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Porcine circovirus diseases

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Abstract

Porcine circovirus type 2 (PCV2) is a member of the family *Circoviridae*, a recently established virus family composed of small, non-enveloped viruses, with a circular, single-stranded DNA genome. PCV2, which is found all over the world in the domestic pig and probably the wild boar, has been recently associated with a number of disease syndromes, which have been collectively named porcine circovirus diseases (PCVD). Postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS) and reproductive disorders are the most relevant ones. Among them, only PMWS is considered to have a severe impact on domestic swine production. PMWS mainly affects nursery and/or fattening pigs; wasting is considered the most representative clinical sign in this disease. Diagnosis of this disease is confirmed by histopathological examination of lymphoid tissues and detection of a moderate to high amount of PCV2 in damaged tissues. Since PMWS is considered a multifactorial disease in which other factors in addition to PCV2 are needed in most cases to trigger the clinical disease, effective control measures have focused on the understanding of the co-factors involved in individual farms and the control or elimination of these triggers. PDNS, an immuno-complex disease characterized by fibrino-necrotizing glomerulonephritis and systemic necrotizing vasculitis, has been linked to PCV2, but a definitive proof of this association is still lacking. PCV2-associated reproductive disease seems to occur very sporadically under field conditions, but it has been characterized by late-term abortions and stillbirths, extensive fibrosing and/or necrotizing myocarditis in fetuses and the presence of moderate to high amounts of PCV2 in these lesions. Taking into account that scientific information on PCV2 and its associated diseases has been markedly expanded in the last 8 years, the objective of this review is to summarize the current state of knowledge of the most relevant aspects of PCV2 biology and PCVD.

Keywords: porcine circovirus type 2, pig, postweaning multisystemic wasting syndrome, porcine dermatitis and nephropathy syndrome, reproductive disease

Introduction

In 1974, researchers in Germany described a new, non-cytopathogenic, picornavirus-like contaminant in the porcine kidney cell line, PK-15 (ATCC-CCL33) (Tischer *et al*., 1974). This 'contaminant' was later shown to be a small icosahedral, non-enveloped virus containing a single-stranded, circular DNA genome, and the name porcine circovirus (PCV) was proposed (Tischer *et al*., 1982). Serological surveys for PCV antibodies in swine in Germany (Tischer *et al*., 1986), Canada (Dulac and Afshar, 1989), Great Britain (Edwards and Sands, 1994) and Northern Ireland (Allan *et al*., 1994) revealed that the virus was widespread in the swine population. However, experimental infections of newborn to 9-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).

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In the late 1990s, an apparently novel PCV-like virus was first isolated from pigs with a wasting disease in Western Canada (Ellis *et al.*, 1998). Shortly thereafter, almost identical viruses to this Canadian isolate were recovered from diseased pigs in other parts of North America and Europe (Allan *et al.*, 1998). Using monoclonal and polyclonal antibodies, and genomic sequence analysis, these PCV virus isolates were shown to be antigenically and genetically distinct from the PCV contaminant of PK-15 cell cultures. Consequently, it was proposed that the PCV isolates from diseased pigs should be designated porcine circoviruses type 2 viruses (PCV2) and the original PCV contaminant of PK-15 cell cultures designated porcine circovirus type 1 virus (PCV1) (Allan *et al.*, 1999b).

PCV2 has been associated with a number of disease syndromes in pigs and the terminology porcine circovirus diseases (PCVD) has recently been proposed to replace existing acronyms (Allan *et al.*, 2002a).

A wasting syndrome in Western Canadian pigs, first identified in 1991 in high health SPF herds, was reported in 1996 (Clark, 1996; Harding, 1996). The authors proposed the term 'postweaning multisystemic wasting syndrome' (PMWS) to describe the clinical condition. PCV nucleic acid and antigen were demonstrated in abundance within the lesions of affected pigs and subsequent isolation and characterization of a PCV2 virus from diseased pigs was reported (Ellis *et al.*, 1998). The PCV genome was also associated with interstitial pneumonia and lymphadenopathy in a 6-week-old pig in California (Daft *et al.*, 1996) and a PCV2 virus was also recovered from this animal (Allan *et al.*, 1998). In 1996/97, a clinical wasting disease associated with PCV2 was described in France (LeCann *et al.*, 1997) and Spain (Segalés *et al.*, 1997). Since these initial reports of PCV2-associated PMWS, the disease has been reported in almost all pig producing countries around the world. PMWS histological lesions associated with an abundance of PCV2 antigen have been retrospectively described in archived tissue samples taken in 1986 from pigs in Spain and the United Kingdom (Rodríguez Arrioja *et al.*, 2003b; Grierson *et al.*, 2004) and in 1989 in Japan (Mori *et al.*, 2000). Moreover, evidence of PCV2 infection in pigs as early as 1969 was provided in a retrospective assessment (Sánchez *et al.*, 2001b). Therefore, PMWS should not be considered as a new disease and PCV2 should not be considered as a new virus.

PCV2 has also been associated with another rapidly expanding disease syndrome of pigs, porcine dermatitis and nephropathy syndrome (PDNS) (Rosell *et al.*, 2000b), which in recent years has reached epizootic proportions in some countries. Although epidemiological evidence strongly suggests that the causes of PMWS and PDNS may be linked, it still remains to be proven that PCV2 is an infectious component of PDNS.

PCV2 is now recognized as a causal agent of reproductive disorders in pigs and has been demonstrated in abundance in heart tissue from pigs with neonatal myocarditis (West *et al.*, 1999).

PCV2 antigen has been demonstrated in abundance in lung lesions from pigs with proliferating and necrotizing pneumonia (PNP) (Allan and Ellis, 2000) and in tissues from sows that suffered from sow abortion and mortality syndrome (SAMS) (Harms *et al.*, 2001). Finally, PCV2 is also considered a contributor to porcine respiratory disease complex (PRDC) (Thacker and Thacker, 2000; Harms *et al.*, 2002).

PCV2-like isolates with 99% genomic homology to isolates from PMWS cases have been reported to be present in lungs of cattle with respiratory disease (Nayar *et al.*, 1999) and the central nervous system of piglets with congenital tremors (CT) (Stevenson *et al.*, 2001). In addition, PCV2 was reported to replicate in mice (Kiupel *et al.*, 2001). However to date, none of these reports on PCV2-like viruses in cattle or CT or their replication in mice have been confirmed by other workers (Quintana *et al.*, 2002; Kennedy *et al.*, 2003; Rodríguez-Arrioja *et al.*, 2003a), nor have they been expanded by the original authors, and PCV2 remains only a virus of the pig without proven association to CT. Available evidence indicates that PCV2 does not represent a zoonotic risk for humans.

Among PCVD, only PMWS is considered to have a severe impact on swine production. It has been estimated that PCVD costs around 600 million Euros per year to the European Union (Armstrong and Bishop, 2004). 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.

Taking into account the marked increase of scientific and practical knowledge on PCV2 infection and its associated diseases in the last 8 years, the purpose of this review is to comprehensively present the most relevant and updated information on this emergent swine infection.

Etiology

PCV2 belongs to the family *Circoviridae*, a recently established virus family composed of small, non-enveloped, isometric DNA viruses with a circular, single-stranded DNA genome. The family *Circoviridae* is divided into two genera on the basis of virion size and genomic organization. PCV2 is classified under the genus *Circovirus* together with PCV1, beak and feather disease virus, goose circovirus, pigeon circovirus, canary circovirus and duck circovirus. Chicken anemia virus is the only member of the second genus, Gyrovirus (McNulty *et al.*, 2000).

The circular PCV2 viral genome contains 1767–1768 nucleotides (Hamel *et al.*, 1998; Meehan *et al.*, 1998; Mankertz *et al.*, 2000) and genomic analysis of PCV2 viruses from diseased and non-diseased pigs around the world has shown that they all belong to a phylogenetic cluster with a nucleotide sequence identity greater than

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94% (Meehan *et al.*, 1998; Mankertz *et al.*, 2000; Larochelle *et al.*, 2002). However, comparison of the genomic sequence of PCV2 isolates with the genomic sequence of PCV1 has shown an 80% overall nucleotide sequence identity. Potentially, six ORFs larger than 200 nucleotides have been suggested for the PCV2 genome (Hamel *et al.*, 1998; Meehan *et al.*, 1998), but proteins seem to be expressed only by ORF1 and ORF2. Analysis of ORF1 (replicase proteins; Rep) and ORF2 (capsid protein; Cap) of PCV2 has shown 83% nucleotide and 86% predicted protein homology with PCV1 for ORF1 and 67% nucleotide and 65% predicted protein homology with PCV1 for ORF2, respectively (Mankertz *et al.*, 2000). Very recently, a third viral gene termed ORF3 has been described (Liu *et al.*, 2005). The ORF3 protein appears to be a highly conserved one, with a greater than 95% identity at the amino acid level among PCV2 strains (Liu *et al.*, 2005). Little is known about the significance of the rest of potential smaller ORFs.

During PCV1 and PCV2 viral replication, a double-stranded, replicative DNA form is generated. The Rep protein is encoded on the viral plus-strand, while the Cap protein is transcribed from the counterclockwise strand (Mankertz *et al.*, 2004). The intergenic region comprises the origin of replication, which encompasses a stem-loop structure with a conserved nonanucleotide and several repeats. Nine specific RNAs are synthesized during PCV2 replication in PK-15 cell culture including the capsid RNA, five Rep-associated RNAs, and three non-essential-associated RNAs (Cheung, 2003; Mankertz *et al.*, 2004). Despite the abundance of viral transcripts, only three proteins have been detected to date. The Rep-gene codes for two protein variants, the full-length Rep protein (312 amino acids), and the truncated, spliced variant Rep' protein (168 amino acids); both proteins are essential for PCV2 replication (Cheung, 2003; Mankertz *et al.*, 2004). During productive infection, viral antigens, RNA transcripts and progeny viruses increase in a time-dependent manner. In cell culture studies, viral antigens are observed in a few cells at 18 h post-infection (PI) and cell-free progeny viruses begin to appear at about 30 h PI (Cheung and Bolin, 2002). On the other hand, ORF3 protein is not essential for PCV2 replication, and seems to play a major role in viral-induced apoptosis in PK-15 cells (Liu *et al.*, 2005); it is not known at this time if this protein can be responsible for apoptosis under *in vivo* conditions in any of the tissues infected with PCV2.

Little data exist on the biological and physicochemical characteristics of PCV2. However, it is known that PCV1 has a buoyant density of 1.37 g ml^{-1} in CsCl, is not able to hemagglutinate erythrocytes from a wide range of species, is resistant to inactivation at pH 3 and by chloroform, and is stable at 70°C for 15 min (Allan *et al.*, 1994). Although not demonstrated for PCV2, it is probable that these properties are common to both PCV1 and PCV2. Exposure of PCV2 for 10 min at room temperature to a number of commercial disinfectants based on chlorhexidine, formaldehyde, iodine and alcohols leads to a 1.8–4.4 log $TCID_{50}$ reduction in virus titer (Royer *et al.*, 2001).

Epidemiology

Geographic distribution

PCV2 is now considered a ubiquitous virus, both in countries where PCVD has or has not been detected (Allan and Ellis, 2000; Segalés *et al.*, 2004b). PCV1 is also considered to have worldwide distribution, but its exact prevalence, which is probably lower than that of PCV2 (Calsamiglia *et al.*, 2002), has not yet been determined as early serological investigations on PCV1 may have detected cross-reactive antibodies elicited by PCV2 (Pogranichniy *et al.*, 2000; Rodríguez-Arrioja *et al.*, 2000).

Susceptible species

Swine appear to be the natural host of PCV1 and PCV2 (Segalés and Domingo, 2002). Along with domesticated pigs, PCV2 antibodies have been demonstrated in 33 and 37% of wild boar sera in Belgium that were examined in 1993 and 2000, respectively, and 48% in Spain (Sanchez *et al.*, 2001b; Vicente *et al.*, 2004). Moreover, a PMWS-like disease has also been described in wild boar from North-America (Ellis *et al.*, 2003) and Europe (Schulze *et al.*, 2004; Vicente *et al.*, 2004). The genomes of PCV2 viruses recovered from diseased wild boar in two of these studies have been sequenced (Ellis *et al.*, 2003; Schulze *et al.*, 2004), showing a nucleotide homology that is almost identical to other PCV2 viruses characterized from domestic swine. While the first serological study in which serum samples from humans, cattle and mice were tested gave positive results (Tischer *et al.*, 1995), recent serological surveys in cattle, goats, sheep, horses, dogs, cats, mice and humans have shown no evidence of infection (Allan *et al.*, 2000b; Ellis *et al.*, 2001; Rodríguez-Arrioja *et al.*, 2003a) and further serological data on a theoretically high risk populations, such as veterinarians, have also yielded negative results (Ellis *et al.*, 2000).

Transmission of PCV2 infection

The oro-nasal route is considered the most likely and frequent route of PCV2 transmission. This is supported by experimental studies on PCV2 infection, which have mainly used the intranasal route of inoculation (Allan *et al.*, 1999a; Balasch *et al.*, 1999; Ellis *et al.*, 1999; Krakowka *et al.*, 2000, 2001; Rovira *et al.*, 2002). Under commercial farm conditions, the majority of pigs sero-convert to PCV2 between 2 and 4 months of age (Larochelle *et al.*, 2003; Sibila *et al.*, 2004), indicating that horizontal transmission of PCV2 between pigs is very

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efficient. Horizontal transmission of PCV2 has been demonstrated under experimental conditions to contact susceptible pigs commingled with already infected pigs (Albina *et al.*, 2001; Bolin *et al.*, 2001).

Intranasal and subcutaneous (Pogranichniy *et al.*, 2000; Bolin *et al.*, 2001) routes of inoculation with PCV2 virus have also been used in attempts to experimentally reproduce PMWS. Importantly, 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).

Transplacental transmission of PCV2 has recently been demonstrated following experimental intranasal infection of sows (Park *et al.*, 2005), indicating that vertical transmission of PCV2 is feasible. However, the frequency of these reproductive alterations under field conditions is apparently variable, since it has been reported as very rare in Europe (Pensaert *et al.*, 2004; Maldonado *et al.*, 2005), but data from Korea showed PCV2 infection in about 13% of aborted fetuses and stillborn piglets (Kim *et al.*, 2004).

Routes of shedding

PCV2 can be detected by polymerase chain reaction (PCR) in nasal cavities, tonsillar and bronchial secretions, feces and urine (Calsamiglia *et al.*, 2004a; Sibila *et al.*, 2004) of both naturally PMWS and non-PMWS affected pigs, although it is known that the virus load present in these potential excretion routes is much higher in diseased pigs (Calsamiglia *et al.*, 2004a). These results are further sustained by experimental infections with PCV2; the virus has been isolated or detected by PCR from nasal, rectal, urinary, salivary, ocular and tonsillar swab specimens (Krakowka *et al.*, 2000; Bolin *et al.*, 2001; Shibata *et al.*, 2003). However, although it is known that PCV2 have been detected in nasal cavities and tonsil of PMWS affected pigs by *in situ* hybridization (ISH) (Segalés *et al.*, 2004b), it has not definitively been proven whether the viral load obtained from nasal or tonsillar swabs corresponds to viral excretion or is due to virus present in the environment that reached those locations.

PCV2 nucleic acid has been demonstrated by PCR in pig semen (Hamel *et al.*, 2000; Larochelle *et al.*, 2000). Under experimental conditions, PCV2 DNA was detected in semen up to day 47 PI, the last day tested in this study (Larochelle *et al.*, 2000). It is important to note that, to date, PCV2 infectious virus has not been demonstrated in semen, since no virus isolation or swine bioassays have been performed on PCR positive samples. Therefore, although artificial insemination and natural mating may be considered as potential routes of dissemination of PCV2 virus in the reproductive stock, further studies are needed to elucidate this possibility.

Persistent infection

PCV2 nucleic acid has been demonstrated in serum from pigs up to 22 weeks of age under field conditions (Rodríguez-Arrioja *et al.*, 2002). However, none of these studies assessed whether pigs were continuously or intermittently viremic. Pigs with persistent infection can occur in both PMWS and non-PMWS affected herds (Larochelle *et al.*, 2003; Sibila *et al.*, 2004). These field data are further supported by experimental studies. PCV2 has been detected in blood and tissues of a high proportion of experimentally inoculated pigs at termination (days 21–71 post-inoculation) of the experiments (Allan *et al.*, 1999a; Balasch *et al.*, 1999; Krakowka *et al.*, 2000; Magar *et al.*, 2000; Pogranichniy *et al.*, 2000; Harms *et al.*, 2001; Rovira *et al.*, 2002; Resendes *et al.*, 2004b), and in one experiment, PCV2 nucleic acid was detected in tissues of a single pig sacrificed at 125 days PI (Bolin *et al.*, 2001). The mechanism by which PCV2 persists in the pig is not known at present.

PCV2 molecular epidemiology

The fact that PCV2 has been detected in both PCVD and non-PCVD affected farms and pigs has raised the question of the possibility that different viral strains may vary in their pathogenicity. A specific study comparing PCV2 sequences from affected and non-affected pigs from France concluded that no viral molecular marker specific to a pathogenic state was identified, even when including other PCV2 variants isolated from PMWS-suffering animals from other countries (De Boisseson *et al.*, 2004). This result is further supported by experimental evidence in which PMWS was reproduced using PCV2 recovered from both diseased (Allan *et al.*, 1999a) and clinically healthy pigs (Allan *et al.*, 2003). However, it is probably too early, based on the available information, to rule out definitively whether differences in pathogenicity among PCV2 isolates exist.

Pathogenesis

PMWS is now recognized as a disease of pigs where PCV2 infection is needed for the full expression of the clinical condition. However, this does not mean that all pigs infected with PCV2 will develop clinical PMWS. Multiple attempts to experimentally reproduce PMWS have been published in the literature. Some early experimental trials using tissue homogenates (Balasch *et al.*, 1999) or PCV2 isolated and propagated in cell culture (Ellis *et al.*, 1999; Magar *et al.*, 2000; Porgraninchniy *et al.*, 2000) reproduced PMWS-like histological lesions of slight to moderate intensity, but not the clinical wasting syndrome. However in a study in colostrum-deprived (CD) pigs,

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clinical disease and lesions consistent with PMWS were reported in 1 of 4 piglets inoculated with PCV2 alone (Allan *et al.*, 1999a; Kennedy *et al.*, 2000) and similar or higher percentage of diseased pigs inoculated with PCV2 alone have been reported by other workers (Bolin *et al.*, 2001; Harms *et al.*, 2001; Okuda *et al.*, 2003). Consequently, it has been suggested that PCV2 infection, linked to other co-factors, is necessary for the consistent development of full clinical disease in pigs. In retrospect, it seems likely that a number of factors such as age and source of pigs, the environmental conditions in which the pigs were held, the genetics of the pigs and the nature of the PCV2 inoculum used, may have played a significant role in the consistent experimental reproducibility of the disease.

More consistent and repeatable PMWS disease models have been obtained using infectious (Allan *et al.*, 1999a, 2003; Krakowka *et al.*, 2000) and non-infectious (Krakowka *et al.*, 2001) co-factors. The reproduction of full clinical disease has been reported in a high percentage of CD and gnotobiotic pigs co-inoculated with PCV2 and porcine parvovirus (PPV), or inoculated with a potent immunostimulant, such as keyhole limpet hemocyanin in incomplete Freund's adjuvant. The mechanism by which other viruses or immunostimulation may trigger the development of PMWS in PCV2-infected pigs is still unknown. It has been speculated that the antigenic stimulation with infectious and non-infectious agents presumably increases the number of permissive cells for PCV2 replication entering the S phase of the cell cycle (Ellis *et al.*, 1999; Krakowka *et al.*, 2000), a condition needed for PCV replication (Tischer *et al.*, 1987). High loads of PCV2 in blood, lymphoid tissues and other tissues are associated with the expression of disease (Kennedy *et al.*, 2000; Liu *et al.*, 2000; Chianini *et al.*, 2003; Olvera *et al.*, 2004).

When clinical PMWS is apparent, damage of the immune system is the main feature in affected pigs (Nielsen *et al.*, 2003; Darwich *et al.*, 2004). Lymphocyte depletion of lymphoid tissues, changes in peripheral blood mononuclear cell (PBMC) subpopulations and altered cytokine expression patterns have been all demonstrated in naturally and experimentally PMWS-affected pigs (Clark, 1997; Rosell *et al.*, 1999; Darwich *et al.*, 2003a, b; Nielsen *et al.*, 2003). Although many aspects of the pathogenesis of PMWS still await a definitive identification and confirmation, an outline of the pathogenesis of PMWS-affected and PCV2-subclinical infected pigs has been proposed in relation to the immunological aspects of the disease (Darwich *et al.*, 2004).

PCV2 is also a hepatitis-inducing virus for pigs (Rosell *et al.*, 2000a; Krakowka *et al.*, 2001), and liver failure is one of the causes of death in PMWS-affected pigs.

Still unresolved is the identification of cells that support PCV2 replication. The large amount of PCV2 virus antigen found in macrophages and dendritic cells of diseased pigs appears to be the result of accumulation of viral particles (Gilpin *et al.*, 2003; Vincent *et al.*, 2003) and not the result of active virus replication in these cells. However, it is still possible that PCV2 replicates in a small, as yet unidentified subpopulation of these cell types.

The entry pathway of PCV2 into target cells in the pig has not been yet determined. However, some preliminary data generated *in vitro* using a porcine monocytic cell line have shown that PCV2 enters those cells predominantly via clathrin-mediated endocytosis and an acidic environment is required for this entry (Misinzo *et al.*, 2005). In addition it has been shown that a non-macropinocytic uptake by mature dendritic cells (DC) and DC precursors allows PCV2 internalization in these cells, in absence of viral replication (Vincent *et al.*, 2005).

The pathogenesis of non-PMWS PCVD is much more obscure. Reproductive disease associated with PCV2 infection has been described under field conditions (West *et al.*, 1999; Ladekjaer-Mikkelsen *et al.*, 2001; O'Connor *et al.*, 2001) and experimental studies have demonstrated a deleterious effect to fetuses when PCV2 was directly inoculated into them (Sanchez *et al.*, 2001a, 2003, 2004; Johnson *et al.*, 2002; Pensaert *et al.*, 2004; Yoon *et al.*, 2004). From these studies it is known that fetuses are susceptible to PCV2 infection and that this virus seems to replicate in tissues or cell types with a high-mitotic rate such as fetal myocardiocytes (Sánchez *et al.*, 2003). This latter finding might explain the heart lesions and the clinical outcome of the reproductive form of PCVD under natural conditions (West *et al.*, 1999). Moreover, it has also been shown that PCV2 target cells change from cardiomyocytes, hepatocytes and macrophages during fetal life to only macrophages postnatally (Sánchez *et al.*, 2003). However, as in PMWS, it is not really known if macrophages support definitively PCV2 viral replication, or simply accumulate viral particles. It has also been suggested that PCV2 titers in lymphoid organs of intrauterine infected fetuses may lead to the development of histological lesions similar to those observed in pigs with PMWS without causing disease (Sánchez *et al.*, 2004).

Attempts to infect fetuses by intranasal inoculation of pregnant sows or artificial insemination route have yielded differing results (Cariolet *et al.*, 2001a, b; Nielsen *et al.*, 2004). However, transplacental transmission of PCV2 has recently been demonstrated following experimental intranasal inoculation of six PCV2 seronegative sows at 3 weeks before the expected farrowing date (Park *et al.*, 2005). Among these sows, three aborted and three farrowed prematurely; PCV2 antigen and nucleic acid were detected in lymphoid and non-lymphoid tissues from stillborn and liveborn piglets (Park *et al.*, 2005). However, no histological lymphoid lesions resembling those from PMWS were observed in any of the studied fetuses. Surprisingly, no heart lesions were observed in the fetuses coming from these aborted or prematurely farrowed sows (Park *et al.*, 2005). This may be explained by the PCV2 infection during late gestation, since gross heart alterations have been described when fetuses were infected at mid gestation (Sánchez *et al.*, 2001a).

Fig. 1. PMWS-affected pig (large arrow) compared to an age-matched, healthy pigs (small arrows). Note the moderate growth retardation and the marked spinal cord and ribs of the affected animal.

Furthermore, PCV2 is also able to replicate in *in vivo* produced zona pellucida-free morulae and blastocysts, suggesting a potential effect of PCV2 in embryonic stages (Mateusen *et al.*, 2004). The importance of this finding in the naturally occurring reproductive disease associated with PCV2 and/or other PCVD scenarios remains to be elucidated.

PDNS is considered to be a type III hypersensitivity reaction, in which the antigen present in the immune complexes is not yet known. It has been speculated that PCV2 could be the antigen; however, to date, studies on tissue sections from pigs with PDNS have failed to consistently demonstrate PCV2 antigen or nucleic acid associated with PDNS lesions. A recent study (Wellenberg *et al.*, 2004) demonstrated significantly higher serum antibody titers to PCV2 in PDNS-affected pigs compared to healthy or PMWS-affected pigs, suggesting that the causative physiological basis for PDNS may be the excessive levels of PCV2 antibodies.

Although important advances in our understanding of the pathogenesis of PCVD have been achieved during the last 5 years, detailed definitive studies are still lacking for many aspects of these diseases.

Clinical signs and lesions

PMWS

Clinical signs

PMWS most commonly affects pigs of 2–4 months of age, although the disease has been described in 1- to 6-month-old pigs. Only one report has described naturally occurring PMWS in nursing 3-day-old piglets (Hirai *et al.*, 2001). PMWS has been described in almost all types of farms, including farrow-to-finish and multi-site operations, and farms from 30- to 10,000-sow herds. Morbidity and mortality on PMWS-affected farms are variable, depending on the farm, type, husbandry and management practices, co-infections, etc. Common rates of morbidity seen in affected farms are 4–30% (with exceptional rates over 50–60%) and 70–80% of affected pigs die; mortality rates on farms vary between 4 and 20% (Segalés and Domingo, 2002). Single pigs suffering from PMWS have also been observed sporadically on farms with very good production records and minimal postweaning mortality.

The major clinical sign of PMWS is wasting (Fig. 1) but it is usually seen concomitantly with other signs such as pallor of the skin, respiratory distress, and diarrhea and, occasionally, icterus (Harding and Clar, 1997). A relatively striking feature of pigs in the early clinical phases of PMWS is the increase in size of the subcutaneous lymph nodes (mainly inguinal superficial lymph node), although it is not always seen (Segalés *et al.*, 2004b).

It has been reported that, on some farms, most of the pigs developing PMWS belong 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 (Corrégé *et al.*, 2001; Rodríguez-Arrioja *et al.*, 2002). Moreover, it has been reported that pigs with lower birth and weaning weights tended to develop PMWS with higher frequencies, as well as the lighter pigs at the beginning of the fattening period (Corrégé *et al.*, 2001).

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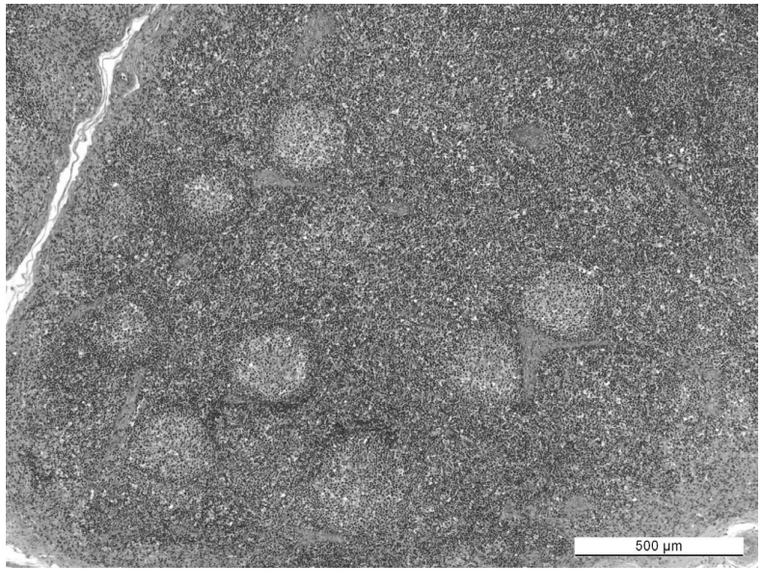

Fig. 2. Inguinal superficial lymph node of a healthy pig. Normal lymphoid tissue showing the follicular and parafollicular areas densely packed with lymphocytes. Hematoxylin and eosin stain.

Other infections or diseases are more commonly found on PMWS-affected farms, when compared to non-affected farms (Ellis *et al.*, 2004). These diseases include pseudorabies (Aujeszky's disease), porcine reproductive and respiratory syndrome (PRRS), PPV infection, Glässer's disease, streptococcal meningitis, salmonellosis, post-weaning colibacillosis, non-specific diarrhea, hepatosis dietetica, and bacterial pneumonia. Whether the increased disease load found on PMWS-affected farms is a consequence of PMWS and the associated immune dysfunction in affected animals or causal of PMWS in association with PCV2 infection 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 various concurrent diseases.

Lesions

The main lesions of PMWS occur in lymphoid tissues, although inflammatory infiltrates associated with PCV2 infection have been detected in a wide range of tissues from affected pigs. A summary of most frequent macroscopic and microscopic lesions of PMWS-affected pigs has been published elsewhere (Segalés and Domingo, 2002).

Enlargement of lymph nodes is the most prominent feature of the early clinical phases of PMWS (Clark, 1997; Rosell *et al.*, 1999). This increase in size is easily recognized at the inguinal and mesenteric lymph nodes, but others may be also enlarged. The cut surface of the nodes is homogeneous pale brown or whitish, and slightly humid. In a small number of affected pigs (approximately in 2% of cases), a necrotizing lymphadenitis is observed (Segalés *et al.*, 2004b). Normal or even atrophic lymph nodes are usually seen in more advanced phases of PMWS (Segalés *et al.*, 2004b) and the thymus is normally atrophic in diseased pigs (Ladekjaer-Mikkelsen *et al.*, 2002; Darwich *et al.*, 2003b).

The histopathological lymphoid lesions observed in PMWS-affected pigs 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 (Figs. 2 and 3) in parafollicular zones, which may vary in intensity from slight to severe (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 dendritic cells (Fig. 4). 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.

Lungs may be enlarged, non-collapsed and rubbery in consistency, following a diffuse or patchy distribution. These findings correspond microscopically to interstitial pneumonia, with alveolar, peribronchial and peribronchiolar mononuclear infiltrates. Obvious damage of epithelial cells lining airways or alveoli is absent. Peribronchial fibrosis and bronchiolitis fibrosa occur in advanced cases (Clark, 1997; Segalés *et al.*, 2004b). Pulmonary interstitial septa are often distended and filled with liquid (interstitial edema). Other frequent lesions in the lungs are catarrhal-purulent bronchopneumonia of the anterior lobes, or even necrotizing pneumonia, attributable to secondary superimposed bacterial infections.

In the majority of PMWS-affected pigs, the liver may appear unchanged or slightly pale, although foci of

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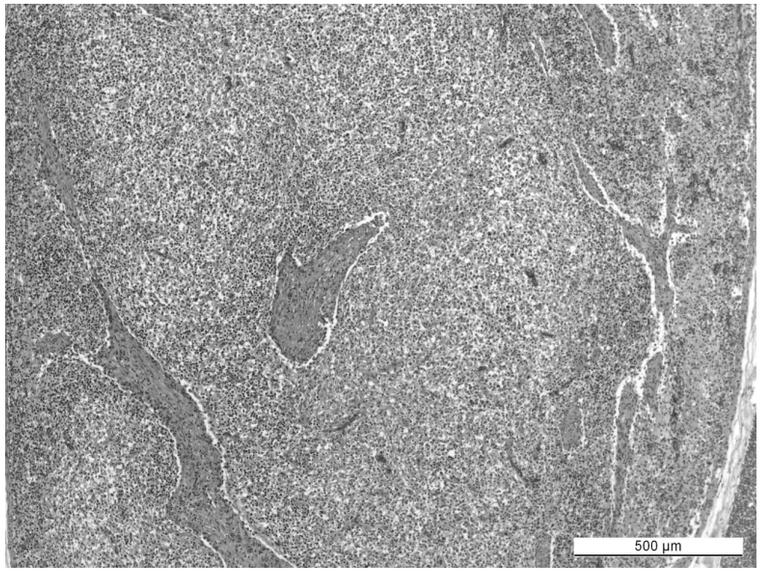

Fig. 3. Superficial inguinal lymph node of a PMWS-affected pig. Marked loss of lymphocytes (depletion) in follicular (note the lack of follicular areas) and interfollicular zones. Hematoxylin and eosin stain.

mononuclear cells infiltrating the liver parenchyma and a variable number of single hepatocytes showing clear signs of apoptosis may be seen in a proportion of animals (Rosell *et al.*, 1999, 2000a). In a few cases, the liver is enlarged, pale, and firm in consistency, with a fine granular surface (Rosell *et al.*, 2000a). This picture corresponds microscopically to a widespread cytopathic change and inflammation, characterized by disruption of liver plates and extensive cytopathic changes of hepatocytes (with cyto- and karyo-megalia, and chromatin margination). 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 lobullar margins, and inflammatory infiltrates through all

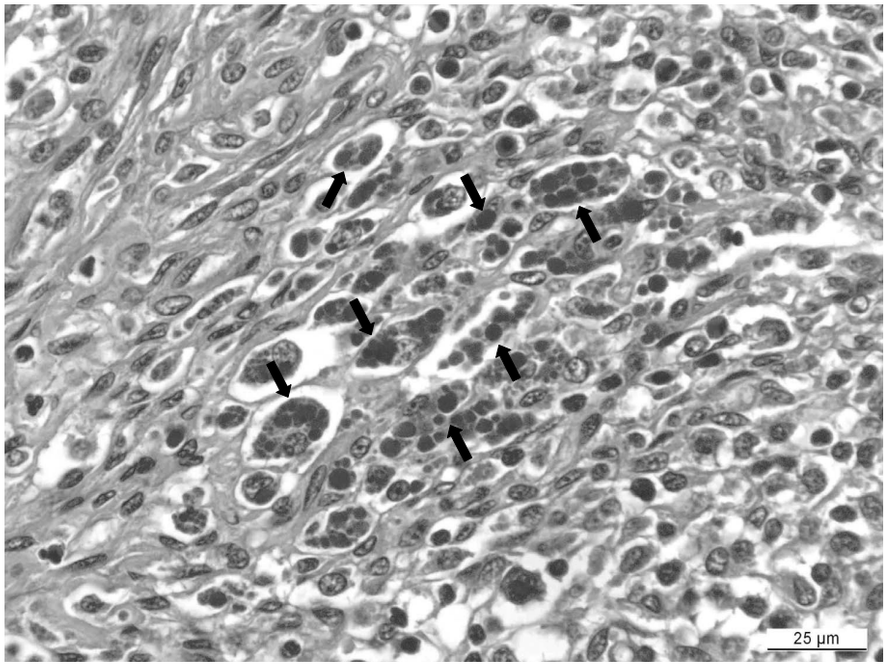

Fig. 4. Section through Peyer's patch from the ileum of a PMWS-affected pig. Massive presence of dark round intracytoplasmic inclusion bodies of variable diameter (arrows) characteristic of PCV2 infection within macrophages. Hematoxylin and eosin stain.

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Fig. 5. PDNS-affected pig. Presence of irregular, red-to-purple macules and papules in the skin, in a generalized distribution but mainly located in limbs and perineal area, where they tend to coalesce. These lesions correspond to necrotizing cutaneous lesions.

the hepatic tissue (Krakowka *et al.*, 2000; Rosell *et al.*, 2000a). Pigs may show generalized icterus at this latter stage.

Some pigs show white spots in the kidney cortex (non-purulent interstitial nephritis), a lesion which, at this age, is found almost exclusively in PMWS cases. Renal clinical disease is not seen, even in cases of widespread inflammatory infiltration, macroscopically visible. Gastric ulceration of the *pars oesophagea* has also been reported frequently in PMWS-affected pigs (Segalés *et al.*, 2000), although no direct relationship between PCV2 infection and this lesion has been established. Other organs such as pancreas, gastric and intestinal mucosa, heart, adrenal glands, salivary glands, bone marrow may show also foci of lympho-histiocytic inflammatory infiltrates, although in a lesser intensity and extension than the main affected organs cited before.

PDNS

Clinical signs

PDNS may affect nursery and growing pigs, and, sporadically, adult animals (Drolet *et al.*, 1999). The prevalence of the syndrome in affected herds is relatively low, being less than 1% (usually between 0.05 and 0.5%) (Segalés *et al.*, 1998). However, higher prevalences have been detected in the United Kingdom and other countries, with case mortality in affected herds ranging from 0.25 to 20%, or even higher (Gresham *et al.*, 2000). Mortality among pigs aged 3-month-old or older approaches 100%, while approximately half of affected pigs aged between 1.5 and 3 months die. Pigs with severe acute disease die within a few days after the onset of clinical signs. Surviving pigs tend to recover and gain weight 7–10 days after the beginning of the syndrome (Segalés *et al.*, 1998).

PDNS-affected pigs have anorexia, depression, prostration, stiff-gait and/or reluctance to move, and normal temperatures or mild pyrexia (Segalés *et al.*, 1998; Drolet *et al.*, 1999). However, the most obvious sign in the acute phase of the disease is the presence of irregular, red-to-purple macules and papules on the skin, mainly located on the hind limbs and perineal area (Fig. 5), which tend to coalesce, and may be of generalized distribution in most severely affected animals. With time, the lesions become covered by dark crusts, and fade gradually (usually in 2–3 weeks), sometimes leaving scars (Drolet *et al.*, 1999).

The cause of death in PDNS-affected pigs is an acute renal failure (Smith *et al.*, 1993; Helie *et al.*, 1995; Segalés *et al.*, 1998), with usually very marked increase in serum levels of creatinine and urea.

Lesions

In the skin, red-to-dark macules and papules correspond microscopically to necrotic tissue associated with necrotizing vasculitis of dermal and hypodermal capillaries and arterioles, and extensive hemorrhages (Helie *et al.*, 1995; Segalés *et al.*, 1998; Thibault *et al.*, 1998). Necrotizing vasculitis is, moreover, a systemic feature, since these lesions can be present in any tissue, although they are more prominent in the skin, kidney pelvis, mesenterium and spleen (Thibault *et al.*, 1998).

Apart from skin lesions, pigs which die acutely with PDNS have bilaterally enlarged kidneys that are firm in consistency, with fine granular cortical surface and edema of the renal pelvis (Helie *et al.*, 1995; Segalés *et al.*, 1998; Thibault *et al.*, 1998). The renal cortex shows multiple small reddish pinpoint lesions, similar to petechial hemorrhages, which microscopically correspond to

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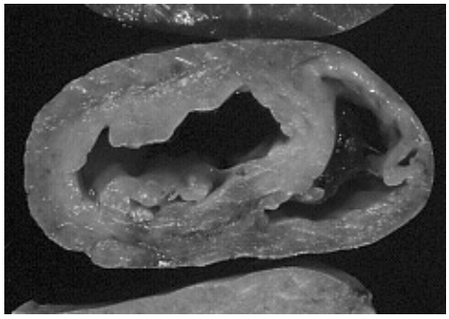

Fig. 6. Transverse section of the heart of an aborted fetus from a case of PCV2-associated reproductive failure. The myocardium shows multiple and irregular pale areas that correspond to histological lesions of necrosis (picture kindly provided by Dr John Ellis, University of Saskatchewan, Canada).

enlarged and inflamed glomeruli, with fibrin, necrotic inflammatory cells and erythrocytes within Bowman's space (fibrino-necrotizing glomerulitis). Histologically, a moderate to severe non-purulent interstitial nephritis with dilation of renal tubules is also seen. Pigs with a prolonged disease course may show chronic glomerulonephritis, probably resulting from progression of the initial glomerular damage (Segalés *et al.*, 1998). Normally, both skin and renal lesions are present in PDNS, but in rare cases, skin or renal lesions may occur alone (Segalés, 2002).

Renal lymph nodes, as well as other nodes, may be enlarged and of red color due to blood drainage from affected zones with hemorrhages (skin mainly). Microscopically, lesions similar to PMWS such as lymphocyte depletion and certain degree of histiocytic and/or multinucleate giant cell infiltration are frequently observed in lymphoid tissues of PDNS-affected pigs (Rosell *et al.*, 2000b).

Spleen infarcts may be also present (Segalés *et al.*, 1998), as a result of necrotizing vasculitis of splenic arteries or arterioles.

Reproductive disease

Clinical signs

PCV2 has been linked to reproductive failure characterized by late term abortions and stillbirths in the absence (West *et al.*, 1999) or presence (O'Connor *et al.*, 2001) of other well-established reproductive pathogens. Almost all of these descriptions of reproductive disorders are from North-America, and very few cases have been reported in Europe (Ladekjaer-Mikkelsen *et al.*, 2001).

Lesions

Stillborn and non-viable neonatal piglets present hepatic chronic passive congestion and cardiac hypertrophy with multifocal areas of myocardium discoloration (Fig. 6) (West *et al.*, 1999; O'Connor *et al.*, 2001). The main microscopic lesion is present in the heart, where a fibrosing and/or necrotizing myocarditis is the hallmark of PCV2-associated reproductive disease (West *et al.*, 1999; O'Connor *et al.*, 2001).

Immunity

Immune response to PCV2 and protection

In experimentally infected pigs, seroconversion to PCV2 has been demonstrated to occur between 14 and 28 days PI (Allan *et al.*, 1999a; Balasch *et al.*, 1999; Pogranichniy *et al.*, 2000; Krakowka *et al.*, 2001). Seroconversion has been demonstrated in experimentally infected pigs with and without clinical disease but some studies have shown that clinically diseased pigs seroconvert at a later stage PI with PCV2 (Bolin *et al.*, 2001; Rovira *et al.*, 2002; Okuda *et al.*, 2003).

Under field conditions, colostral antibodies typically decline during the lactating and nursery periods, followed by an active seroconversion (Rodríguez-Arrioja *et al.*, 2002; Rose *et al.*, 2002; Blanchard *et al.*, 2003b; Larochelle *et al.*, 2003). This seroconversion usually occurs around 7–12 weeks of age, and antibodies may last at least until 28 weeks of age (Rodríguez-Arrioja *et al.*, 2002). PMWS is not usually observed in pigs younger than 4 weeks of age (Rodríguez-Arrioja *et al.*, 2002), which may be associated with protective maternal immunity against the development of PMWS, based on field and experimental studies (Allan *et al.*, 2002b; Calsamiglia *et al.*, 2004b; Ostanello *et al.*, 2005). However, another field study has shown no significant protective effect against PMWS in association with high levels of colostral-derived serum antibodies to PCV2 (Hassing *et al.*, 2004). Although a humoral immune response to PCV2 in the field takes place around 2–3 months of age, a variable percentage of growing or finishing pigs may be viremic, suggesting that the PCV2 antibodies are not fully protective against the infection (Rodríguez-Arrioja *et al.*, 2002; Larochelle *et al.*, 2003; Sibila *et al.*, 2004) This situation also seems to occur in adult pigs under field conditions, since they can be infected but do not show apparent or detectable clinical signs (Calsamiglia *et al.*, 2002). Whether this is due to humoral immunity to PCV2 or natural age-resistance is not known at present.

Only one report has dealt with sero-neutralizing antibodies to PCV2 (Pogranichniy *et al.*, 2000). Virus-neutralizing antibodies were not detected until day 28 PI. As neutralizing antibodies developed, cross-reactivity with PCV1 also developed using this serological test. The neutralizing activity to PCV2 was previously assessed using monoclonal antibodies (McNeilly *et al.*, 2001), but it has recently being characterized, and it seems that amino acid sequences spanning residues 47 to 57 and residues 165 to 200 of the capsid protein (codified by ORF2) may

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be involved in constituting at least one neutralizing epitope of the virus (Lekcharoensuk *et al.*, 2004).

Interaction between PCV2 and the immune system in PMWS

The effect of PCV2 on the immune system in PMWS-affected and PCV2-subclinically infected pigs has not been fully characterized. It has shown experimentally that stimulation and/or activation of the immune system of PCV2-infected pigs by some viruses or non-infectious factors up-regulate PCV2 replication and increases viral loads in tissues and serum (Allan *et al.*, 1999a; Krakowka *et al.*, 2000, 2001; Harms *et al.*, 2001; Rovira *et al.*, 2002), indicating that PCV2 infection and immunostimulation can be pivotal events in the development of PMWS (Krakowka *et al.*, 2001). Conversely, typical microscopic lymphoid lesions in tissues from PMWS-affected pigs (Clark, 1997; Rosell *et al.*, 1999), the association of the disease with opportunistic pathogens (Clark 1997; Carrasco *et al.*, 2000; Nuñez *et al.*, 2003; Segalés *et al.*, 2003c), and other changes in immune cell subpopulations of lymphoid tissues and PBMC (Segalés *et al.*, 2001; Darwich *et al.*, 2002, 2003b; Chianini *et al.*, 2003; Nielsen *et al.*, 2003) are regular features of PMWS in severely affected pigs, suggesting an immunosuppressive status in diseased pigs (Segalés *et al.*, 2004a).

Recent studies on field cases of PMWS have shown that pigs with clinical disease have an evident and significant alteration of the cytokine mRNA expression patterns of several pro-inflammatory and regulatory cytokines in different lymphoid tissues (Darwich *et al.*, 2003b). However, it is not clear from these studies whether these altered profiles were related to the development of PMWS or were a consequence of the severely altered cell population dynamics in lymphoid tissues of diseased animals, when compared to non-diseased animals. In these studies, a significant overexpression of IL-10 mRNA in the thymus of PMWS-affected pigs, compared to non-affected pigs, was reported, which was associated with thymic depletion and atrophy of this organ (Darwich *et al.*, 2003b). In contrast, Sipos *et al.* (2004) reported no significant differences in the expression of cytokines in blood and tissue samples from field cases of PMWS-affected pigs when compared to non-affected pigs, and these workers concluded that their results did not support either a Th1 profile response to viral infection or a profile indicative of T cell immunosupression. However, the same authors suggested that animals under investigation were probably at the remission stage of the disease, potentially ameliorating differences between both groups (Sipos *et al.*, 2004). Studies on sequential blood samples from pigs experimentally infected with PCV2 have also indicated an increase in IL-10 production in PMWS-affected inoculates, compared to inoculates that remained subclinically infected; this increase was only detected late in the infection and disease process and was deemed by the authors to be reflecting the effects of clinical PMWS development rather than contributing to the initiation of the disease study (Stevenson *et al.*, 2004). However, in the same study a consistent down-regulation of interferon was noted early in infected pigs that developed PMWS when compared to infected pigs that remained subclinically infected. It was concluded that the inability of some PCV2-infected pigs to produce interferon early in the infection process may be a key factor in an inappropriate immune response to PCV2 infection, leading to disease (Stevenson *et al.*, 2004). Reduction in interferon production and increase of IL-10 production in PCV2 experimentally infected pigs that develop PMWS have now been confirmed by other workers using a different experimental model (Hasslung *et al.*, 2005). These effects are probably related to the CpG motifs identified on the PCV2 genome that inhibit interferon alpha production by other CpG motifs and other viruses (Hasslung *et al.*, 2003).

In vitro studies on PBMC from healthy and PMWS-diseased pigs have revealed substantial and specific effects on the functional capabilities of PBMC of PMWS pigs in terms of cytokine release (Darwich *et al.*, 2003a). On the other hand, no specific differences were seen in expression of cell surface markers of PBMC or alveolar macrophages exposed *in vitro* to PCV2 when compared to mock-infected controls (Gilpin *et al.*, 2001; Vincent *et al.*, 2003).

In vitro PCV2 infection of blood and plasmacytoid DC as well as DC precursors has shown that the virus does not inhibit DC differentiation, and the ability to process and present antigen to T lymphocytes by the cells is maintained (Vincent *et al.*, 2005). However, PCV2 seems to be able to impair the effect of CpG-ODN on plasmacytoid DC (natural interferon producing cells, NIPC) by inducing inhibition of IFN-α and TNF-α. An immunomodulatory effect of PCV2 on NIPC has been described, suggesting that PCV2-infected pigs, or at least those with PMWS, would be more susceptible to concomitant infections (Vincent *et al.*, 2005).

The mechanisms responsible for lymphocyte depletion and immunodeficiency associated with PCV2 infection have been poorly investigated. Some authors have pointed out apoptosis as a mechanism for B cell depletion in lymph nodes (Shibahara *et al.*, 2000); however, opposite results have been published (Mandrioli *et al.*, 2004). A very recent study has further supported the fact that apoptosis is not a remarkable feature in PMWS lymphoid lesion development (Resendes *et al.*, 2004a). Moreover, there is evidence that lymphocyte proliferation could be inhibited in the thymus and secondary lymphoid organs (Darwich *et al.*, 2003b; Mandrioli *et al.*, 2004). Therefore, altogether, the most recent findings suggest that apoptosis is not a significant mechanism that would explain the hallmark lesions in lymphoid tissues of PMWS-affected pigs.

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The results generated by different workers on the interactions of PCV2 with the porcine immune system to date are controversial and further studies are required. These studies should focus on the interactions following infection and prior to the development of clinical disease in an attempt to elucidate the pathways that determine clinical and/or subclinical infections.

Immune system and non-PMWS PCVD

Minimal information is available in regards the immune system and other non-PMWS PCVD conditions.

It has been suggested that excessive PCV2 antibody titers may trigger the development of PDNS (Wellenberg *et al.*, 2004), but no experimental demonstration of this has been achieved as yet. On the other hand, it has been suggested that cytokine profiles and hematological data of PDNS-affected pigs indicates a pro-inflammatory condition, supporting a Th1 bias in these animals (Sipos *et al.*, 2005). However, in this study no detailed pathological description of the animals was given and a surprising lack of differences in urea and creatinine levels between PDNS and PMWS was noted (Sipos *et al.*, 2005).

The reproductive form of PCVD appears to be a rare event under field conditions (Pensaert *et al.*, 2004). It has been suggested that this is probably due to the widespread PCV2 seroprevalence in adult pigs, which could result in the breeding herd being immune to the virus and, therefore, not susceptible to the clinical disease.

Diagnosis

Differential diagnosis

The differential diagnosis list for PMWS can be very extensive depending on the dominant clinical sign in each farm. The first and most important entity to be included in the list is the respiratory form of PRRS; however, the widespread distribution of PRRS in most countries makes the differentiation between PRRS and PMWS very difficult, unless an appropriate battery of laboratory tests for PRRS virus and PCV2 is used at the same time. Moreover, all diseases and conditions that cause wasting have to be included in the differential diagnosis list (Harding and Clark, 1997). Besides PRRS, conditions like PRDC, Glässer's disease, classical swine fever, pseudorabies, blue eye disease, carbadox/olaquindox toxicity, postweaning colibacillosis, swine dysentery, porcine colonic spirochetosis, porcine intestinal adenomatosis, *pars* esophagica gastric ulceration, and eperythrozoonosis should be, depending on the country of origin, included in the list.

The differential diagnosis list for PDNS should include those conditions causing red to dark discoloration of the skin as well as those that cause petechial hemorrhages in the kidneys (Segalés, 2002). Special emphasis should be made on the gross lesional similarities between PDNS and classical/African swine fever, but septicemic salmonellosis or erysipelas should also be considered in the differential diagnosis list.

The reproductive form associated with PCV2 can clinically be indistinguishable from other swine diseases that cause late-term abortions and stillbirths such as PRRS, pseudorabies, PPV infection, swine influenza, blue eye disease, enterovirus infections, classical swine fever, erysipelas and leptospirosis, among others.

Diagnosis of PCVD

PMWS

The diagnostic criteria for PMWS in single animals are now well established (Sorden, 2000; Segalés, 2002). Classically, a pig or a group of pigs suffer from PMWS if they fulfill the following criteria (Sorden, 2000):

(1) Clinical signs including growth retardation and wasting, frequently with dyspnea and enlargement of inguinal lymph nodes, and occasionally with jaundice.
(2) Presence of characteristic histopathological lesions in lymphoid tissues (lymphocyte depletion together with granulomatous inflammation, and presence of inclusion bodies in a proportion of affected pigs).
(3) Detection of moderate to high amounts of PCV2 within the lesions in lymphoid and other tissues of affected pigs.

This case definition does not exclude the concomitant presence of other diseases together with PMWS. It also implies that neither clinical signs nor gross lesions observed in suspected PMWS-affected pigs are sufficient to diagnose the disease, although they are indicative. However, the presence of clinical signs, PMWS compatible gross lesions and the demonstration of high levels of PCV2 by different methods (immunocytochemistry on cryostat sections, quantitative antigen capture or quantitative PCR) can be considered diagnostic in the absence of access to histopathological expertise.

A herd case definition for PMWS should include the occurrence of a clinical process, characterized mainly by wasting and mortality, in excess of the expected and/or historical level for each farm, and the establishment of individual diagnoses, as described above, of the disease in a number of pigs (Segalés *et al.*, 2003a). However, the herd case definition for PMWS has not been formally established as yet.

PDNS

The case definition for PDNS is relatively simple (Smith *et al.*, 1993; Helie *et al.*, 1995) and includes two main criteria:

(1) Presence of hemorrhagic and necrotizing skin lesions, mainly located on the hind limbs and perineal area, and/or swollen and pale kidneys with generalized cortical petechia.

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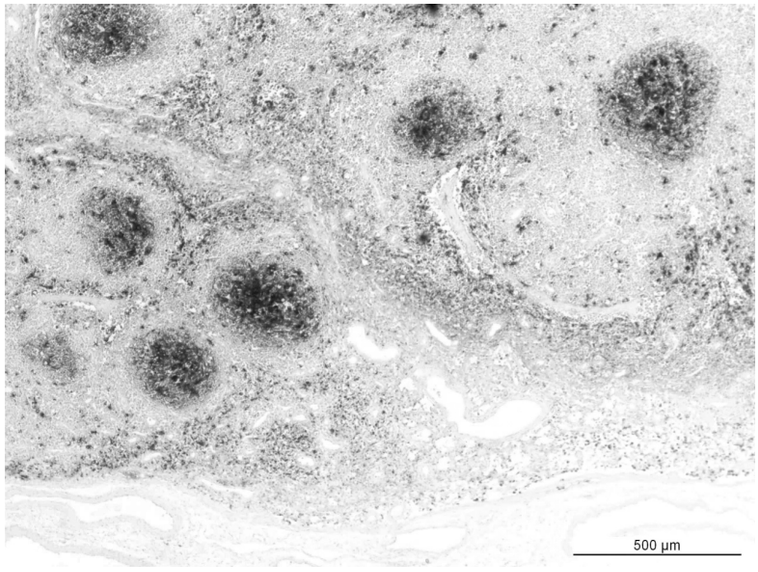

Fig. 7. Superficial inguinal lymph node of a PMWS-affected pig. Moderate to marked presence of PCV2 nucleic acid in the cytoplasm of macrophages and DC (dark stained cells) in a severe case of lymphocyte depletion and granulomatous inflammation. ISH to detect PCV2; fast green counterstain.

(2) Presence of systemic necrotizing vasculitis, and necrotizing and fibrinous glomerulonephritis.

From a diagnostic point of view, detection of PCV2 is not currently included in the diagnostic criteria for PDNS.

Reproductive disease

The case definition for reproductive problems associated with PCV2 infection has not been formally established, but taking into account the clinico-pathological features of the described cases (West *et al.*, 1999), it should include three main criteria:

(1) Late-term abortions and stillbirths, sometimes with evident hypertrophy of the fetal heart.
(2) The presence of heart lesions characterized by extensive fibrosing and/or necrotizing myocarditis.
(3) Presence of high amounts of PCV2 in the myocardial lesions and other fetal tissues.

Laboratory confirmation

Methods of virus detection

Several methods have been developed to detect PCV2 in tissues and to correlate its detection with the presence of lesions. Among them, ISH and immunohistochemistry are the most widely used tests (McNeilly *et al.*, 1999; Rosell *et al.*, 1999) for the diagnosis of PCVD. PCV2 nucleic acid or antigen in PMWS and PDNS affected pigs is usually found in the cytoplasm of histiocytes, multinucleate giant cells and other monocyte/macrophage lineage cells such as alveolar macrophages, Kupffer cells and follicular DC of lymphoid tissues (Rosell *et al.*, 1999, 2000a; Allan and Ellis, 2000). It is also possible to detect viral nucleic acid or antigen in the cytoplasm of renal and respiratory epithelia, vascular endothelium, pancreatic acinar and ductular cells and nuclei of monocyte/macrophage lineage cells, smooth muscular cells, pancreatic acinar and ductular cells, hepatocytes and enterocytes (Kiupel *et al.*, 1999; McNeilly *et al.*, 1999; Rosell *et al.*, 1999, 2000a, b; Sirinarumitr *et al.*, 2000). The main cell containing PCV2 in fetuses is the myocardiocyte (Sánchez *et al.*, 2001a).

A strong correlation has been observed between the amount of PCV2 nucleic acid or antigen and the severity of microscopic lymphoid lesions in PMWS (Fig. 7) (Rosell *et al.*, 1999; Quintana *et al.*, 2001). The detection of PCV2 antigen or nucleic acid in tissues from clinically healthy pigs or diseased pigs without clinical signs and gross lesions consistent with PMWS should be interpreted with caution since subclinical PCV2 infection with viremia occurs in almost all farms (Rosell *et al.*, 2003b).

Since the amount of PCV2 demonstrated in tissue samples with lesions is the major difference between PMWS-affected pigs and PCV2 subclinically infected pigs, techniques that allow the quantification of virus in tissues and/or serum, such as quantitative PCR methods, antigen capture ELISA and immunocytochemical analysis of cryostat sections could potentially be used to diagnose PMWS (McNeilly *et al.*, 2002; Olvera *et al.*, 2004). This does not apply to PDNS (Olvera *et al.*, 2004), but probably applies to the PCV2-associated reproductive disease, since the amount of virus observed in tissues with lesions is high (West *et al.*, 1999).

The non-quantitative, gel-based PCR technique has been shown as a very sensitive test for the detection of PCV2 (Larochelle *et al.*, 1999; Hamel *et al.*, 2000; Kim and Chae, 2001; Calsamiglia *et al.*, 2002; Quintana *et al.*, 2002) but virus

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detection of PCV2 nucleic acid in serum or inguinal lymph node has not been shown to be correlated with the typical microscopic PMWS lymphoid lesions of the syndrome when compared with ISH (Calsamiglia *et al.*, 2002). Non-quantitative PCR methods should not be used to diagnose PCVD.

Methods of antibody detection

Several serological techniques to detect antibodies to PCV2 have been developed (Rodríguez-Arrioja *et al.*, 2000; Walker *et al.*, 2000; Nawagitgul *et al.*, 2002; Blanchard *et al.*, 2003b; McNair *et al.*, 2004). These tests are based on cell cultures infected with PCV2 (immunoperoxidase monolayer and indirect immunofluorescence assays) or ELISA methods (plates coated with a monolayer of PCV2 infected cell culture or baculovirus-expressed PCV2 capsid protein). Most of these tests have been developed by PCV2 research groups to monitor PCV2 infections in experimental studies and epidemiological investigations, but no commercial availability of PCV2 serology exists at present. This is probably due to the fact that PCV2 is ubiquitous and the seroconversion pattern is relatively similar in PMWS affected and non-affected farms (Rose *et al.*, 2002; Larochelle *et al.*, 2003; Sibila *et al.*, 2004), which do not allow use of serological techniques for diagnostic purposes.

Prevention and control

Because PCV2 infection is ubiquitous, to date, minimal efforts have been directed to the control of the infection. Among PCVD, PMWS is the disease scenario with the major economic impact on swine production. As described above, PMWS is now defined as a multifactorial disease which involves infection of pigs with PCV2 and the influence of infectious and non-infectious factors or triggers for the development of clinical disease. Consequently, effective control measures to date for PMWS, without the control of PCV2 infection, have focused on the understanding of the co-factors and triggers involved in individual farms and the control or eradication of these triggers. The most studied co-factors and triggers in relation to disease progression or protection are outlined below.

Management measures

Prospective studies carried out in France from 1998 (Madec *et al.*, 2000) have clearly shown that management deviations occurred in severely PMWS-affected farms. As a result of these studies it was suggested that several environmental conditions might be necessary in association with PCV2 infection to lead to the clinical expression of the disease. The implementation of what is today known as the Madec's 20-point plan (a list of management measures to lower the impact of the disease) significantly decreased the percentage of mortality in severely affected farms (Madec *et al.*, 2001). These measures were designed to reduce 'infection pressure' in regard to PCV2 and any other infections, improve hygiene and to reduce stress at the different production stages (Madec *et al.*, 2000; Madec and Waddilove, 2002). Significant positive results have been obtained when these management measures were applied and a significant improvement in loss rates was achieved when the rate of compliance with the recommended measures was high (Guilmoto and Wessel-Robert, 2000).

The effect of concurrent viral infections

Viral co-infections with PCV2 have been used to experimentally reproduce PMWS (Allan *et al.*, 1999a; Krakowka *et al.*, 2000; Harms *et al.*, 2001; Rovira *et al.*, 2002). These experimental results have been further supported by epidemiological data (Rose *et al.*, 2003b) and a wide spectrum of infectious agents has been observed concomitant with PCV2 infection in PMWS-affected farms (Rodríguez-Arrioja *et al.*, 1999; Pallares *et al.*, 2002; Pogranichniy *et al.*, 2002; Segalés *et al.*, 2002; Segalés and Domingo, 2002; Ellis *et al.*, 2004). Therefore, the control of concurrent viral infections in the post-weaning area should decrease the incidence of PMWS. From a practical point of view, attempts to control PMWS with PPV vaccination on finishing sites in the USA with confirmed PPV circulation have been repeatedly successful (Halbur, 2001). However, this positive effect of PPV vaccination in reducing the clinical incidence of PMWS has not been experimentally proven (Opriessnig *et al.*, 2004b). To date, no published results are available on the control of PRRSV infection (by vaccination or other systems) to mitigate the effects of PMWS.

The stimulation of the immune system

The induction of clinical disease following immunostimulation in PCV2 experimentally infected pigs (Krakowka *et al.*, 2001; Opriessnig *et al.*, 2003, 2004c) has also been supported by a number of on-farm studies, where PCV2 infection and the use of certain commercially available pig vaccines (mainly bacterins containing *Mycoplasma hyopneumoniae* or *Actinobacillus pleuropneumoniae*) or immunomodulators have acted as apparent triggering factors for PMWS (Allan *et al.*, 2001; Kyriakis *et al.*, 2002). Although other experiments have failed to confirm the effect of immunostimulation (Ladekjaer-Mikkelsen *et al.*, 2002; Resendes *et al.*, 2004b), these results indicate that immune activation may be an important triggering factor of PMWS and a pivotal event in the pathogenesis of this disease on some farms.

Recently, it has been experimentally shown that the ability of vaccine-induced enhancement of PCV2 replication and PCVD seems to depend on the type of

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vaccine adjuvant (Krakowka *et al.*, 2005) and also on the timing of the administration of the vaccine product (Opriessnig *et al.*, 2004c). Regarding this last point, no or minimal PCV2-associated lesions were developed when pigs were vaccinated with a commercially available *M. hyopneumoniae* vaccine 2–4 weeks prior to the expected PCV2 exposure, at about 8 weeks of age (Opriessnig *et al.*, 2004c). On the other hand, in gnotobiotic piglets, it has been observed that mineral oil-based adjuvants are able to potentiate PCV2 replication, thus promoting induction of PMWS. In contrast, plant oil-based and alum-based adjuvants seemed to have minimal apparent effects upon PCV2 replication or the induction of PMWS (Krakowka *et al.*, 2005).

From a practical point of view, to exclude the use of vaccines from sanitary programmes may be inappropriate, since the risk of eliminating effective vaccines may be greater than the risk of inducing PMWS in a low percentage of pigs in a given pig population. Therefore, based on the available results, producers with PMWS-affected herds should consider determining the approximate timing of PCV2 infection, with the objective of re-scheduling the timing of vaccination as a potential plan to minimize the disease (Opriessnig *et al.*, 2004c).

The infectious PCV2 status and the serological titers to PCV2 of the sow at farrowing

Initial observations from a study on PMWS-affected farms in France indicated that pigs that develop PMWS corresponded to a few litters, suggesting a possible litter effect (Madec *et al.*, 2000). In further studies (Allan *et al.*, 2002b; Calsamiglia *et al.*, 2004b), it was reported that PCV2 infection or low serological titers to PCV2 in sows at farrowing had a significant effect on the overall mortality of its offspring due to PMWS. Conversely, 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 protection from PMWS in the piglets derived from these animals (Hassing *et al.*, 2004). However, the protective effect of maternal passive immunity on PMWS development is supported by the fact that disease occurs once these titers 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. From a practical point of view, these possibilities could potentially be achieved by PCV2 vaccination of gilts and sows, and/or controlled PCV2 infection of gilts during the acclimatization period.

Role of nutrition on PMWS

Partial control of epizootic PMWS has been achieved on some farms in the United Kingdom by changes in the diet of affected pigs (Donadeu *et al.*, 2003). These changes included an increase in the nutrient density of young pig diets and addition of commercial feed additives, most of them with anti-oxidant effects. However, these results have not been confirmed by other workers. On the other hand, a recent study has shown that conjugated linoleic acid (CLA) ameliorates PCV2 experimental infection (Bassaganya-Riera *et al.*, 2003). Finally, it has been suggested that the addition of vitamin E and/or selenium in the feed may be of benefit in those farms with PMWS (Baebko *et al.*, 2004). Overall, although some preliminary field and experimental data on nutrition suggest that certain nutritional factors might favor a decrease in PMWS outcome, there is not enough scientific information available to establish the real effect of nutrition in this particular disease.

PCV2 vaccination

An inactivated, ajuvanted PCV2 vaccine for use in sows and gilts (Reynaud *et al.*, 2004a, b) is now commercially available and in use under special license in some European countries. The efficacy of this vaccine in controlling PMWS under field conditions remains to be determined.

The use of an experimental inactivated PCV2 vaccine in postweaning piglets has already shown clinical efficacy (Pogranichniy *et al.*, 2004), but no further experiments have been performed.

Experimental PCV2 vaccine prototypes, including recombinant and DNA vaccines have also been tested (Blanchard *et al.*, 2003a). In this study, in a first trial, a group of piglets received a first injection with plasmids directing ORF2 protein and granulocyte-macrophage colony-stimulating factor (GM-CSF) expression, followed by a second injection of baculovirus-expressed ORF2 protein. In a second trial, protection induced by a subunit vaccine (including the baculovirus-expressed ORF2 protein) was even better than that induced by the DNA vaccine, since PCV2 replication was completely inhibited. In both cases, significant protection was achieved as determined by measurement of body weight and rectal temperatures after PCV2 challenge in piglets. Although these vaccine strategies could be of interest, it is presumed that cost of a vaccine would not be acceptable under commercial circumstances.

A chimeric infectious DNA clone containing the immunogenic ORF2 capsid gene of PCV2 cloned into the non-pathogenic PCV1 genetic backbone induced antibody response to PCV2 capsid when inoculated in pigs and was shown to be attenuated compared to the PCV2 virus (Fenaux *et al.*, 2003). These latter results are promising in regards the potential use of this chimeric PCV as a vaccine candidate, especially due to the fact that protective immunity against the wild-type PCV2 challenge in pigs vaccinated with the chimeric virus has been

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reported (Fenaux *et al.*, 2004a). On the other hand, it has been recently demonstrated that a PCV2 isolate serially passaged in PK-15 cells (120 times) had two amino acid mutations in the capsid protein (Fenaux *et al.*, 2004b). These mutations enhanced the growth ability of PCV2 *in vitro* and attenuated the virus *in vivo*, suggesting potential implications for PCV2-attenuated vaccine development.

'Serum-therapy'

Subcutaneous injection of PCV2 hyperimmune sera from commercial slaughterhouse age pigs in suckling or nursery pigs has been reported as successfully reducing mortality in several PMWS-affected farms (Ferreira *et al.*, 2001; Waddilove and Marco, 2002). However, success of this procedure has been variable, and the use of 'serum-therapy' in some farms did not result in any significant effect. It is important to note that strict precautions must be applied when performing serum-therapy. Blood collection must be from the same affected farm to avoid any risk of introducing other pathogenic agents and bleeding and injections must be carried out under maximal hygiene conditions. The mechanism of action of serum-therapy has not been elucidated as yet.

Genetics

Field observations from farmers and veterinarians have suggested that certain genetic lines of pigs, specifically in relation to boar lines, are more or less susceptible to PMWS. This observation has been supported by recent experimental studies where Landrace pigs were experimentally shown to be more susceptible to development of PMWS lesions than Duroc and Large White pigs (Oppriessnig *et al.*, 2004a). Other studies have shown contradictory results with the use of Pietrain boar line; while the use of this genetic line did not seem to have any effect on the offspring in one study (Rose *et al.*, 2003a), another study showed lower general postweaning and PMWS-associated mortalities (López-Soria *et al.*, 2004). These findings with regard to the role of genetics in susceptibility/resistance to PCVD 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).

Discussion

On the basis of the current knowledge on PCV2 infection, it is evident that the clinical and pathological scope of this viral infection has been expanded since its initial association with PMWS. Little doubts on the association and causality of PCV2 on PMWS and reproductive disease exist based on experimental evidence, but the remaining clinical conditions have been reported as retrospective/prospective studies (PDNS, PNP) and clinical cases (PRDC, CT), and no experimental studies have clarified the exact role of PCV2 on them.

Among these non-experimentally tested conditions, this review has included PDNS because the available literature since the year 2000 shows a strong link with PCV2 (Rosell *et al.*, 2000b; Allan *et al.*, 2002a) although in some cases together with other concomitant viral infections (Choi and Chae, 2002). However, it must be also noted that one group of researchers has linked PDNS to infection with strains of *Pasteurella multocida* showing a certain pulsed-field gel electrophoresis pattern (Lainson *et al.*, 2002). Definitive evidence that PCV2 (or other infectious or non-infectious agent) is the antigen associated with the immune-complexes observed in PDNS is still lacking.

PRDC, PNP and CT have not been included in this review on PCVD. PRDC is characterized by slow and uneven growth, reduced feed intake, increased gain conversion rates, cough and clinical pneumonia (Thacker and Thacker, 2000). Major pathogens involved in PRDC are PRRSV and *M. hyopneumoniae*. Taking into account that an important number of bacterial and viral pathogens may contribute to PRDC outbreaks, PCV2 should be considered as just one more of these agents. Furthermore, the relative importance of this agent among the complex etiology of PRDC has not been established. In most cases, no clear-cut differences exist between PRDC and PMWS cases, since the clinical picture can be very similar or overlapping (Harms *et al.*, 2002) due to the usual respiratory component in PMWS (Segalés and Domingo, 2002). In addition, clinical cases diagnosed as PRDC with PCV2 in the lungs, where lymphoid tissues have not been examined could be, potentially, cases of PMWS. Therefore, there is a blurred border between PRDC and PMWS, basically due to the very general definition of the former syndrome (Segalés *et al.*, 2003b).

PNP is a lung lesion associated with respiratory problems in postweaning pig units (Segalés *et al.*, 2004b). This condition is, again, considered of multietiological origin, since PRRSV, swine influenza virus and PCV2 have been found in the affected lungs (Drolet *et al.*, 2003). However, it has been demonstrated that this latter viral infection is not essential for the development of PNP lesions (Drolet *et al.*, 2003).

CT was linked to PCV2 by Stevenson *et al.* (2001) in the USA. However, no further results have been generated by North-American researchers, while studies in Europe (Kennedy *et al.*, 2003) and South-East Asia (Ha and Chae, 2005) showed a lack of association between PCV2 and CT.

Finally, it is important to note that since the initial outbreaks of PMWS in Canada and Europe debates have been on-going with regard to the causal agent of the disease being PCV2 or some other infectious agent. In fact, many laboratories around the world have been

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examining a wide range of tissue samples from diseased and non-diseased pigs for evidence of a common new infectious trigger for PMWS. Recent retrospective epidemiological studies in New Zealand (Stone, 2004), Great Britain (Woodbine, 2005) and Denmark (Vigre *et al*., 2005) have suggested that, in these countries, the introduction and subsequent spread of PMWS would not be associated with PCV2 but with another, as yet unidentified, 'exotic agent'. In contrast, a recent prospective study on the outbreak of PMWS in Sweden (Wallgren *et al*., 2004) has concluded that the source of the outbreak of disease in this country was not due to the importation of an exotic infectious agent. To date, no such agent has been recovered or reported in the literature. The failure of researchers over the last 8 years to recover and identify a common new infectious agent that is consistently associated with PMWS-affected herds and individual animals does not, of course, mean that such an agent does not exist. However, the fact that PCV2 infection with certain viral load is always associated with PMWS in field outbreaks of disease, that the disease can be reproduced experimentally in a number of different models using PCV2 as the only infectious agent, and the lack of laboratory-based epidemiological evidence to support the 'exotic agent hypothesis', are strongly supportive of PCV2 being the infectious causal agent of PMWS.

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