (MEM) (Biosciences, Dublin, Ireland) with Earls salts supplemented with 5% foetal calf serum (FCS) and 100μg/ml gentamicin, were dispersed from confluent flasks using trypsin. The cells were resuspended in Hanks balanced salt solution (HBSS) without phenol red (Biosciences, Dublin, Ireland, Gibco cat. no. 14065-031) containing 1% FCS. To the cell suspension PCV2 was added and incubated on a roller with continuous mixing for 2-4 hours, at 37°C. The cells were gently pelleted at 805 X *g* for 5 minutes and resuspended in 300-mM D(+)-glucosamine ($C_6H_{13}NO_5$ HCl, Sigma-Aldrich cat. no. G4875) prepared in HBSS. This suspension was incubated at 37°C for a further 10 minutes with continuous mixing. D-glucosamine (Sigma-Aldrich, Dublin, Ireland) stimulates the progression of cells in the S phase of mitosis, which accelerates the replication of PCV2 (Tischer *et al.*, 1987). However, care must be taken when carrying out this step due to its high level of cellular toxicity, and once the cells have been treated all traces of the glucosamine must be removed. The cells were then pelleted again as before and resusupended in MEM growth medium. The cells were resuspended at a concentration, which gave a near confluent monolayer within 72 hours of seeding the microtitre plates. The plates were harvested by removing the supernatant and washing the monolayer once with phosphate buffered saline and then leaving them to air dry completely. They were

then sealed and stored at least overnight at -20°C prior to use in order to adhere cells to the plates.

In order to perform the antibody titrations the plates were removed from the freezer and allowed to come to room temperature and to completely dry. A 4% paraformaldehyde (PFA) (Sigma-Aldrich) solution in distilled water was added to each well for approximately 10 minutes in order to fix the cells followed by methanol containing 0.3% hydrogen peroxide for five minutes to destroy endogenous peroxidase. Serum dilutions from 1 in 50 to 1 in 156,250 were prepared in PBS containing 0.05% Tween 80 and 10% horse serum (PBS-TH) and 50µl of each dilution was added to the wells of the seeded microtitre plates and incubated for one hour at 37°C. 50µl of a 1 in 200 dilution of Peroxidase-conjugated rabbit anti-swine immunoglobulins (DAKO Cytomation Ltd. P0164) was added to each well and incubated for a further hour at 37°C. Finally, 50µl enzyme substrate chromagen (AEC Zymed cat. no. 00-2007) was added to the plates and incubated at room temperature in the dark for 15 minutes. The reaction was stopped by washing the plates four times with distilled water. Finally, 100µl of distilled water was then added to each well prior to viewing under the microscope. Antibody reacting with PCV2-infected cells was seen as red coloured staining, predominantly in the cell nucleus. The titre was determined as the last dilution at which staining was still visible. All tests contained a positive control of known titre (provided by Veterinary Sciences Division, DARDNI, Stromont, Belfast), the test being valid if the results fell within a 2-fold dilution of this known titre.

2.2 Indirect Flourescent Antibody Staining of Pig Cryostat Tissue Sections for PCV2

In order to detect the presence of PCV2 antigen in fresh tissue samples, an IFA was performed, as previously described (Allan *et al.*, 1994b), on snap frozen cryostat tissue sections. Fresh tissue samples were first frozen in isopentane cooled in liquid nitrogen. Frozen tissues were then stored at -20°C until required for sectioning. These sections were cut to 6μm onto clean glass slides in a cryostat set at -16°C. Tissue sections were treated with PCV2-specific monoclonal antibody F217 (1 in 100 dilution in 0.01M PBS pH 7.2; (McNeilly *et al.*, 2001) and incubated for one hour at 37°C followed by rabbit anti-mouse FITC (DAKO F216), 1 in 100 diluted in 0.01M PBS pH 7.2, containing 10% PCV2 antibody free normal pig serum for a further hour at 37°C. Slides were examined using x40 objective lens, using incident UV illumination. Scoring ranged from a negative to a +4. Where no fluorescence was observed the tissue was considered negative, slight to moderate fluorescing, tissues were scored 1+ and 2+, respectively. Tissues where most areas or all were fluorescing were scored 3+ and 4+ and were considered to be from a PMWS diseased animal.

2.3 Polymerase Chain Reaction (PCR)

Various pig tissue samples (lymph nodes, liver, spleen, lung etc.), faeces and sera collected from pigs with and without PMWS, as confirmed by IFA, IHC and IPMA, were used. Viral DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen, cat. no. 69506) and QIAmp DNA Blood Mini Kit (Qiagen, cat. no. 51106), respectively, as per manufacturer's instructions. Extracted products were then stored at -20°C until required. PCR primer pairs were selected from work published by Fenaux *et al.*, (2000). These primers were reported to amplify two overlapping fragments representing the entire genome of PCV2. The first set of primers, CV1 and CV2, amplified a 989-bp fragment, and the second set of primers, CV3 and CV4 amplified a 1,092-bp fragment.

CV1: 5'-AGGGCTGTGGCCTTTGTTAC-3'

CV2: 5'-TCTTCCAATCACGCTTCTGC-3'

CV3: 5'-TGGTGACCGTTGCAGAGCAG-3'

CV4: 5'-TGGGCGGTGGACATGATGAG-3'

PCR reactions were prepared as follows using HotStarTaq® PCR Kit (Qiagen, cat. no. 203203):

PCR Mastermix:

PCR Component	Volume (µl)
10x Buffer	5.0
25 mM $MgCl_2$	1.5
DNTP mix (10mM)	8.0
Rnase/Dnase free H_2O	28.0
Taq DNA polymerase	0.5
Primer 1	1.0
Primer 2	1.0
DNA Sample	5.0

Amplification reactions were performed in the DNA Engine PTC-200 as follows: 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 3 minutes, followed by a terminal extension at 72°C for 7 minutes. This annealing temperature was 51°C for primer pairs CV1 and CV2. All PCR products were visualised by electrophoresis on a 2% high resolution agarose gel. PCR products of expected size were cleaned using QIAquick PCR Purification Kit (Qiagen, cat. no. 28104) according to manufacturer's instructions and were sequenced by MWG Biotech (Ebersberg, Germany). The sequences were then analyzed using the internet based bio-informatic software http://www.ebi.ac.uk/services/.

Analyzed sequenced products were found to not always overlap at expected regions, indicating that the entire genome had not been sequenced. Primer pairs were

then designed in order to amplify the area of the genome where the original primer pairs did not overlap using the internet based software http://bibiserv.techfak.uni-bielefeld.de. These primers pairs were called CV5, CV6, CV7 and CV8 and each amplified a 600-bp fragment (figure 2.1).

CV5: 5'-GAGGAAGGACGAACACCTCAC-3'

CV6: 5'-GAGAGCTTCTACAGCTGGGAC-3'

CV7: 5'-GAACAATCCACGGAGGAAGGG-3'

CV8: 5'-TGGGCGGTGGACATGATGAG-3'

PCR reactions were carried out as above.

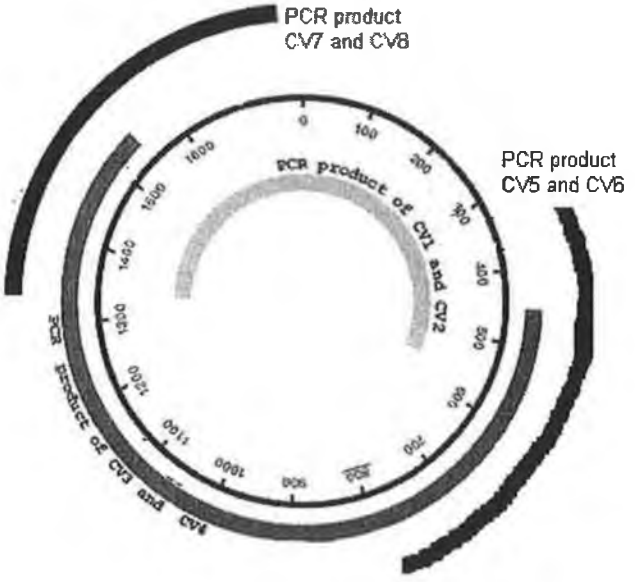

Figure 2.1: Genome of PCV2. Overlapping PCR fragments used to determine the complete genome of PCV2 are indicated in the circular map.

2.4 Immunohistochemistry

Immunohistochemistry was carried out in order to detect the level of PCV2 antigen on tissue sections stored in 10% Neutral Buffered Formalin (NBF). The tissue sections were first dewaxed in two xylene baths for five minutes in each bath followed by two baths of methanol for five minutes each bath. Endogenous peroxidase was blocked with 0.5% H_2O_2 in methanol for 20 minutes, then briefly washed in distilled water. Tissue sections were then washed twice for 10 minutes in triphosphate buffer solution (TBS) and were incubated in 0.05% Protease 14 (Protease 14-P5147 SIGMA) in TBS, for 15 minutes at 37°C and briefly washed in distilled water. Sections were then washed twice for 10 minutes in TBS then incubated in 10% normal goat serum (Biotylinated goat anti-mouse, streptavidin peroxidase conjugate, Zymed Histostain Mouse SP Kit Catalogue no. 95-6543B) for 30 minutes. Sections were incubated in PCV monoclonal F217 at 1/250 in PBS overnight at room temperature. Following overnight incubation sections were washed twice for 10 minutes in TBS and incubated in biotylinated goat anti-mouse antibody for 30 minutes followed by washing twice for 10 minutes in TBS. Sections were then incubated in streptavidin peroxidase conjugate reagent for 30 minutes and washed twice for 10 minutes in TBS, incubated in DAB substrate chromagen for approx. 5 minutes and washed in distilled water. Sections were then counterstained with haematoxylin, cleared, mounted and examined by bright light microscopy. Scoring of sections for PCV2 antigen ranges from negative to a +4. A tissue score equal or greater than 3+ suggested the presence of PMWS (Ellis *et al.*, 1998; McNeilly *et al.*, 1999).

2.5 Longitudinal Field Studies

2.5.1 *Sampling the herd*

Two PMWS-affected and one non-affected control herd were identified and studied in ROI and one positive herd in NI. A full history was taken from each. Approximately 5 litters per herd were identified and tagged on the first week of life and used for the study. Immediatley after farrowing the sows were also bled and a serum sample stored. The piglets were raised "normally" and blood sampled approximately every 10 days. Sera were collected on a total of 7 sampling dates.

Samples taken from the piglets included clotted blood, tonsil and faecal swabs in viral transport medium (VTM) for virus isolation studies and a bacterial faecal swab in Aimes charcoal transport medium.

On arrival at the laboratory, blood samples were centrifuged at 805 X *g* for 10 minutes and the serum removed. Sera were then tested to determine PCV2 antibody titre (IPMA) and the presence of antibodies to PPV by ELISA. All sera were stored at -20°C until required. A cohort of approximately 20 serum samples was chosen from each farm for anlaysis, this cohort included any animals, which had died during the study and a selection of animals from each litter and pen.

Faecal and tonsil swabs in VTM were vortexed and stored frozen at -70°C on the day of sampling. After all samples from each herd were collected a cohort of samples was then chosen. This cohort was chosen at random, including all pigs that had died

during the study and a number of healthy animals were chosen from each litter. These samples were then subjected to two freeze-thaw cycles, clarified by centrifugation at 805 X *g* for 30 minutes and the supernatant removed, aliquoted and stored frozen at -70°C prior to virus isolation studies. Faecal swabs collected using Transport swabs (Medical Supply Co. Ltd., Dublin, Ireland) containing Aimes Charcoal for bacteriology were tested for the isolation of heamolytic *E. coil*, *Campylobacter* spp. and *Salmonella* spp. immediately upon arrival in the laboratory.

2.5.2 *Protocol for the Isolation of Haemolytic E. coli from faecal swabs*

McConkey agar (Oxoid, Hampshire, UK) and Blood Agar (Oxoid, Hampshire, UK) plates were prepared according to the manufacturers instructions. Faecal swabs were streaked directly onto McConkey plates. The plates were then incubated aerobically at 37°C overnight. Presumptive positive pink colonies were then restreaked onto blood agar plates and incubated aerobically at 37°C overnight. A zone of haemolysis surrounding the streak suggested the presence of haemolytic *E.coli* in the faecal swab. Colonies suggestive of haemolytic *E. coli* were stored on protecta beads (Technical Services Consultants Limited, England) and stored at -20°C for later species confirmation.

Three beads from each stored haemolytic suspect *E. coli* isolate were plated onto blood agar plates and incubated aerobically at 37°C overnight. A bacterial suspension was prepared in API NaCl 0.85% medium (Biomerieux, UK) and API 20E tests were carried out according to the manufacturers instructions in order to identify the culture.

2.5.3 *Protocol for the Isolation of Campylobacter spp.*

Modified charcoal cefoperazone deoxycholate agar (mCCDA) (Mast Preston blood-free agar DM251 containing one Camp "Selectavial" supplement SV18/litre, Mast Diagnostics, Merseyside, U.K.) plates were prepared according to the manufacturer's instructions. Addition of CCDA Selective Supplement inhibited growth of enterobacteriaceae, yeasts and fungi and made it more selective for *C. jejuni, C. coli and C. lari*. Faecal swabs were streaked onto CCDA plates to obtain single colonies. The plates were incubated at 42°C for 48 hours in a microaerophilic atmosphere of approximately 6% (v/v) O_2, 7% (v/v) CO_2, 7% (v/v) H_2, 80% (v/v) N_2. This was achieved using commercial catalyst-free gas generating kits (Genbox microaer gas packs, Biomerieux, U.K.). Presumptive *Campylobacter* colonies were defined as colonies which appeared grey and had a watery appearance. Ten persumptive *Campylobacter* colonies were subcultured onto blood agar and incubated microaerobically at 42°C for 48 hours. The Biochemical tests to identify the species of *Campylobacter* were carried-out according to manufactures instructions (Camp ID™, Mast Diagnostics, Merseyside, England). This 3-test biochemical profiling system for the presumptive identification of thermophilic *Campylobacter* spp. was based on the detection of hippurate hydrolysis, indoxyl acetate hydrolysis and urease activity and is differentiates for *C. jejuni*, *C. coli* and *C. lari*.

2.5.4 *Protocol for the Isolation of Salmonella spp.*

Faecal swabs were placed into sterile universal containers containing 10mls pre-enrichment broth, Buffered Peptone Water (BPW) (Oxoid, U.K.) and incubated overnight at 37°C. Following incubation, 100µl of the pre-enrichment media containing the swab was transferred into 10mls of Rappaport-Vassiliadis Broth (RV) (Oxoid, U.K.) and incubated overnight at 42°C. Following incubation, cultures were streaked onto both modified Brillant Green Agar (BGA) (Oxoid, U.K.) and Mannitol Lysine Crystal Violet Blue Agar (MLCB) (Oxoid, U.K.) plates and incubated overnight at 37°C. Presumptive positive colonies were seen as red-pink-white opaque coloured colonies on the BGA plates and large purple black colonies of H_2S positive Salmonellae or black "bulls-eye" colonies on MLCB plates. Confirmatory tests were applied to suspect colonies. Suspect colonies were streaked onto MacConkey plates and incubated overnight at 37°C. To determine if these colonies were poly 'O', positive samples were tested using Murex Salmonella Poly 'O' group A-G antiserum (Cruinn Diagnostics Ltd., Ireland) according to manufacturers instructions.

2.5.5 Virus Isolation from tonsil and faecal swabs

All virus isolation work was carried out on Primary Pig Kidney cells (PK-05). Freshly obtained trysinised PK-05 cells were grown at 37°C for 3 days in MEM, in a 5% CO_2 atmosphere, until a semi-confluent growth of cells was visible, prior to inoculation with 100µl of either tonsil or faecal VTM aliquots. Tubes were then incubated on rollers at 37°C for approximately 6 days. Cells were checked for cytopathic effect (CPE) 3, 4 and 5 days following inoculation. Monolayers which had been completely lysed were

immediately frozen. Cells which showed some or no CPE were subjected to freezing on day six and then frozen and thawed twice more prior to being passaged onto fresh 3 day old primary PK-05 cells and incubated at 37°C on rollers. The cells were again checked for CPE on days 3, 4 and 5 following incoulation. Following this passage, all cells showing viral CPE were passaged into 25ml flasks and incubated at 37°C for a further three days and then checked again for CPE on days 3, 4 and 5. At this stage all cells showing good CPE were checked by PCR for the presence of enteroviruses 1 and 2 reovirus and adenoviruses.

2.5.6 Serum Analysis

2.5.6.1 Enzyme Linked Immunosorbent Assay (ELISA)

A commercially available ELISA, Svanovir™ (cat. no. 10-7400-02, Uppsala, Sweeden) for PPV Antibody was used to detect antibodies to PPV in serum. This test was carried out according to the manufacturers instructions. The kit consisted of microplate strips coated with non-infectious PPV antigen, positive and negative control sera, PBS-Tween solution, conjugate, TMB substrate chromagen and stop solution. The principle of the test is based on a competitive ELISA. In this procedure sera were reacted with noninfectious PPV antigen coated wells on microtitre strips. Serum samples, controls and the antibody conjugate were added simultaneously. PPV antibodies (if present) compete with the enzyme-conjugated antibodies for the antigen binding sites in the wells. In the absence of PPV antibodies the enzyme conjugated antibodies are bound, giving rise to a colour change when a substrate chromagen solution was subsequently added. Therefore, the absence of colour indicated a positive result. The optical density

(OD) was measured at 450nm and mean OD values were calculated for each of the controls and sera samples. The percent inhibition (PI) values for controls as well as sera samples was calculated using the following formula:

$$PI = 100 - \frac{(\text{Mean OD}_{\text{samples/control}} \times 100)}{\text{Mean OD}_{\text{negative control}}}$$

A sample was regarded negative when PI < 50, positive when PI 50 – 80 and a strong positive when PI > 80. To ensure validity of the test the negative control should have an OD value greater than 0.6 and the positive control should have an OD less than 0.3.

2.5.6.2 PCV2 Antibody Detection and Titration

The testing for PCV2 antibody titre was performed on a cohort of serum samples from each of the three herds by the IPMA method as described above, section 2.1.

Chapter 3: Determination of the Presence and Extent of PCV2 Infection and Associated Disease in Pig Herds in Ireland

3.1 Abstract

A serological survey was performed to determine the extent of PCV2 infection in both parts of Ireland. Serum samples were tested using the IPMA method, this technique is based on PCV-free PK15 cell cultures infected with PCV2 virus. Ten serum samples from fifty randomly selected herds in the south of Ireland and ten to nineteen serum samples from 20 herds in the North of Ireland were tested. Of the 70 farms tested only four herds were negative for PCV2 antibody. These four negative herds were all small hobby herds (less than 12 pigs) from ROI. These findings indicate that there is widespread seroconversion to PCV2 in both parts of Ireland. There was a significant variation in PCV2 antibody titers from animal to animal within the same herd and from herd to herd.

To establish the current level of PMWS in Ireland a study was performed to determine the incidence of PMWS in both parts of Ireland over a 12 month period. Pig veterinarians were asked to submit tissue samples (lymph nodes, liver, spleen, kidney and lung) from four necropsied pigs from suspect herds. Suspect herds were identified as herds that showed clinical signs consistent with PMWS such as an increase in weaner mortality, wasting or ill-thrift, respiratory disease, jaundice, pallor and sudden death in weaned pigs (Segalés and Domingo, 2002). A PMWS case was confirmed by testing with a combination of indirect fluorescent antibody staining of pig cryostat tissue sections and/or immunohistochemistry and histopathology. In some cases both IFA and IHC were performed in order to ensure the accuracy of results when determining PCV2 levels. This study ran from 01/01/2004 to the 31/12/2004. In this year there was a total of 30 suspect

herds in the South of Ireland and 31 in the North of Ireland. All herds tested were positive for the presence of PCV2 antigen, however, of the 30 herds in the South of Ireland 24 had high PCV2 levels and histological lesions consistent with PMWS and 6 had lower PCV2 levels, which were considered negative for PMWS. In the North of Ireland there was a total of 14 herds positive for PMWS and 17 herds negative based on PCV2 levels.

The results of this study indicate that PCV2 is widespread in Ireland and that new cases of PMWS are occurring throughout the island. Therefore PMWS is a significant economic and welfare risk to the Irish pig industry.

3.2 Introduction

Post Weaning Multisystemic Wasting Syndrome (PMWS) is a new and emerging disease in the pig industry worldwide (Chae, 2004). It was first described in western Canada in a specific pathogen free (SPF) herd in 1991 (Clark, 1997; Harding, 1997). PMWS has since spread throughout most of the world (except Australia). This syndrome has significant consequences for the pig industry worldwide from a welfare and economic viewpoint. It has been estimated that PMWS costs around €600 million per year to the European Union (Armstrong, 2004).

PMWS was first reported in Republic Ireland in 1998 (Spillane *et al.*, 1998) and Northern Ireland in 1997 (Kennedy *et al.*, 1998). An apparent increase in the number of PMWS cases in Irish pig herds as observed by veterinarians at the Central Veterinary Research Laboratory (CVRL) during 2003 prompted the present study to establish the current status of this disease in Ireland. To the author's knowledge this is the first detailed report of the prevalence of PCV2 infection in pigs and the incidence of PMWS in the Republic of Ireland.

The main objectives of this study were (1) to estimate the prevalence of PCV2 infection in Irish pigs by testing for the presence of antibody to PCV2 and (2) to provide laboratory confirmation of suspect clinical PMWS on submitted samples from notified suspect herds throughout the island of Ireland, by quantifying the amount of PCV2 antigen present in tissues from affected pigs.

3.3 Materials and Methods

3.3.1 *Serological Survey to determine the Prevalence of PCV2 Infection in Irish Pigs*

Sera were tested for the presence of PCV2-specific antibodies using the immunoperoxidase monolayer assay (IPMA) as described in chapter two. Five-fold dilutions of each serum sample were performed (beginning with a lowest dilution of 1 in 50 and ending with a highest dilution of 1 in 156,250). In Northern Ireland two-fold dilutions were used (beginning with an initial dilution of 1 in 50 and a final dilution of 1 in 102,400). The antibody titre for each serum sample was the last dilution at which red/pink staining of cells was still visible. The mean titre was then calculated for each herd (as the average titre for all sera tested in that herd).

In the Republic of Ireland (21 of the 26 counties) 426 sera from 50 herds were chosen at random from 1149 registered pig herds. Up to ten sera were tested from each of these herds; sera were taken from pigs of different ages and from both breeding sows and fatteners. In Northern Ireland, 212 sera were collected from 20 herds; 10 sera were collected from 16 of the 20 herds, 11 sera from 3 herds and 19 sera from one herd; all of these sera were collected from fattener pigs that were randomly selected at slaughter.

3.3.2 Study of PCV2 infected herds to establish the number of PMWS cases from submitted clinically suspect herds

This study was performed over a one-year period (01/01/2004 to 31/12/2004) in order to determine the extent of the disease in Ireland. It involved working on a collaborative basis with private veterinary practitioners (PVPs) and the regional

veterinary laboratories (RVLs) of the Department of Agriculture and Food (DAF). In the case of each herd in which PMWS was suspected, the veterinarian investigating the herd was asked to perform a necropsy on at least four affected pigs and to submit tissues (lymph nodes, liver, spleen, kidney and lung) for laboratory analyses. Herds in which PMWS was suspected were those with persistent clinical disease and high mortality; clinical signs which were observed and are consistent with a diagnosis of PMWS included an increase in weaner mortality, wasting or ill-thrift, respiratory disease, jaundice, pallor and sudden death in weaned pigs. The basis for the confirmation of PMWS was the diagnostic criteria reported from the meeting of the American Association of Swine Practitioners in Quebec (Canada) in March 1997; namely, (1) the existence of characteristic clinical signs, (2) the presence of characteristic microscopic lesions and (3) the detection of PCV2 within these lesions (Clark, 1997). Thus a clinical diagnosis of PMWS was confirmed in the laboratory using a combination of histopathology and either immunoflouresence assay (IFA) or immunohistochemistry (IHC) to demonstrate the presence of PCV2 antigen by immunological means as previously described in chapter 2. PCV2 virus has been present in pigs for many years prior to the recognition of PMWS (Staebler *et al.*, 2005), without being associated with any specific disease syndrome and PCV2 antigen is now evident in tissues collected from most pigs. However, a pig was only confirmed as PMWS positive if high levels of PCV2 antigen were displayed by immunological means and characteristic histopathalogical lesions were present. In some cases both tests were not performed, in this situation a diagnosis was made based on the result of the test used and the clinical signs observed. In a case where conflicting results arose between the two tests (i.e IFA was negative for

PMWS and IHC positive or vice versa) a positive result was given as enough antigen was present on the tissue section to indicate the animal was PMWS positive.

3.4 Results

3.4.1 Serological Survey

3.4.1.1 *Republic of Ireland*

In the Republic of Ireland forty-six of the 50 sampled herds tested positive for antibodies to PCV2 (by IPMA). Of the 426 sera tested, 363 (85.2%) were antibody positive and 63 (15%) negative. The reciprocal mean titre varied considerably between herds, ranging from 230 to 88,550 (Table 3.1). There was also a wide variation in reciprocal antibody titres for the individual pigs within herds (Figure 3.1a and b). In most herds, at least eight of the ten pigs tested were sero-positive (Figure 3.2). All four sero-negative herds, accounting for 17 of the 63 sera that tested negative in this survey, were small "hobby" herds, each of which consisted of 12 pigs or less and were located in counties Clare, Kildare, Mayo and Westmeath.

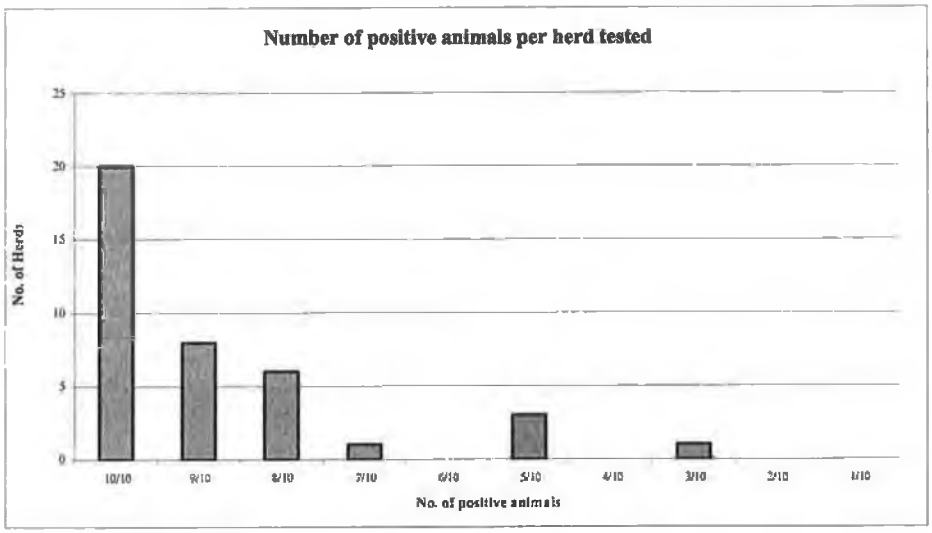

Figure 3.2: Number of PCV2 antibody-positive animals per herd in ROI

Farm No.	Type of Pig	Sera +ve/ sera tested	Min Reciprocal Herd Titre	Max Reciprocal Herd Titre	Arithmetic Mean Reciprocal Herd Titre
1	Sows	8/10	250	6,250	1,300
2	Finishers	10/10	6,250	156,250	28,650
3	Sows	9/10	6,250	250	1,925
4	Fattener	10/10	6,250	156,250	48,575
5	Gilts/Sows/Fatteners	10/10	250	156,250	25,075
6	Fatteners	10/10	156,250	6,250	88,550
7	Gilts/Sows/Fatteners	9/10	0	31,250	9,075
8	Sow/Boar	0/2	0	0	0
9	Fatteners	10/10	1,250	156,250	48,075
10	Fatteners	8/10	0	156,250	22,465
11	Fatteners	10/10	156,250	6,250	38,675
12	NA	2/3	0	1,250	500
13	Fatteners/Sows	10/10	250	156,250	22,975
14	Suckler	10/10	50	250	230
15	Fatteners/Sows	8/10	0	156,250	19,650
16	Sows	0/1	0	0	0
17	Fatteners/Sows	10/10	250	156,250	20,400
18	Sows	10/10	6,250	156,250	38,550
19	Finishers	10/10	6,250	156,250	88,550
20	Sows	10/10	250	31,250	9,100
21	Fatteners	10/10	1,250	31,250	6,725
22	Sows	3/3	6,250	6,250	6,250
23	Sows/Finishers	10/10	250	156,250	21,500
24	Sows	10/10	250	6,250	4,550
25	NA	5/10	0	156,250	21,950
26	Fatteners/Sows	0/10	0	0	0
27	NA	1/1	0	31,250	250
28	Sows	9/10	250	250	8,575
29	NA	7/10	0	156,250	19,725
30	NA	10/10	250	156,250	26,550
31	Fatteners/Sows	10/10	31,250	50	6,605
32	NA	1/10	6,250	6,250	6,250
33	NA	9/10	0	6,250	2,425
34	NA	9/10	0	31,250	19,875
35	NA	10/10	1,250	156,250	64,750
36	Gilts	10/10	250	156,250	58,000
37	Sows	9/10	0	31,250	5,500
38	Finishers/Sows	8/11	0	6,250	1,727
39	Fatteners/Gilts	5/10	0	31,250	12,625
40	Finishers/Sows	9/10	0	6,250	2,425
41	Fatteners/Sows	8/10	0	156,250	84,325
42	Sows	5/10	0	156,250	22,550
43	Fatteners	0/2	0	0	0
44	Sows	8/10	0	31,250	8950
45	Sows	9/10	0	31,250	13,025
46	Fatteners/Sows	5/10	0	156,250	17,100
47	Sows	3/10	0	31,250	3,350
48	Fatteners	10/10	1,250	250	950
49	NA	3/3	31,250	1,250	12,833.33
50	NA	3/3	31,250	250	20,750

Table 3.1: Serological Survey to Determine the Prevalence of PCV2 Infection in the Republic of Ireland.

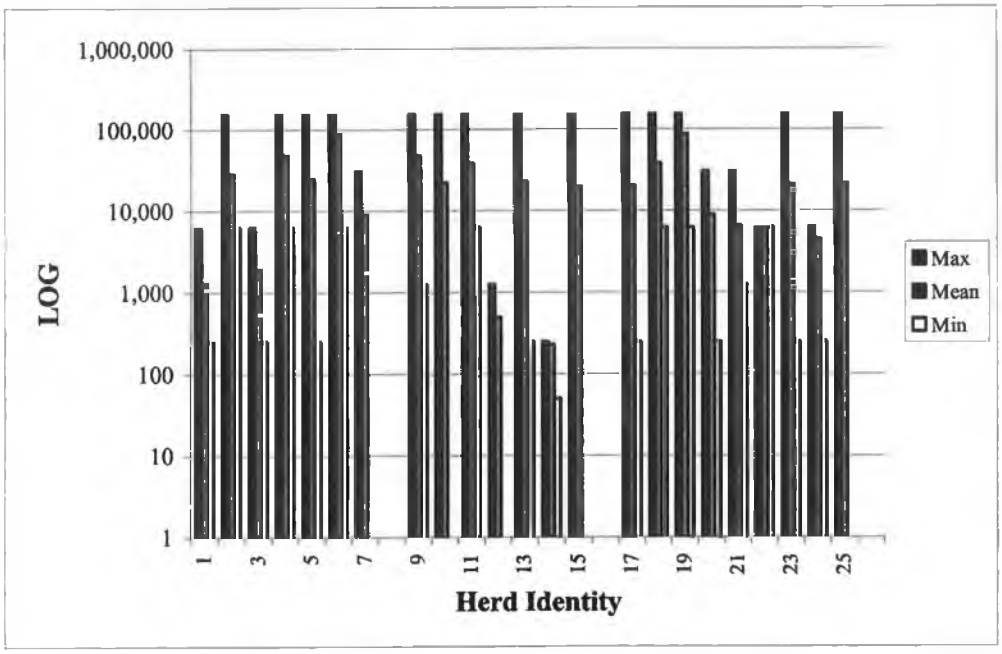

Figure 3.1a: Maximum, Minimum and Mean Reciprocal Antibody Titre per Herd in ROI herds 1 – 25

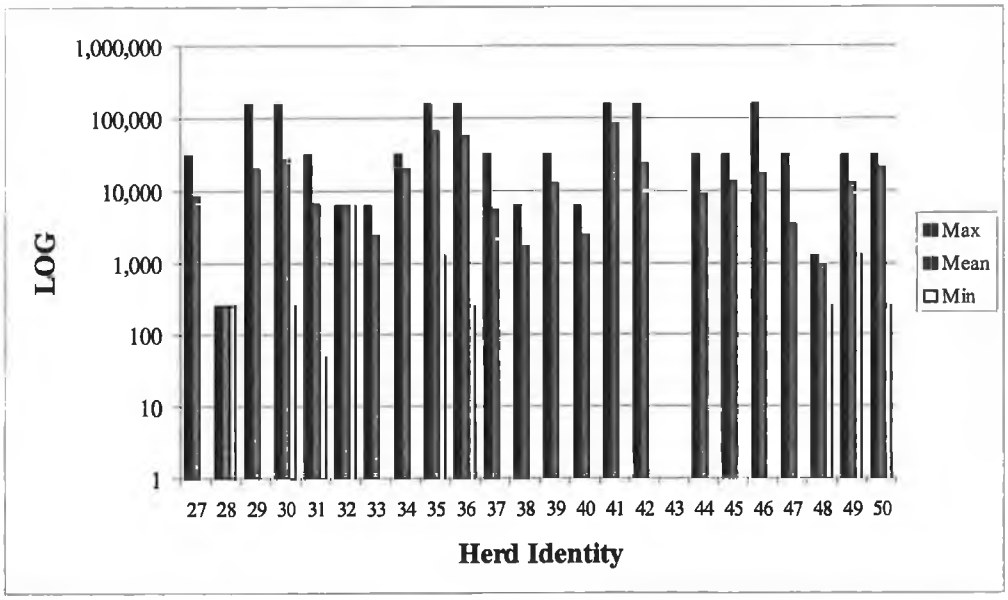

Figure 3.1b: Maximum, Minimum and Mean Reciprocal Antibody Titre per Herd in ROI herds 27-50.

3.4.1.2 Northern Ireland

In Northern Ireland all of the sampled pigs were sero-positive for PCV2 antibodies. The majority of herds displayed high antibody titres. Low antibody titres were only observed in one of the herds in which the reciprocal antibody titres ranged from 400 to 12,800 (Table 3.2). A wide variation in antibody titre was observed between individual animals in each herd (Figure 3.3).

Farm No.	Type of Pig	Sera +ve sera tested	Min reciprocai Antibody Titre	Max reciprocal Antibody Titre	Arithmetic Mean reciprocal Antibody Titre
1	Fattener	10/10	6,400	102,400	45,440
2	Fattener	10/10	3,200	102,400	61,760
3	Fattener	10/10	1,600	51,200	25,120
4	Fattener	10/10	6,400	102,400	68,072
5	Fattener	10/10	51,200	102,400	88,436
6	Fattener	10/10	12,800	102,400	57,600
7	Fattener	10/10	51,200	102,400	87,040
8	Fattener	10/10	6,400	102,400	58,240
9	Fattener	10/10	6,400	102,400	39,680
10	Fattener	11/11	3,200	102,400	62,545
11	Fattener	10/10	800	102,400	41,360
12	Fattener	11/11	3,200	51,200	15,709
13	Fattener	11/11	800	102,400	35,418
14	Fattener	10/10	400	12,800	3,720
15	Fattener	10/10	51,200	102,400	97,280
16	Fattener	10/10	25,600	102,400	89,600
17	Fattener	10/10	51,200	102,400	92,160
18	Fattener	19/19	3,200	102,400	72,252
19	Fattener	10/10	3,200	51,200	21,760
20	Fattener	10/10	1,600	102,400	30,240

Table 3.2: Serological Survey to Determine the Prevalence of PCV2 Infection in Northern Ireland.

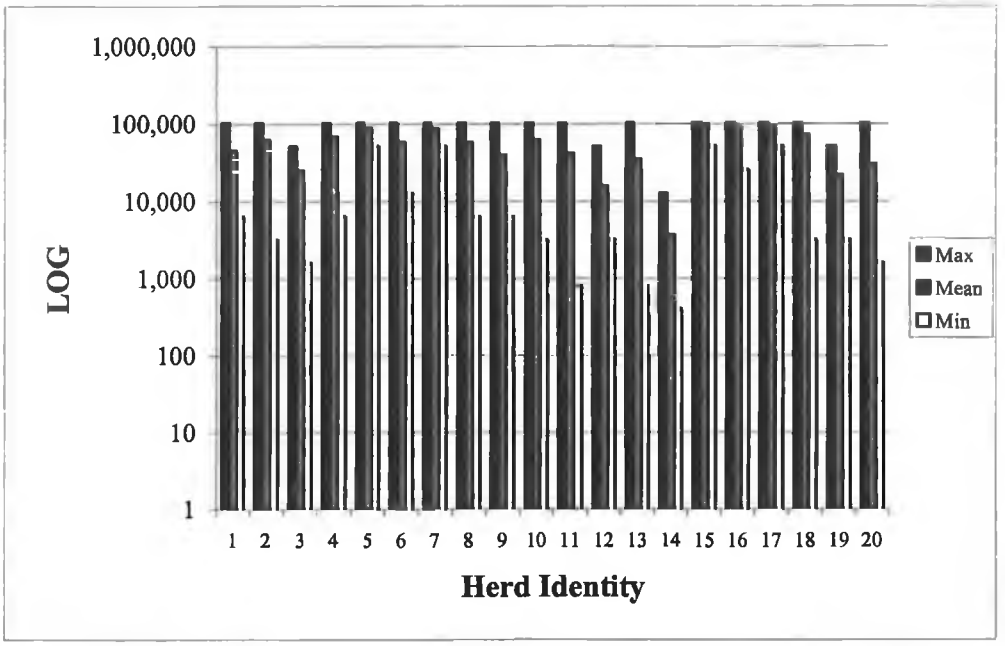

Figure 3.3: Maximum, Minimum and mean Reciprocal Antibody Titre per Herd in NI herds.

3.4.2 *Detection of PMWS In Ireland (North and South) from clinically suspect PMWS cases*

In the Republic of Ireland an average of four pigs from each of a total of 30 herds in which PMWS was suspected were submitted to the CVRL, DAF. Twenty-four (80%) of these herds were confirmed in the laboratory to have PMWS. An average of 4 pigs from 31 problem herds in Northern Ireland were submitted to the Veterinary Sciences Division (VSD), DARDNI as suspect PMWS cases; PMWS was confirmed as present in 14 (45.2%) of these herds. IFA, IHC and histopatholoigcal findings from positive and negative herds in the ROI are shown (Tables 3.3) and IFA and histopathological results for NI (Table 3.4). The main clinical signs in the herds in which PMWS was confirmed included wasting, respiratory distress, increased mortality and diarrhoea, while the

principal necropsy finding was lymph node enlargement (Table 3.5). Similar clinical and gross pathological findings were evident in herds in which PMWS was not confirmed in the laboratory. Mortality in positive herds (those which scored 3+ or 4+ by IFA and/or IHC) ranged from 2% to 19%, and in negative herds (those which scored a 0, 1+ or 2+ by IFA and IHC) from 6% to 20% in 5 to 14 week old pigs. The principal histopathological lesions observed in pigs from PMWS positive herds included lymphocytic depletion; histiocytic infiltration and the presence of giant cells in the lymph nodes; bronchointerstitial or bronchopneumonia featuring occasional syncytia and the presence of inclusion bodies in the lungs; pulmonary oedema and lymphohistiocytic interstitial nephritis and infiltrates of mononuclear cells in subcapsular and pelvic areas of kidney.

In Northern Ireland the clinical and necropsy findings were similar to those reported in the Republic of Ireland from positive PMWS herds (Table 3.6); mortality in affected herds ranged from 2% to 13%. The principal histopathological lesions in pigs from PMWS-affected herds was lymphocytic depletion in secondary lymphoid organs (lymph nodes, spleen and tonsil) and necrosis; aggregates of syncytial cells and typical PCV2 type inclusion bodies were prominent features in these lymphoid tissues. Interstitial pneumonia and bronchopneumonia were accompanied by interlobular and alveolar oedema. Interstitial renal oedema and focal lymphocytic interstitial nephritis were observed, as were cytoplasmic inclusion bodies typical of PCV2 infection. As can be seen from tables 3.3 and 3.4 herds with high levels of PCV2 antigen were also found to have histopathological lesions associated with PMWS showing a good correlation between the two tests.

Farm Reference No.	No. of Animals Submitted to Laboratory	IFA[1]	IHC[2]	Histopathological lesions	PMWS Herd Result
1	4	0/4	0/4	B, L	Neg
2	4	0/4	NT	NT	Neg
3	4	1/4	3/4	L, I	Pos
4	3	2/3	1/3	L	Pos
5	2	0/2	1 /2	L, G, I	Pos
6	3	1/3	1/3	NT	Pos
7	4	3/4	3/4	B, L	Pos
8	6	5/6	5/6	NT	Pos
9	3	2/3	2/3	B, G, M	Pos
10	2	0/2	2/2	L, G, I, IN	Pos
11	4	3/4	2/4	I	Pos
12	6	5/6	NT	NT	Pos
13	3	2/3	3/3	B, L, G, I	Pos
14	4	1/3	1/3	I, O	Pos
15	2	0/2	NT	H	Neg
16	1	1/1	NT	NT	Pos
17	6	3/6	3/6	I, G, L, O	Pos
18	3	1/3	1/3	L, I, IB	Pos
19	2	1/2	NT	NT	Pos
20	5	1/5	NT	NT	Pos
21	3	2/3	3/3	B, L, IN	Pos
22	2	0/2	0/2	LN, H	Neg
23	1	0/1	NT	NT	Neg
24	4	3/4	1/4	M, G, L	Pos
25	2	2/2	1/2	I, L	Pos
26	3	3/3	3/3	L, I, G	Pos
27	3	0/3	0/3	I	Neg
28	2	2/2	2/2	NT	Pos
29	2	2/2	1/2	I, L, IN,	Pos
30	1	NT	1/1	L, B	Pos

1 = No. of animals which tested positive for PMWS by IFA over the number of animals tested by IFA in this herd.
2 = No. of animals which tested positive for PMWS by IHC over the number of animals tested by IHC in this herd.
NT= Not tested
NO = no significant abnormality
L = lymphocytic depletion
G = giant cells in lymph nodes
I = interstitial pneumonia
B = bronchopneumonia
IN = interstitial nephritis
O = oedema
M = mononuclear cells in pelvic areas of kidney
H = hepatitis
LN = Lythic necrosis
IB = inclusion bodies

Table 3.3: Detection of PCV2 Antigen by FAT & IHC in PMWS-affected Herds in the Republic of Ireland.

Farm Reference No.	No. of Animals Submitted to Laboratory	IFA[1]	Histopathological lesions	PMWS Herd Result
8	3	1/3	I, L, O, IB	Pos
9	7	4/7	IB, LN	Pos
10	4	0/4	I, O, B, L, IB	Neg
11	1	1/1	L	Pos
12	2	1/1	IB, B	Pos
13	2	1/1	B, L	Pos
14	4	1/2	I, O, IB, L, LN	Pos
15	1	1/1	IN, L, IB	Pos
16	2	1/2	L	Pos
17	1	0/1	B	Neg
18	2	0/2	IB	Neg
19	1	0/1	B, O	Neg
20	4	1/2	O	Pos
21	1	0/1	NO	Neg
22	5	2/5	B, IN, L	Pos
23	1	0/1	B	Neg
24	4	1/4	L, IB, L	Pos
25	1	0/1	O, B	Neg
26	4	0/4	IN, B	Neg
27	2	0/2	B	Neg
28	8	5/8	LN, L, IB, O, B	Pos
30	2	0/2	O	Neg
31	1	0/1	NO	Neg
32	1	0/1	NT	Neg
33	3	0/3	B, O, L	Neg
34	1	0/1	L	Neg
35	4	2/4	L, I	Pos
37	5	0/1	NO	Neg
38	1	0/1	H, I, IN	Neg
39	3	0/1	Men	Neg
46	1	1/1	L	Pos

1 = No. of animals which tested positive for PMWS by IFA over the number of animals tested by IFA in this herd.
NT = Not tested
NO = no significant abnormality
L = lymphocytic depletion
G = giant cells in lymph nodes
I = interstitial pneumonia
B = bronchopneumonia
IN = interstitial nephritis
O = oedema
M = mononuclear cells in pelvic areas of kidney
H = hepatitis
LN = Lythic necrosis
IB = inclusion bodies
Men = meningitis

Table 3.4: Detection of PCV2 Antigen by IFA in PMWS-Positive and Negative Herds in NI.

	Positive Farms	Negative Farms
Wasting	16/24	3/6
Jaundice	6/24	
Respiratory Disease	14/24	3/6
Pallor	11/24	3/6
Lymph Node Enlargement	17/24	3/6
Diarrhoea	18/24	3/6
Pneumonia	12/24	3/6
Oedema	2/24	1/6
GIT Ulceration	1/24	2/6
Kidney Lesions	8/24	1/6
Sudden Death	1/24	1/6

Table 3.5: The Principal Clinical and Necropsy Findings Observed on Problem farms in the Republic of Ireland.

	Positive Farms	Negative Farms
Wasting	14/14	9/17
Jaundice	1/14	1/17
Respiratory Disease	6/14	5/17
Pallor		6/17
Lymph Node Enlargement	9/14	6/17
Diarrhoea	11/14	3/17
Pneumonia	6/14	7/17
Oedema	3/14	8/17
GIT Ulceration		
Kidney Lesions		
Sudden Death		

Table 3.6: The Principal Clinical and Necropsy Findings Observed on Problem farms in Northern Ireland.

3.5 Discussion

In this study serum antibodies to PCV2 were found to be ubiquitous in Irish pigs, with 85% of herds testing sero-positive in Republic of Ireland and 100% testing sero-positive in NI. These findings are consistent with previous reports elsewhere (Clark, 1997; Sibila *et al.*, 2004). Several separate studies have indicated that a high PCV2 seroprevalence is a worldwide phemomenon (Segalés and Domingo, 2002). In Sweden, two separate serological surveys found 96% and 81% of porcine sera to be positive for antibodies to PCV2 (Wallgren *et al.*, 2004). A similar situation was observed in The Netherlands where 94% of Dutch pig herds were antibody positive (Wellenberg *et al.*, 2004) and in Taiwan 83.5% of pigs were sero-positive (Chun *et al.*, 2004). PCV2 infection is present in almost 100% of Spanish pig herds (López-Soria *et al.*, 2005) and in Canada the presence of virus has been demonstrated in all pig herds examined (Harding, 2004). In a retrospective study carried out in Northern Ireland, 78% of porcine sera collected between 1973 to 1999 were shown to be PCV2 antibody positive, with more animals positive in the latter years i.e. 100% in 1998 compared to 69.1% in 1973 (Walker *et al.*, 2000).

Results of this study indicate that PCV2 is widespread in pig herds in Ireland, regardless of whether PMWS occurs in these herds. Active seroconversion generally occurs 3 to 4 weeks after weaning at 8 to 12 weeks of age (Clark, 1997). A very large variation was observed in antibody titres within herds; this was seen to vary from the lowest (<1/250) to the highest titre (1/156,250) detectable with this test. This may reflect variation in the immune responses of individual animals to PCV2 and/or it may reflect

differences in the amount of time elapsed since the animals were exposed to the virus. Inadequate colostrum intake may allow primary infection in pigs when younger thereby advancing the disease, while maternal antibody would therefore delay infection.

An interesting observation in this study was that the four PCV2 sero-negative herds were all small hobby/backyard herds. A total of 10 hobby herds were tested for the presence of PCV2 antibodies. This indicates that PCV2 infection is predominantly a commercial herd problem in Ireland. However, from this study it can be concluded that PCV2 infection is widespread in the commercial pig population of Ireland. A large proportion of Irish herds are sero-positive and a high within-herd seroprevalence is found in positive herds and is present in both large commercial fattening units and some small backyard or "hobby" herds. It has also been reported elsewhere that individual pigs with PMWS can be found either in herds with high mortality and pig losses or in herds with very good productive levels and minimal mortality, in large fattening units or small backyard herds (Segales and Morvan, 2004). From this study it can be seen that PCV2 infection is present in both commercial herds and hobby herds, however it is more prevalent in commercial herds.

Confirmed PMWS in Ireland was determined over a one year period from the suspect herd samples submitted. The proportion that were deemed to be PMWS-affected in the ROI and NI were 80% and 45.2% respectively. The percentages of suspect herds that were shown to be PMWS-affected in both the North and South of Ireland were large in comparison to other studies that have been reported. However this may be a reflection

of the selectivity of submissions of suspect cases in the present study. There are a total of 1149 active Irish pig herds in ROI and one possibility is that suspect PMWS herds were not submitted as frequently in the South of Ireland. Although laboratory confirmation of the disease is necessary it is possible that many specialist pig veterinarians may have clinically recognized PMWS in the herd and made a diagnosis without submitting suspect samples to the laboratory. In a study carried out by Kim *et al.*, (2002) over a two year period (1999 and 2000), 8.1% of herds were PMWS positive in Korea (these samples were selected as they were submitted for diagnosis to the Department of Veterinary Pathology, Seoul University) (JungHyun *et al.*, 2002). In a Danish study (October 2001 to September 2003) 277 of the 6,624 herds (4.1%) from which specimens were submitted were PMWS positive; this represents 1.3% of the total number of pig herds registered in Denmark (Vigre *et al.*, 2005).

The principal clinical and necropsy findings observed in positive herds in the North and South of Ireland included wasting, respiratory distress, lymph node enlargement, diarrhoea and pneumonia. Wasting was the most common finding in both NI and ROI. There were some reported findings in the ROI which included pallor, GIT ulceration, kidney lesions and sudden death which were not reported in Northern Ireland in PMWS affected herds. Clinical signs observed and post-mortem changes coincided with those described in the literature for PMWS (Harding *et al.*, 1998; Allan and Ellis, 2000a; Allan, 2002). Many of the findings reported were common to both affected and non-affected herds, but more frequent in the affected herds. Many of the clinical signs of PMWS can correspond to signs associated with other diseases such as acute classical

swine fever (CSF) or PRRS. Therefore a differential diagnosis and laboratory confirmation of infection/disease is crucial when investigating field cases of PMWS (Jemersic *et al.*, 2004).

The histopathological lesions observed on PMWS-affected herds in ROI and NI coincided with those already described in the literature for PMWS (Segalés and Domingo, 2002). A strong correlation was observed between histopathological lesions and the level of PCV2 antigen present in the tissue (IFA and IHC) in PMWS positive herds. Some of the lesions were common to both PMWS positive and negative herds, namely bronchopneumonia and lymphocytic depletion. It is possible in cases where slight lymphoid lesions and low amounts of PCV2 were detected that the pigs were only at the initial stages of the disease or the animals may have been in a convalescent period after suffering from PMWS (Segalés and Domingo, 2002).

As can be seen from Table 3.3 the results of IFA and IHC do not always agree on an animal-to-animal basis in PMWS-affected herds. However, on a herd unit basis these results do agree, i.e. is the herd affected by PMWS. Deviations may often occur in tissue sections, which may result in one part of the tissue having a higher level of the PCV2 antigen than another. Therefore, the authors recommend that all tissue samples be tested in duplicate or as was the case in this study both IFA and IHC be performed together with histopathology. This supports the importance of the three criteria (Clark, 1997) when diagnosing a herd with PMWS, and if all tests are performed then the risk of misdiagnosing an animal is reduced. Where conflicting results did arise a positive result

was given as long as there was sufficient antigen present on the tissue to indicate PMWS together with characteristic histopathological lesions. PCV2 is an important pathogen whose clinical expression in swine is associated with a number of different syndromes and diseases in pigs such as PDNS, reproductive failure, PRDC, granulomatous enteritis, necrotizing lymphadenitis and possibly exudative epidermitis (Chae, 2005). Until recently PMWS often remained overlooked and undiagnosed, many findings being attributed to stress or other disease phenomena. Retrospective studies have also demonstrated that PCV2 associated cases of PMWS have been present from at least 1985 onwards and were not recognized as PMWS (Toplak *et al.*, 2004). Such retrospective studies have shown that PMWS has occurred since at least 1986 in Spain, 1989 in Japan, and 1993 in Thailand (Staebler *et al.*, 2005).

Chapter 4: Genome sequence analysis of PCV2 isolates from PMWS-affected and non-affected pigs in Ireland

4.1 Abstract

The entire genome of Irish isolates (ROI and NI) of PCV2 from both diseased and non-diseased animals were amplified by PCR and sequenced. To determine the extent of genetic diversity of Irish isolates from diseased and non-diseased animals and from isolates originating from other countries a phylogenetic analyses was performed on Irish PCV2 sequences and compared to sequences already published in GenBank. Sequence analysis of the complete genome indicated that the Irish PCV2 isolates analysed in the present study are closely related to each other but also to other PCV2 strains originating from Western Canada, USA, other parts of Europe and Asia. Phylogenetic analyses of all isolates revealed one large cluster composed of isolates from USA, Canada, Spain, Germany and Taiwan. A closely related second cluster was composed of ROI isolates from non-diseased animals and two ROI isolates from diseased animals. Isolates from France, Nethlerlands, UK and NI were found to be closely related to each other.

Sequence analysis of ORF1 and ORF2 genes of the 21 Irish isolates revealed that there were very few nucleotide changes between isolates from both parts of Ireland and PMWS-negative isolates within ORF2; most nucleotide changes occurred within the ORF1 gene.

4.2 Introduction

In order to determine the genetic heterogeneity among PCV2 isolates from diseased and non-diseased pigs in Ireland, the complete genome of PCV2 was amplified by PCR and sequenced. A phylogenetic analysis of these PCV2 genomes was then carried out in order to assess the possibility of the circulation of virulent and non-virulent strains of the virus resulting in the epizootic form of the disease or the presence of a novel genotype of PCV2. To more fully understand the genetic diversity of the virus a phylogentic analysis was also performed on strains from Ireland and other countries from sequences already published in GenBank including starains from Europe, Canada, America and Asia.

4.3 Materials & Methods

Sample Sources: Various tissue samples (lymph nodes, lung, liver) were collected from pigs, which were submitted to the CVRL (DAF) as suspect PMWS cases as described in chapter 3. These tissues were confirmed either positive or negative for PMWS by IFA and/or IHC, histopathology and PCR and stored at -70°C until use. The complete PCV2 genome was amplified by PCR and sequenced from tissues from seven PMWS-affected and three non-affected animals from ROI. Isolates N4, N5 and N6 were from PMWS negative pigs and isolates 2, 3, 5, 6, 7, 10 and 12 were from confirmed field cases of PMWS (isolate reference number does not relate to farm number in chapter 3). From NI DNA was extracted from ten tissue samples (samples A – J) from PMWS-positive field cases and a faecal sample from one negative case (V2) (as analysed by DARDNI) were amplified by PCR and sequenced.

Isolation of DNA from tissues: DNA was extracted from the various tissue samples with the Dneasy Tissue Kit (Qiagen Inc.) according to the manufacturers instructions. For each DNA extraction 25mg of tissue sample was used.

PCR amplification of the complete genome of PCV2: Four sets of primer pairs were used, as described in Chapter 2. These primer pairs amplified overlapping fragments that represent the entire genome of PCV2. The extracted DNA was amplified by PCR with HotStarTaq® PCR Kit (Qiagen Inc.). The PCR consisted of 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute (primers CV3,4,5,6,7,8) or 51°C (primer pair CV1,2) and extension at 72°C for 3 minutes, followed by a terminal

extension at 72°C for 7 minutes. PCR products of expected size were purified by electrophoresis on a 2% agarose gel, followed by a clean up using the QIAquick PCR Purification Kit (Qiagen). Purified PCR products were sequenced in both directions by MWG BIOTECH, Germany.

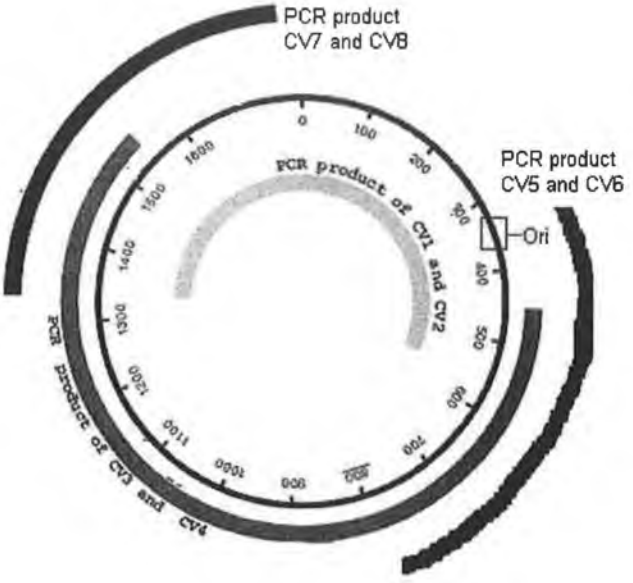

Figure 4.1: Genome of PCV2. Overlapping PCR fragments used to determine the complete genome of PCV2 and origin of replication are indicated in the circular map.

Sequence Analysis: The nucleotide sequences were aligned using the ClustalW version 1.83 multiple sequence alignment programme http://www.ebi.ac.uk/clustalw/. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. A phylogenetic analyses was first performed on aligned data from ROI and NI in order to determine if there was much

difference in nucleotide sequence between isolates from pigs which were affected and those non-affected by PMWS on the island of Ireland. A phylogenetic analysis was performed on the aligned data set and an unrooted tree was constructed. Besides the 21 PCV2 sequences reported in this study, 12 PCV2 genomic sequences published in GenBank database, listed in table 4.1 with their geographic origin, were also included in an additional phylogenetic analysis. Alignments of the complete viral genomes were performed.

4.4 Results

4.4.1. Sequence Analysis

On the island of Ireland the complete genomic nucleotide sequences of 10 PCV2 isolates from ROI and 11 PCV2 isolates from NI from diseased and non-diseased animals (table 4.1) were determined and found to be 1767 nucleotides (nt) in length. ROI and NI isolates were closely related to each other displaying 97-100% overall nucleotide homology (figure 4.2). When these PCV2 isolates were aligned along with other isolates chosen randomly from GenBank (table 4.2) PCV2 isolates showed 94-100% overall nucleotide homology (figure 4.3).

Isolate ID	PMWS Status	Isolate ID	PMWS Status
2	Pos	A	Pos
3	Pos	B	Pos
5	Pos	C	Pos
6	Pos	D	Pos
7	Pos	E	Pos
10	Pos	F	Pos
12	Pos	G	Pos
N4	Neg	H	Pos
N5	Neg	I	Pos
N6	Neg	J	Pos
		V2	Neg

Table 4.1: Identification of PCV2 isolates from suspect field cases of PMWS in Ireland

4.4.2 Phylogenetic Analysis of PCV2 isolates

In the phylogenetic analysis of all genomic sequences, trees were constructed by the ClustalW programme and are presented in phylogram format. A phylogenetic tree resulting from the analysis of the total viral genomes of PCV2 isolates from both ROI and NI was created (figure 4.4). This tree displayed a large cluster divided into small groups containing sequences from the PMWS negative pigs from ROI in one small group close to pigs B and 12. Another small group off this main cluster contained isolates D and E with another group containing isolate A. All remaining pigs were aligned along

the main branch of the tree. A small group existed on this main branch containing isolates F, G, I, J and V2.

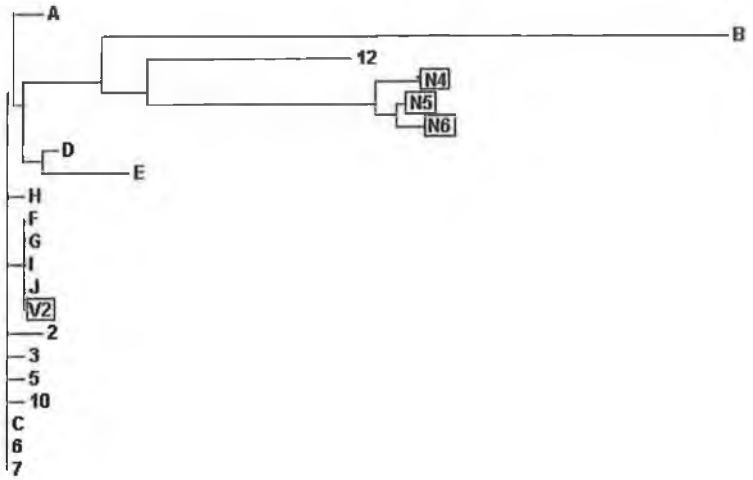

Figure 4.4: Phylogenetic analyses of PCV2 complete genomes from aligned sequences of 11 ROI sequences and 11 NI sequences. PMWS negative sequences are shown in boxes.

The final tree constructed (figure 4.5) consisted of genomic sequences from both parts of Ireland and other sequences found in GenBank (table 4.2) in order to compare Irish PCV2 sequences with others worldwide. This phylogram contained 3 clusters. One large cluster consisted of four small groups. The first group was divided into two sub-groups, the first contained isolates from the US, Canada and Germany, the other sub-group contained isolates from Spain and Taiwan. Isolate B from NI and 12 from ROI were both in groups on their own. The negative sequences from ROI were also in a sub-

group together. Two small clusters could be observed off the main branch, the first was divided into two groups containing strains from France and the Netherlands and NI in the other sub-group. In the other small cluster off the main branch NI strains (V2, G, F, I and J) were situated in the other cluster. The remaining NI and ROI strains were positioned off the main branch of the tree.

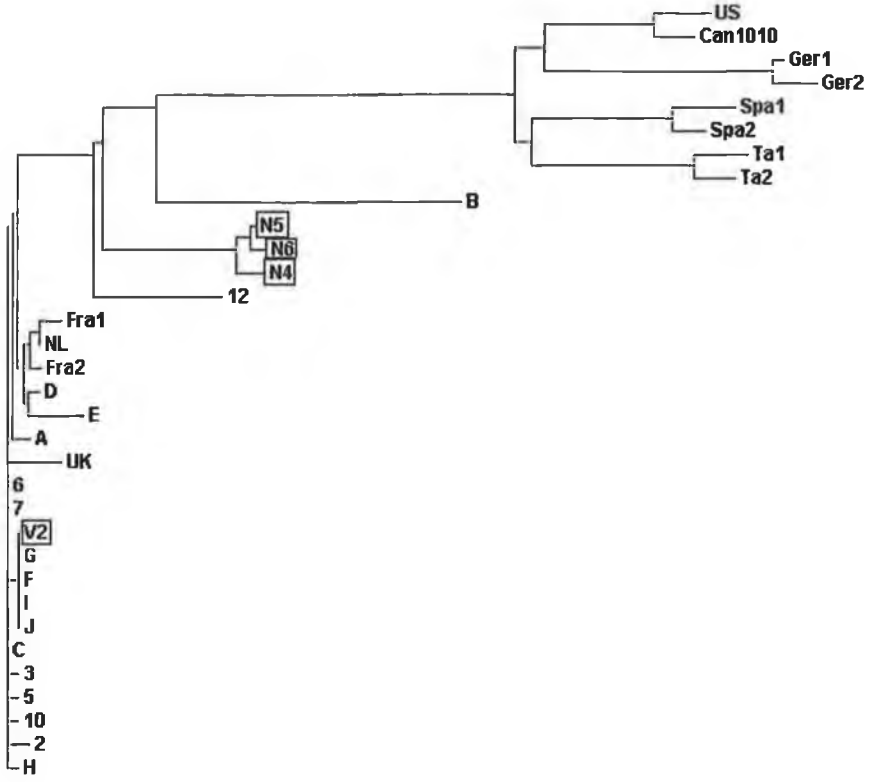

Figure 4.5: Phylogenetic analysis of PCV2 complete genomes. Tree was constructed from aligned nucleic acid sequences of 10 ROI strains, 11 sequences from NI strains and 12 sequences from GenBank. PMWS negative sequences are shown in boxes.

Isolate ID/ Original name	Geographic origin	Genbank Accession no.	Reference
Imp.999	US	AF055391	Meehan *et al.*, 1998
Imp.1010-Stoon (archetype strain)	Canada	AF055392	Meehan *et al.*, 1998
Imp1011-48121 (FRA1)	France	AF055393	Meehan *et al.*, 1998
Imp.1011-48285 (FRA2)	France	AF055394	Meehan *et al.*, 1998
GER 1	Germany	AF201305	Mankertz *et al.*, 2000
GER2	Germany	AF201306	Mankertz *et al.*, 2000
SPA1	Spain	AF201308	Mankertz *et al.*, 2000
SPA2	Spain	AF201309	Mankertz *et al.*, 2000
MLTW98 (TA1)	Taiwan	AF154679	Kuo *et al.*, unp*
Taiwan (TA2)	Taiwan	AF166528	Yang *et al.*, unp*
24657 NL	Netherlands	AF201897	Wellenberg *et al.*,unp*
Imp. 1147	UK	AJ293869	Meehan *et al.*, 2001

* unpublished

Table 4.2: Identification of PCV2 isolates previously reported and for which sequence was obtained from GenBank

4.4.3 Analysis of ORF's

The positions of the 6 open reading frames (ORF's) of PCV2 are outlined in figure 4.8. Sequence comparisons of the ORF2 gene coding for the putative capsid protein of the PCV2 ROI and NI isolates revealed that the nucleotide sequence homology ranged between 95 – 100% (figure 4.2). ORF2 of PCV2 is 702 nt's in length. Irish isolates were translated using Transeq (http://www.ebi.ac.uk/emboss/transeq/). When

translated ORF2 genes were found to encode proteins of 233 aa residues with a deletion at the end of the sequence, except for isolates 2 and 7 from ROI which encoded a protein of 234 aa residues. When aligned these aa sequences showed an overall aa homology of 93 - 100% (figure 4.6).

Sequence comparisons of the ORF1 gene coding for the Rep protein of the PCV2 ROI and NI isolates revealed that the nucleotide sequence homology ranged between 97-100% (figure 4.2). ORF1 of PCV2 is 945 nt's in length when translated ORF1 genes were found to encode proteins of 314 aa's with a deletion at the end of the sequence. When aligned these aa sequences showed an overall aa homology of 98-100% (figure 4.7).

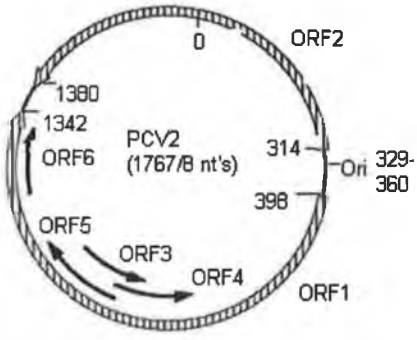

Figure 4.8: Genome organisation of PCV2. The two major ORF's are indicated by hatched boxes, the nt positions of these major ORF's are shown, black arrows show the positions and orientations of the smaller ORF's.

4.5 Discussion

Sequence analysis of the complete genome of PCV2 isolates from diseased and non-diseased pigs in Ireland indicated that they are very closely related to one another (lowest homology 96%) and to other PCV2 isolates originating from Europe, USA, Canada and Asia (lowest homology 94%).

Most phylogenetic analyses of PCV2 isolates reported to date have been performed on PCV2 isolates identified from PMWS cases (Larochelle *et al.*, 2002). In this study we report the phylogenetic analyses of Irish PCV2 isolates identified from PMWS affected and non-affected pigs and these were then compared with 12 PCV2 sequences from GenBank, representing isolates from PMWS cases from different countries. In the phylogenetic analyses when ROI isolates were compared to NI isolates one large cluster was revealed and showed that isolate B from a PMWS affected pig from NI was found closely related to the PCV2 isolates from non-PMWS pigs from ROI and PMWS-positive pig 12. Isolates A, D and E were also found in sub-clusters of this. All other isolates analysed were found along the main branch of the tree. One small group was revealed that contained strain V2 identified from a healthy pig in NI along with strains F, V, I and J from field cases of PMWS pigs from NI.

When these PCV2 isolates were analysed along with sequences already published in GenBank it could be seen that that they are closely related to each other showing a lowest homology of 94%. It was seen that isolates from the US, Canada, Germany, Spain and Taiwan were closely related to one another and positioned together in a sub-cluster.

This sub-cluster was found positioned closely to the sub-cluster containing ROI sequences N4, N5, N6 and 12 and NI sequence B. Sequences from the Netherlands and France were found to be closely related to sequences D, E and A from NI. All remaining sequences from ROI, NI and the UK were spread in small groupings along the phylogenetic tree.

PCV2 contains 6 ORF's larger than 200nt's but proteins seem to be expressed only by ORF1 and ORF2. The ORF1 is 945 nt in length and encodes a protein of 35.7 kDa involved in viral replication, the Rep protein. The DNA replicase is a protein of 314 aa for PCV2 (Meehan *et al.*, 1998). PCV2 DNA requires 2 proteins (Rep and Rep') for replication (Cheung, 2004a). The PCV2 ORF2 is 702 nt in length and has been shown to encode the major capsid protein (Allan and Ellis 2000a) of approximately 30 kDa (Lekcharoensuk *et al.*, 2004). The capsid protein contains a nuclear localization sequence which binds to cell nucleoli presumably facilitating encapsidation of viral DNA. The origin of PCV DNA replication (Ori) is contained within a conserved single stem loop configuration at nt positions 329-360 (figure 4.1) (Meehan *et al.*, 1998). Recombinant PCV2 ORF2 protein self-assembles into icosahedral structures and at least three immunoreactive epitopes have been identified (Mahé *et al.*, 2000). As can be seen from figure 4.2 there were no nt changes at the Ori in all isolates in this study.

Sequence analysis of Irish PCV2 ORF2 gene, which encodes for the major structural capsid protein indicated that the ORF2 gene encodes a protein of 233 and 234 amino acid residues and revealed a lowest nucleotide homology of 95% and lowest

amino acid homology of 93%. Most nt sequence changes appeared to occur in the ORF2 between isolates when compared to other countries (figure 4.3), however there were very few nt changes between isolates from both parts of Ireland and PMWS-negative isolates were very similar to PMWS-positive isolates within ORF2 (figure 4.2). A deletion of a T nt was found on position 1389 (within ORF2), this nt was present in isolates from US, Canada, Germany, Spain and Taiwan but was absent in all other isolates from ROI, NI (diseased and non-diseased animals), France, The Netherlands and UK. Within the ORF2 no nt substitutions occoured in isolate V2 compared to diseased isolates from Ireland and there was only one nt change in N4, N5 and N6 at nt 1514 where a T was substituted for a C. An aa alignment showed that at aa 190 an A was substituted for a T in non-diseased animals in ROI and there was no change in the aa sequence for the non-diseased animal in NI. Sequence analysis of more isolates from negative herds in NI is required in order to determine if this substitution is significant, more work in this area is currently being undertaken by DARDNI.

Sequence analysis of Irish PCV2 ORF1 gene, which encodes for the Rep protein indicated that the ORF1 gene encodes a protein of 314 amino acid residues and revealed a lowest nucleotide homology of 97% and lowest amino acid homology of 98%. Most nt changes in N4, N5 and N6 occurred within ORF1 at nt's 514, 565, 601, 604, 619, 664, 685, 711, 714 and 736. Only one nt substitution existed in isolate V2 this occoured at nt 994. An aa alignment showed that at aa 34 of the ORF1 a D was substituted for an E in non-diseased animals (N4, N5 and N6) this substitution was also in PMWS-affected animal 12 from ROI. Other aa changes in non-diseased animals occurred at aa's 74

where a K was substituted for an N and aa 77 where an L was substituted for an F. There were no aa substitutions in V2 in ORF1.

As the ORF2-encoded protein is the major structural antigen of the virus, changes to its primary sequence may result in an altered immune response to PCV2, either due to an alteration of T cell immunoreatcive epitopes or the binding sites for neutralising antibodies. Other genomic changes, whilst not affecting immunogenicity per se, may affect the pathogenicity of a given PCV2 isolate by leading to changes in mRNA or protein stability, potentially leading to altered rates of viral replication. Investigating the impact of such changes would require a strategy involving the incorporation of specific sequence changes into recombinant PCV2 followed by experimental infection of pigs.

Figure 4.2: Nucleotide sequences alignment of the complete genome of PCV2 from ROI and NI isolates from diseased and non-diseased animals. The positions of the two major ORF's, ORF1 and ORF2 and the Origin of Replication are indicated. Changes in the nucleotide sequence are highlighted.

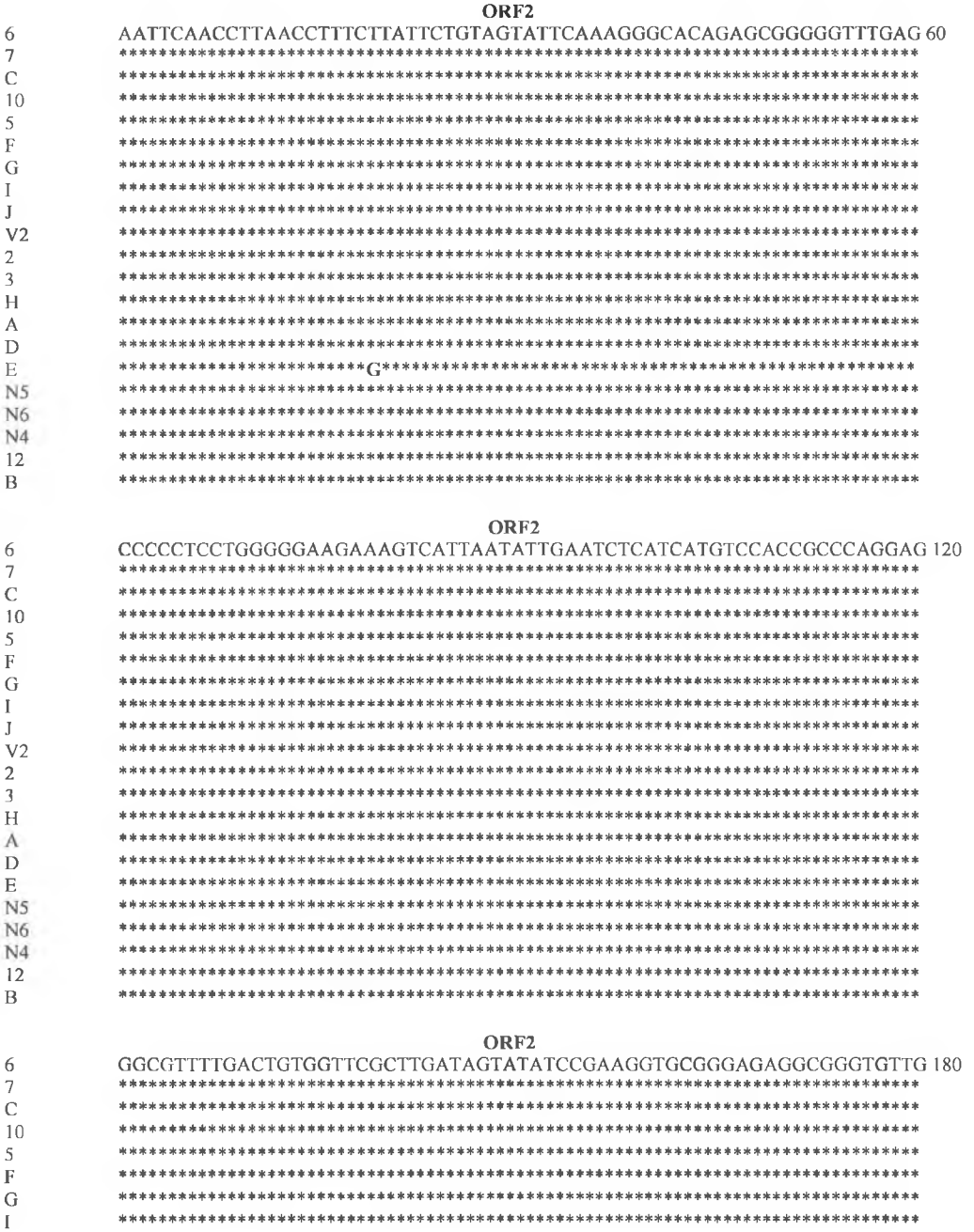

Figure 4.2: Continued

```
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     ********************************************************************************
A     ********************************************************************************
D     GGCGTTCTGACTGTGGTTCGCTTGATAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
E     GGCGTTCTGACTGTGGTTCGCTTGATAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
N5    ********************************************************************************
N6    ********************************************************************************
N4    GGCGTTTTGACTGTGGTTCGCTTGATAGTATATCCGAAGGTTCGGGAGAGGCGGGTGTTG
12    ********************************************************************************
B     ********************************************************************************

                                        ORF2
6     AAGATGCCATTTTTCCTTCTCCAGCGGTAACGGTGGCGGGGGTGGACGAGCCAGGGGCGG 240
7     ********************************************************************************
C     ********************************************************************************
10    ********************************************************************************
5     ********************************************************************************
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     ********************************************************************************
A     ********************************************************************************
D     ********************************************************************************
E     ********************************************************************************
N5    ********************************************************************************
N6    ********************************************************************************
N4    ********************************************************************************
12    ********************************************************************************
B     ********************************************************************************

                                        ORF2
6     CGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGGCGGTGTCTTCTTCTCCGGTAACGCCTC 300
7     ********************************************************************************
C     ********************************************************************************
10    ********************************************************************************
5     ********************************************************************************
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     ********************************************************************************
A     ********************************************************************************
D     ********************************************************************************
E     ********************************************************************************
N5    ********************************************************************************
N6    ********************************************************************************
N4    ********************************************************************************
12    ********************************************************************************
B     ********************************************************************************

        ORF2          ]               [              Ori                ]
6     CTTGGATACGTCATATCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC 360
7     ********************************************************************************
C     ********************************************************************************
10    ********************************************************************************
5     CTGGGATACGTCATATCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC
F     ********************************************************************************
```

Figure 4.2: Continued

```
G           ********************************************************************************
I           ********************************************************************************
J           ********************************************************************************
V2          ********************************************************************************
2           ********************************************************************************
3           ********************************************************************************
H           ********************************************************************************
A           ********************************************************************************
D           ********************************************************************************
E           ********************************************************************************
N5          ********************************************************************************
N6          ********************************************************************************
N4          ********************************************************************************
12          ********************************************************************************
B           ********************************************************************************

                                                      |          ORF1
6           GGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCCAGCAAGAAGAATGGAAG 420
7           ************************************************************************
C           ********************************************************************************
10          ********************************************************************************
5           ********************************************************************************
F           ********************************************************************************
G           ********************************************************************************
I           ********************************************************************************
J           ********************************************************************************
V2          ********************************************************************************
2           ********************************************************************************
3           ********************************************************************************
H           ********************************************************************************
A           ********************************************************************************
D           ********************************************************************************
E           ********************************************************************************
N5          GGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCTAGCAAAAAGAATGGAAG 420
N6          GGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCTAGCAAAAAGAATGGAAG 420
N4          GGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCTAGCAAAAAGAATGGAAG 420
12          GGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAAAATGCCCAGCAAGAAGAATGGAAG 420
B           ********************************************************************************

                                                ORF1
6           AAGCGGACCCCAACCCCATAAAAGGTGGGTGTTCACTCTGAATAATCCTTCCGAAGACGA 480
7           ************************************************************************
C           ********************************************************************************
10          ********************************************************************************
5           ********************************************************************************
F           ********************************************************************************
G           ********************************************************************************
I           ********************************************************************************
J           ********************************************************************************
V2          ********************************************************************************
2           ********************************************************************************
3           ********************************************************************************
H           ********************************************************************************
A           ********************************************************************************
D           ********************************************************************************
E           ********************************************************************************
N5          AAGCGGACCCCAACCACACAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
N6          AAGCGGACCCCAACCACACAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
N4          AAGCGGACCCCAACCACACAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
12          ************************************************************************
B           ********************************************************************************

                                                ORF1
6           GCGCAAGAAAATACGGGATCTTCCAATATCCCTATTTGATTATTTTATTGTTGGCGAGGA 540
7           ************************************************************************
C           ********************************************************************************
10          ********************************************************************************
5           ********************************************************************************
```

Figure 4.2: Continued

```
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     ********************************************************************************
A     ********************************************************************************
D     ********************************************************************************
E     ********************************************************************************
N5    GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTTTTTGATTATTTTATTGTTGGCGAGGA
N6    GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTTTTTGATTATTTTATTGTTGGCGAGGA
N4    GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTTTTTGATTATTTTATTGTTGGCGAGGA
12    GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTGTTTGATTATTTTATTGTTGGCGAGGA
B     ********************************************************************************

                                          ORF1
6     GGGTAATGAGGAAGGACGAACACCTCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA 600
7     ********************************************************************************
C     ********************************************************************************
10    GGGTAATGAGGAAGGACGAACACCTCACCTCCCGGGGTTCGCTAATTTTGTGAAGAAGCA 600
5     ********************************************************************************
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     GGGTAATGAGGAAGGACGAACACCTCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAACA
A     GGGTAATGAGGAAGGACGAACACCTCACCTTCAGGGGTTCGCTAATTTTGTGAAGAAGCA
D     GGGTAATGAGGAAGGACGAACACCTCACCTTCAGGGGTTCGCTAATTTTGTGAAGAAGCA
E     ********************************************************************************
N5    GGGTAATGAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA
N6    GGGTAATGAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA
N4    GGGTAATGAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA
12    GGGTAATGAGGAAGGACGAACACCTCACCTTCAGGGGTTCGCTAATTTTGTGAAAAAGCA
B     ********************************************************************************

                                          ORF1
6     GACTTTTAATAAAGTGAAGTGGTATTTGGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG 660
7     ********************************************************************************
C     ********************************************************************************
10    ********************************************************************************
5     ********************************************************************************
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     ********************************************************************************
A     ********************************************************************************
D     ********************************************************************************
E     ********************************************************************************
N5    AACATTTAATAAAGTGAAATGGTATTTCGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG
N6    AACATTTAATAAAGTGAAATGGTATTTCGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG
N4    AACATTTAATAAAGTGAATTGGTATTTCGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG
12    AACTTTTAATAAAGTGAAGTGGTATCTTGGTGCCCGCTGCCACATCGAGAAAGCCAAAGG
B     GACTTTTAATAAAGTGAAGTGGTATTTGGGTGCTCGCTGCCACATCGAGAAAGCGAAAGG
```

Figure 4.2: Continued

ORF1

```
6    AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTGATCGAGTGTGG 720
7    ************************************************************
C    ************************************************************
10   ************************************************************
5    ************************************************************
F    ************************************************************
G    ************************************************************
I    ************************************************************
J    ************************************************************
V2   ************************************************************
2    ************************************************************
3    ************************************************************
H    ************************************************************
A    ************************************************************
D    AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTGATGGAGTGTGG
E    AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTGATGGAGTGTGG
N5   AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATAGAATGTGG
N6   AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATAGAATGTGG
N4   AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATAGAATGTGG
12   AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTTATTGAGTGTGG
B    AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATGGAATGTGG
```

ORF1

```
6    AGCTCCTAGATCTCAGGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA 780
7    ************************************************************
C    ************************************************************
10   ************************************************************
5    ************************************************************
F    ************************************************************
G    ************************************************************
I    ************************************************************
J    ************************************************************
V2   ************************************************************
2    ************************************************************
3    ************************************************************
H    ************************************************************
A    ************************************************************
D    ************************************************************
E    ************************************************************
N5   AGCTCCTAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
N6   AGCTCCTCGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
N4   AGCTCCTAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
12   AGCTCCTAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
B    ************************************************************
```

ORF1

```
6    GAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACCCTGTAACGTTTGTCAGAAATTTCCG 840
7    ************************************************************
C    ************************************************************
10   ************************************************************
5    ************************************************************
F    ************************************************************
G    ************************************************************
I    ************************************************************
J    ************************************************************
V2   ************************************************************
2    ************************************************************
3    ************************************************************
H    ************************************************************
A    ************************************************************
D    ************************************************************
E    GAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACCCTGTAACGTTTGTCAGAAATTACCG
N5   ************************************************************
N6   ************************************************************
N4   ************************************************************
```

Figure 4.2: Continued

```
12          ********************************************************************************
B           ********************************************************************************

                                                ORF1
6           CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACTAA 900
7           ********************************************************************************
C           ********************************************************************************
10          ********************************************************************************
5           ********************************************************************************
F           ********************************************************************************
G           ********************************************************************************
I           ********************************************************************************
J           ********************************************************************************
V2          ********************************************************************************
2           ********************************************************************************
3           ********************************************************************************
H           ********************************************************************************
A           ********************************************************************************
D           ********************************************************************************
E           ********************************************************************************
N5          CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACGAA
N6          CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAA
N4          CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACGAA
12          CGGGCTGGCTGAACTTTTGAAAGTAAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAA
B           CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACGAA

                                                ORF1
6           TGTACACGTCATTGTGGGGCCACCTGGGTGTGGTAAAAGCAAATGGGCTGCTAATTTTGC 960
7           ********************************************************************************
C           ********************************************************************************
10          ********************************************************************************
5           ********************************************************************************
F           ********************************************************************************
G           ********************************************************************************
I           ********************************************************************************
J           ********************************************************************************
V2          ********************************************************************************
2           ********************************************************************************
3           ********************************************************************************
H           ********************************************************************************
A           ********************************************************************************
D           ********************************************************************************
E           ********************************************************************************
N5          ********************************************************************************
N6          ********************************************************************************
N4          ********************************************************************************
12          ********************************************************************************
B           TGTACACGTCATTGTGGGGCCACCTGGGTGTGGCAAAAGCAAATGGGCTGCTAATTTTGC

                                                ORF1
6           AGACCCGGAAACCACATACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATGG 1020
7           ********************************************************************************
C           ********************************************************************************
10          ********************************************************************************
5           ********************************************************************************
F           AGACCCGGAAACCACATACTGGAAACCACCTAGGAACAAGTGGTGGGATGGTTACCATGG
G           AGACCCGGAAACCACATACTGGAAACCACCTAGGAACAAGTGGTGGGATGGTTACCATGG
I           AGACCCGGAAACCACATACTGGAAACCACCTAGGAACAAGTGGTGGGATGGTTACCATGG
J           AGACCCGGAAACCACATACTGGAAACCACCTAGGAACAAGTGGTGGGATGGTTACCATGG
V2          AGACCCGGAAACCACATACTGGAAACCACCTAGGAACAAGTGGTGGGATGGTTACCATGG
2           ********************************************************************************
3           ********************************************************************************
H           ********************************************************************************
A           ********************************************************************************
D           ********************************************************************************
E           ********************************************************************************
N5          ********************************************************************************
```

Figure 4.2: Continued

```
N6      ********************************************************************************
N4      ********************************************************************************
12      ********************************************************************************
B       AGACCCGGAAACCACATACTGGAAACCACCTAGAAATAAGTGGTGGGATGGTTACCATGG

                                        ORF1
6       TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCCTGGGATGATCTACTGAG 1080
7       ********************************************************************************
C       ********************************************************************************
10      ********************************************************************************
5       ********************************************************************************
F       ********************************************************************************
G       ********************************************************************************
I       ********************************************************************************
J       ********************************************************************************
V2      ********************************************************************************
2       ********************************************************************************
3       ********************************************************************************
H       ********************************************************************************
A       ********************************************************************************
D       ********************************************************************************
E       TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGTTGCCCTGGGATGATCTACTGAG
N5      ********************************************************************************
N6      ********************************************************************************
N4      ********************************************************************************
12      TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAG
B       TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAG

                                        ORF1
6       ACTGTGTGATCGATATCCATTGACTGTAGAGACTAAAGGTGGAACTGTACCTTTTTTGGC 1140
7       ********************************************************************************
C       ********************************************************************************
10      ********************************************************************************
5       ********************************************************************************
F       ********************************************************************************
G       ********************************************************************************
I       ********************************************************************************
J       ********************************************************************************
V2      ********************************************************************************
2       ********************************************************************************
3       ********************************************************************************
H       ********************************************************************************
A       ********************************************************************************
D       ********************************************************************************
E       ********************************************************************************
N5      ********************************************************************************
N6      ********************************************************************************
N4      ********************************************************************************
12      ********************************************************************************
B       ACTGTGTGATCGGTATCCATTGACTGTAGAGACTAAAGGTGGAACTGTACCTTTTTTGGC

                                        ORF1
6       CCGCAGTATTCTGATTACCAGCAATCAGACCCCGTTGGAATGGTACTCCTCAACTGCTGT 1200
7       ********************************************************************************
C       ********************************************************************************
10      ********************************************************************************
5       ********************************************************************************
F       ********************************************************************************
G       ********************************************************************************
I       ********************************************************************************
J       ********************************************************************************
V2      ********************************************************************************
2       ********************************************************************************
3       ********************************************************************************
H       ********************************************************************************
A       ********************************************************************************
D       ********************************************************************************
```

Figure 4.2: Continued

```
E     ********************************************************************************
N5    ********************************************************************************
N6    ********************************************************************************
N4    ********************************************************************************
12    ********************************************************************************
B     CCGCAGTATTCTGATTACCAGCAATCAGACCCCGTTGGAATGGTACTCCTCGGCTGCTGT

                                        ORF1
6     CCCAGCTGTAGAAGCTCTTTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC 1260
7     ********************************************************************************
C     ********************************************************************************
10    ********************************************************************************
5     ********************************************************************************
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     ********************************************************************************
A     ********************************************************************************
D     ********************************************************************************
E     ********************************************************************************
N5    ********************************************************************************
N6    ********************************************************************************
N4    CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC
12    ********************************************************************************
B     CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC

                                        ORF1
6     AGAACAATCCACGGAGGAAGGGGGCCAGTTCGTCACCCTTTCCCCCCCATGCCCTGAATT 1320
7     ********************************************************************************
C     ********************************************************************************
10    ********************************************************************************
5     ********************************************************************************
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     ********************************************************************************
A     ********************************************************************************
D     ********************************************************************************
E     ********************************************************************************
N5    ********************************************************************************
N6    ********************************************************************************
N4    ********************************************************************************
12    ********************************************************************************
B     AGAACAGTCCACGGAGGAAGGGGGCCAGTTCGTCACCCTTTCCCCCCCATGCCCTGAATT

                ORF1            ]                                       [
6     TCCATATGAAATAAATTACTGAGTCTTTTTTATCACTTCGTAATGGTTTTTATTATTCAT 1380
7     ********************************************************************************
C     ********************************************************************************
10    ********************************************************************************
5     ********************************************************************************
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     ********************************************************************************
```

Figure 4.2: Continued

```
A     **********************************************************************
D     **********************************************************************
E     **********************************************************************
N5    TCCATATGAAATAAATTACTGAGTCCTTTTTATCACTTCGTAATGGTTTTTATTATTTAT
N6    TCCATATGAAATAAATTACTGAGTCCTTTTTATCACTTCGTAATGGTTTTTATTATTTAT
N4    TCCATATGAAATAAATTACTGAGTCCTTTTTATCACTTCGTAATGGTTTTTATTATTTAT
12    **********************************************************************
B     **********************************************************************

                                        ORF2
6     TAAGGGTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTACA 1440
7     **********************************************************************
C     **********************************************************************
10    **********************************************************************
5     **********************************************************************
F     **********************************************************************
G     **********************************************************************
I     **********************************************************************
J     **********************************************************************
V2    **********************************************************************
2     TAAGGGTTAAGTGGGGGGTCTTGAGGATTAAATTCTCTGAATTGTACATACATGGTTACA
3     **********************************************************************
H     **********************************************************************
A     **********************************************************************
D     **********************************************************************
E     **********************************************************************
N5    **********************************************************************
N6    **********************************************************************
N4    **********************************************************************
12    TAAGGGTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATAGTTACA
B     TTAGGGTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTACA

                                        ORF2
6     CGGATATTGTATTCCTGGTCGTATATACTGTTTTCGAACGCAGTGCCGAGGCCTACGTGG 1500
7     **********************************************************************
C     **********************************************************************
10    **********************************************************************
5     **********************************************************************
F     **********************************************************************
G     **********************************************************************
I     **********************************************************************
J     **********************************************************************
V2    **********************************************************************
2     **********************************************************************
3     CGGATATTGTATTCCTGGTCGTATATACTGTTTTTGAACGCAGTGCCGAGGCCTACGTGG
H     **********************************************************************
A     CGGATGTTGTATTCCTGGTCGTATATACTGTTTTCGAACGCAGTGCCGAGGCCTACGTGG
D     **********************************************************************
E     **********************************************************************
N5    CGGATATTGTATTCCTGGTCGTATATACTGTTTTCGAACGCAGTGCCTAGGCCTACGTGG
N6    CGGATATTGTATTCCTGGTCGTATATACTGTTTTCGAACGCAGTGCCTAGGCCTACGTGG
N4    **********************************************************************
12    **********************************************************************
B     CGGATATTGTAGTCCTGGTCGTATTTACTGTTTTCGAAGGCAGTGCCGAGGCCTACATGG

                                        ORF2
6     TCTACATTTCCAGCAGTTTGTAGTCTCAGCCACAGCTGATTTCTTTTGTTGTTTGGTTGG 1560
7     **********************************************************************
C     **********************************************************************
10    **********************************************************************
5     **********************************************************************
F     **********************************************************************
G     **********************************************************************
I     **********************************************************************
J     **********************************************************************
V2    **********************************************************************
2     **********************************************************************
```

Figure 4.2: Continued

```
3     ********************************************************************************
H     ********************************************************************************
A     ********************************************************************************
D     ********************************************************************************
E     ********************************************************************************
N5    TCTACATTTCCAGTAGTTTGTAGTCTCAGCCACAGCTGATTTCTTTTGTTGTTTGGTTGG
N6    TCTACATTTCCAGTAGTTTGTAGTCTCAGCCACAGCTGATTTCTTTTGTTGTTTGGTTGG
N4    TCTACATTTCCAGTAGTTTGTAGTCTCAGCCACAGCTGATTTCTTTTGTTGTTTGGTTGG
12    ********************************************************************************
B     TCTACATTTCCAGTAGTTTGTAGTCTCAGCCAGAGTTGATTTCTTTTGTTATTGGGTTGG

                                        ORF2
6     AAGTAATCAATAGTGGAATCTAGGACAGGTTTGGGGGTAAAGTAGCGGGAGTGGTAGGAG 1620
7     ********************************************************************************
C     ********************************************************************************
10    ********************************************************************************
5     ********************************************************************************
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     ********************************************************************************
A     ********************************************************************************
D     ********************************************************************************
E     ********************************************************************************
N5    ********************************************************************************
N6    ********************************************************************************
N4    ********************************************************************************
12    AAGTAATCAATAGTGGAATCTAGGACAGGTTTGGGGGTAAAATAGCGGGAGTGGTAGGAG
B     AAGTAATCGATTGTCCCATCAAGGATAGGTTTGGGGGTGAAATAGCGGGAGTGGTAGGAG

                                        ORF2
6     AAGGGCTGGGTTATGGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTGAGGGCT 1680
7     ********************************************************************************
C     ********************************************************************************
10    ********************************************************************************
5     ********************************************************************************
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
2     ********************************************************************************
3     ********************************************************************************
H     ********************************************************************************
A     ********************************************************************************
D     ********************************************************************************
E     ********************************************************************************
N5    ********************************************************************************
N6    ********************************************************************************
N4    ********************************************************************************
12    ********************************************************************************
B     AAGGGCTGGGTTATGGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTTAGGGCT

                                        ORF2
6     GTGGCCTTTGTTACAAAGTTATCATCTAGAATAACAGCACTGGAGCCCACTCCCCTGTCA 1740
7     ********************************************************************************
C     ********************************************************************************
10    ********************************************************************************
5     ********************************************************************************
F     ********************************************************************************
G     ********************************************************************************
I     ********************************************************************************
J     ********************************************************************************
V2    ********************************************************************************
```

Figure 4.2: Continued

```
2       ********************************************************************************
3       ********************************************************************************
H       ********************************************************************************
A       ********************************************************************************
D       ********************************************************************************
E       ********************************************************************************
N5      ********************************************************************************
N6      ********************************************************************************
N4      ********************************************************************************
12      ********************************************************************************
B       GTGGACTTTGGATAAAAGTTATCATCTAGAATAACAGCACTGGATCCAACTCCCCTGTCA

                              ORF2
6       CCCTGGGTGATCGGGGAGCAGGGCCAG 1767
7       ****************************
C       ****************************
10      ****************************
5       ****************************
F       ****************************
G       ****************************
I       ****************************
J       ****************************
V2      ****************************
2       ****************************
3       ****************************
H       ****************************
A       ****************************
D       ****************************
E       CCCTGAGTGATCGGGGAGCAGGGCCAG
N5      ****************************
N6      ****************************
N4      ****************************
12      ****************************
B       CCCTGGGTGATTGGGGAGCAGGGCCAG
```

Figure 4.3: Nucleotide sequence alignment of the complete genome of Irish isolates of PCV2 with isolates from other countries already present in GenBank, changes in nt sequence are highlighted in red. Deletions are shown by dashes. Only 2 isolates from diseased animals in ROI and 4 from NI are represented as no changes existed in other sequences

```
V2          AATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGCACAGAGCGGGGGTTTGAG 60
C           ************************************************************
6           ************************************************************
UK          ************************************************************
A           ************************************************************
Fra1        ************************************************************
NL          ************************************************************
Fra2        ************************************************************
D           ************************************************************
E           AATTCAACCTTAACCTTTCGTATTCTGTAGTATTCAAAGGGCACAGAGCGGGGGTTTGAG 60
12          ************************************************************
N5          ************************************************************
N6          ************************************************************
N4          ************************************************************
B           ************************************************************
US          AATTCAACCTTAACCTTTTTTATTCTGTAGTATTCAAAGGGTATAGAGATTTTGTTGGTC
Can1010     AATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGTATAGAGATTTTGTTGGTC
Ger1        AATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGTATAGAGATTTTGTTGGTC
Ger2        AATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGTATAGAGATTTTGTTGGTC
Spa1        AATTCTACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGTATAGAGATTTTGTTGGTC
Spa2        AATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGTATAGAGATTTTGTTGGTC
Ta1         AATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGTATAGAGATTTTGTTGGTC
Ta2         AATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGTATAGAGATTTTGTTGGTC

V2          CCCCCTCCTGGGGGAAGAAAGTCATTAATATTGAATCTCATCATGTCCACCGCCCAGGAG 120
C           ************************************************************
6           ************************************************************
UK          ************************************************************
A           ************************************************************
Fra1        ************************************************************
NL          ************************************************************
Fra2        ************************************************************
D           ************************************************************
E           ************************************************************
12          ************************************************************
N5          ************************************************************
N6          ************************************************************
N4          ************************************************************
B           ************************************************************
US          CCCCCTCCCGGGGGAACAAAGTCGTCAATATTAAATCTCATCATGTCCACCGCCCAGGAG
Can1010     CCCCCTCCCGGGGGAACAAAGTCGTCAATTTTAAATCTCATCATGTCCACCGCCCAGGAG
Ger1        CCCCCTCCCGGGGGAACAAAGTCGTCAAGATTAAATCTCAGCATGTCCACCGCCCAGGAG
Ger2        CCCCCTCCCGGGGGAACAAAGTCGTCAAGATTAAATCTCAGCATGTCCACCGCCCAGGAG
Spa1        CCCCCTCCCGGGGGAACAAAGTCGTCAATATTAAATCTCATCATGTCCACCGCCCAGGAG
Spa2        CCCCCTCCCGGGGGAACAAAGTCGTCAATATTAAATCTCATCATGTCCACCGCCCAGGAG
Ta1         CCCCCTCCCGGGGGAACAAAGTCGTTAATATCAAATCTCATCATGTCCACCGCCCAGGAG
Ta2         CCCCCTCCCGGGGGAACAAAGTCGTTAATATTAAATCTCATCATGTCCACCGCCCAGGAG

V2          GGCGTTTTGACTGTGGTTCGCTTGATAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG 180
C           ************************************************************
6           ************************************************************
UK          GGCGTCTTGACTGTGGTTCGCTTGATAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
A           ************************************************************
Fra1        GGCGTTCTGACTGTGGTTCGCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
NL          GGCGTTCTGACTGTGGTTCGCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
```

Figure 4.3 Continued

```
Fra2        GGCGTTTTGACTGTGGTTCGCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
D           GGCGTTCTGACTGTGGTTCGCTTGATAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
E           GGCGTTCTGACTGTGGTTCGCTTGATAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
12          ************************************************************************
N5          ************************************************************************
N6          ************************************************************************
N4          GGCGTTTTGACTGTGGTTCGCTTGATAGTATATCCGAAGGTTCGGGAGAGGCGGGTGTTG
B           ************************************************************************
US          GGCGTTCTGACTGTGGTAGCCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
Can1010     GGCGTTGTGACTGTGGTACGCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
Ger1        GGCGTGCTGACTGTGGTAGCCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGCGTTG
Ger2        GGCGTGCTGACTGTGGTAGCCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGCGTTG
Spa1        GGCGTTCTGACTGTGGTAGCCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
Spa2        GGCGTTCTGACTGTGGTAGCCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
Ta1         GGCGTTCTGACTGTGCTAGCCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG
Ta2         GGCGTTCTGACTGTTGTAGCCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTG

V2          AAGATGCCATTTTTCCTTCTCCAGCGGTAACGGTGGCGGGGGTGGACGAGCCAGGGGCGG 240
C           ************************************************************************
6           ************************************************************************
UK          ************************************************************************
A           ************************************************************************
Fra1        ************************************************************************
NL          ************************************************************************
Fra2        ************************************************************************
D           ************************************************************************
E           ************************************************************************
12          ************************************************************************
N5          ************************************************************************
N6          ************************************************************************
N4          ************************************************************************
B           ************************************************************************
US          AAGATGCCATTTTTCCTTCTCCAACGGTAGCGGTGGCGGGGGTGGACGAGCCAGGGGCGG
Can1010     AAGATGCCATTTTTCCTTCTCCAACGGTAGCGGTGGCGGGGGTGGACGAGCCAGGGGCGG
Ger1        ************************************************************************
Ger2        ************************************************************************
Spa1        ************************************************************************
Spa2        ************************************************************************
Ta1         AAGATGCCATTTTTCCTTCTCCAGCGGTAACGCTGGCGGGGGTGGACGAGCCAGGGGCGG
Ta2         ************************************************************************

V2          CGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCTTCTCCGGTAACGCCTC 300
C           ************************************************************************
6           ************************************************************************
UK          ************************************************************************
A           ************************************************************************
Fra1        ************************************************************************
NL          ************************************************************************
Fra2        ************************************************************************
D           ************************************************************************
E           ************************************************************************
12          ************************************************************************
N5          ************************************************************************
N6          ************************************************************************
N4          ************************************************************************
B           ************************************************************************
US          CGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCTTCTGCGGTAACGCCTC
Can1010     CGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCTTCTGCGGTAACGCCTC
Ger1        CGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCGTCTGCGGAAACGCCTC
Ger2        CGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTGCGTCTGCGGAAACGCCTC
Spa1        CGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCGTCTGCGGAAACGCCTC
Spa2        CGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCGTCTGCGGAAACGCCTC
Ta1         CGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCGTCTGCGGAAACGCCTC
Ta2         CGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCGTCTGCGGAAACGCCTC
```

Figure 4.3 Continued

```
V2      CTTGGATACGTCATATCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC 360
C       ************************************************************
6       ************************************************************
UK      CTTGGATACGTCATATCTGAAAACGAAAGAAGTGCGCT-TAAGTATTACCAGCGCACTTC 359
A       ************************************************************
Fra1    ************************************************************
NL      ************************************************************
Fra2    ************************************************************
D       ************************************************************
E       CTTGCATACGTCATATCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC 360
12      ************************************************************
N5      ************************************************************
N6      ************************************************************
N4      ************************************************************
B       ************************************************************
US      CTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC
Can1010 CTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC
Ger1    ************************************************************
Ger2    CTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC
Spa1    CTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC
Spa2    CTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC
Ta1     CTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC
Ta2     CTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGTAAGTATTACCAGCGCACTTC

V2      GGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCCAGCAAGAAGAATGGAAG 420
C       ************************************************************
6       ************************************************************
UK      ************************************************************
A       ************************************************************
Fra1    ************************************************************
NL      ************************************************************
Fra2    ************************************************************
D       ************************************************************
E       ************************************************************
12      GGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAAAATGCCCAGCAAGAAGAATGGAAG
N5      GGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCTAGCAAAAAGAATGGAAG
N6      GGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCTAGCAAAAAGAATGGAAG
N4      GGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCTAGCAAAAAGAATGGAAG
B       GCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCCAGCAAGAAGAATGGAAG
US      ************************************************************
Can1010 ************************************************************
Ger1    ************************************************************
Ger2    ************************************************************
Spa1    ************************************************************
Spa2    ************************************************************
Ta1     ************************************************************
Ta2     ************************************************************

V2      AAGCGGACCCCAACCCCATAAAAGGTGGGTGTTCACTCTGAATAATCCTTCCGAAGACGA 480
C       ************************************************************
6       ************************************************************
UK      ************************************************************
A       ************************************************************
Fra1    ************************************************************
NL      ************************************************************
Fra2    ************************************************************
D       ************************************************************
E       ************************************************************
12      ************************************************************
N5      AAGCGGACCCCAACCACACAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
N6      AAGCGGACCCCAACCACACAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
N4      AAGCGGACCCCAACCACACAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
B       ************************************************************
US      AAGCGGACCCCAACCACATAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
Can1010 AAGCGGACCCCAACCACATAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
Ger1    AAGCGGACCCCAACCACATAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
Ger2    AAGCGGACCCCAACCACATAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
```

Figure 4.3 Continued

```
Spa1    AAGCGGACCCCAACCACACAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
Spa2    AAGCGGACCCCAACCACACAAAAGGTGGGTGTTCACGCTGAATAATCCTTCCGAAGACGA
Ta1     AAGCGGACCCCAACCACATAAAAGGTGGGTGTTCACGCTCAATAATCCTTCCGAAGACGA
Ta2     AAGCGGACCCCAACCACATAAAAGGTGGGTGTTCACGCTCAATAATCCTTCCGAAGACGA

V2      GCGCAAGAAAATACGGGATCTTCCAATATCCCTATTTGATTATTTTATTGTTGGCGAGGA 540
C       ************************************************************************
6       ************************************************************************
UK      ************************************************************************
A       ************************************************************************
Fra1    ************************************************************************
NL      ************************************************************************
Fra2    ************************************************************************
D       ************************************************************************
E       ************************************************************************
12      GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTGTTTGATTATTTTATTGTTGGCGAGGA
N5      GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTTTTTGATTATTTTATTGTTGGCGAGGA
N6      GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTTTTTGATTATTTTATTGTTGGCGAGGA
N4      GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTTTTTGATTATTTTATTGTTGGCGAGGA
B       GCGCAAGAAAATACGGGATCTTCCAATATCCCTATTTGATTATTTTATTGTTGGCGAGGA
US      GCGCAAGAAAATACGGGAGCTCCCAATCTCCCTATTTGATTATTTTATTGTTGGCGAGGA
Can1010 GCGCAAGAAAATACGGGAGCTCCCAATCTCCCTATTTGATTATTTTATTGTTGGCGAGGA
Ger1    GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTGTTTGATTATTTTATTGTTGGCGAGGA
Ger2    GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTGTTTGATTATTTTATTGTTGGCGAGGA
Spa1    GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTGTTTGATTATTTTATTGTTGGCGAGGA
Spa2    GCGCAAGAAAATACGGGAGCTTCCAATCTCCCTTTTTGATTATTTTATTGTTGGCGAGGA
Ta1     GCGCAAGAAAATACGGGAGCTCCCAATCTCCCTATTTGATTGTTTTATTGTTGGCGAGGA
Ta2     ************************************************************************

V2      GGGTAATGAGGAAGGACGAACACCTCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA 600
C       ************************************************************************
6       ************************************************************************
UK      ************************************************************************
A       GGGTAATGAGGAAGGACGAACACCTCACCTTCAGGGGTTCGCTAATTTTGTGAAGAAGCA
Fra1    ************************************************************************
NL      ************************************************************************
Fra2    ************************************************************************
D       GGGTAATGAGGAAGGACGAACACCTCACCTTCAGGGGTTCGCTAATTTTGTGAAGAAGCA
E       ************************************************************************
12      GGGTAATGAGGAAGGACGAACACCTCACCTTCAGGGGTTCGCTAATTTTGTGAAAAAGCA
N5      GGGTAATGAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA
N6      GGGTAATGAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA
N4      GGGTAATGAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA
B       ************************************************************************
US      ************************************************************************
Can1010 ************************************************************************
Ger1    GGGTAATGAGGAAGGACGAACACCTCACCTCCAGGGGTTCGCTAATTTTGTGAAAAAGCA
Ger2    GGGTAATGAGGAAGGACGAACACCTCACCTCCAGGGGTTCGCTAATTTTGTGAAAAAGCA
Spa1    GGGTAATGAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA
Spa2    GGGTAATGAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA
Ta1     GGGTAATGAGGAAGGCCGAACACCCCACCTACAGGGGTTCGCTAATTTTGTGAAGAAGCA
Ta2     GGGTAATGAGGAAGGCCGAACACCCCACCTACAGGGGTTCGCTAATTTTGTGAAGAAGCA

V2      GACTTTTAATAAAGTGAAGTGGTATTTGGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG 660
C       ************************************************************************
6       ************************************************************************
UK      ************************************************************************
A       ************************************************************************
Fra1    ************************************************************************
NL      ************************************************************************
Fra2    ************************************************************************
D       ************************************************************************
E       ************************************************************************
12      AACTTTTAATAAAGTGAAGTGGTATCTTGGTGCCCGCTGCCACATCGAGAAAGCCAAAGG
N5      AACATTTAATAAAGTGAAATGGTATTTCGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG
N6      AACATTTAATAAAGTGAAATGGTATTTCGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG
N4      AACATTTAATAAAGTGAATTGGTATTTCGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG
```

Figure 4.3 Continued

```
B          GACTTTTAATAAAGTGAAGTGGTATTTGGGTGCTCGCTGCCACATCGAGAAAGCGAAAGG
US         AACTTTTAATAAAGTGAAGTGGTATTTGGGTGCCCGCTGCCACATCGAGAAAGCCAAAGG
Can1010    AACTTTTAATAAAGTGAAGTGGTATTTGGGTGCCCGCTGCCACATCGAGAAAGCCAAAGG
Ger1       AACATTTAATAAAGTGAAGTGGTATCTTGGTGCCCGCTGCCACATCGAGAAAGCCAAAGG
Ger2       GACATTTAATAAAGTGAAGTGGTATCTTGGTGCCCGCTGCCACATCGAGAAAGCCAAAGG
Spa1       AACATTTAATAAAGTGAAATGGTATTTGGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG
Spa2       AACATTTAATAAAGTGAAATGGTATTTGGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG
Ta1        AACTTTTAATAAAGTGAAGTGGTATTTTGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG
Ta2        AACTTTTAATAAAGTGAAGTGGTATTTTGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG

V2         AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTGATCGAGTGTGG 720
C          ************************************************************************
6          ************************************************************************
UK         ************************************************************************
A          ************************************************************************
Fra1       AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTGATGGAGTGTGG
NL         AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTGATGGAGTGTGG
Fra2       AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTGATGGAGTGTGG
D          AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTGATGGAGTGTGG
E          AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTGATGGAGTGTGG
12         AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTTATTGAGTGTGG
N5         AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATAGAATGTGG
N6         AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATAGAATGTGG
N4         AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATAGAATGTGG
B          AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATGGAATGTGG
US         AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTTATTGAATGTGG
Can1010    AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTTATTGAATGTGG
Ger1       AACAGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTTATTGAGTGTGG
Ger2       AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTTATTGAGTGTGG
Spa1       AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATTGAATGTGG
Spa2       AACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATTGAATGTGG
Ta1        AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTTATCGAGTGTGG
Ta2        AACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTACTTATCGAGTGTGG

V2         AGCTCCTAGATCTCAGGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA 780
C          ************************************************************************
6          ************************************************************************
UK         ************************************************************************
A          ************************************************************************
Fra1       ************************************************************************
NL         ************************************************************************
Fra2       ************************************************************************
D          ************************************************************************
E          ************************************************************************
12         AGCTCCTAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
N5         AGCTCCTAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
N6         AGCTCCTCGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
N4         AGCTCCTAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
B          ************************************************************************
US         AGCTCCTCGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
Can1010    AGCTCCTCGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
Ger1       AGCTCCTAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
Ger2       AGCTCCTAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
Spa1       ************************************************************************
Spa2       AGCTCCTAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
Ta1        AGCTCCTAGATCTCAAGGACAACGCAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA
Ta2        AGCTCCTAGATCTCAAGGACAACGCAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGA

V2         GAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACCCTGTAACGTTTGTCAGAAATTTCCG 840
C          ************************************************************************
6          ************************************************************************
UK         ************************************************************************
A          ************************************************************************
Fra1       ************************************************************************
NL         ************************************************************************
Fra2       ************************************************************************
D          ************************************************************************
E          GAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACCCTGTAACGTTTGTCAGAAATTACCG
```

Figure 4.3 Continued

```
12          GAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACCCTGTAACGTTTGTCAGAAATTTCCG
N5          ********************************************************************************
N6          ********************************************************************************
N4          ********************************************************************************
B           ********************************************************************************
US          ********************************************************************************
Can1010     ********************************************************************************
Ger1        ********************************************************************************
Ger2        ********************************************************************************
Spa1        ********************************************************************************
Spa2        ********************************************************************************
Ta1         ********************************************************************************
Ta2         ********************************************************************************

V2          CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACTAA 900
C           ********************************************************************************
6           ********************************************************************************
UK          ********************************************************************************
A           ********************************************************************************
Fra1        ********************************************************************************
NL          ********************************************************************************
Fra2        ********************************************************************************
D           ********************************************************************************
E           ********************************************************************************
12          CGGGCTGGCTGAACTTTTGAAAGTAAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAA
N5          CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACGAA
N6          CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAA
N4          CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACGAA
B           CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACGAA
US          CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAA
Can1010     CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAA
Ger1        CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAA
Ger2        CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAA
Spa1        CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACGAA
Spa2        CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACGAA
Ta1         CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAA
Ta2         CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAA

V2          TGTACACGTCATTGTGGGGCCACCTGGGTGTGGTAAAAGCAAATGGGCTGCTAATTTTGC 960
C           ********************************************************************************
6           ********************************************************************************
UK          ********************************************************************************
A           ********************************************************************************
Fra1        ********************************************************************************
NL          ********************************************************************************
Fra2        ********************************************************************************
D           ********************************************************************************
E           ********************************************************************************
12          ********************************************************************************
N5          ********************************************************************************
N6          ********************************************************************************
N4          ********************************************************************************
B           TGTACACGTCATTGTGGGGCCACCTGGGTGTGGCAAAAGCAAATGGGCTGCTAATTTTGC
US          ********************************************************************************
Can1010     ********************************************************************************
Ger1        ********************************************************************************
Ger2        ********************************************************************************
Spa1        ********************************************************************************
Spa2        ********************************************************************************
Ta1         ********************************************************************************
Ta2         ********************************************************************************

V2          AGACCCGGAAACCACATACTGGAAACCACCTAGGAACAAGTGGTGGGATGGTTACCATGG 1020
C           AGACCCGGAAACCACATACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATGG
6           ********************************************************************************
UK          ********************************************************************************
A           ********************************************************************************
Fra1        ********************************************************************************
```

Figure 4.3 Continued

```
NL      AGACCCGGAAACCACATACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATGG 1020
Fra2    ************************************************************
D       ************************************************************
E       ************************************************************
12      ************************************************************
N5      ************************************************************
N6      ************************************************************
N4      ************************************************************
B       AGACCCGGAAACCACATACTGGAAACCACCTAGAAATAAGTGGTGGGATGGTTACCATGG
US      ************************************************************
Can1010 ************************************************************
Ger1    ************************************************************
Ger2    ************************************************************
Spa1    ************************************************************
Spa2    ************************************************************
Ta1     AGACCCGGAAACCACATACTGGAAACCACCTCGAAACAAGTGGTGGGATGGTTACCATGG
Ta2     AGACCCGGAAACCACATACTGGAAACCACCTCGAAACAAGTGGTGGGATGGTTACCATGG

V2      TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCCTGGGATGATCTACTGAG 1080
C       ************************************************************
6       ************************************************************
UK      ************************************************************
A       ************************************************************
Fra1    ************************************************************
NL      ************************************************************
Fra2    ************************************************************
D       ************************************************************
E       TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGTTGCCCTGGGATGATCTACTGAG
12      ************************************************************
N5      ************************************************************
N6      ************************************************************
N4      ************************************************************
B       TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAG
US      TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAG
Can1010 TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAG
Ger1    TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAG
Ger2    TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAG
Spa1    TGAAGAAGTGGTGGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAG
Spa2    TGAAGAAGTGGTGGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAG
Ta1     TGAAGAAGTGGTTGTTATTGATGACTTTTATGGTTGGCTGCCGTGGGATGATCTACTGAG
Ta2     TGAAGAAGTGGTTGTTATTGATGACTTTTATGGTTGGCTGCCGTGGGATGATCTACTGAG

V2      ACTGTGTGATCGATATCCATTGACTGTAGAGACTAAAGGTGGAACTGTACCTTTTTTGGC 1140
C       ************************************************************
6       ************************************************************
UK      ************************************************************
A       ************************************************************
Fra1    ************************************************************
NL      ************************************************************
Fra2    ************************************************************
D       ************************************************************
E       ************************************************************
12      ************************************************************
N5      ************************************************************
N6      ************************************************************
N4      ************************************************************
B       ACTGTGTGATCGGTATCCATTGACTGTAGAGACTAAAGGTGGAACTGTACCTTTTTTGGC
US      ************************************************************
Can1010 ************************************************************
Ger1    ************************************************************
Ger2    ************************************************************
Spa1    ACTGTGTGATCGGTATCCATTGACTGTAGAGACTAAAGGTGGAACTGTACCTTTTTTGGC
Spa2    ACTGTGTGATCGGTATCCATTGACTGTAGAGACTAAAGGTGGAACTGTACCTTTTTTGGC
Ta1     ************************************************************
Ta2     ************************************************************
```

Figure 4.3 Continued

```
V2        CCGCAGTATTCTGATTACCAGCAATCAGACCCCGTTGGAATGGTACTCCTCAACTGCTGT 1200
C         ************************************************************
6         ************************************************************
UK        ************************************************************
A         ************************************************************
Fra1      ************************************************************
NL        ************************************************************
Fra2      ************************************************************
D         ************************************************************
E         ************************************************************
12        ************************************************************
N5        ************************************************************
N6        ************************************************************
N4        ************************************************************
B         CCGCAGTATTCTGATTACCAGCAATCAGACCCCGTTGGAATGGTACTCCTCGGCTGCTGT
US        ************************************************************
Can1010   ************************************************************
Ger1      ************************************************************
Ger2      ************************************************************
Spa1      ************************************************************
Spa2      ************************************************************
Ta1       ************************************************************
Ta2       ************************************************************

V2        CCCAGCTGTAGAAGCTCTTTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC 1260
C         ************************************************************
6         ************************************************************
UK        ************************************************************
A         ************************************************************
Fra1      ************************************************************
NL        ************************************************************
Fra2      ************************************************************
D         ************************************************************
E         ************************************************************
12        ************************************************************
N5        ************************************************************
N6        ************************************************************
N4        CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC
B         CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC
US        CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC
Can1010   CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC
Ger1      CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC
Ger2      CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTGTTTTGGAAGAATGCTAC
Spa1      CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC
Spa2      CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC
Ta1       CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC
Ta2       CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGCTAC

V2        AGAACAATCCACGGAGGAAGGGGGCCAGTTCGTCACCCTTTCCCCCCCCATGCCCTGAATT 1320
C         ************************************************************
6         ************************************************************
UK        AGAACAATCCACGGAGGAAGGGGGCCAGTTCGTCACCCTCTCCCCCCCCATGCCCTGAATT 1319
A         ************************************************************
Fra1      ************************************************************
NL        ************************************************************
Fra2      ************************************************************
D         ************************************************************
E         ************************************************************
12        ************************************************************
N5        ************************************************************
N6        ************************************************************
N4        ************************************************************
B         AGAACAGTCCACGGAGGAAGGGGGCCAGTTCGTCACCCTTTCCCCCCCCATGCCCTGAATT
US        ************************************************************
Can1010   ************************************************************
Ger1      ************************************************************
Ger2      ************************************************************
```

Figure 4.3 Continued

```
Spa1    AGAACAATCCACGGAGGAAGGGGGCCAGTTCGTCACCCTTTCCCCCCCATGCCCTGAATT 1320
Spa2    ************************************************************
Ta1     ************************************************************
Ta2     ************************************************************

V2      TCCATATGAAATAAATTACTGAGTCTTTTTTATCACTTCGTAATGGTTTTTATTATTCAT 1380
C       ************************************************************
6       ************************************************************
UK      ************************************************************
A       ************************************************************
Fra1    ************************************************************
NL      ************************************************************
Fra2    ************************************************************
D       ************************************************************
E       ************************************************************
12      ************************************************************
N5      TCCATATGAAATAAATTACTGAGTCCTTTTTATCACTTCGTAATGGTTTTTATTATTTAT
N6      TCCATATGAAATAAATTACTGAGTCCTTTTTATCACTTCGTAATGGTTTTTATTATTTAT
N4      TCCATATGAAATAAATTACTGAGTCCTTTTTATCACTTCGTAATGGTTTTTATTATTTAT
B       ************************************************************
US      ************************************************************
Can1010 ************************************************************
Ger1    ************************************************************
Ger2    ************************************************************
Spa1    TCCATATGAAATAAATTACTGAGTCTTTTTTATCACTTCGTAATGGTTTTTATTATTAAT
Spa2    TCCATATGAAATAAATTACTGAGTCTTTTTTATCACTTCGTAATGGTTTTTATTATTAAT
Ta1     ************************************************************
Ta2     ************************************************************

V2      TAAGGGTT-AAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTAC 1439
C       ************************************************************
6       ************************************************************
UK      ************************************************************
A       ************************************************************
Fra1    ************************************************************
NL      ************************************************************
Fra2    ************************************************************
D       ************************************************************
E       ************************************************************
12      ************************************************************
N5      ************************************************************
N6      ************************************************************
N4      ************************************************************
B       TTAGGGTT-AAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTAC
US      TTAGGGTTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTAC 1440
Can1010 TTAGGGTTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTAC
Ger1    TTAGGGTTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTAC
Ger2    TTAGGGTTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTAC
Spa1    TTAGGGTTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTAC
Spa2    TTAGGGTTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTAC
Ta1     TTAGGGTTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATAGTTAC
Ta2     TTAGGGTTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATAGTTAC

V2      ACGGATATTGTATTCCTGGTCGTATATACTGTTTTCGAACGCAGTGCCGAGGCCTACGTG 1499
C       ************************************************************
6       ************************************************************
UK      ************************************************************
A       ACGGATGTTGTATTCCTGGTCGTATATACTGTTTTCGAACGCAGTGCCGAGGCCTACGTG
Fra1    ************************************************************
NL      ************************************************************
Fra2    ************************************************************
D       ************************************************************
E       ************************************************************
12      ************************************************************
N5      ACGGATATTGTATTCCTGGTCGTATATACTGTTTTCGAACGCAGTGCCTAGGCCTACGTG
N6      ACGGATATTGTATTCCTGGTCGTATATACTGTTTTCGAACGCAGTGCCTAGGCCTACGTG
N4      ************************************************************
B       ACGGATATTGTAGTCCTGGTCGTATTTACTGTTTTCGAAGGCAGTGCCGAGGCCTACATG
```

Figure 4.3 Continued

```
US          ACGGATATTGTAGTCCTGGTCGTATATACTGTTTTCGAACGCAGTGCCGAGGCCTACGTG 1500
Can1010     ACGGATATTGTAGTCCTGGTCGTATTTACTGTTTTCGAACGCAGCGCCGAGGCCTACGTG
Ger1        ACGGATATTGTAGTCCTGGTCGTATTTACTGTTTTCGAACGCAGTGCCGAGGCCTACGTG
Ger2        ACGGATATTGTAGTCCTGGTCGTATTTACTGTTTTCGAACGCAGTGCCGAGGCCTACGTG
Spa1        ACGGATATTGTAGTCCTGGTCGTATTTACTGTTTTCGAAGGCAGTGCCGAGGCCTACGTG
Spa2        ACGGATATTGTAGTCCTGGTCGTATTTACTGTTTTCGAAGGCAGTGCCGAGGCCTACGTG
Ta1         ACGGATATTGTAGTCCTGGTTGTATTTACTGTTTTCGAACGCAGTGCCGAGGCCTACGTG
Ta2         ACGGATATTGTAGTCCTGGTCGTATTTACTGTTTTCGAACGCAGTGCCCAGGCCTACGTG

V2          GTCTACATTTCCAGCAGTTTGTAGTCTCAGCCACAGCTGATTTCTTTTGTTGTTTGGTTG 1559
C           ***********************************************************************
6           ***********************************************************************
UK          GTCTACATTTCCAGCAGCTTGTAGTCTCAGCCACAGCTGATTTCTTTTGTTGTTTGGTTG
A           ***********************************************************************
Fra1        ***********************************************************************
NL          ***********************************************************************
Fra2        ***********************************************************************
D           ***********************************************************************
E           ***********************************************************************
12          ***********************************************************************
N5          GTCTACATTTCCAGTAGTTTGTAGTCTCAGCCACAGCTGATTTCTTTTGTTGTTTGGTTG
N6          GTCTACATTTCCAGTAGTTTGTAGTCTCAGCCACAGCTGATTTCTTTTGTTGTTTGGTTG
N4          GTCTACATTTCCAGTAGTTTGTAGTCTCAGCCACAGCTGATTTCTTTTGTTGTTTGGTTG
B           GTCTACATTTCCAGTAGTTTGTAGTCTCAGCCAGAGTTGATTTCTTTTGTTATTGGGTTG
US          GTCCACATTTCTAGAGGTTTGTAGCCTCAGCCAAAGCTGATTCCTTTTGTTATTTGGTTG 1560
Can1010     GTCCACATTTCCAGAGGTTTGTAGTCTCAGCCAAAGCTGATTCCTTTTGTTATTTGGTTG
Ger1        GTCCACATTTCTACTGGTTTGGAGTCTCATCCACAGCTGATTCCTTTTGTTATTTGGTTG
Ger2        GTCCACATTTCTACTGGTTTGGAGTCTCATCCACAGCTGATTCCTTTTGTTATTTGGTTG
Spa1        GTCCACATTTGCAGAGGTTTGTAGCCTCAGCCAAAGCTGATTCCTTTTGTTATTTGGTTG
Spa2        GTCCACATTTGCAGAGGTTTGTAGCCTCAGCCAAAGCTGATTCCTTTTGTTATTTGGTTG
Ta1         GTCCACATTTGCCGAGGTTTGTAGCCTCAGCCAAAGCTGATTCCTTTTGTTGTTTGGTTG
Ta2         GTCCACATTTGCCGAGGTTTGTAGCCTCAGCCAAAGCTGATTCCTTTTGTTGTTTGGTTG

V2          GAAGTAATCAATAGTGGAATCTAGGACAGGTTTGGGGGTAAAGTAGCGGGAGTGGTAGGA 1619
C           ***********************************************************************
6           ***********************************************************************
UK          GAAGTAATCAATAGTGGAATCTAGGACAGGTTTGGGGGTAAAGTAACGGGAGTGGTAGGA
A           ***********************************************************************
Fra1        ***********************************************************************
NL          ***********************************************************************
Fra2        ***********************************************************************
D           ***********************************************************************
E           ***********************************************************************
12          GAAGTAATCAATAGTGGAATCTAGGACAGGTTTGGGGGTAAAATAGCGGGAGTGGTAGGA
N5          ***********************************************************************
N6          ***********************************************************************
N4          ***********************************************************************
B           GAAGTAATCGATTGTCCCATCAAGGATAGGTTTGGGGGTGAAATAGCGGGAGTGGTAGGA
US          GAAGTAATCAATAGTGGAGTCAAGAACAGGTTTGGGTGTGAAGTAACGGGAGTGGTAGGA 1620
Can1010     GAAGTAATCAATAGTGGAGTCAAGAACAGGTTTGGGTGTGAAGTAACGGGAGTGGTAGGA
Ger1        GAAGTAATCAATAGTGGAATCAAGAACAGGTTTGGGTGTGAAGTAACGGGAGTGGTAGGA
Ger2        GAAGTAATCAATAGTGGAATCAAGAACAGGTTTGGGTGTGAAGTAACGGGAGTGGTAGGA
Spa1        GAAGTAATCAATAGTGGAATCAAGAACAGGTTTGGGGGTAAAGTACCGGGAGTGGTAGGA
Spa2        GAAGTAATCAATAGTGGAATCAAGAACAGGTTTGGGGGTAAAGTACCGGGAGTGGTAGGA
Ta1         GAAGTAATCAATAGTGGAATCAAGGACAGGTTTGGGGGTAAAGTACCGGGAGTGGTAGGA
Ta2         GAAGTAATCAATAGTGGAATCAAGGACAGGTTTGGGGGTAAAGTACCGGGAGTGGTAGGA

V2          GAAGGGCTGGGTTATGGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTGAGGGC 1679
C           ***********************************************************************
6           ***********************************************************************
UK          ***********************************************************************
A           ***********************************************************************
Fra1        ***********************************************************************
NL          ***********************************************************************
Fra2        ***********************************************************************
D           ***********************************************************************
E           ***********************************************************************
12          ***********************************************************************
```

Figure 4.3 Continued

```
N5          GAAGGGCTGGGTTATGGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTGAGGGC 1679
N6          ************************************************************
N4          ************************************************************
B           GAAGGGCTGGGTTATGGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTTAGGGC
US          GAAGGGTTGGGGGATTGTATGGCGGGAGGAGTAGTTTACATATGGGTCATAGGTTAGGGC 1680
Can1010     GAAGGGTTGGGGGATTGTATGGCGGGAGGAGTAGTTTACATATGGGTCATAGGTTAGGGC
Ger1        GAAGGGTTGGGGGATTGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTTTGGGC
Ger2        GAAGGGTTGGGGGATTGTATGCCGGGAGGAGTAGTTTACATAGGGGTCATAGGTTTGGGC
Spa1        GAAGGGTTGGGGGTATGGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTTAGGGC
Spa2        GAAGGGTTGGGGGATTGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTTAGGGC
Ta1         GAAGGGTTGGGGGATTGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTTAGGGC
Ta2         GAAGGGTTGGGGGATTGTATGGCGGGAGGAGTAGTTTACATACGGGTCATAGGTTAGGGC


V2          TGTGGCCTTTGTTACAAAGTTATCATCTAGAATAACAGCACTGGAGCCCACTCCCCTGTC 1739
C           ************************************************************
6           ************************************************************
UK          ************************************************************
A           ************************************************************
Fra1        ************************************************************
NL          ************************************************************
Fra2        ************************************************************
D           ************************************************************
E           ************************************************************
12          ************************************************************
N5          ************************************************************
N6          ************************************************************
N4          ************************************************************
B           TGTGGACTTTGGATAAAAGTTATCATCTAGAATAACAGCACTGGATCCAACTCCCCTGTC
US          TGTGGCCTTTGTTACAAAGTTATCATCTAGAATAACAGCAGTGGAGCCCACTCCCCTATC 1740
Can1010     TGTGGCCTTTGTTACAAAGTTATCATCTAGAATAACAGCAGTGGAGCCCACTCCCCTATC
Ger1        TGTGGCCTTTATTACAAAGTTGTCATCTAGAATAATAGCACTGGATCCAACTCCCCTGTC
Ger2        TGTGGCCTTTATTACAAAGTTGTCATCTAGAATAATAGCACTGGATCCAACTCCCCTGTC
Spa1        TGTGGACTTTGGAAAAAAGTTATCATCTAGAATAACAGCAGTGGAGCCCACTCCCCTATC
Spa2        TGTGGCCTTTGTTACAAAGTTATCATCTAGAATAACAGCAGTGGAGCCCACTCCCCTATC
Ta1         TGTGGCCTTAGTTACAAAGTTATCATCTAGAATAACAGCAGTGGAGCCCACTCCCCTGTC
Ta2         TGTGGCCTTAGTTACAAAGTTATCATCTAGAATAACAGCAGTGGAGCCCACTCCCCTGTC


V2          ACCCTGGGTGATCGGGGAGCAGGGCCAG 1767
C           ************************************  1767
6           ************************************ 1767
UK          ACCCTGGGTGATCGGGGAGCAGGGCCAA 1766
A           ************************************ 1767
Fra1        ************************************ 1767
NL          ************************************ 1767
Fra2        ************************************ 1767
D           ************************************ 1767
E           ACCCTGAGTGATCGGGGAGCAGGGCCAG 1767
12          ************************************ 1767
N5          ************************************ 1767
N6          ************************************ 1767
N4          ************************************ 1767
B           ACCCTGGGTGATTGGGGAGCAGGGCCAG 1767
US          ACCCTGGGTGATGGGGGAGCAGGGCCAG 1768
Can1010     ACCCTGGGTGATGGGGGAGCAGGGCCAG 1768
Ger1        ************************************ 1768
Ger2        ************************************ 1768
Spa1        ACCCTGGGTGATTGGGGAGCAGGGCCAG 1768
Spa2        ACCCTGGGTGATTGGGGAGCAGGGCCAG 1768
Ta1         ACCCTGGGTGATTGGGGAGCAGGGCCAG 1768
Ta2         ACCCTGGGTGATTGGGGAACAGGGCCAG 1768
```

Figure 4.6: Amino Acid Alignment of ORF2 of PCV2 of ROI and NI isolates. AA changes are highlighted in red.

```
N5      MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTIKRT 60
N6      ************************************************************
N4      ************************************************************
3       ************************************************************
5       ************************************************************
12      ************************************************************
D       ************************************************************
E       MTYARRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTIKRT
V2      ************************************************************
10      ************************************************************
6       ************************************************************
J       ************************************************************
I       ************************************************************
H       ************************************************************
G       ************************************************************
F       ************************************************************
C       ************************************************************
A       ************************************************************
2       ************************************************************
7       ************************************************************
B       ************************************************************

N5      TVKTPSWAVDMMRFNINDFLPPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGS 120
N6      ************************************************************
N4      ************************************************************
3       ************************************************************
5       ************************************************************
12      ************************************************************
D       TVRTPSWAVDMMRFNINDFLPPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGS
E       TVRTPSWAVDMMRFNINDFLPPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGS
V2      ************************************************************
10      ************************************************************
6       ************************************************************
J       ************************************************************
I       ************************************************************
H       ************************************************************
G       ************************************************************
F       ************************************************************
C       ************************************************************
A       ************************************************************
2       ************************************************************
7       ************************************************************
B       ************************************************************

N5      SAVILDDNFVTKATALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLDSTIDYFQPNNKR 180
N6      ************************************************************
N4      ************************************************************
3       ************************************************************
5       ************************************************************
12      ************************************************************
D       ************************************************************
E       ************************************************************
V2      ************************************************************
10      ************************************************************
6       ************************************************************
J       ************************************************************
I       ************************************************************
H       ************************************************************
G       ************************************************************
F       ************************************************************
C       ************************************************************
```

Figure 4.6 Continued

```
A     SAVILDDNFVTKATALTYDPYVNYSSRHTITQPFSYHSRYFTPKPVLDSTIDYFQPNNKR 180
2     ************************************************************************
7     ************************************************************************
B     SAVILDDNFYPKSTALTYDPYVNYSSRHTITQPFSYHSRYFTPKPILDGTIDYFQPNNKR

N5    NQLWLRLQTTGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP- 233
N6    NQLWLRLQTTGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP-
N4    NQLWLRLQTTGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP-
3     NQLWLRLQTAGNVDHVGLGTAFKNSIYDQEYNIRVTMYVQFREFNLKDPPLNP-
5     ************************************************************************
12    ************************************************************************
D     ************************************************************************
E     ************************************************************************
V2    ************************************************************************
10    ************************************************************************
6     ************************************************************************
J     ************************************************************************
I     ************************************************************************
H     ************************************************************************
G     ************************************************************************
F     ************************************************************************
C     ************************************************************************
A     ************************************************************************
2     NQLWLRLQTAGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFRELILKTPHLTLX  234
7     NQLWLRLQTAGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKTPHLTLX  234
B     NQLWLRLQTTGNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP- 233
```

Figure 4.7: Amino Acid Alignment of ORF1 PCV2 from ROI and NI isolates. AA changes are highlighted in red.

```
2     MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRDLPISLFDYFIVGEEGNEEGRTPHLQG 60
3     ************************************************************
5     ************************************************************
6     ************************************************************
7     ************************************************************
I     ************************************************************
J     ************************************************************
H     ************************************************************
G     ************************************************************
F     ************************************************************
C     ************************************************************
A     ************************************************************
V2    ************************************************************
B     ************************************************************
E     ************************************************************
D     ************************************************************
10    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRDLPISLFDYFIVGEEGNEEGRTPHLPG
N5    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRELPISLFDYFIVGEEGNEEGRTPHLQG
N6    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRELPISLFDYFIVGEEGNEEGRTPHLQG
N4    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRELPISLFDYFIVGEEGNEEGRTPHLQG
12    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRELPISLFDYFIVGEEGNEEGRTPHLQG

2     FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS 120
3     ************************************************************
5     ************************************************************
6     ************************************************************
7     ************************************************************
I     ************************************************************
J     ************************************************************
H     ************************************************************
G     ************************************************************
F     ************************************************************
C     ************************************************************
A     ************************************************************
V2    ************************************************************
B     FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLMECGAPRSQGQRSDLS
E     FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLMECGAPRSQGQRSDLS
D     FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLMECGAPRSQGQRSDLS
10    ************************************************************
N5    FANFVKKQTFNKVKWYFGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS
N6    FANFVKKQTFNKVKWYFGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS
N4    FANFVKKQTFNKVNWYFGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS
12    ************************************************************

2     TAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK 180
3     ************************************************************
5     ************************************************************
6     ************************************************************
7     ************************************************************
I     ************************************************************
J     ************************************************************
H     ************************************************************
G     ************************************************************
F     ************************************************************
C     ************************************************************
A     ************************************************************
V2    ************************************************************
B     ************************************************************
E     TAVSTLLESGSLVTVAEQHPVTFVRNYRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK
D     ************************************************************
10    ************************************************************
N5    ************************************************************
```

Figure 4.7 Continued

```
N6          TAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK 180
N4          ************************************************************************
12          ************************************************************************

2           SKWAANFADPETTYWKPPRNKWWDGYHGEEVVVIDDFYGWLPWDDLLRLCDRYPLTVETK 240
3           ************************************************************************
5           ************************************************************************
6           ************************************************************************
7           ************************************************************************
I           ************************************************************************
J           ************************************************************************
H           ************************************************************************
G           ************************************************************************
F           ************************************************************************
C           ************************************************************************
A           ************************************************************************
V2          ************************************************************************
B           ************************************************************************
E           ************************************************************************
D           ************************************************************************
10          ************************************************************************
N5          ************************************************************************
N6          ************************************************************************
N4          ************************************************************************
12          ************************************************************************

2           GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT 300
3           ************************************************************************
5           ************************************************************************
6           ************************************************************************
7           ************************************************************************
I           ************************************************************************
J           ************************************************************************
H           ************************************************************************
G           ************************************************************************
F           ************************************************************************
C           ************************************************************************
A           ************************************************************************
V2          ************************************************************************
B           GGTVPFLARSILITSNQTPLEWYSSAAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT 300
E           ************************************************************************
D           ************************************************************************
10          ************************************************************************
N5          ************************************************************************
N6          ************************************************************************
N4          ************************************************************************
12          ************************************************************************

2           LSPPCPEFPYEINY 314
3           **************
5           **************
6           **************
7           **************
I           **************
J           **************
H           **************
G           **************
F           **************
C           **************
A           **************
V2          **************
B           **************
E           **************
D           **************
10          **************
N5          **************
N6/N4/12    **************
```

Chapter 5: Longitudinal Cohort Study of PMWS-affected and non-affected Herds in Ireland

5.1 Abstract

A longitudinal study was performed on four herds from Ireland. Three of these herds were from ROI, two PMWS positive herds and one negative control herd. The fourth herd was a PMWS positive herd from NI. This study was undertaken in order to elucidate any infectious/non-infectious "triggers" that may potentiate the enhanced PCV2 replication in infected pigs, which is associated with PMWS. Five litters (approximately ten pigs per litter) from each of the four herds were tagged on the first week of life, raised "normally" and were followed for ten consecutive weeks. Serum samples for PCV2 antibody titre and the presence of PPV antibody, tonsil and faecal swabs for virus isolation and faecal swabs in Aimes charcoal for bacteriological isolation were taken at each visit.

All pigs were positive for antibodies to PCV2 on the first week of sampling and most pigs were seen to respond serologically by the final herd visit. In PMWS positive herds disease appeared to occur in animals once maternal titres had declined. All herds were PPV antibody positive. In all herds isolation of haemolytic *E. coli* and *Campylobacter* spp. appeared to be ubiquitous throughout the ten weeks, while isolation of *Salmonellae* spp. was more sporadic with none isolated from the non-diseased herd and from the NI herd. The main viruses isolated from these herds were Enterovirus 1 and 2, reovirus and adenovirus.

5.2 Introduction

It has been established from experimental infections that PCV2 is the causative agent of PMWS (Allan *et al*, 1999a; Allan *et al.*, 2003; Krakowka *et al.*, 2000; Krakowka *et al.*, 2001)). However the pathogenesis of the disease is not fully understood and the evidence suggests that additional co-factors are necessary (Darwich *et al.*, 2004). This longitudinal study was undertaken in order to elucidate the infectious/non-infectious "triggers" that may potentiate the enhanced PCV2 replication in infected pigs, which is associated with PMWS.

Nutritional deficiencies and excesses may both give rise to clinical diseases in pigs, affecting productivity in both breeding and growing animals. Current porcine nutritional standards are based on research in pigs of very different genotypes to those commonly found in herds nowadays. There are some concerns that dietary requirements at critical stages could contribute to the severity of PMWS, however, there is not enough scientific information available to establish the real effect of nutrition in this disease (Allan *et al.*, 2005). It has also been suggested that the water supply could act as a potential pathway for the spread of PCV2 or other potential disease causing organisms. The quality of the water supplied and access to water has also the potential to affect growth rates (Marco, 2005). It is also known that maternal immunity and colostrum intake may have a significant bearing on progeny survival and that cross-fostering may play a part in PMWS (Allan, 2005; Marco, 2005). Reducing stress on the suckler and newly weaned pig may help control the amount of PMWS in a herd and consequent mortality. There may therefore be a relationship between the number of potential stress

factors, whether they occur sequentially or concurrently, and subsequent mortality due to PMWS (Madec, 2001; Marco, 2005). Although most breeder/feeders practice all-in/all-out management in the farrowing and weaner stages, many practice continuous throughput in their finishing houses and this compromises herd health. Some pig farmers are now successfully practicing all-in/all-out management in their finishing houses after changing to batch farrowing and they report improved herd health (Marco, 2005). The risk that PMWS could be spread by natural mating or through AI cannot be ruled out. Traces of PCV2 have been found in boar semen but it is not known if the virus can survive there (Hamel *et al.*, 2000). In France it was found that herds which solely used AI (no natural service) were at less risk of developing PMWS. However, others have suggested that PMWS was associated with a change in the sourcing of semen (Ohlinger and Pesch, 2000). Although the cofactors associated with PMWS remain a mystery, other diseases unquestionably exacerbate the clinical picture. Most commonly implicated are PRRS and PPV-associated illness.

A longitudinal study was therefore undertaken on four Irish herds to determine if PCV2 antibody titre, or other co-factors such as PPV, other viruses or bacterial organisms were involved in exacerbating the disease in Ireland.

5.3 Materials & Methods

Four herds, three from ROI and one NI herd were chosen to take part in this study. In ROI two herds had been confirmed positive for PMWS by the laboratory (CVRL) and one was a negative control herd. In NI a confirmed PMWS-positive herd was studied. A full history of each herd was taken on the first date of sampling and five litters (approximately ten pigs per litter) from each of the four herds were tagged during the first week of life, raised "normally" and were followed for ten consecutive weeks.

Herd A: Herd "A" was an indoor (on slats) integrated herd located in County Cavan which finished over 20,000 pigs per year. A PMWS outbreak occurred in this herd in Febuary 2004. The syndrome was initially suspected by staff and confirmed by clinical and laboratory examination. The clinical signs included laboured breathing, loss of condition, wasting, nervous signs (loss of balance, paralysis) pneumonia, diarrhoea and sudden death. Pre-PMWS, post weaning mortality on average was 3.5%. At the time of the outbreak the mortality rate observed post-weaning to 30kg was 7% with a high of 8%. The mortality rate in pigs over 30kg was 2%. These high mortality rates continued for 2-3 months. All animals were fed purchased compound. Dry food pellets were fed to weaners and rearers and wet food to dry and lactating sows, growers and finishers. The water source was an on-farm bore hole and disinfectants were not used when flushing out the water system. Pigs were cross-fostered after 48 hours and farrowings were managed by hormones. Batch farrowing was carried out weekly. Around the time of farrowing, pigs were closed away from sows in a creep area until dry. No changes were made with regard to cross-fostering and farrowing methods as a result of PMWS. Piglets were

initially injected with iron within 24 hours of birth. However, after the PMWS outbreak this was no longer practiced. Tail docking and teeth clipping were still performed on the first day of life. Piglets were weaned at 29 to 30 days of age and were removed from suckling pens immediately afterwards and placed in flat decks and fully slatted pens. Originally, rearers were grouped according to size within one week of weaning, but following the outbreak they were kept as groups of 2 to 3 litters, with 21 to 30 weaners in each group, until 15 to 21 days after weaning. An all-in-all-out management system by room was in operation. However, dry sows were not removed under this system. All sows were mated using purchased semen and the suppliers had not being changed in the past 8 years. The breed make-up of the weaner/slaughter group was Landrace and Large White. All pigs were vaccinated at weaning against enzootic pneumonia, erysipelas and *E. coli* disease.

Herd B: Herd 'B' was an indoor (on slats) integrated herd located in County Cork which finished over 20,000 pigs per year. PMWS was diagnosed clinically in May 2004 and confirmed by laboratory examination. Clinical signs included fever, joint problems, laboured breathing, loss of condition and wasting. Prior to the PMWS outbreak, weaner mortality was 2%. This increased to 3.5% and then decreased again to 2.5%. The mortality remained at the highest level for 10 to 12 months. All pigs were fed a home mix and by-products while rations were analysed annually both before and after the outbreak. The home milling and mixing used purchased wheat, barley and soya and the content of this food was not changed as a result of PMWS. The source of water for pigs was an on-farm bore hole and Virkon S™ (Antec®, DuPont Animal Health Solutions,

United Kingdom) was used for disinfecting. The water source was not altered as a result of the outbreak. It was noticed on the farm by staff that infected pigs had a preference for dirty water off the solid floor. Piglets were cross-fostered after 4 to 6 hours ingestion of their mother's colostrum. Farrowing was managed by the use of hormones and it was ensured that each pig had suckled well before fostering. At the time of farrowing pigs were closed away from the sow in the creep area until dry and batch farrowing was not in operation. No change in management at farrowing occurred as a result of PMWS. Piglets were injected with iron, had their tails docked and their teeth clipped between 3 to 5 days of age and were weaned at 27 to 28 days. Fifty-one to 75 pigs were placed in each rearer group. An all-in-all-out management system was operated by room, with over 300 pigs sharing the same common air space. This system remained in operation following the outbreak. Sows were mated by natural service (up to 10%) and by purchased semen (90 to 100%). The source of AI semen remained the same after the outbreak. Purchased replacement gilts were introduced on average every 6 to 9 months. Pigs were vaccinated against parvovirus reproductive disease (2 doses plus a booster), enzootic pneumonia and enterotoxaemia (*Clostridium perfringens*) when aged 22 to 28 days.

Herd C: Herd 'C', the negative control herd in this study, was an integrated indoor herd also located in County Cork, which finished 5,000 to 10,000 pigs per year. Pigs were fed a homemade mix, with rations being analysed for mineral content (calcium, phosphorous, salt and zinc) and vitamin content (vitamins A, B12, D3 and E) every 6 months. Cross-fostering occured after 4 to 6 hours ingestion of their mother's colostrum and farrowings were managed by the use of hormones. Pigs remained with the sows

when farrowing. Iron was injected between 3 to 5 days of age and tails were docked and teeth clipped within 24 hours of birth. Piglets were weaned at 27 to 28 days of age and were immediately removed from their suckling pen and placed in kennels. Litters were mixed within one week of weaning, with 11 to 20 weaners in each rearer group. Natural service (10 to 20%) and AI (80 to 90%) were used for mating with replacement boars being introduced every 12 to 18 months on average. Pigs were vaccinated against parvovirus reproductive disease and enzootic pneumonia.

Herd D: This herd is located in NI and had an on-going PMWS problem. Information regarding the history of this herd was unavailable to the author as all work on this herd was carried out by VSD staff, Stormont and they did not complete the survey provided.

Samples from each of the four herds were taken approximately every ten days for ten weeks (a total of 7 visits). At each visit, the following samples were taken; serum samples [or PCV2 antibody titre (IPMA) and presence of PPV antibody (ELISA)] tonsil and feacal swabs in VTM for virus isolation and faecal swabs in Aimes charcoal buffer/transport medium for bacteriological isolation. Serum samples and swabs in VTM were stored at -20°C and -70°C respectively and a retrospective analysis was performed on a cohort of approximately 20 animals chosen from herds A, B and C; all samples from herd D were tested. The cohorts included diseased and non-diseased pigs chosen at random from each litter. All bacteriological samples were tested immediately upon arrival at the laboratory. The methods used are as outlined in chapter 2; section 2.5.

5.4 Results

5.4.1 *Serum Analysis*

5.4.1.1 *PCV2 Antibody Titre*

The reciprocal antibody titre was determined on serum samples from a cohort of animals from each of the three herds in ROI using the IPMA with 5 fold dilutions (herd A, B and C). All samples from herd D were tested using 2 fold dilutions as described in chapter 2.

Herd A: Six pigs (pigs no. 23, 28, 34, 32 56, 51) died during the study. Pigs 23, 28, 32 and 34 died after the first herd visit, post mortem revealed bleeding the pigs at such a young age as the cause of death. Only one (pig no. 56) of these pigs was confirmed positive for PMWS by IFA and IHC, as described in chapter 2. IFA on cryostat sections demonstrated high levels of PCV2 antigen. All other pigs that died had insufficient PCV2 antigen on cryostat sections to indicate PMWS. All pigs had maternal antibodies to PCV2 in the first week of life. Most pigs were seen to respond serologically showing a four fold increase in titre on the fourth or fifth sampling date, approximately 40 to 50 days of age (figure 5.1). The initial antibody titre in the first week of life varied between pigs, ranging from 250 to 31,250 reciprocal antibody titre while the final antibody titre recorded for each pig was usually 156,250 or 31,250 except in two cases, pig 19, which had a final titre of 1,250 and pig 15 which had a final tire of 6,250. Pig 56 had a reciprocal antibody titre of 1,250 in the first week of life and on the 5^{th} sampling date this had reduced to 50 and on the 6^{th} sampling date, at the time of death the reciprocal antibody titre was 0 (does not appear on a log scale graph).

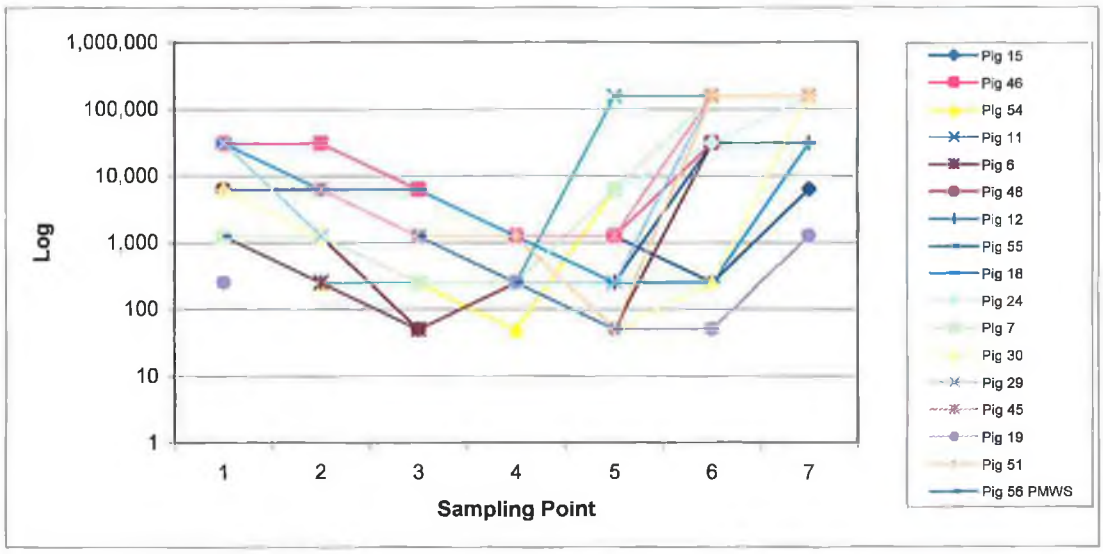

Figure 5.1: PCV2 antibody titre of cohort of samples from herd A over the observation period of ten weeks

Herd B: Six pigs died during the study in Herd 'B' (pigs no. 25, 36, 37, 40, 42, 49). However, only two (pig no. 36, 42) of these were confirmed positive for PMWS by IFA and IHC. IFA on cryostat sections demonstrated high levels of PCV2 antigen. All other pigs that died had insufficient PCV2 antigen on cryostat sections to indicate PMWS. Pigs were only bled from the second visit to the herd. All pigs had maternal antibodies to PCV2 (figure 5.2). In this herd most pigs were seen to respond serologically, with a four-fold increase in titre on the 6th farm visit, at around 60 days of age. Both PMWS-affected pigs did not appear to mount an active immune response to PCV2. Pig 36 died on the 4th herd visit and had a reciprocal antibody titre of 250 while pig 42 died on the final herd visit and had a reciprocal antibody titre of 50.

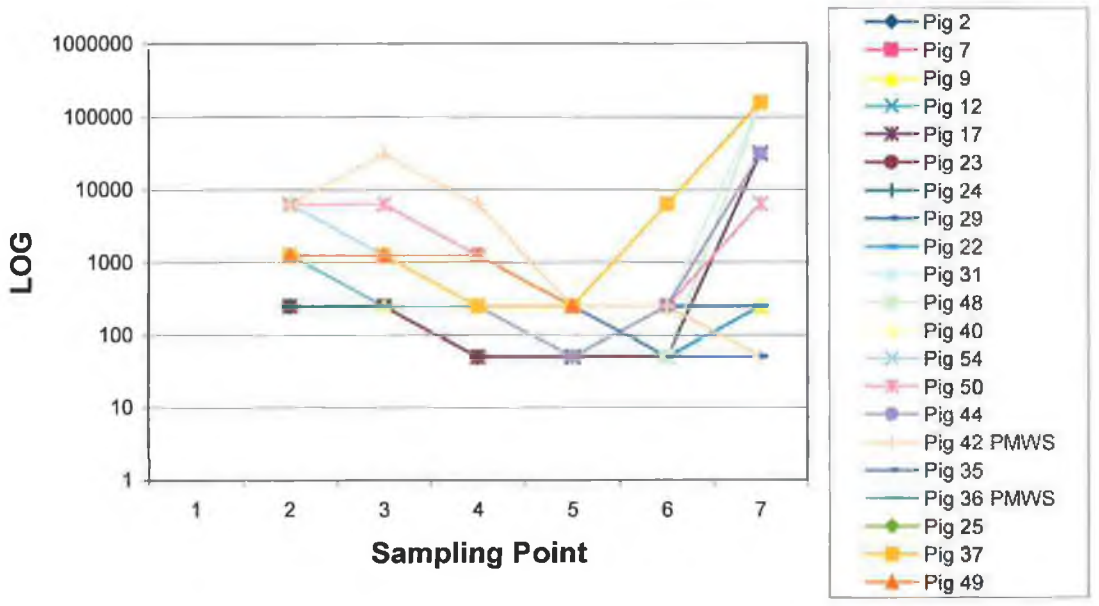

Figure 5.2: PCV2 antibody titre of cohort of samples from herd B over the observation period of ten weeks

Herd C: One pig died (pig no. 5) during this study. However, it was confirmed as PMWS negative by IFA and IHC as there was insufficient PCV2 antigen on cryostat sections to indicate PMWS. Low levels of PCV2 antigen were observed in tissues. All pigs had maternal antibodies to PCV2 and were seen to serologically respond on the 6th herd visit, at approximately 60 days of age (Figure 5.3) except pigs 35 and 24 who had a final titre of 50.

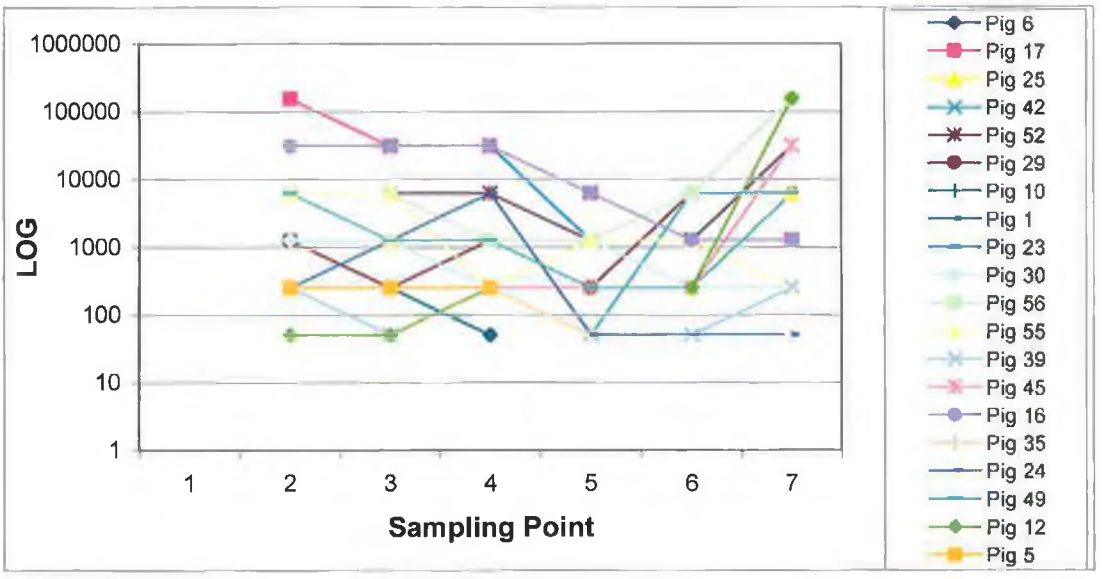

Figure 5.3: PCV2 antibody titre of cohort of samples from herd C over the observation period of ten weeks

Herd D: Ten pigs died in this herd during the study (pigs no. 42, 47, 1, 9, 4, 5, 31, 43, 46 and 16). Pigs 42 and 47 died during the first week and their bodies were discarded. Five pigs (pigs no. 9, 4, 31, 43, 46) were confirmed positive for PMWS. IFA on cryostat sections demonstrated high levels of PCV2 antigen. Two pigs (pigs no. 1, 5) demonstrated moderate/high levels of PCV2 antigen and were described as pre-clinical and would likely develop full-blown disease. One pig (pig no. 16) had insufficient PCV2 antigen on cryostat sections to indicate PMWS. All pigs initially had maternal antibodies to PCV2. On the 5th herd visit these maternal antibodies appeared to decline in titre. Most pigs were then seen to respond serologically with a four-fold increase in titre by the 8th farm visit, around 70 days of age (figure 5.4a, b, c, d and e). All healthy pigs exhibited seroconversion, but many PMWS affected animals failed to do so.

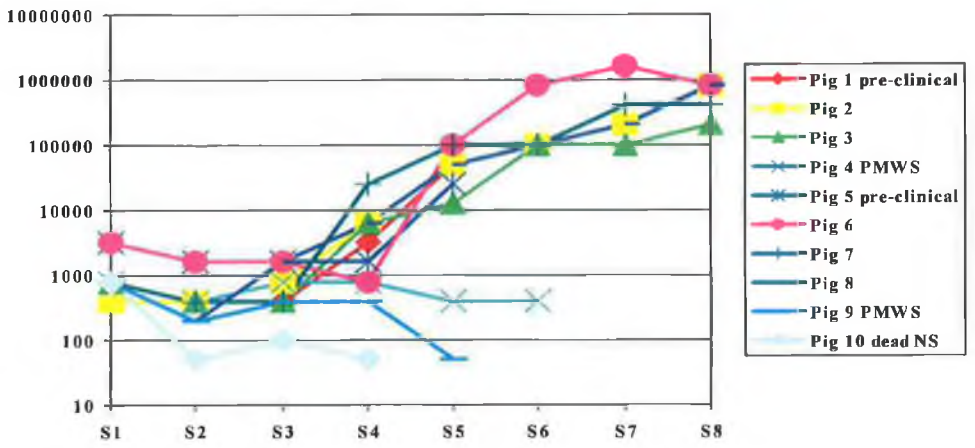

Figure 5.4a: PCV2 antibody titre of cohort of samples from herd D over the observation period of ten weeks in litter 1

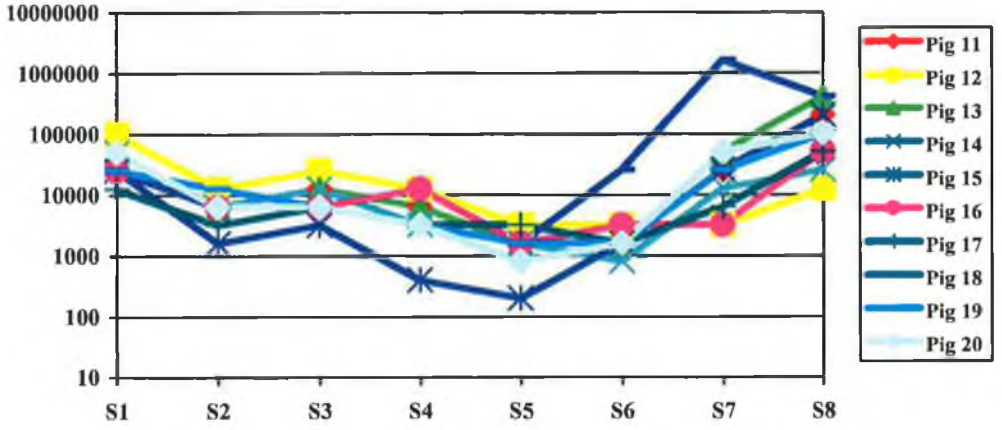

Figure 5.4b: PCV2 antibody titre of cohort of samples from herd D over the observation period of ten weeks in litter 2

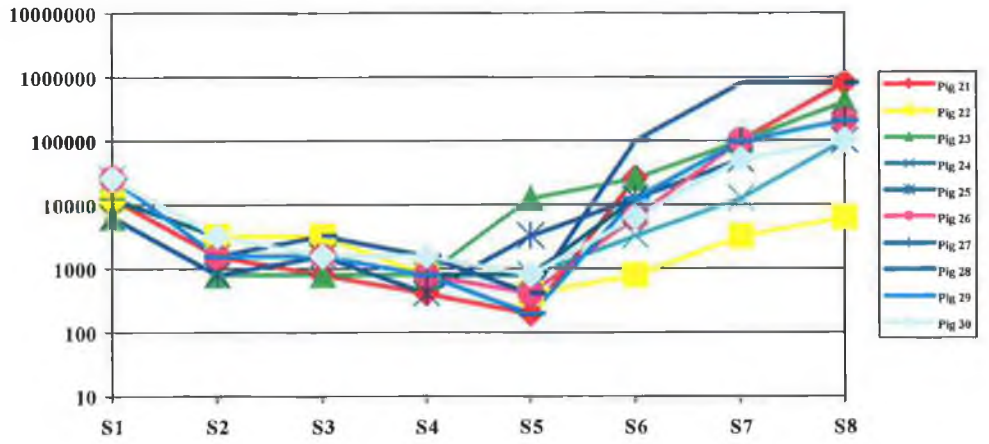

Figure 5.4c: PCV2 antibody titre of cohort of samples from herd D over the observation period of ten weeks in litter 3

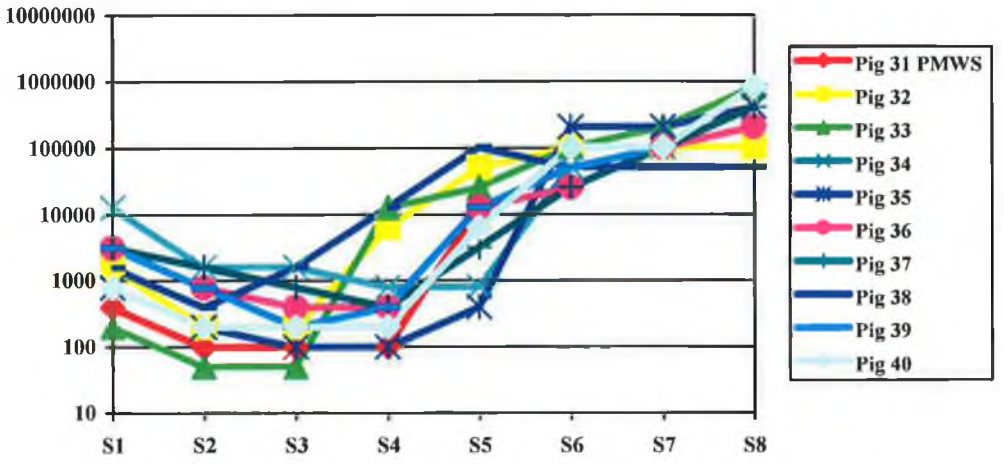

Figure 5.4d: PCV2 antibody titre of cohort of samples from herd D over the observation period of ten weeks in litter 4

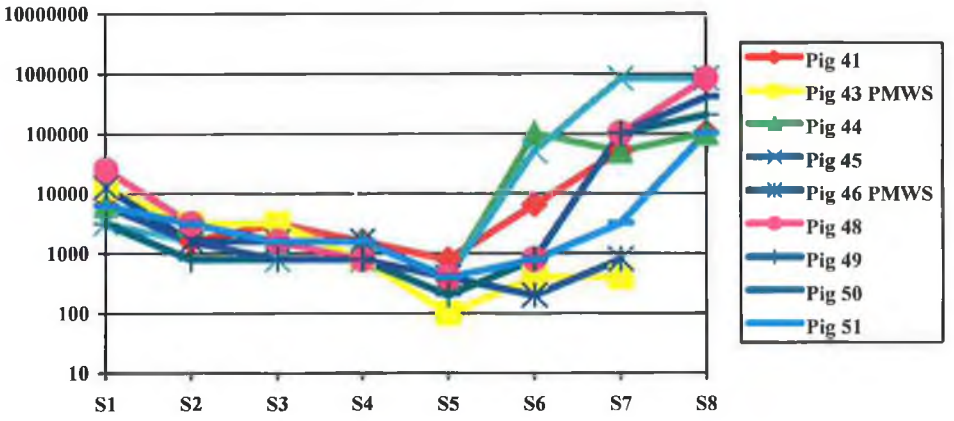

Figure 5.4e: PCV2 antibody titre of cohort of samples from herd D over the observation period of ten weeks in litter 5

5.4.1.2 PPV Analysis

To determine if PPV infection levels play a role in the pathogenesis of field cases of PMWS, serum samples were tested using an ELISA for the presence of PPV antibodies over the same time-course as for PCV2 Antibody titre. Results were classified as negative, positive and strongly positive as detailed in chapter 2.5.6.1.

Herd A: Three of the pigs from the cohort tested were PPV antibody negative. Pig 56 and 51 had declining antibody levels when they died. All other pigs were strongly positive for PPV antibodies, (figure 5.5).

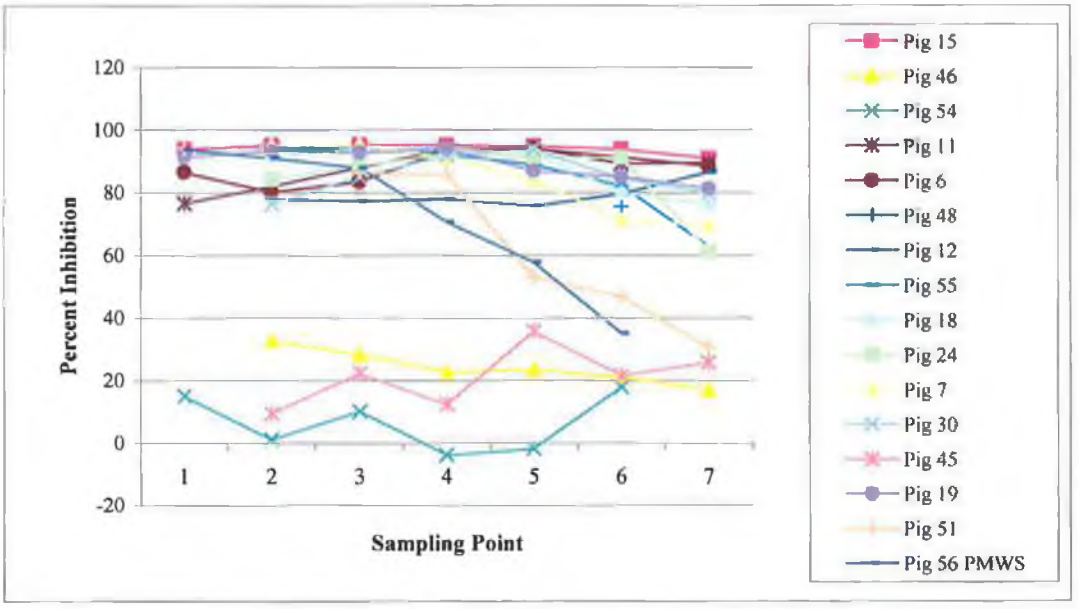

Figure 5.5: PPV percent inhibition in herd A

Herd B: All pigs were strongly positive for antibodies to PPV on the first date they were bled, i.e. 2nd herd visit. Most pigs in the cohort retained their maternal antibodies until towards the end of the 10 weeks (figure 5.6). All pigs had been vaccinated for PPV. Seven of the pigs did lose their maternal antibodies and on the 7th visit they were PPV antibody negative. One of these animals was pig no. 42, which was confirmed as PMWS positive.

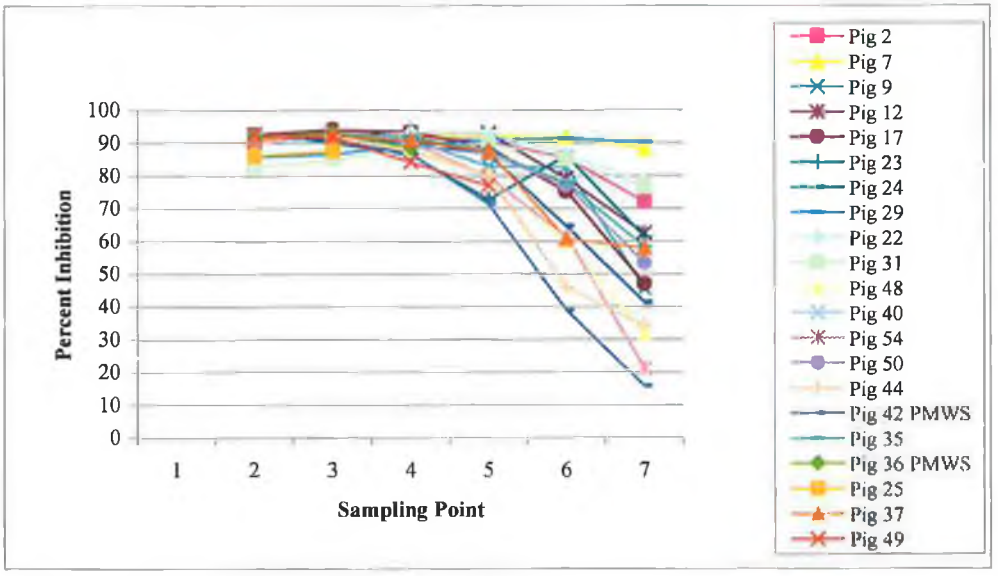

Figure 5.6: PPV percent inhibition of PPV in herd B

Herd C: Four of the twenty pigs tested from this herd were PPV antibody negative. One of these pigs (pig no. 35) was seen to respond serologically. All pigs had been vaccinated for PPV. Pig no. 23 was also seen to respond serologically after the 5th sampling date, (Figure 5.7).

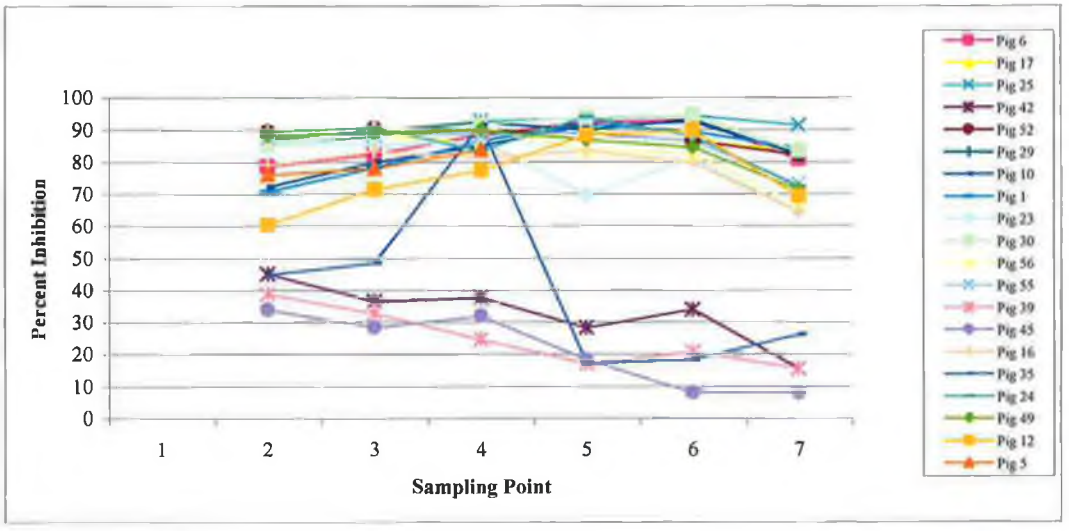

Figure 5.7: PPV percent inhibition of PPV in herd C

Herd D: Eighteen of the 51 pigs tested from herd D were antibody negative on the first bleed and were not demonstrated to respond serologically. All PPV antibody positive pigs showed a decline in maternal antibodies throughout the 10 weeks (figure 5.8 and 5.9). Twenty-six of these antibody positive pigs lost all their maternal antibodies and did not serologically respond. Seven of the pigs retained their maternal antibodies; however 5 of these 7 died during the 10 weeks observation period.

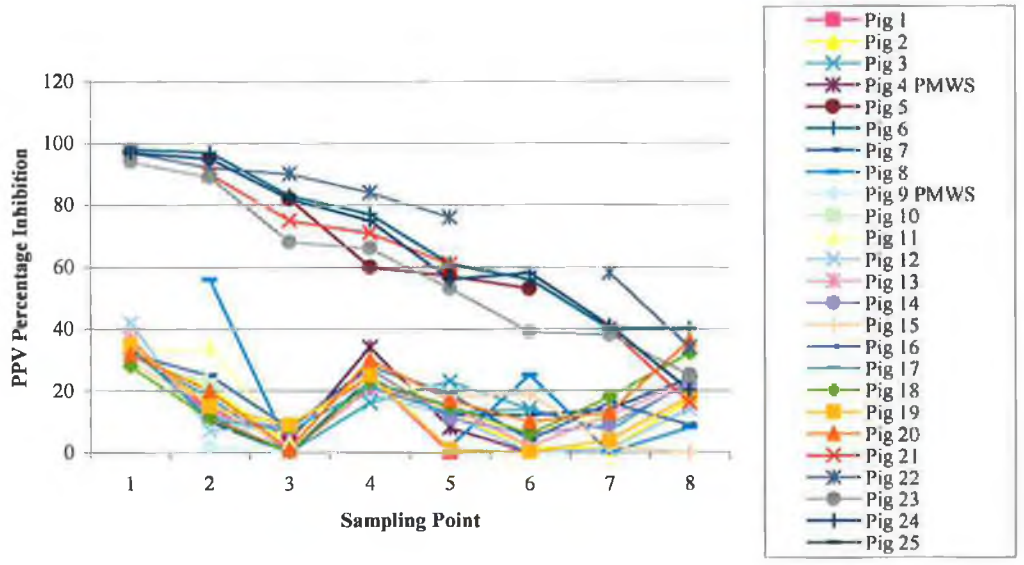

Figure 5.8: Percent Inhibition of PPV in herd D pigs 1-25

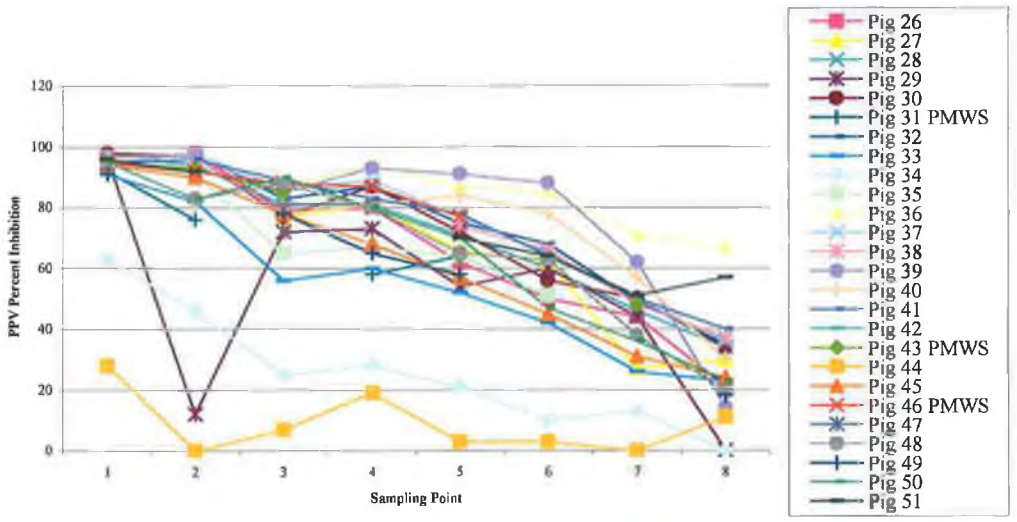

Figure 5.9: Percent Inhibition of PPV in herd D pigs 26-51

5.4.2 Isolation of haemolytic *E.coli, Campylobacter and Salmonella* species from faecal swabs

These swabs were analysed immediately upon arrival into the laboratory as described in chapter 2 and 5.2. There appeared to be an increase in the isolation of haemolytic *E.coli* with time in all four herds, resulting in a 96 – 100% isolation rate from faeces in this study. Suspect haemolytic *E. coli* isolates were stored on beads at -20°C until all samples had been analysed. In order to identify the most commonly isolated *Enterobacteriaceae* an API® 20E test was performed on stored isolates. *E. coli 1* was the most common isolate, with sporadic isolation of *E. coli 2* from all four herds. On one occasion in herd "B" (pig 42) *Citrobacter braakii* was identified. *Campylobacter* species (with *Campylobacter coli* the most commonly isolated) were almost commonly isolated

throughout the 10 weeks. The isolation of *Salmonella* was sporadic and none were detected in herds "C" and "D" (Table 5.1, 5.2, 5.3 and 5.4).

Haemolytic *E. coli* was isolated from pig 56 herd A from the first herd visit, *Campylobacter* spp. were not isolated until the third visit and *Salmonella* spp. were isolated on the fourth herd visit. In herd B, pig 36, haemolytic *E. coli* and *Salmonella* spp. were isolated from the first herd visit and *Campylobacter* spp. was isolated from the second visit. In the case of pig 42, haemolytic *E. coli* was isolated from the first visit, *Campylobacter* spp. were isolated from the second visit and there was no *Salmonella* species isolated from this pig. In herd D, haemolytic *E. coli* were isolated from the first visit in pigs 4, 9 and 46 and *Campylobacter* spp. was isolated from the first visit and in pigs 31 and 43 *Campylobacter* spp. was isolated from the second visit. No *Salmonella* was isolated from herd D.

Sampling No.	No. of haemolytic *E. coli* isolated/No. examined	No. *Campylobacter spp.* Isolated/No. examined	No. *Salmonella spp.* Isolated/No. examined
1	18/55	40/55[1], 30%[2],60%[3],10%[4]	0/55
2	17/52	49/52[1], 60%[2],40%[3]	1/52
3	30/52	46/52[1],100%[2]	0/52
4	46/50	50/50[1], 94%[2],6%[3]	1/50
5	41/52	51/52[1], 80%[2],20%[3]	0/52
6	46/52	52/52[1], 100%[2]	1/52
7	49/51	50/51[1], 100%[2]	4/52

[1] No. of positive Campylobacter isolates
[2] % of *Campylobacter coli* isolates from 10 tested
[3] % of *Campylobacter jejuni* isolates from 10 tested
[4] % of *Campylobacter lari* isolates from 10 tested

Table 5.1: Isolation of haemolytic *E. coli*, *Campylobacter* spp. and *Salmonella* spp. from faecal swabs in herd A over the observation period

Sampling No.	No. of haemolytic *E. coli* isolated/No. examined	No. *Campylobacter spp.* isolated/No. examined	No. *Salmonella spp.* Isolated/No. examined
1	38/51	42/51[1]	5/51
2	47/49	43/49[1]	1/49
3	47/51	51/51[1]	0/51
4	40/50	50/50[1]	1/50
5	48/48	48/48[1]	1/48
6	46/46	46/46[1]	0/46
7	45/47	47/47[1]	0/47

[1] No. of positive Campylobacter isolates
[2] % of *Campylobacter coli* isolates from 10 tested
[3] % of *Campylobacter jejuni* isolates from 10 tested
[4] % of *Campylobacter lari* isolates from 10 tested

Table 5.2: Isolation of haemolytic *E. coli*, *Campylobacter* spp. and *Salmonella* spp. from faecal swabs in herd B over the observation period

Sampling No.	No haemolytic *E. coli* isolated/No. examined	No. *Campylobacter spp.* Isolated/No. examined	No. *Salmonella spp.* Isolated/No. examined
1	42/55	42/51[1]	0/55
2	51/55	39/55[1]	0/55
3	53/55	54/55[1]	0/55
4	52/55	52/55[1]	0/55
5	51/54	46/54[1]	0/54
6	54/54	30/54[1]	0/54
7	38/54	51/54[1]	0/54

[1] No. of positive Campylobacter isolates
[2] % of *Campylobacter coli* isolates from 10 tested
[3] % of *Campylobacter jejuni* isolates from 10 tested
[4] % of *Campylobacter lari* isolates from 10 tested

Table 5.3: Isolation of haemolytic *E. coli*, *Campylobacter* spp. and *Salmonella* spp. from faecal swabs in herd C over the observation period

Sampling No.	No. of haemolytic *E. coli* isolated/No. examined	No. *Campylobacter spp.* Isolated/No. examined	No. *Salmonella spp.* Isolated/No. examined
1	31/51	40/51[1], 100%[2]	0/51
2	30/49	48/49[1], 50%[2],40%[3] 10%[4]	0/49
3	27/49	49/49[1],80%[2] 20%[3]	0/49
4	36/49	45/49[1], 90%[2],10%[3]	0/49
5	44/48	47/48[1], 100%[2]	0/48
6	34/46	39/46[1], 80%[2] 20%[3]	0/46
7	28/42	42/42[1], 100%[2]	0/42

[1] No. of positive Campylobacter isolates
[2] % of *Campylobacter coli* isolates from 10 tested
[3] % of *Campylobacter jejuni* isolates from 10 tested
[4] % of *Campylobacter lari* isolates from 10 tested

Table 5.4: Isolation of haemolytic *E. coli*, *Campylobacter* spp. and *Salmonella* spp. from faecal swabs in herd D over the observation period

5.4.3 Virus Isolation

Virus isolation was performed on tonsil and faecal swabs, stored in VTM, on primary pig kidney cells. Samples from herds B, C and D were examined.

Herd B: In herd B the main virus isolated was enterovirus 1 with sporadic isolates of enterovirus 2 and reovirus. Pigs 36 and 42 were the only two animals confirmed as PMWS positive in this herd; porcine enterovirus 1 and 2 (now known as porcine teschovirus 1 and 2) were isolated on sampling dates 6 and 7 from pig 42 while no virus was detected from pig 36 (Table 5.5).

Pig ID		SAMPLING 1	SAMPLING 2	SAMPLING 3	SAMPLING 4	SAMPLING 5	SAMPLING 6	SAMPLING 7
B.102	Tonsil	NVI	NVI	NT	NVI		NVI	Entero 1
	Faeces	NT	NVI	NVI	NVI		Entero 1	Entero 1/Reo
B.107	Tonsil	NVI	NVI	NVI	Entero 1		NVI	NVI
	Faeces	bact	NVI	NVI	NVI		NT	Entero 1
B.109	Tonsil	NVI	NVI	NVI	NVI		Entero 1	Entero 1
	Faeces	NVI	NVI	NVI	NVI		Entero 1	NVI
B.112	Tonsil	NVI	NVI	NVI	NVI		NVI	Entero 1
	Faeces	NVI	NVI	NVI	NVI		Entero 1	Entero 1
B.117	Tonsil	NVI	NVI	NVI	NVI		NVI	Entero 1
	Faeces	NVI	NVI	NVI	NVI		Entero 1	Entero 1
B.122	Tonsil	NVI	NVI	NVI	NVI		NVI	Entero 1
	Faeces	NVI	NVI	NVI	NVI	No Samples	NT	Entero 1
B.123	Tonsil	NVI	NVI	NVI	NVI		NVI	NVI
	Faeces	NVI	NVI	NVI	NVI		NT	Entero 1
B.124	Tonsil	NVI	NVI	NVI	NVI		NVI	Entero 1
	Faeces	NVI	NVI	NVI	NVI		Entero 1	Entero 1
B.125	Tonsil	NT	NVI	NVI	NT		NT	NT
	Faeces	NVI	NVI	NVI	NT		NT	NT
B.129	Tonsil	NVI	NVI	NVI	Entero 1		NVI	NVI
	Faeces	NVI	NVI	NVI	Entero 1		Entero 1	Bact
B.131	Tonsil	NVI	NVI	NVI	NVI		NVI	Entero 1
	Faeces	NVI	NVI	NVI	fungus		Entero 1	Entero 2
B.135	Tonsil	NVI	NVI	NVI	NT		NVI	NVI
	Faeces	NVI	NVI	NVI	NVI		NVI	Entero 2/Reo
B.136	Tonsil	NVI	NVI	NT	NVI		PMWS	
	Faeces	NVI	NVI	NVI	NVI			
B.137	Tonsil	NVI	NVI	NVI	NVI		Entero 1	NVI
	Faeces	NVI	NT	NVI	NVI		Entero 1	NVI
B.140	Tonsil	NVI	NVI	NVI	NVI		NT	NT
	Faeces	NVI	NVI	NT	NVI		NT	NT
B.142	Tonsil	NVI	NVI	NVI	NVI		Entero 1	Entero 1 & 2
	Faeces	NVI	NVI	NVI	NVI		Entero 1	Entero 1
B.144	Tonsil	NVI	NVI	NVI	NVI		NT	Entero 1
	Faeces	NVI	NVI	NVI	Bact		NT	Reo
B.148	Tonsil	NVI	NVI	NVI	NVI		Entero 1	NVI
	Faeces	NVI	NVI	NVI	NVI		Entero 1	Entero 1, 2 & Reo
B.149	Tonsil	NVI	NVI	NVI	NT		NT	NT
	Faeces	NVI	NVI	NVI	Entero 2		NT	NT
B.150	Tonsil	NVI	NVI	NVI	NVI		NVI	Reo
	Faeces	NVI	NVI	NVI	NVI		Entero 1	Entero 1
B.154	Tonsil	NVI	NVI	NVI	Entero 1		NVI	Entero 1
	Faeces	NVI	NVI	NVI	Entero 1		Entero 1	Reo

NVI: No virus isolated
NT: Not tested

Table 5.5: Virus Isolation from tonsil and faecal swabs in VTM in herd B

Herd C: In herd C, porcine enterovirus 1 was the main virus isolated and this was on the 4th, 5th, 6th and 7th sampling dates. Adenovirus was also isolated from pigs 45 and 49 on the 5th and 6th sampling dates. Porcine enterovirus 2 was not isolated from this herd (Table 5.6).

Pig ID		Sampling 1	Sampling 2	Sampling 3	Sampling 4	Sampling 5	Sampling 6	Sampling 7
C.1	Tonsil	NVI	NVI	NVI	Entero 1	NVI	Bact	Entero 1
	Faeces	NVI	NVI	NVI	NVI	NVI	Entero 1	NVI
C.5	Tonsil	NVI	NVI	NVI	NVI	Died		
	Faeces	NVI	NVI	NVI	NVI			
C.6	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	Entero 1	Entero 1
C.10	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	Entero 1
	Faeces	NVI	NVI	NVI	NVI	NVI	Entero 1	Entero 1
C.16	Tonsil	NVI	NVI	bact	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	bact	Entero 1
C.17	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	Entero 1
	Faeces	NVI	NVI	NVI	NVI	Entero 1	Entero 1	Entero 1
C.23	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	NVI	NVI
C.24	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	NVI	NVI
C.25	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	Entero 1	Entero 1
C.29	Tonsil	NVI	NVI	NVI	NVI	Entero 1	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	Entero 1	NVI
C.30	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	Entero 1	NVI
C.35	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	NVI	Entero1/PCV2
C.39	Tonsil	NVI	NVI	NVI	NT	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	Entero 1	NVI
C.42	Tonsil	NVI	NVI	NVI	NVI	Entero 1	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	Entero 1	Entero 1	Entero 1
C.45	Tonsil	NVI	NVI	NVI	NVI	NVI	Adeno/PCV2	Entero 1
	Faeces	NVI	NVI	NVI	NVI	Entero 1	ND	Entero 1
C.49	Tonsil	NVI	NVI	NVI	Entero 1	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	Entero1/Adeno	NVI	NVI
C.52	Tonsil	NVI	NVI	NVI	NVI	Entero 1	Entero 1	? NVI
	Faeces	NVI	NVI	ND	Entero 1	NVI	NVI	Entero 1
C.55	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	NVI	Entero 1
C.56	Tonsil	NVI	NVI	NVI	Entero 1	Entero 1	NVI	Entero 1
	Faeces	NVI	NVI	NVI	NVI	Entero 1	Entero 1	NVI

NVI: No virus Isolated
NT: Not tested

Table 5.6: Virus Isolation from tonsil and faecal swabs in VTM in herd C

Herd D: Adenovirus was isolated from this herd as early as the 1st sampling date. Porcine enterovirus 1 and 2 and reovirus were also isolated. Reovirus was only isolated on the final herd visit. Enteroviruses 1 and 2 were isolated on visits 4, 5, 6 and 7 (Table 5.7). In pig 9, adenovirus was isolated on sampling date 3 and enterovirus 2 was isolated on sampling date 4. In pig 4, enterovirus 1 was isolated on sampling date 6. No virus was isolated from pig 31. In pig 43, adenovirus was isolated on sampling date 3 and enterovirus 1 was isolated on sampling dates 5 and 6. In pig 46, enterovirus 2 was isolated on sampling date 5 and enterovirus 1 on sampling date 6.

Piglet ID	Sample ID	Primary pig kidney virus isolation						
		Sampling 1	Sampling 2	Sampling 3	Sampling 4	Sampling 5	Sampling 6	Sampling 7
D.1	Tonsil	NVI	NVI	NVI	NVI	NVI	In poor condition euthanased after sampling 5 Pre-clinical	
	Faeces	NVI	**ADENO**	**Adeno-cpe**	**entero cpe 2**	NVI		
D.2	Tonsil	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	REO
	Faeces	NVI	NVI	**ADENO**	**entero cpe 1**	NVI	NVI	**entero cpe 1**
D.3	Tonsil	NVI	NVI	NVI	**entero cpe 2**	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	**entero cpe 1**	NVI	**entero cpe 1**
D.4	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	PMWS
	Faeces	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	
D.5	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	PRE-CLIN
	Faeces	NVI	NVI	**ADENO**	NVI	**entero cpe 1**	NVI	
D.6	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**
	Faeces	NVI	ADENO	**ADENO**	NVI	**entero cpe 1**	NVI	**entero cpe 1**
D.7	Tonsil	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	NVI
	Faeces	NVI	NVI	**Adeno-cpe**	NVI	NVI	**entero cpe 1**	**REO**
D.8	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	**Re-inoc**	**entero cpe 1**	**entero cpe 1**
D.9	Tonsil	NVI	NVI	NVI	NVI	NVI	PMWS	
	Faeces	NVI	NVI	**Adeno-cpe**	**entero cpe 2**	NVI		
D.10	Tonsil	NVI	NVI	NVI	NVI	Very small pig in v. poor condition died; discarded by farmer.		
	Faeces	NVI	NVI	NVI	NVI			
D.11	Tonsil	NVI	NVI	NVI	**entero cpe 2**	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	**Adeno-cpe**	**entero cpe 2**	**entero cpe 1**	NVI
D.12	Tonsil	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	**entero cpe 1**

NVI: No virus Isolated
NT: Not tested

Table 5.7: Virus Isolation from tonsil and faecal swabs in VTM in herd D

Piglet ID	Sample ID	Sampling 1	Sampling 2	Sampling 3	Sampling 4	Sampling 5	Sampling 6	Sampling 7 Wat-85 5/7/04
D.13	Tonsil	NVI	NVI	NVI	NVI	entero cpe 2	entero cpe 1	REO
	Faeces	NVI	NVI	NVI	NVI	NVI	NVI	REO
D.14	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	ADENO	? mod entero type 3	?? only weak cpe 2	entero cpe 1	entero cpe 1
D.15	Tonsil	NVI	NVI	NVI	NVI	NVI	entero cpe 1	REO
	Faeces	ADENO	NVI	ADENO	entero cpe 2	NVI	NVI	NVI
D.16	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	REO
	Faeces	NVI	NVI	NVI	entero cpe 2	NVI	entero cpe 1	entero cpe 1
D.17	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	NVI	NVI
D.18	Tonsil	NVI	NVI	NVI	Adeno-cpe	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	entero cpe 3	NVI	entero cpe 1	REO
D.19	Tonsil	NVI	NVI	NVI	NVI	NVI	entero cpe 1	NVI
	Faeces	NVI	NVI	NVI	entero cpe 2	Re-inoc	entero cpe 1	entero cpe 1
D.20	Tonsil	NVI	NVI	NVI	Adeno-cpe	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	NVI	entero cpe 1
D.21	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	REO
	Faeces	NVI	NVI	NVI	NVI	NVI	entero cpe 1	REO
D.22	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	entero cpe 1	NVI
D.23	Tonsil	NVI	NVI	NVI	ADENO	NVI	entero cpe 1	REO
	Faeces	NVI	NVI	NVI	NVI	entero cpe 1	NVI	NVI
D.24	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	REO
	Faeces	NVI	NVI	NVI	NVI	NVI	entero cpe 1	entero cpe 1

NVI: No virus Isolated
NT: Not tested

Table 5.7: Virus Isolation from tonsil and faecal swabs in VTM in herd D (continued)

Piglet ID	Sample ID	Primary pig kidney virus isolation						
		Sampling 1 Wat-50 5/5/04	Sampling 2 Wat-52 17/5/04	Sampling 3 Wat-56 27/5/04	Sampling 4 Wat-69 7/6/04	Sampling 5 Wat-72 16/6/04	Sampling 6 Wat-79 24/6/04	Sampling 7 Wat-85 5/7/04
D.25	Tonsil	NVI	NVI	NVI	**ADENO**	NVI	**entero cpe 1**	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	**entero cpe 1 & REO**
D.26	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	**Re-inoc**	NVI	**entero cpe 1**	**entero cpe 1**
D.27	Tonsil	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	**Re-inoc**
	Faeces	NVI	NVI	NVI	**Re-inoc**	NVI	**entero cpe 1**	**entero cpe 1**
D.28	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	**REO**
	Faeces	NVI	NVI	NVI	**Re-inoc**	**entero cpe 1**	**entero cpe 1**	NVI
D.29	Tonsil	NVI	NVI	NVI	NVI	**entero cpe 2**	NVI	NVI
	Faeces	NVI	NVI	NVI	**entero cpe 1**	NVI	**entero cpe 1**	NVI
D.30	Tonsil	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	NVI
	Faeces	NVI	NVI	NVI	**entero cpe 1**	NVI	NVI	**entero cpe 1**
D.31	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	
	Faeces	NVI	NVI	NVI	NVI	NVI	NVI	**PMWS**
D.32	Tonsil	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	**REO**
	Faeces	NVI	NVI	NVI	NVI	**entero cpe 1**	NVI	**REO**
D.33	Tonsil	NVI	NVI	NVI	**ADENO**	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	**entero cpe 1**	**entero cpe 1**	**entero cpe 1**
D.34	Tonsil	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	NVI
	Faeces	**Re-inoc**	NVI	NVI	**entero cpe 1**	**entero cpe 1**	**entero cpe 1**	**REO**
D.35	Tonsil	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	NVI
	Faeces	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	NVI

NVI: No virus Isolated
NT: Not tested

Table 5.7: Virus Isolation from tonsil and faecal swabs in VTM in herd D (continued)

Piglet ID	Sample ID	Primary pig kidney virus isolation						
		Sampling 1 Wat-50 5/5/04	Sampling 2 Wat-52 17/5/04	Sampling 3 Wat-56 27/5/04	Sampling 4 Wat-69 7/6/04	Sampling 5 Wat-72 16/6/04	Sampling 6 Wat-79 24/6/04	Sampling 7 Wat-85 5/7/04
D.36	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	**Re-inoc**	**entero cpe 1**	**entero cpe 1**	**entero cpe 1**
D.37	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	**ADENO**	**ADENO**	NVI	NVI	NVI	**entero cpe 1**
D.38	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	NVI	NVI	**entero cpe 1**	**entero cpe 1**	**entero cpe 1**
D.39	Tonsil	NVI	NVI	NVI	NVI	**entero cpe 1**	**entero cpe 1**	NVI
	Faeces	NVI	NVI	NVI			NVI	NVI
D.40	Tonsil	NVI	NVI	NVI	**ADENO**	NVI	NVI	**REO**
	Faeces	NVI	NVI	NVI	NVI		**entero cpe 1**	**REO**
D.41	Tonsil	NVI	NVI	NVI	**Re-inoc**	**entero cpe 2**	NVI	NVI
	Faeces	NVI	NVI	NVI		**entero cpe 3**	NVI	**REO**
D.42	Tonsil	NVI	**Died shortly after first bleed; not necropsied**					
	Faeces	NVI						
D.43	Tonsil	NVI	NVI	NVI	NVI	**entero cpe 1**	NVI	NVI
	Faeces	NVI	NVI	**ADENO**	**Re-inoc**	NVI	**entero cpe 1**	NVI
D.44	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	NVI
	Faeces	NVI	NVI	**ADENO**	NVI	**entero cpe 1**	**entero cpe 1**	**entero cpe 1**
D.45	Tonsil	NVI	NVI	NVI	NVI	NVI	**NVI**	**REO**
	Faeces	NVI	NVI	**ADENO**	NVI	NVI	NVI	**REO**
D.46	Tonsil	NVI	NVI	NVI	NVI	**entero cpe 2**	NVI	ND
	Faeces	NVI	NVI	NVI	NVI	NVI	**entero cpe 1**	ND

NVI: No virus Isolated
NT: Not tested

Table 5.7: Virus Isolation from tonsil and faecal swabs in VTM in herd D (continued)

Piglet ID	Sample ID	Primary pig kidney virus isolation						
		Sampling 1 Wat-50 5/5/04	**Sampling 2 Wat-52 17/5/04**	**Sampling 3 Wat-56 27/5/04**	**Sampling 4 Wat-69 7/6/04**	**Sampling 5 Wat-72 16/6/04**	**Sampling 6 Wat-79 24/6/04**	**Sampling 7 Wat-85 5/7/04**
D.47	Tonsil	NVI	**Died shortly after first bleed; not necropsied**					
	Faeces	NVI						
D.48	Tonsil	NVI	NVI	NVI	NVI	NVI	NVI	**?? Only weak cpe 3**
	Faeces	NVI	NVI	NVI	**entero cpe 2**	NVI	NVI	NVI
D.49	Tonsil	NVI	NVI	NVI	NVI		NVI	**REO**
	Faeces	**Re-inoc**	NVI	NVI	NVI	**entero cpe 1**	**entero cpe 1**	NVI
D.50	Tonsil	NVI	NVI	NVI	**ADENO**	NVI	NVI	**REO**
	Faeces	NVI	NVI	NVI	**entero cpe 1**	**entero cpe 1**	NVI	NVI
D.51	Tonsil	NVI	NVI	NVI	NVI	**Adeno-cpe**	**entero cpe 1**	NVI
	Faeces	NVI	NVI	NVI	**entero cpe 3**	**entero cpe 1**	**entero cpe 1**	**entero cpe 1**

NVI: No virus Isolated
NT: Not tested

Table 5.7: Virus Isolation from tonsil and faecal swabs in VTM in herd D (continued)

5.5 Discussion

All pigs tested from the four herds were positive for maternal antibodies to PCV2. Most pigs were seen to respond serologically by the final herd visit. In herds B and D those pigs which were confirmed as PMWS positive did not appear to mount an active immune response as no increase in titre was observed. In herd D, pigs 1 and 5, which were described as pre-clinical, did appear to respond serologically. Active seroconversion to PCV2 generally occurs at 8 to twelve weeks of age (Segalés and Domingo, 2002). In herds B and C, pigs were seen to respond serologically at sixty to seventy days of age (approximately 8 to 10 weeks) and in herds A and D from 40 to 60 days of age (approximately 6 to 8 weeks). It has been reported that PCV2 infection or low serological titres to PCV2 in sows at farrowing had a significant effect on the overall mortality of their offspring due to PMWS (Allan *et al.*, 2005). Conversely, more recent studies have shown that high levels of antibody to PCV2 in sows and gilts does not result in protection from PMWS in the piglets derived from these animals (Hassing *et al.*, 2004; Allan *et al.*, 2005). However, the protective effect of maternal passive immunity on PMWS development is supported by the fact that disease occurs once titres have declined (Allan *et al.*, 2005). This is supported by the findings in herds B and D. Therefore measures that increase maternal immunity and decrease sow viraemia at farrowing may diminish PMWS impact on pig mortality.

However, it has been reported elsewhere that seroprofiles from positive and negative herds are not very different (Rodríguez-Arrioja *et al.*, 2000; Sibila, 2001; Larochelle *et al.*, 2003) and the prevalence and distribution of PCV2 in positive and

negative herds are not very different (Ladekjaer-Mikkelsen, 2003). For this reason it is necessary to maintain the health balance in a herd, measures employed to maintain the health balance, such as Madec's 20 principles, will also assist in avoiding spread between animals (Marco, 2005).

PPV is endemic in swine populations and many weaned pigs may be infected with PPV. It has been extensively reported through experimental models that concurrent infection with PPV and PCV2 has increased the severity of lesions, suggesting this and other agents may play an important role in modifying the clinical and pathological expression of PCV2 infection (Kennedy *et al.*, 2000). In order to determine if PPV enhanced PCV2 infection and so played a role in field cases of PMWS in Ireland a commercial ELISA (Svanovir™) was used on the four herds in this study to determine PPV antibody titre. A decline in maternal antibodies was observed in pig 56 in herd A; in herd B, pig 42 had lost all maternal antibodies and pig 36 was still antibody positive; in herd D pigs 9 and 4 were PPV antibody negative throughout the ten weeks and pigs 31, 43 and 46 were PPV antibody positive throughout the ten weeks. From the results in the present study it can be concluded that although in some cases PPV may lead to an increase in the severity of the disease it is not one of the crucial co-factors.

Haemolytic *E. coli* and *Campylobacter* spp. were isolated from faeces from all four herds and *Salmonella* spp. was isolated sporadically from faeces from herds A and B. There appeared to be an increase in the isolation of *E. coli* with time in all four herds and *Campylobacters* appear to be ubiquitious in the pig population, with *Campylobacter*

coli the most commonly isolated species in this study. These organisms may cause diarrhoea and oedema in pigs; however there appeared to be no difference in the isolation rate of these organisms from diseased and non-diseased pigs in Irish herds.

The most commonly isolated viruses in this study were found to be porcine enterovirus 1 and 2 and reovirus in ROI herds and porcine enterovirus 1 and 2, reovirus and adenovirus in NI herds. Porcine enteroviruses are enteric viruses, host specific to the pig, that are included in the group called "SMEDI viruses" (Stillbirth, Mummification, Embryonic Death and Infertility). The enteroviruses are subdivided into serotypes of which at least 11 are known. Four of these, serotypes 1, 3, 6 and 8, have been implicated in reproductive problems in pigs. Serotype 1 is the Teschen/Talfan virus which can also cause paralysis in pigs. Usually, each pig herd has an array of different serotypes which circulate sub-clinically in weaned and young growing pigs which are protected by circulatory antibodies derived from their dam's colostrum. By the time they reach breeding age they are solidly immune (http://www.thepigsite.com/pighealth/article/139/enteroviruses-smedi). The common mode of enterovirus transmission is by oral exposure to contaminated faeces. Enteroviruses may contaminate semen via aerosols during collection of semen and can cause foetal and neonatal death. (Guérin and Pozzi, 2005). Reoviruses have been implicated in relation to infertility or reproductive disorders in pigs (Guérin and Pozzi, 2005). These viruses can be readily detected in faeces, and may also be recovered from pharyngeal or nasal secretions, urine, cerebrospinal fluid and blood. Adenoviruses are a group of viruses that infect the membranes (tissue linings) of the respiratory tract, the

eyes, the intestines, and the urinary tract; adenoviruses are a cause of acute respiratory infections and are a frequent cause of diarrhoea in pigs (Guérin and Pozzi, 2005).

In herd B, of the pigs affected with PMWS, porcine enterovirus 1 and 2 were isolated from pig 42, while no virus was isolated from pig 36. In herd D enterovirus 1 was isolated from pig 4, adenovirus and enterovirus 2 was isolated from pig 9, no virus was detected in pig 31, adenovirus and porcine enterovirus 1 was isolated from pig 43 and porcine enterovirus 1 and 2 were isolated from pig 46. There was no common virus isolated from every pig which died as a result of PMWS. It is therefore unlikely that these viruses, porcine enterovirus 1 and 2, reovirus and adenovirus when co-infected with PCV2 act as a trigger for PMWS.

Although there was no common CPE virus isolated from all diseased animals in the farms under study, all animals were probably co-infected with other viruses, animals 36 from herd B and 31 from herd D were PPV antibody positive when tested by an ELISA. No unidentified CPE was found on any sample. All animals under study were infected with haemolytic *E. coli* and *Campylobacter* spp., however, only *Salmonella* spp. was isolated from pig 56 in herd A and pig 36 in herd B. It can be concluded therefore from these results that the PMWS animals under study had no distinguishing co-infection over otherwise healthy animals in the four herds tested in Ireland. The only major difference in husbandry management between the PMWS and non-PMWS herds and the PMWS free herd was the fact that it was smaller in size. It has been reported that factors such as environmental conditions, the genetics of the pigs, the introduction of

bioflavonoids, vitamin E, selenium and antioxidants in the diet and meal rather than pellet in food may assist in controling the disease and its effects. However in some units there have been conflicting results (Marco, 2005).

The practice of intensive pig farming where sow stalls are used for lower production costs has led to significant animal welfare issues along with the possible introduction of new diseases and syndromes. Indoor pig systems allow the pigs condition to be monitored ensuring minimum fatalities and increased productivity. Buildings are ventilated and their temperature regulated. Most domestic pig varieties are susceptible to heat stress, and all pigs lack sweat glands and cannot cool themselves. Pigs have a limited tolerance to high temperatures and heat stress can lead to death. Maintaining a more specific temperature within the pig-tolerance range also maximizes growth and growth to feed ratio. Intensive piggeries control temperature through ventilation or drip water systems (dropping water to cool the system). The overcrowding of pigs in barren environments can exacerbate problems that may exist in the pig population and recurrent viral diseases have emerged and can be more readily spread.

Chapter 6: Overall Discussion

6.1 Overall Discussion

This study involved the determination of the presence and extent of PCV2 infection and PMWS, a newly emerged disease syndrome in weaned pigs, in both parts of Ireland. To date no such work has been has been performed on pigs in ROI on this economically important disease which has affected countries in Europe, America, Asia (Chae, 2004) and has recently been reported from New Zealand (Laval, 2004).

The seroprevalence of PCV2 infection in Irish pig herds was determined on a cohort of randomly chosen serum samples from the pig herd population. PCV2 was found to be widespread in Irish pig herds whether or not PMWS occurs in them, with 85% of pigs testing positive in ROI and 100% in NI. Four of the 50 herds tested in ROI were antibody negative to PCV2; these 4 herds were all small hobby herds. A further four hobby herds were antibody positive, indicating that PCV2 may exist and spread in both large commercial herds and small hobby herds. However, it may be that PCV2 requires the circumstances found in commercial herds to effectively contribute to causation of PMWS, such as early weaning and small pens. This would suggest that although the risks of infection are lower in smaller herds it is still extremely important to adhere to strict husbandry practices, an improvement in hygiene and a reduction of stress.

A very large variation was observed in PCV2 antibody titres within herds; this was seen to vary from the lowest (<1/250) to the highest titre (1/156,250) detectable with this test. A large proportion of Irish herds were sero-positive and a high within-herd seroprevalence was found in positive herds. It has been reported elsewhere that

seroprofiles from PMWS-positive and negative herds are not very different (Marco, 2005).

Specimens from herds clinically suspected of PMWS were submitted to the laboratory for investigation over a one-year period. Of the 30 herds examined in ROI, 24 were confirmed to have PMWS and in NI, of the 31 submitted, 14 were confirmed as PMWS positive. This showed a much higher incidence of PMWS in the herds examined in ROI than in NI at this time, 80% and 45.2% respectively. Work on PCV2 infection and PMWS has been ongoing in NI since the emergence of the disease and therefore beneficial changes may have already occurred in husbandry and management practices there which were not yet established in ROI. It may also be a reflection of the selectivity of submissions of suspect cases in the present study. There are a total of 1149 active Irish pig herds in ROI, and it may be the case that samples from suspect PMWS herds were not submitted as frequently in the South of Ireland. Although laboratory confirmation of the disease is necessary it is possible that many specialist pig veterinarians may have clinically recognized PMWS or other diseases in herds and therefore made diagnoses without submitting suspect samples to the laboratory, thereby having the effect of reducing the number of submissions.

The principal clinical and necropsy findings observed in positive herds in Northern Ireland and the Republic of Ireland included wasting, respiratory distress, lymph node enlargement, diarrhoea and pneumonia. Wasting was the most common finding in both NI and ROI. The principal histopathology findings included lymphocytic

depletion, bronchopneumonia, renal oedema and cytoplasmic inclusion bodies typical of PCV2 infection. Clinical signs observed, post-mortem changes and histopathology findings accorded with those described in the literature for PMWS (Segalés and Domingo, 2002).

Sequencing of PCV2 from Irish herds was performed. Isolates from tissues from field cases of PMWS and from non-diseased animals were amplified by PCR and sequenced in order to determine if differences existed between them, or if Irish isolates differed from isolates in other countries. The complete genome of PCV2 isolates were aligned using ClustalW version 1.83 multiple sequence alignment programme http://www.ebi.ac.uk/clustalw/. Aligned data demonstrated that all the isolates from Ireland showed a minimum homology of 97% with each other, and when compared to those from other countries, showed a minimum homology of 94%.

The two major open reading frames of PCV2, ORF1 and ORF2 were examined. ORF1 codes for the Rep protein (Cheung, 2004a), while ORF2 codes for the capsid protein (Allan and Ellis, 2000a). An amino acid alignment was also preformed on these ORF's using the ClustalW programme. Within ORF1 the minimum homology observed between isolates from both parts of Ireland was found to be 98% and in ORF2 the minimum homology was 94%. When comparing PCV2 isolates from diseased animals with those from non-diseased animals, it was observed that some amino acid changes existed within these ORFs. As the ORF2-encoded protein is the major structural antigen of the virus, changes to its primary sequence may result in an altered immune response to

PCV2, either due to an alteration of T cell immunoreactive epitopes or to the binding sites for neutralising antibodies. No nucleotide changes were observed within the stem loop structure of the origin of replication of Irish PCV2 isolates.

A longitudinal study was performed on four herds in Ireland, comprising 2 diseased herds and one non-diseased herd from ROI and one diseased herd from NI. Pigs from 5 litters were included in the study. To determine if any other infectious agent was present and so involved in potentiating PCV2 to cause disease, these animals were tested for PCV2 and PPV antibody and for the presence of haemolytic *E. coli*, *Campylobacter* spp., *Salmonella* spp. and other viral pathogens. There did not appear to be any difference in the isolation rates of viruses and bacteria between the diseased and non-diseased animals and herds. Therefore it can be concluded that the PMWS animals under study did not appear to have a distinguishing co-infection over otherwise healthy animals in the four herds tested in Ireland. A complete history was taken from each herd in ROI in order to determine if there were any obvious difference in husbandry and management, from the information given the only major difference between the PMWS affected and non-affected herds was size. This would suggest that the most effective control measures are improved management and control of other pathogens and to reduce stresses that may be brought about by overcrowding. It may be worth investigating the size of pig herds as a whole and its implication in the spread of disease.

PMWS has had major economic, public health and animal welfare impacts on the pig meat industry. Economic looses have been reported as more than €600 million per

year to the European Union (Allan *et al.*, 2005). Factors underlying the economic losses include fewer pigs at slaughter, reduced feed conversion rates, increased costs for management, medication of sick pigs and costs of secondary diseases following PMWS associated immunosupression. The potential impact of PMWS in terms of food safety and veterinary public health is only beginning to emerge. PMWS associated immunosuppression may result in increased carcase contamination by food-borne pathogens (eg. *Salmonella typhimurium*) arising from potentially increased prevalence of infection on-farm and increased shedding at slaughter. A second public health concern is an associated increase in the use of antimicrobial agents in the attempted control of PMWS-associated disease, with consequently increased potential for selecting for antimicrobial resistant bacteria and drug residues in pig meat. In the UK, where PMWS emerged between 1998 and 1999, the use of antimicrobials in pig production increased markedly from 83 tonnes/year in 1998 to 109 tonnes/year in 2001; a 31% increase despite a 7% reduction in the UK breeding pig population. The introduction of PMWS is a likely driver behind this increased antibiotic use. The impact of PMWS on pig welfare is manifest through prolonged increases in mortality rates and increased numbers of ill-thriven pigs requiring hospitalization (Tucker et al., 2006).

There are many control measures that can be used with PMWS. These can be combined successfully. However, there is no general protocol that will suit in exactly all herds the same way. Each individual herd will need to take a slightly different approach depending on their facilities, size and health status.

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Chapter 8: Appendix

Preparation of Buffer Solutions

Hanks balanced Salt Solution (HBSS) 10X:	Biosciences, Dublin 4mls HBSS diluted in 36mls of sterile distilled water with a few drops of foetal calf serum (FCS)
Phosphate Buffered Saline (PBS):	Sigma-Aldrich, Dublin, Ireland Contents of one pouch, when dissolved in one liter of distilled or deionized water, will yield 0.01 M phosphate buffered saline (NaCl 0.138 M; KCl - 0.0027 M); pH 7.4, at 25 °C.
D-glucosamine:	Sigma-Aldrich, Dublin, Ireland 300-mM: 2.588g diluted in 36mls sterile distilled water + 4mls of HBSS filtered through a 0.22 micron filter.

PBS with Tween and Horse Serum (PBS-TH):	400mls of PBS containing 4mls of 0.05% Tween 80 and 40mls of horse serum.
Neutral Buffered Formalin (NBF):	10% (pH 7) in 1 litre: dissolve sodium phosphate salts in 500ml of water, add 100 ml formalin with stirring, adjust pH to pH 7.0 with NaOH. Make up to 1 liter with remaining water. Filter through 0.45um filter and store tightly capped in refrigerator.
Triphosphate buffered solution (TBS):	IHC Select® 20X TBS Rinse Buffer, Chemicon, cat. No. 20845 dilute this 20X Rinse Buffer to 1X with distilled or deionized water it is recommended that Tween 20® be added to the 1X Rinse Buffer at a final concentration of 0.1%.