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Antigenic characterisation of porcine circovirus 2 strains and outcome of experimental porcine circovirus 1 and 2 infections in porcine foetuses and in pigs

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List of abbreviations

aa	amino acid
Ab	antibody
AMP	anti microbial proteins
ATCC	American Type Culture Collection
b or bp	base(s) or base pair(s)
BVD	bovine viral diarrhoea
BVDV	bovine viral diarrhoea virus
Cap	capsid
CD	cluster of differentiation
CD/CD	caesarean-derived colostrum-deprived
CT	congenital tremor
DABCO	1,4-diazobicyclo-2.2.2.-octane
DC	dendritic cells
DNA	deoxyribonucleic acid
dpi	days post inoculation
dpsi	days post super-inoculation
EAV	equine arteritis virus
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
HCV	hog cholera virus
HE	hematoxylin and eosin
HI	haemagglutination inhibition
hpi	hours post inoculation
ICTV	International Committee on Taxonomy of Viruses
ICFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
IIF	indirect immunofluorescence staining
IL	interleukin
IPMA	immuno-peroxidase monoloayer assay
kDa	kilodalton
KLH	keyhole limpet hemocyanin
M	molar
mAbs	monoclonal antibodies
MEM	minimum essential medium
mg	milligram
mRNA	messenger RNA
N	neutralisation
NK	natural killer cells
nm	nanometer
ORF	open reading frame
OD	optical density
P	probability
pAb	polyclonal antibody
PASC	pairwise sequence comparisons
PBMC	peripheral blood mononuclear cells

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV	porcine circovirus
PCVD	porcine circovirus diseases
PCVAD	porcine circovirus-associated diseases
PDB	protein data bank
PDNS	porcine dermatitis and nephropathy syndrome
PEDV	porcine epidemic diarrhoea virus
PI	percent inhibition
PK	porcine kidney
PMWS	postweaning multisystemic wasting syndrome
PNP	proliferative and necrotising pneumonia
PPV	porcine parvovirus
PRDC	porcine respiratory disease complex
PRRSV	porcine reproductive and respiratory syndrome virus
qPCR	quantitative PCR
Rep	replicase protein
RNA	ribonucleic acid
SD	standard deviation
SN	seroneutralisation
SPF	specific pathogen free
ss	single-stranded
$TCID_{50}$	50% tissue culture infectious dose
TTV	torque teno virus
U	unit
VWF	Von Willebrand factor
wt/vol	weight by volume
ZP	zona pellucida

Chapter 1. Introduction

1.1. History of porcine circoviruses

A non-cytopathic, picornavirus-like contaminant of the PK-15 cell line (ATCC-CCL33) was first discovered in Germany, supposed to contain an RNA genome (Tischer et al., 1974). This picornavirus-like contaminant was subsequently described as a small, non-enveloped, icosahedral virus containing a circular single-stranded DNA genome and was named as porcine circovirus (PCV) (Tischer et al., 1982). Serological surveys demonstrated a high prevalence of anti-PCV antibodies in the swine population worldwide (Tischer et al., 1982; Tischer et al., 1986; Dulac and Afshar, 1989; Horner, 1991; Allan et al., 1994; Edwards and Sands, 1994; Tischer et al., 1995), although no disease association could be found with this virus either in PCV-positive pig farms and in experimental PCV-inoculated pigs (Tischer et al., 1986; Allan et al., 1995). Therefore, it was generally accepted that PCV is non-pathogenic to pigs (Tischer et al., 1986; Allan et al., 1995; Tischer et al., 1995).

In 1991, postweaning multisystemic wasting syndrome (PMWS), a newly emerging disease in pigs was first reported in Western Canada (Clark, 1996; Harding 1996). Later on, a new DNA-virus, morphologically similar to the PK-15 origin PCV, was isolated from the tissues of PMWS-affected pigs in Europe and North America (LeCann et al., 1997; Nayar et al., 1997; Segalés et al., 1997; Allan et al., 1998; Ellis et al., 1998; Harding et al., 1998; Kennedy et al., 1998; Kiupel et al., 1998). Despite morphological similarities, the distinct genetic and antigenic differences were noted between these two DNA viruses (Allan et al., 1998; Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998; Allan et al., 1999). Due to these differences, the PK-15 originated PCV was named as porcine circovirus type 1 (PCV1) while the new DNA-virus was designated as porcine circovirus type 2 (PCV2) (Allan et al., 1998; Meehan et al., 1998).

1.2. Taxonomy of porcine circoviruses

Porcine circoviruses (PCVs) belong to the single-stranded DNA (ssDNA) viruses (Tischer et al., 1982). According to the International Committee on Taxonomy of Viruses (ICTV), the ssDNA viruses are divided into seven different families on the basis of their genome nature (circular or linear) and host range. The *Inoviridae* and *Microviridae* infect bacteria; the *Anelloviridae* and *Circoviridae* infect vertebrates; the *Parvoviridae* infect vertebrates and invertebrates; and the *Geminiviridae* and *Nanoviridae* infect plants. The viruses of the *Parvoviridae* family have linear genomes while the viruses of other families have circular

genomes. There are a number of additional ssDNA viruses that have not been classified to date - the parvovirus like viruses. These viruses have linear ssDNA genomes but unlike the parvoviruses their genome is bipartite. A new family *Bidensoviridae* has been proposed for this group but this proposal has not been ratified by the ICTV to date (wikipedia.org/ssDNA viruses).

The *Circoviridae* family is divided into the genera *Circovirus* and *Gyrovirus* based on their morphology and genomic organisation (Pringle, 1999). Besides PCV1 and PCV2, the genus *Circovirus* includes beak and feather disease virus (Ritchie et al., 1989), pigeon circovirus (Woods et al., 1993), canary circovirus (Phenix et al., 2001), goose circovirus (Todd et al., 2001), duck circovirus (Hattermann et al., 2003), finch circovirus (Shivaprasad et al., 2004), starling circovirus (Johne et al., 2006), gull circovirus (Smyth et al., 2006) and swan circovirus (Halami et al., 2008). Raven circovirus (Stewart et al., 2006) and two recently identified fish circoviruses, barbel circovirus 1 and 2 (Lorincz et al., 2011), have not been included in the ICTV taxonomy list yet. Chicken anaemia virus (Yuasa et al., 1979) is the only species of the genus *Gyrovirus*.

1.3. Genome organisation of porcine circoviruses

PCVs are non-enveloped and the smallest animal viruses with a diameter of 17±1.3 nm (Tischer et al., 1982) and contain a covalently closed circular ssDNA genome. The virion has an icosahedral T=1 lattice containing 60 capsid protein subunits arranged in 12 flat pentamer clustered units (Crowther et al., 2003; Khayat et al., 2011). The circular PCV genome consists of 1,758-1,760 nucleotides for PCV1 and 1,766-1,769 nucleotides for PCV2 (Fig. 1) (Allan et al., 1998; Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998; Fenaux et al., 2000; Huang et al., 2011). The overall DNA sequence identity within PCV1 or PCV2 isolates is greater than 90%, while the identity between the PCV1 and PCV2 isolates is 68 to 76% (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998; Cheung and Bolin, 2002). Both PCV1 and PCV2 have a similar genomic organisation, which contains three major open reading frames (ORFs): ORF1, ORF2 and ORF3 (Fig. 1).

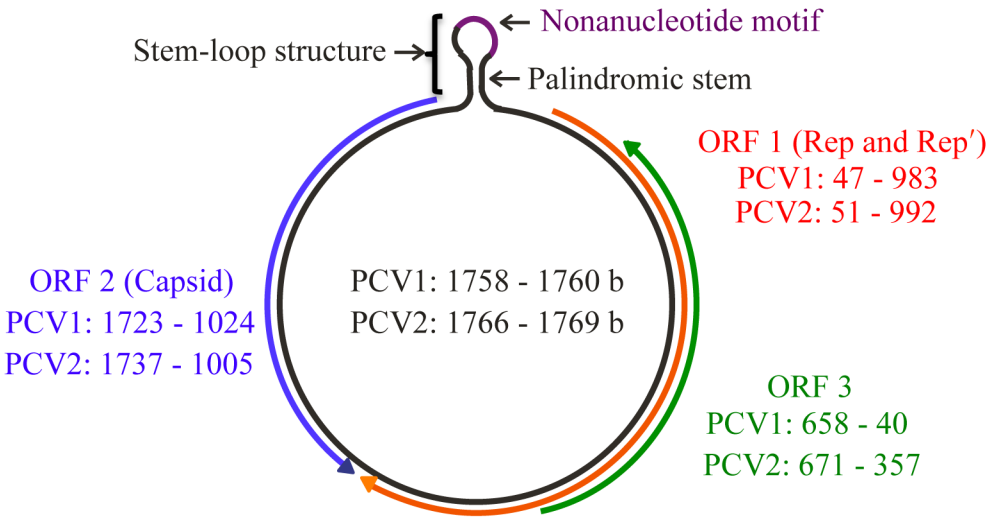

Fig. 1. Genomic organisation of PCVs. PCVs genome sizes and open reading frame (ORF) sizes are shown. Red arrow: Open Reading Frame 1 (ORF1), clockwise oriented and located on the positive strand, which encodes Rep and Rep' proteins. Blue arrow: Open Reading Frame 2 (ORF2), counter-clockwise oriented, located on the negative strand, which encodes the capsid protein. Green arrow: Open Reading frame 3, counter-clockwise oriented, located on the negative strand, which overlaps the ORF1. The figure was adapted from G. Misinzo, PhD thesis, Ghent University.

ORF1 is located on the positive strand of the viral DNA genome, and is oriented in clockwise direction (Fig. 1). It encodes for the non-structural proteins Rep (35.7 kDa) and its alternatively spliced frame-shifted variant Rep' (20 kDa), which are involved in virus replication (Mankertz et al., 1998; Mankertz and Hillenbrand, 2001; Cheung, 2003a; Mankertz et al., 2003; Shang et al., 2009). PCV1 Rep and Rep' proteins consist of 312 and 168 amino acids (aa), respectively and PCV2 Rep and Rep' proteins consist of 314 and 178 aa, respectively (Hamel et al., 1998). ORF1 is more conserved between PCV1 and PCV2 (Mankertz et al., 2004; Steinfeldt et al., 2007), with 83% nucleotide identity and 86% amino acid identity (Meehan et al., 1998; Morozov et al., 1998). Since Rep and Rep' proteins are involved in virus replication, mutations in Rep or truncated Rep' proteins of PCV can cause more than 99% reduction in viral protein synthesis and completely shut down viral DNA replication (Cheung, 2003b; Cheung 2004).

ORF2 is located on the negative strand of the viral genome and is oriented in counter-clockwise direction (Fig. 1), and encodes a structural capsid protein (Cap) (27.8 kDa) (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998; Mahé et al., 2000; Mankertz et al., 2000; Nawagitgul et al., 2000; Truong et al., 2001). The capsid protein consists of 230-233 aa

for PCV1 and 233-236 aa for PCV2 (Hamel et al., 1998; Nawagitgul et al., 2000; Lefebvre et al., 2008a; Lefebvre et al., 2009; Guo et al., 2010; Huang et al., 2011). ORF2 is more variable between PCV1 and PCV2 and have approximately 67% and 65% identity at nucleotide and amino acid levels, respectively (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998; Fenaux et al., 2000; Grierson et al., 2004a; Knell et al., 2005). Since Cap is the most variable protein, a link between capsid protein variation and the pathogenicity of PCV2 has been proposed (Larochelle et al., 2002; Todd et al., 2002).

ORF3 is completely overlapped with ORF1 and located on the complementary strand with counter-clockwise orientation (Fig. 1) (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998). It encodes a non-structural protein of 206 aa (23.2 kDa) for PCV1 and 104 aa (11.9 kDa) for PCV2. ORF3 proteins have 62% identity between PCV1 and PCV2 (Hamel et al., 1998; Meehan et al., 1998). PCV2 ORF3 has been shown to be involved with apoptosis *in vitro* and viral pathogenesis *in vivo* (Liu et al., 2005; Liu et al., 2006), but these results are highly controversial since other laboratories could not reproduce these findings (as described and reviewed by Juhan et al., 2010). However, in the same laboratory, it has recently been demonstrated that apoptosis induced by the PCV2 ORF3 enhances the release and spread of the virus *in vitro* and monocyte-mediated systemic spread of the virus *in vivo* (Karuppannan and Kwang, 2011). PCV1 ORF3 has been shown to be more toxic to different cell types and induced more apoptosis than PCV2 ORF3 (Chaiyakul et al., 2010). Further research is needed to confirm these findings.

1.4. Phylogenetic and antigenic features of PCV2

According to their genomic or amino acid sequences, or to restriction fragment length polymorphism patterns, several genotyping studies on PCV2 have been performed (De Boisséson et al., 2004; Grierson et al., 2004a; Grierson et al., 2004b; Wen et al., 2005; Gagnon et al., 2007; Martins Gomes de Castro et al., 2007; Olvera et al., 2007; Carman et al., 2008; Dupont et al., 2008; Grau-Roma et al., 2008; Timmusk et al., 2008; Wang et al., 2009) and at least three different phylogenetic groups of PCV2 have been recognised (Gagnon et al., 2007; Dupont et al., 2008, Grau-Roma et al., 2008; Timmusk et al., 2008). Olvera et al. (2007) proposed a classification system that divides the PCV2 strains into two groups: group 1, which is further subdivided into 3 clusters (PCV2-1A to PCV2-1C), and group 2, subdivided into 5 clusters (PCV2-2A to PCV2-2E) (Fig. 2). PCV2 genomes of group 1 consist of 1,766-1,767 nucleotides and PCV2 genomes of group 2 comprise 1,768-1,769

nucleotides (Olvera et al., 2007; Huang et al., 2011). Afterwards, Grau-Roma et al. (2008) proposed a non-arbitrary definition of PCV2 genotypes and divided the PCV2 strains into two genotypes (genotypes 1 and 2, based on the groups 1 and 2 of Olvera et al., 2007). Later on, a third PCV2 genotype was also noticed in archived tissue samples from Denmark (Dupont et al., 2008). The definition of PCV2 genotypes was based on the proportion of nucleotide differences between pairwise sequence comparisons (PASC) of PCV2 capsid proteins. In general, the capsid protein consists of 233 aa, except for the PCV2 strains of cluster 1C, that contains 234 aa (Olvera et al., 2007). However, recently Huang et al. (2011) demonstrated that few PCV2 strains belonging to the clusters 1A/1B and 2B (according to Olvera et al., 2007) contain 235-236 aa in the capsid protein. For phylogenetic analysis, the *cap gene* showed to be a reliable phylogenetic marker in contrast to the *rep gene* and the phylogenetic tree that was constructed with the whole viral genome, the same tree could be reconstructed with the *cap gene*, but not with the *rep gene*, due to frequent recombination in the *rep gene* (Olvera et al., 2007). Therefore, the genotyping for PCV2 was performed based on the *cap gene* (ORF2) sequences by applying PASC analysis and a distance threshold of 0.035 was considered to differentiate PCV2 genotypes (Grau-Roma et al., 2008) and consequently, three PCV2 genotypes have been defined (Segalés et al., 2008). Two of these genotypes were already defined by the different research groups using several methods of phylogenetic analysis and their definitions were fitting to each other (Segalés et al., 2008). For example, genotypes ‘1’ and ‘2’ (Olvera et al., 2007; Grau-Roma et al., 2008) correspond, respectively, to groups ‘I’ and ‘II’ (De Boisséson et al., 2004), ‘b’ and ‘a’ (Gagnon et al., 2007), ‘A’ and ‘B’ (Martins Gomes de Castro et al., 2007), ‘SG3’ and ‘SG1/SG2’ (Timmusk et al., 2008) and the restriction fragment length polymorphism patterns ‘321’ and ‘422’ (Carman et al., 2008). However, all these classifications create scientific confusions on genotype names. In order to correct the scientific confusions, the EU consortium on porcine circovirus diseases proposed a unified nomenclature for PCV2 genotypes and the European nomenclature of genotypes 1, 2 and 3 (Grau-Roma et al., 2008; Dupont et al., 2008) has been adapted from the Canadian nomenclature, which was based on the PCV2 entire genome sequences (Gagnon et al., 2007). Nowadays, genotype 1 is called PCV2b, genotype 2 is called PCV2a and genotype 3 is called PCV2c (Segalés et al., 2008; Cortey et al., 2011a). Recently, two new PCV2 genotypes (PCV2d and PCV2e) have been reported (Wang et al., 2009; Guo et al., 2010; Zhai et al., 2011). However, the definition of these two new PCV2 genotypes was consequently found to be wrong (Cortey et al., 2011a) since this definition was solely based on the distance among the complete genomes using the threshold calibrated

for the *cap gene* and no PASC analysis was performed to adapt the threshold calibrated for the *cap gene* to the complete genomes. Accordingly, PCV2d and PCV2e sequences are not considered as new genotypes. Moreover, PCV2d and PCV2e sequences of Wang et al. (2009) belong to genotypes PCV2b and PCV2a, respectively (Cortey et al., 2011a). Several retro-prospective studies have demonstrated that PCV2a was the most circulating genotype in the field from the early 90s until 2000, whereas PCV2b have become more common from 2003-04 onwards in Asia, Europe and North America (Allan et al., 2007a; Gagnon et al., 2007; Dupont et al., 2008; Timmusk et al., 2008; Wang et al., 2009; Cortey et al., 2011b). It indicates a gradual genotypic switch from PCV2a to PCV2b over time (Dupont et al., 2008; Cortey et al., 2011b). To date, only three sequences fit into the genotype PCV2c (Dupont et al., 2008).

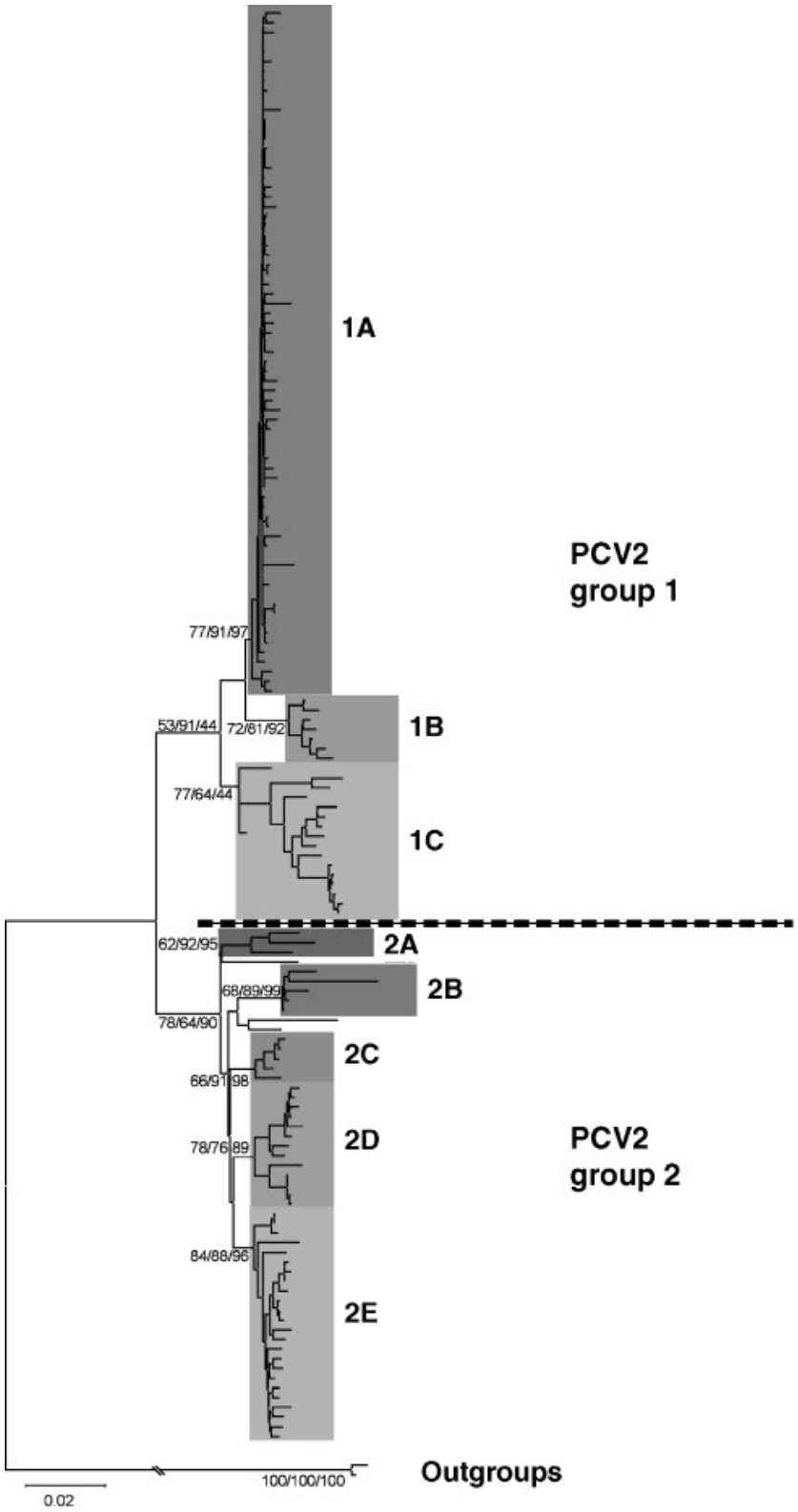

Fig. 2. Phylogenetic tree based on the Neighbor-Joining method for the 148 PCV2 sequences plus 2 outgroups used in the study of Olvera et al. (2007). Numbers along the branches refer to the percentages of confidence in the Maximum Likelihood, Maximum Parsimony and Neighbor-Joining analyses. PCV2 can be divided into two main groups (groups 1 and 2) and 8 clusters (1A to 1C and 2A to 2E), based on full-length genomic sequences. Figure obtained from Olvera et al. (2007).

Among various strains of PCV2, the Rep and Cap have 97-100% and 91-100% identity, respectively at the nucleotide level and 97-100% and 89-100% identity, respectively at the protein level (Larochelle et al., 2002). Amino acid alignment using capsid protein sequences of PCV2a and PCV2b strains reveals three major heterogenic regions (residues 57-91, 121-136 and 180-191), of which two have a high immunogenic index (Larochelle et al., 2002; de Boisséson et al., 2004; Grau-Roma et al., 2008). Despite the presence of considerable genetic diversity between different PCV2 strains, no major antigenic differences between different PCV2 genotypes or strains could be revealed using PCV2-specific monoclonal (mAbs) and polyclonal antibodies (pAbs) (Allan et al., 1999; McNeilly et al., 2001). And therefore, it was initially thought that no antigenic differences exist among the different PCV2 strains. Later on, existence of antigenic differences between PCV2 genotypes and strains has been demonstrated using mAbs directed against the PCV2 capsid protein (Lefebvre et al., 2008a; Shang et al., 2009; Guo et al., 2011; Huang et al., 2011); however, antigenic diversity among PCV2 isolates could not be completely explored since PCV2 strains from a few PCV2 genetic clusters were used. To date, no studies have been performed to antigenically subtype PCV2 strains enclosing all eight PCV2 clusters (PCV2b-1A to PCV2b-1C and PCV2a-2A to PCV2a-2E) by applying a large panel of mAbs raised against both PCV2a or PCV2b. Therefore, a follow-up study of Lefebvre et al. (2008a) has been reported here to shed some light on the antigenic diversity among PCV2 genotypes or strains. Several antigenic domains have been identified on the PCV2 capsid protein by using porcine pAbs (Mahé et al., 2000; Truong et al., 2001) or mouse mAbs (Lekcharoensuk et al., 2004; Shang et al., 2009). Genotype-specific domains (six aa residues at positions 86-91 and four aa residues at positions 190, 191, 206 and 210) on the PCV2 capsid protein have also been identified using multiple sequence alignment (Cheung et al., 2007; Cheung and Greenlee, 2011). Lekcharoensuk et al. (2004) identified at least five overlapping conformational epitopes within residues 47-85, 165-200 and 230-233 of the PCV2 capsid protein using chimeric PCV1/PCV2 infectious clones. PEPSCAN analysis using anti-PCV2 swine polyclonal serum revealed 6 linear epitopes within the residues 25-43, 69-83, 113-127, 117-131, 169-183 and 193-207 of the PCV2 capsid protein (Mahé et al., 2000). However, epitopes' competition analysis and the spatial relationships among the epitopes of the capsid protein have not been studied yet.

PMWS-affected pigs have significantly lower levels of PCV2-neutralising antibodies when compared to sub-clinically infected animals (Meerts et al., 2006; Fort et al., 2007), suggesting

a crucial role for neutralising antibodies in the prevention of PMWS. Several authors have described mAbs with neutralising activity to the PCV2 capsid protein (McNeilly et al., 2001; Lekcharoensuk et al., 2004; Zhou et al., 2005; Lefebvre et al., 2008a; Shang et al., 2009; Guo et al., 2010; Huang et al., 2011) and Lefebvre et al. (2008a) demonstrated that several mAbs were able to recognise seven PCV2 isolates of both PCV2a and PCV2b in immuno-peroxidase monolayer assay (IPMA+); however, some of them were neutralised (N+) and some isolates were not neutralised (N-) by mAbs in sensitive neutralisation assay irrespective of their genotypes. This points to the existence of two distinct PCV2 neutralisation phenotypes: phenotype α (mAb recognition with neutralisation; IPMA+N+) and phenotype β (mAb recognition without neutralisation; IPMA+N-). Alignments of the capsid proteins of these PCV2 isolates within a genotype revealed few aa differences (Lefebvre et al., 2008a) and these particular aa differences could be of importance to determine the neutralisation capacity of mAbs and to switch the neutralisation phenotypes of PCV2 (α to β or β to α). This thesis will deal with these issues. Despite the presence of genetic and antigenic diversity between PCV2 genotypes or strains, the immunity induced by a PCV2a infection can cross-protect pigs upon a subsequent infection with PCV2b and vice-versa (Fort et al., 2008; Fort et al., 2009; Opriessnig et al., 2009a; Segalés et al., 2009; Beach et al., 2011; Beach and Meng, 2012).

1.5. Pathogenesis of porcine circoviruses

1.5.1. Porcine circovirus type 1

Very little is known about the pathogenesis of PCV1 infections in pigs, although PCV1 infections are widely distributed around the world (Allan et al., 1998; Allan and Ellis, 2000; Fenaux et al., 2000). The seroprevalence of PCV1 at herd level varies between 10 % (Puvanendiran et al., 2011) and 100 % (Labarque et al., 2000) although no disease association could be found with this virus in the field (Tischer et al., 1986; Allan et al., 1995; Krakowka et al., 2000; Fenaux et al., 2003). The transmission of PCV1 presumably occurs via ingestion and/or inhalation (Tischer et al., 1986). Recently, it has also been shown that PCV1 is shed into the milk of sows and transmitted to newborn pigs through colostrum (Shibata et al., 2006). Few experimental studies have been performed in the past to study the pathogenesis of PCV1 infections in 1 day old, 2 days old, 1 month old and 9 months old pigs (Tischer et al., 1986; Allan et al., 1995; Krakowka et al., 2000; Fenaux et al., 2003) but no disease could be produced following experimental PCV1 inoculation. Nonetheless, PCV1

could be isolated from nasal secretions starting from 3 days post inoculation (dpi) and from faecal samples at 13 or 14 dpi (Tischer et al., 1986) but neither any signs of disease, nor any increase in body temperature is noticed in pigs over a period of 3 weeks after experimental PCV1 inoculation. The isolation of PCV1 from the serum and plasma samples indicates a transient viraemia following PCV1 infection and it is believed that the distribution of PCV1 to the different organs occurs via blood through infected monocytes (Allan et al., 1995). Primary replication sites of PCV1 are still unknown. Starting from 1 to 3 dpi, PCV1 antigens could be detected in nasal mucosa, lung, liver, spleen, thymus, and mesenteric and bronchial lymph nodes and from 5 dpi in small intestine and distribution of PCV1 antigens are substantially greater at 7 to 9 dpi (Allan et al., 1995). Afterwards, the amount of PCV1 antigens start to decrease, as the PCV1-specific antibodies with neutralising abilities start to appear at 7 dpi and the antibodies rapidly increase during 2 to 5 weeks post inoculation. Then, the antibody titres start to decline but still remain detectable until 39 to 47 weeks post inoculation (Tischer et al., 1986).

Although PCV1 antigens are detected in different tissue types, they are predominantly present in the lungs, spleen and thymus (Allan et al., 1995). PCV1 antigens are mainly detected in non-epithelial cell types with morphological characteristics similar to those described for macrophages, histiocytes, and interdigitating dendritic cells or antigen presenting cells (Allan et al., 1995). However, it is still to be determined which specific cell types are associated with PCV1 replication.

It is generally accepted that PCV1 is non-pathogenic to pigs (Tischer et al., 1986; Allan et al., 1995; Tischer et al., 1995; Allan et al., 2000a; Finsterbusch and Mankertz, 2009; Beach et al., 2010). However, PCV1 DNA has been detected in lymph nodes of a piglet in France with a wasting condition (LeCann et al., 1997). PCV1 has also been detected in cases of congenital tremors in newborn pigs and aborted/stillborn piglets, indicating the possible occurrence of vertical transmission of PCV1 (Allan et al., 1995; Stevenson et al., 2001; Choi et al., 2002). In contrast, no evidence of PCV1 infection was found in piglets affected with congenital tremors in an 11 years retro-prospective study (Kennedy et al., 2003). Up till now, nothing is known about the outcome of experimental PCV1 infections in porcine foetuses. In this thesis, the pathogenesis of PCV1 in porcine foetuses with identification of specific cell types that are associated with PCV1 replication will be described.

1.5.2. Porcine circovirus type 2

PCV2 can infect pigs prenatally at different stages of embryonic and foetal development and postnatally at different ages, resulting in different outcomes (Segalés et al., 2005a). Embryos remains protected to PCV2 infection as long as they are covered by the zona pellucida (ZP) (Mateusen et al., 2004). After hatching, the embryos become susceptible to PCV2 infection and PCV2 replication in embryos may lead to embryonic death, subsequent resorption in the uterus and return to oestrus (Mateusen et al., 2007). During foetal life, the heart is the main target organ of PCV2 replication, followed by liver, spleen, and other lymphoid organs and lungs. The main target cells are cardiomyocytes, hepatocytes and cells of the monocyte/macrophage lineage (Sanchez et al., 2001a; Sanchez et al., 2003). Extensive PCV2 replication in the heart results in heart failure, which leads to the development of ascites, hydrothorax, hydropericardium, oedema, congestion and finally death and mummification of the foetuses (Pensaert et al., 2004; Madson et al., 2009a). The effect that PCV2 replication in the thymus, spleen and lymph nodes has on the functional development of the immune system is not known. However, PCV2 replication decreases considerably with increasing age of the foetuses, due to the development of the adaptive humoral immune response in porcine foetuses after 70 days of gestation and the reduction of mitotic activity of the cells with the progression of gestation. Indeed, PCV2 needs cellular DNA polymerases of actively dividing cells to replicate (Tischer et al., 1987; Gassmann et al., 1988) and cells from early foetal life possess more mitotic activity than the cells from the late foetal life or postnatal life (Awad and Gruppuso, 2000; Sanchez et al., 2003). Postnatally, PCV2 antigens are rarely observed in the heart and liver. In early postnatal life, PCV2 mainly focuses on cells of the monocyte/macrophage lineage and lymphoblasts (Sanchez et al., 2004).

In pigs, the majority of PCV2 infections occur via oro-nasal route. PCV2 can be shed through the nasal, oral and ocular secretions, as well as through the faeces, urine, milk and semen (Krakowka et al., 2000; Larochelle et al., 2000; Kim et al., 2001; Shibata et al., 2003; Segalés et al., 2005b; McIntosh et al., 2006; Shibata et al., 2006; Ha et al., 2009a; Park et al., 2009; Ha et al., 2010). Clinically affected pigs shed PCV2 in higher quantities as compared to sub-clinically infected pigs (Segalés et al., 2005b). The natural vertical transmission of PCV2, which is a rare event due to the high seroprevalence in gilts and sows, has also been reported (Ladekjaer-Mikkelsen et al., 2001; Stevenson et al., 2001; Maldonado et al., 2005). Feeding pork products produced from PCV2-infected muscles and bone marrow to naive piglets for 3

days can also cause PCV2 infections (Opriessnig et al., 2009b). Once PCV2 enters the host, primary replication of PCV2 may start in the tonsils and other lymphoid organs of the head region (Rosell et al., 1999). The distal part of the small intestine, colon, caecum and mesenteric lymph nodes could also be the primary replication sites of PCV2 (McNair et al., 2007). PCV2 causes both cell-associated and cell-free viraemia, which is detectable between 7 and 14 dpi (Pensaert et al., 2004; McIntosh et al., 2006; Fort et al., 2008; Fort et al., 2009; Beach et al., 2011; Podgórska and Stadejek, 2011), and viral titres increase and reach a peak level between 14 and 21 dpi (Rovira et al., 2002; Resendes et al., 2004b; Meerts et al., 2005a; Opriessnig et al., 2008a). Within this time, there is a dissemination of PCV2 throughout the body. PCV2 predominantly replicates in a large range of lymphoid organs affecting the B- and T-lymphocyte populations (Choi and Chae, 1999; Yu et al., 2007; Lefebvre et al., 2008b). In the affected lymphoid organs, PCV2 causes depletion of lymphocytes, which can be caused by the lysis of infected lymphocytes due to high viral replication (Sanchez et al., 2004; Yu et al., 2007), apoptosis (Shibahara et al., 2000; Karuppannan and Kwang, 2011), decreased cellular proliferation (Mandrioli et al., 2004) or the disruption of cell signaling pathways (Vincent et al., 2005). Then, the dendritic cells and monocyte/macrophage lineage cells infiltrate in the lymphocyte-depleted areas and phagocytise the virus particles (Opriessnig et al., 2007; Karuppannan and Kwang, 2011; Nauwynck et al., 2012). A large amount of virus remains accumulated in these cells since apparently there is no degradation of virus particles (Allan and Ellis, 2000; Sorden, 2000; Chianini et al., 2001). Because of the mobility of dendritic cells, it is also believed that dendritic cells may be a cause of systemic dissemination of PCV2 (Vincent et al., 2003). Besides the monocyte/macrophage lineage cells and lymphocytes, PCV2 antigens are also detected in enterocytes, pneumocytes, bronchial, bronchiolar, biliary, pancreatic acinar and ductular and renal tubular epithelial cells, hepatocytes, striated and smooth muscle cells, fibroblasts, neurons and vascular endothelial cells (Rosell et al., 1999; Kennedy et al., 2000; Shibahara et al., 2000; Stevenson et al., 2001; Sanchez et al., 2004).

As mentioned above, macrophages phagocytise the virus particles, which rarely leads to productive viral infection (reviewed by Nauwynck et al., 2012). In contrast, it has been shown that productive viral replication is a frequent event in macrophages since significant ultrastructural alterations were seen in different organells of cells together with the presence of inclusion bodies (both cytoplasmic and nuclear) in histiocytes of lymph nodes of PMWS-affected pigs (Rodriguez-Cariño and Segalés, 2009; Rodriguez-Cariño et al., 2010). On the

other hand, immunostimulated lymphoblasts are susceptible targets for PCV2 replication (Lefebvre et al., 2008b). The larger the number of blasts, the faster and higher the primary replication of PCV2 is in its host. This results in an induction of immune response (Nauwynck et al., 2012). Depending on the ability of pigs to mount an adequate adaptive immune response, more specifically the presence or absence of PCV2-neutralising antibodies determines the progress of PCV2 infection. In experimentally inoculated pigs, the neutralising antibodies start to appear from 10 to 15 dpi and reach to a peak level at 21 dpi (Meerts et al., 2005a; Meerts et al., 2006). If the immune response is strong, the pigs remain sub-clinically infected and the amount of PCV2 decreases and typically ends up with the resolution of viraemia (Resendes et al., 2004b; Meerts et al., 2005a; Fort et al., 2007; Opriessnig et al., 2008a). In sub-clinically infected pigs, low level of PCV2 is detected in tissues with minor or no alterations of the immune system. It is important to note that the long-lasting PCV2 viraemia and/or detection of PCV2 in tissues have been reported in experimentally sub-clinically infected pigs despite the presence of high PCV2-specific antibody titres (Opriessnig et al., 2010). In contrast, a good correlation is observed between the absence or low level of neutralising antibodies (poor humoral immune responses) against PCV2 and the occurrence of a high virus replication and the development of PMWS (Meerts et al., 2006). To our knowledge, it is not known why pigs that develop PMWS are not able to mount a protective neutralising antibody response. Previously, Truong et al. (2001) have identified linear B-cell epitopes within the residues 69-83, 117-131 and 169-183 of the PCV2 capsid protein using porcine hyperimmune sera, but it is not known whether the absence of neutralising antibodies is correlated with an immunological tolerance for these particular epitopes. The cell-mediated immunity is correlated with the level of PCV2 replication and/or PMWS since a decreased level of T-cell immune responses are observed in PMWS-affected pigs (Darwich et al., 2002; Nielsen et al., 2003). The fact that both the humoral and the cellular immune response seem to be ineffective in pigs that develop PMWS might suggest that their immune system is to some extend tolerant for PCV2. Artificially induced immunosuppression in gnotobiotic pigs with cyclosporine A or dexamethasone potentiates the level of PCV2 replication (Krakowka et al., 2002; Kawashima et al., 2003; Meerts et al., 2005a). The number of $CD4^+$T cells in peripheral blood mononuclear cells (PBMC) is reduced in PCV2-inoculated pigs treated with dexamethasone compared to the pigs inoculated with PCV2 alone (Kawashima et al., 2003), which indicates the contribution of T-cell responses to the control of PCV2 infection. It has also been shown that PCV2-inoculated pigs with a high level of IFN-γ mRNA expression in PBMC are less susceptible to PCV2

replication (Meerts et al., 2005a). Another indication of cellular immune response against PCV2 is that pigs immunised with a chimeric PCV1-2 infectious DNA clone do not develop a humoral immune response against PCV2; however, these pigs remain protected against subsequent PCV2 infection (Fenaux et al., 2004). Previously, Stevenson et al. (2007) have shown that T-lymphocyte responses to PCV2 are primarily directed towards epitopes of the non-structural ORF1 (aa residues 81-200 and 201-220) and ORF3 (aa residues 31-50) proteins (Stevenson et al., 2007). To our knowledge, it has however not been shown that the development of PMWS is correlated with a natural or induced immunological tolerance for these particular T-cell epitopes.

In the case of hog cholera virus (HCV), it has been shown that the infection of sows with a less virulent strain, at the moment that the foetuses are still immuno-incompetent, may result in the birth of live-born HCV-infected piglets that do not have neutralising antibodies against HCV (Meyer et al., 1981; Frey et al., 2010). It is not known whether porcine foetuses inoculated with a less virulent PCV2 strain before the development of immunocompetence could be viable and immunotolerant to PCV2 at birth. Nor it is known what would happen if such a pig would be postnatally super-infected with a closely related highly virulent PCV2 strain.

1.6. PCV2-associated diseases

The most common form of PCV2-associated disease is the subclinical infection (Gillespie et al., 2009; Segalés, 2012). Besides subclinical infection, PCV2 infection in pigs has been associated with various clinical syndromes with postweaning multisystemic wasting syndrome (PMWS) as the most significant manifestation (Gillespie et al., 2009; Segalés, 2012). Other PCV2-associated clinical diseases include porcine dermatitis and nephropathy syndrome (PDNS) (Rosell et al., 2000), reproductive failure (West et al., 1999; Ladekjær-Mikkelsen et al., 2001; O'Connor et al., 2001; Farnham et al., 2003; Brunborg et al., 2007; Pittman, 2008), enteritis (Kim et al., 2004a; Jensen et al., 2006; Opriessnig et al., 2011a), proliferative and necrotising pneumonia (PNP) (Drolet et al., 2003; Grau-Roma and Segalés, 2007), porcine respiratory disease complex (PRDC) (Kim et al., 2003a). All these clinical conditions including the PCV2 subclinical infection are collectively called porcine circovirus diseases (PCVD) in Europe (Segalés et al., 2005a; Segalés, 2012) or porcine circovirus-associated diseases (PCVAD) in North America (Opriessnig et al., 2007). PCV2 has also been linked with congenital tremor (CT) type AII (Stevenson et al., 2001; Choi et al., 2002);

however, the association of PCV2 with CT is still controversial since other researchers did not find any evidence of PCV2 infection in piglets with congenital tremors (Kennedy et al., 2003; Ha et al., 2005).

1.6.1. Postweaning multisystemic wasting syndrome (PMWS)

1.6.1.1. Epidemiology and clinical features

PCV2 infection of pigs has been detected as early as 1962 (Jacobsen et al., 2009). The characteristic histopathological lesions of PMWS together with the PCV2 antigen have been identified in pigs in 1985 (Jacobsen et al., 2009). However, the first cases of PMWS have been reported in Western Canada in 1991 (Clark, 1996; Harding, 1996). Since then, PCV2 infection in pigs has been found ubiquitous around the world (Allan and Ellis, 2000) and PMWS has been diagnosed in all major pig-producing countries (Grau-Roma et al., 2011) and most recently in Australia (O'Dea et al., 2011).

PMWS is a multifactorial disease that usually appears in weaned pigs between 7 and 16 weeks old (Harding et al., 1998; Allan and Ellis, 2000; Segalés and Domingo, 2002; Harding, 2004). The majority of the PCV2-infected pigs does not show the clinical form of the disease and remain sub-clinically infected. Only a small proportion of the PCV2-infected pigs develop the clinical form of disease (Segalés, 2012).

Pigs affected with PMWS exhibit severe growth retardation or wasting. Other symptoms may include lethargy, severe respiratory distress, dark-coloured diarrhoea, palpable or visible lymphadenopathy, pallor of the skin, or occasionally icterus (Allan and Ellis, 2000; Segalés and Domingo, 2002; Gillespie et al., 2009; Ge et al., 2011). Coughing, pyraexia, nervous disorders and sudden death have occasionally been reported (Harms, 1999). Morbidity is associated with the development of viraemia and lymphopaenia in pigs followed by the clinical manifestations of the disease (Gillespie et al., 2009). In PMWS-affected farms, morbidity varies from 5 to 20% and mortality can reach up to 80% (Allan and Ellis, 2000; Segalés and Domingo, 2002, Darwich et al., 2004).

1.6.1.2. Pathology

Macroscopic lesions associated with PMWS are quite non-specific and variable (Darwich et al., 2004). Generalised lymphadenopathy and lung lesions are the most consistent findings in PMWS-affected pigs. The lung lesions vary from failure to collapse and increased firmness

to extensive to diffuse mottling areas of consolidation in the anterior ventral pulmonary areas. Hepatic atrophy and white foci in the kidney are less commonly observed (Rosell et al., 1999; Allan and Ellis, 2000; Ladekjaer-Mikkelsen et al., 2002; Segalés and Domingo, 2002; Darwich et al., 2003; Kim et al., 2003b). Occasionally, pallor, oedema, and non-haemorrhagic ulceration of the pars oesophagea of the stomach and fluid-filled, thin-walled intestines, especially the ileum and spiral colon have also been described (Allan and Ellis, 2000).

Microscopic lesions that are typical for PMWS can be observed in almost all lymphoid tissues including different lymph nodes, tonsils, Peyer's patches, spleen and thymus (Darwich et al., 2004). They include a variable degree of lymphocyte depletion with infiltration of histiocytic and/or multinucleated giant cells into the lymphocyte-depleted areas. In early to mid-stages of disease, clusters of variably sized, intensely basophilic, intracytoplasmic inclusion bodies (containing PCV2 antigens) are observed in cells of the monocyte/macrophage lineage, including various types of dendritic cells (Allan et al., 1998; Ellis et al., 1998; Kiupel et al., 1998; Morozov et al., 1998; Rosell et al., 1999; Allan and Ellis, 2000; Darwich et al., 2004).

1.6.1.3. Diagnosis

To diagnose individual cases of PMWS, three internationally accepted individual case definition criteria must be taken into consideration (Sorden, 2000; Segalés et al., 2005a): (a) presence of compatible clinical signs of PMWS (growth retardation and/or wasting) (Fig. 3A), (b) moderate to severe lymphocyte depletion with lymphohistiocytic to granulomatous inflammation of the lymphoid tissues (Fig. 3B) and (c) a moderate to high level of PCV2 antigens within the lymphoid lesions (Fig. 3C). Since clinical signs of PMWS are non-specific and variable, the presence of PCV2 DNA, antigens or virus in the lymphoid tissues together with the characteristic histopathological lesions can be used as criteria for the diagnosis of PMWS (Chae, 2004).

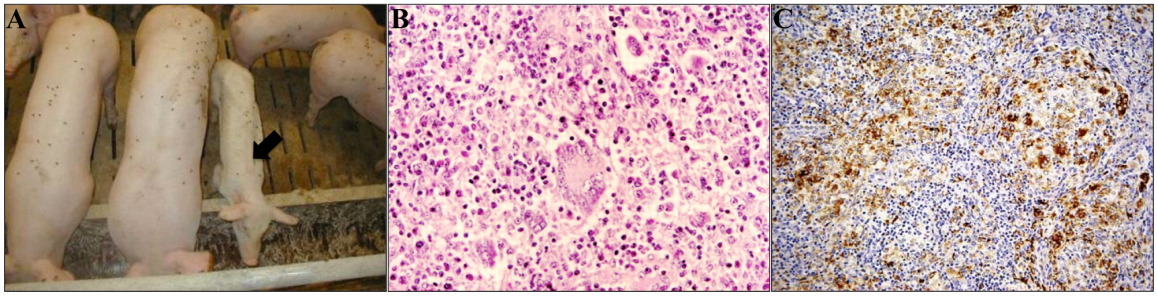

Fig. 3. Diagnostic features of PMWS. (A) A PMWS-affected pig (indicated by arrow) showing severe growth retardation and wasting (Dr. J. Carr, Murdoch University) (http://stdavids-pig.com). (B) Depletion of lymphocytes with infiltration of histiocytes and multinucleated giant cells in lymphoid tissue. (C) PCV2 antigens in lymphoid tissue (brown staining). Fig. B and Fig. C were adapted from Ha et al. (2009b) and Borba et al. (2011), respectively.

Serology cannot be used for the diagnosis of PMWS since many clinically healthy pigs are seropositive (Gillespie et al., 2009). PCR is a sensitive technique for the detection of minimal amounts of PCV2 DNA in pig tissues (Calsamiglia et al., 2002). Nevertheless, PCR cannot be accurately used for the diagnosis of PMWS either (Chae, 2004) since the vast majority of the PCV2 infections occur in pigs without the progression to disease (subclinical infection) and the possibility of contamination with PCV2 is difficult to control during tissue sampling at necropsy on farms or in postmortem facilities (Allan and Ellis, 2000). However, PCR can provide an alternative confirmatory PMWS diagnostic tool, when detection of PCV2 in lymphoid tissues by PCV2 PCR is combined with the characteristic histopathological lesions (Chae, 2004). Recently, The diagnostic value of using serology (to detect PCV2-specific antibodies) and quantitative PCR (qPCR for PCV2 DNA detection) for the diagnosis of PCV2-associated diseases has been evaluated in PMWS-affected pigs and non-PMWS-affected pigs and it has been concluded that neither the qPCR nor the serology nor the combination of both techniques can be used for the diagnosis of PMWS in individual pigs or in herds because of their low diagnostic sensitivity and specificity (Fort et al., 2007; Turner et al., 2008; Grau-Roma et al., 2009; Woodbine et al., 2010).

The diagnosis of PMWS in a herd can be controversial, as individual animals can be affected on farms with excellent production data and without economic losses due to PMWS (Meerts et al., 2004; Jorsal et al., 2006; Nielsen et al., 2008). Epidemiological surveys show that 32 to 55% of the suspected cases do not fulfill the diagnostic criteria as mentioned above (Grau-Roma et al., 2009; Sarli et al., 2009; Grau-Roma et al., 2011). Therefore, the following two diagnostic criteria have been proposed to diagnose PMWS in a herd (Grau-Roma et al.,

2012): (a) a significant increase in postweaning mortality, compared to the historical background in the herd, must be observed in association with clinical signs compatible with PMWS and (b) PMWS must be diagnosed in at least one out of three to five pigs at necropsy concurrently with the increase in mortality. Moreover, the diagnostic exclusion of other potential causes of high mortality is required. This herd case definition for PMWS is an expansion of the diagnostic criteria that were initially proposed by the EU Consortium on PCVDs in 2008 to diagnose PMWS in a herd (http://www.pcvd.eu/news.php).

1.6.1.4. Factors responsible for the development of PMWS

Although PCV2 infection has been shown to be essential for the full expression of PMWS, the majority of the PCV2 infections are subclinical (Gillespie et al., 2009; Segalés, 2012). It has been traditionally difficult to reproduce the full clinical expression of the disease observed under field conditions through experimental infections using PCV2 alone (reviewed by Tomas et al., 2008). Therefore, it is generally accepted that other co-factors are important (Segalés et al., 2005a; Nauwynck et al., 2012). The main factors that might influence the progression of PCV2 infection towards the development of PMWS are co-infections, immunomodulation, viral factors, host factors and management related factors (Fig. 4) (Gillespie et al., 2009; Grau-Roma et al., 2011; Opriessnig and Halbur, 2012).

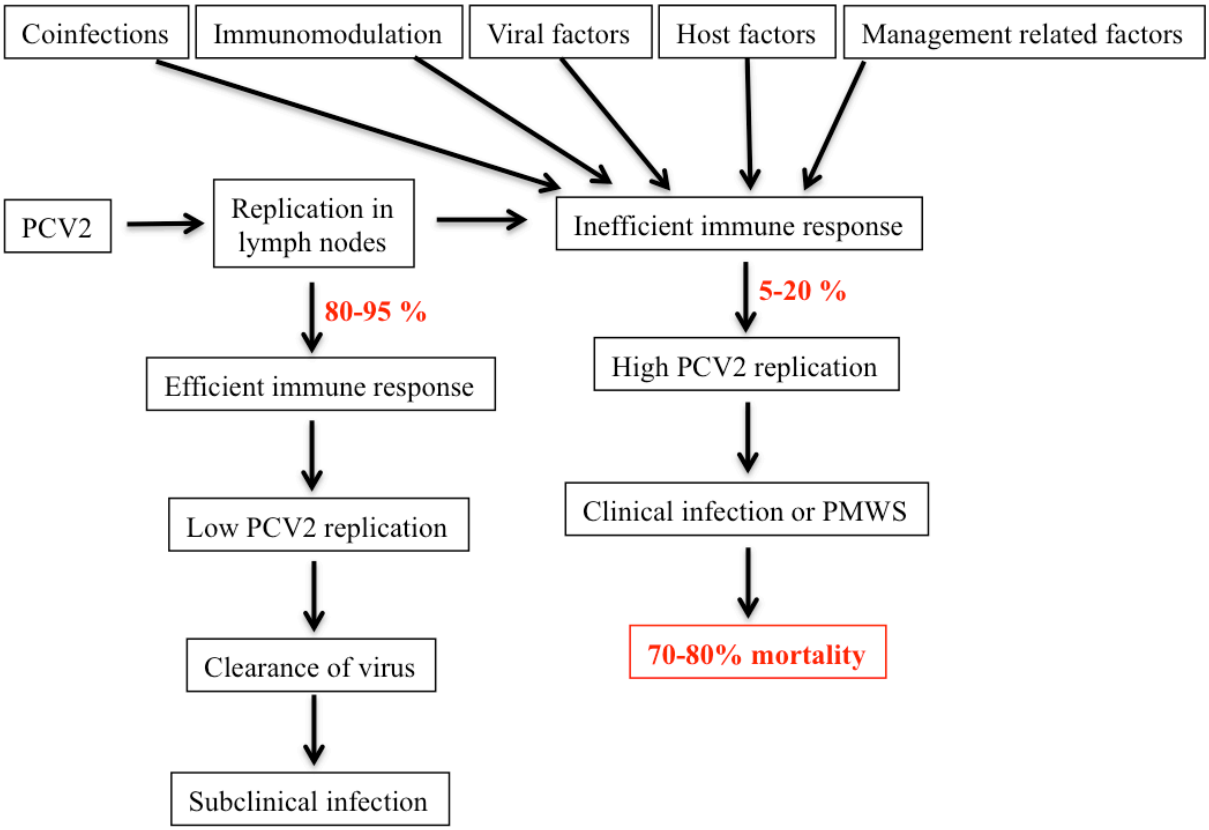

Fig. 4. A pathogenesis model to understand the effect of co-factors that might influence PCV2 infection towards the development of either clinical or subclinical form of disease.

1.6.1.4.1. Co-infections

PMWS-affected pigs exhibit a wide spectrum of concomitant infections. Co-infecting agents that have been isolated from field cases of PMWS are viruses (porcine parvovirus, porcine reproductive and respiratory syndrome virus, porcine epidemic diarrhoea virus, Aujeszky's disease virus, hepatitis E virus, torque teno sus virus, porcine teschovirus and swine influenza virus); mycoplasmas (*Mycoplasma hypopneumoniae*, *Mycoplasma hyorhinitis* and *Eperythrozoon suis*); bacteria (*Salmonella Choleraesuis*, *Escherichia coli* and *Haemophilus parasuis*); and some opportunistic pathogens (*Pneumocystis carinii*, *Candida albicans*, *Aspergillus* spp., *Cryptosporidium parvum*, *Chlamydia* spp. and *Zygomycetes* spp.) (reviewed by Grau-Roma et al., 2011; Opriessnig and Halbur, 2012). All of these co-infecting agents may enhance the PCV2-associated lesions and increase the incidence of PMWS under experimental and field conditions (Pogranichniy et al., 2002; Rose et al., 2003; Dorr et al., 2007).

Under experimental conditions, PMWS can be more successfully reproduced when PCV2 is inoculated in combination with other infectious agents than with PCV2 alone. Co-infection with several other viral and bacterial pathogens, such as porcine parvovirus (PPV) (Allan et al., 2003; Opriessnig et al., 2004a; Kim et al., 2006; Shen et al., 2010a; O'Neill et al., 2011; Opriessnig et al., 2011b), porcine reproductive and respiratory syndrome virus (PRRSV) (Harms et al., 2001; Rovira et al., 2002; Opriessnig et al., 2006a; Opriessnig et al., 2008b; Shen et al., 2010a; Sinha et al., 2010), *Mycoplasma hyopneumponiae* (Opriessnig et al., 2004b; Krakowka et al., 2007), and recently, genogroup 1 torque teno virus (TTV) (Ellis et al., 2008) results in a high level of PCV2 replication and an increased incidence of PMWS. From the above-mentioned experimental studies, it has been shown that PMWS always occurs with characteristic histopathological lesions in the lymphoid organs and these lesions are strongly associated with the high PCV2 load in these tissues; however, not all pigs with high viral load and severe histopathological lesions show the clinical form of PMWS (Silva et al., 2011). A meta-analysis on 44 experimental PCV2 inoculation studies had been performed in order to identify the factors that have a major influence on the successful development of PMWS under experimental conditions. It was concluded that the highest possibility to achieve PMWS in an experiment should include colostrum-deprived pigs

younger than 3 weeks of age, high doses (>$10^{5.0}$ $TCID_{50}$/ pig) of PCV2b (genotype 1), and preferably co-infection with another porcine pathogen (Tomas et al., 2008).

1.6.1.4.2. Immunomodulation

Co-infections with other infectious agents that activate the PCV2 infection into the clinical expression of PMWS might be associated with immunostimulation (Gillespie et al., 2009). Stimulation of the pigs' immune system either by the injection of immunostimulating products such as keyhole limpet hemocyanin in incomplete Freund's adjuvant (KLH/ICFA) (Krakowka et al., 2001; Krakowka et al., 2002; Ladekjær-Mikkelsen et al., 2002; Grasland et al., 2005; Krakowka et al., 2007; Wang et al., 2007), an inactivated Parapoxvirus based immunomodulator (Baypamun) (Kyriakis et al., 2002), concanavalin A (Lefebvre et al., 2008b) or immunostimulation by *Mycoplasma hyopneumponiae*, *Actinobacillus pleuropneumoniae*, PRRSV and classical swine fever virus vaccines (Kyriakis et al., 2002; Opriessnig et al., 2003; Allan et al., 2007b; Krakowka et al., 2007; Ha et al., 2009b) may also potentiate the level of PCV2 replication and/or increase the severity of PMWS. These data indicate that stimulation of the immune system is a key factor to achieve a high level of PCV2 replication. However, immunostimulation does not always assure the enhanced level PCV2 replication and/or PMWS (Allan et al., 2000a; Allan et al., 2000b; Ladekjær-Mikkelsen et al., 2002; Stockhofe-Zurwieden et al., 2003; Opriessnig et al., 2004a; Resendes et al., 2004b; Loizel et al., 2005; Ostanello et al., 2005; Haruna et al., 2006; Allan et al., 2007b; Fernandes et al., 2007; Krakowka et al., 2007; Harding et al., 2008; Harding et al., 2010). In contrast, induction of immunosuppression with cyclosporine or dexamethasone also results in an increased level of PCV2 replication in pigs without the development of clinical form of PMWS (Krakowka et al., 2002; Kawashima et al., 2003; Meerts et al., 2005a); however, recently it has been suggested that induced immunosuppression by interleukin 10 (IL-10) is associated with the development of clinical form of PMWS (Crisci et al., 2010; Doster et al., 2010). Induced immunosuppression can also lead to the development of some co-infections such as *Aspergillus* spp. (Segalés et al., 2003), *Candida albicans* (Zlotowski et al., 2006) or *Chlamydia* spp. (Carrasco et al., 2000).

1.6.1.4.3. Viral factors

PCV2 isolates have been distinguished genetically (Olvera et al., 2007), antigenically (Lefebvre et al., 2008a; Shang et al., 2009) and biologically (virus replication characteristics

in vitro and *in vivo*) (Meerts et al., 2005b; Opriessnig et al., 2006b). Epidemiological studies indicate that PCV2b (genotype 1) may be more virulent than PCV2a (genotype 2), because PCV2b is most frequently isolated from naturally occurring cases of PMWS (Carman et al., 2006; Allan et al., 2007a; Gagnon et al., 2007; Dupont et al., 2008; Takahagi et al., 2008; Timmusk et al., 2008; Chiarelli-Neto et al., 2009; Cortey et al., 2011b). Besides the less frequent isolation of genotype PCV2a than PCV2b from PMWS-affected farms, there are few reports where PCV2a has also been isolated from non-affected farms (Cheung et al., 2007; Carman et al., 2008; Grau-Roma et al., 2008; Horlen et al., 2008; Wiederkehr et al., 2009). However, PMWS has been experimentally reproduced with both genotypes and several experimental studies also indicate that both PCV2a (genotype 2) and PCV2b (genotype 1) are equally pathogenic (Lager et al., 2007; Madson et al., 2008; Opriessnig et al., 2008a; Gauger et al., 2011). It has also been demonstrated that PCV2 strains isolated from non-affected farms can cause PMWS under experimental conditions (Allan et al., 2003; Hasslung et al., 2005). Phylogenetic analysis suggests that PCV2a is older in evolutionary terms than PCV2b (Grau-Roma et al., 2008) and a genotypic shift from PCV2a to PCV2b has been implicated in outbreaks of PMWS (Dupont et al., 2008; Timmusk et al., 2008; Wiederkehr et al., 2009; Cortey et al., 2011b; Grau-Roma et al., 2011).

The presence of different PCV2 sequences *in vivo* in one pig (de Boisséson et al., 2004; Cheung et al., 2007; Grau-Roma et al., 2008; Hesse et al., 2008; Cheung, 2009; Kim et al., 2009) or *in vitro* in cell culture (Lefebvre et al., 2008a) has been reported. Mixed PCV1/PCV2a infection in pigs has also been recently described in Canada (Gagnon et al., 2010). However, none of these authors defined any clear association between mixed PCV infections and the development of PMWS. Recently, multiple PCV2 strains of genotypes PCV2a and PCV2b isolated from a diseased or PMWS-affected pig, which indicates that the co-infection with different PCV2 strains might contribute to the development of PMWS in pigs (Zhai et al., 2011). In a recent study, pigs infected with heterologous (PCV2a/PCV2b or PCV2b/PCV2a) or homologous (PCV2b/PCV2b) strains developed clinical disease and the severity of clinical disease was significantly higher in pigs inoculated with the heterologous strains than homologous strains (Harding et al., 2010). In contrast, in another study clinical disease could not be reproduced in pigs following dual infection with homologous (PCV2a/PCV2a) or heterologous (PCV2a/PCV2b) strains (Opriessnig et al., 2010). Consequently, no clear link has been established yet between a certain PCV2 genotype and PMWS. It is also not clear if the increased prevalence of PCV2b in the field is associated with

a change in virulence. Further research is required to elucidate the relationship between PCV2b infection and the development of PMWS. This thesis will focus on this issue.

1.6.1.4.4. Host factors

An important host factor may be the immunity. Despite the presence of IPMA antibodies, neutralising antibodies could not be found or were present at low titers in experimentally and naturally PMWS-affected animals (Meerts et al., 2006; Fort et al., 2007). The basis for this observation is not clear. Absence of neutralising antibodies can be considered as the cause of the high replication (genetically determined absence of recognition of the neutralising epitope) or as a consequence of the high replication (inhibition of the production of neutralising antibodies by the accumulation of PCV2 antigens in antigen presenting cells or by the deletion of antibody producing cells). Further research is ongoing to find an answer to this question.

All breeds of pigs appear to be susceptible to PCV2 infection (Gillespie et al., 2009). However, in PMWS-affected farms, when the sows from a same genetic line (37.5% Large White x 37.5% Duroc x 25% Landrace) inseminated with three different boar lines such as, 100% Piétrain, 50% Large White x 50% Piétrain and 25% Large white x 75% Duroc, the piglets born from the pure Piétrain boar line had the lowest postweaning mortality than the others (López-Soria et al., 2011). In contrast, another field observation has suggested that breed-related differences in mortality do not exist (Rose et al., 2005). Experimental investigations have demonstrated that the Landrace pigs might be more susceptible to PCV2-associated diseases compared to Duroc, Large White and Piétrain pigs (Opriessnig et al., 2006c; Opriessnig et al., 2009c).

Maternally derived immunity via colostrum is also an important host factor which protect pigs against the development of PMWS and this protection is considered to be antibody-titre dependent (McKeown et al., 2005). A meta-analysis of data from experimental infection studies has indicated that colostrum-deprived pigs are more susceptible to PMWS (Tomas et al., 2008). Piglets from sows with low antibody titres have a higher mortality rate than piglets born from sows with high antibody titres (Calsamiglia et al., 2007).

1.6.1.4.5. Management related factors

The management related factors that could be linked to the development of PMWS are shown in Table 1 (Grau-Roma et al., 2011).

Table 1: A summary of the management related factors.

	Factors increasing the risk of PMWS	Factors decreasing the risk of PMWS
Facilities	– Large number of sows – Large pens at nursery and growing ages – Proximity to other pig farms	– Separate manure pits for adjacent fattening rooms – Shower facilities
Management practices	– High level of cross-fostering – Short empty periods at weaning and fattening – Large range in age and weight entering to nursery – Continuous flow through nursery – Purchase of replacement gilts – Sows with neck injuries due to poor injection technique – Early weaning (<21 days of age)	– Sorting pigs by sex at nursery stage – Greater minimum weight at weaning – Group housing sows during pregnancy – Visitors avoiding contact with pigs for several days before visiting farm – Use of semen from an insemination centre
Vaccination/treatment /nutrition	– Vaccination of gilts against PRRSV – Vaccination of sows against E. coli – Use of separate vaccines against Erysipelas and parvovirus on gilts	– Vaccination of sows against atrophic rhinitis – Regular treatment for ectoparasitism – Use of oxytocin during farrowing – Use of spray-dried plasma in initial nursery ration

1.6.1.5. Prevention and control

The triggering factors involved in the development of PMWS can be controlled by the implementation of 'Madec's 20-point plan' (Madec et al., 2001). The four main key rules of Madec's recommendations are: a) limiting direct and indirect contact between piglets from different litters, b) reducing all types of stress, including physical stress and exposure to other pathogens c) improving hygienic conditions by optimising cleaning and disinfection procedures and d) providing good nutrition for the development of the immune system (http://www.organicvet.co.uk/Pigweb/disease/PMWS/). Madec's plan has been designed to minimise the PCV2 infection pressure and stress and to control other pathogens. The implementation of Madec's plan has effectively reduced the PMWS-associated economic losses (Grau-Roma et al., 2011).

Besides implementing the Madec's plan, vaccination against PCV2 can be used as an extra measure to prevent the disease outbreaks by controlling PCV2 infection. A list of commercially available PCV2 vaccines is shown in Table 2.

Table 2. List of commercially available PCV2 vaccines in the European Union.

PCV2 vaccine	Manufacturer	Antigen	Dosage
Ingelvac CircoFLEX®	Boehringer Ingelheim	PCV2a capsid protein	1 ml IM, single dose, piglets ≥ 2 weeks old
Circovac®	Merial	Inactivated PCV2a virus	Gilts and sows: 2 ml IM a) Primary vaccination Gilts - two doses, 3 to 4 weeks apart, second dose at least 2 weeks before mating. One further injection must be given at least 2 weeks before farrowing Sows - two doses, 3 to 4 weeks apart, second dose at least 2 weeks before farrowing b) Revaccination One dose at each gestation, at least 2 to 4 weeks before farrowing Piglets: 0.5 ml IM, one dose, ≥ 3 weeks old
Porcilis PCV®	Intervet International BV	PCV2a capsid protein	Low to medium levels of maternally derived anti-PCV2 antibodies: 2 ml IM, single dose, piglets ≥ 3 weeks old High level of maternally derived anti-PCV2 antibodies: 2 ml IM, two doses, first dose at 3-5 days old and the second dose 2-3 weeks later

PCV2 vaccination in pigs has been proved to be effective to increase the feed conversion ratio, average daily weight gain and carcass weight, and to reduce the mortality rate, PCV2 viraemia and incidence of PMWS under both experimental and field conditions (Fachinger et al., 2008; Horlen et al., 2008; Desrosiers et al., 2009; Segalés et al., 2009; Pejsak et al., 2010; Takahagi et al., 2010; Jacela et al., 2011; Kristensen et al., 2011; Martelli et al., 2011; O'Neill et al., 2011; Venegas-Vargas et al., 2011; Young et al., 2011). Furthermore, vaccination also reduces the number of co-infections with PRRSV and *Mycoplasma hyorhinis* (Kixmöller et al., 2008) and the severity of clinical disease (Segalés et al., 2009; O'Neill et al., 2011; Opriessnig et al., 2011b; Opriessnig et al., 2011c). Experimental studies have demonstrated that vaccination reduces the level of PCV2 viraemia and severity of lymphoid lesions, even if vaccination is performed in the presence of maternal antibodies (Fort et al., 2008; Opriessnig et al., 2008c; Fort et al., 2009; Opriessnig et al., 2009a; Beach et al., 2011). The currently available PCV2 vaccines are mostly based on PCV2a strains (Beach and Meng, 2012) and recently a PCV2b-based vaccine has also been developed (Beach et al., 2011). PCV2a-based vaccines can protect pigs against subsequent challenges with PCV2b strains and vice-versa (Fort et al., 2008; Fort et al., 2009; Beach et al., 2011; Beach and Meng, 2012). However, it is important to note that PCV2 vaccines based on one particular genotype produce somewhat better results in homologous situations. When PCV2b-

vaccinated animals are challenged with PCV2a, then they have higher viral DNA loads at 21 days post-challenge than when they are challenged with PCV2b (Beach et al., 2011). On the other hand, when PCV2a-vaccinated animals are challenged with PCV2b, then a higher percentage of animals are viral DNA positive in nasal and faecal swabs at 20 days post-challenge than when they are challenged with PCV2a (Fort et al., 2008).

1.6.2. PCV2-associated reproductive failure

1.6.2.1. Field observations

The first case of PCV2-associated reproductive failure has been described in 1999 in Canada (West et al., 1999). In this report, PCV2 was isolated from a litter of aborted piglets from a newly established farm entirely stocked with first parity gilts and experiencing late-term abortions and stillbirths. Severe diffuse myocarditis was present in one piglet associated with extensive accumulation of PCV2 antigens. Variable amounts of PCV2 antigen were also present in liver, lung, and kidney of multiple foetuses. The presence of other agents associated with foetal lesions and abortion in swine, including porcine parvovirus, porcine reproductive respiratory syndrome virus, encephalomyocarditis virus, and enterovirus, were not found (West et al., 1999), which indicates that the reproductive failure in this herd was associated with PCV2 infection. Afterwards, PCV2 has been isolated from several aborted and mummified foetuses and stillborn piglets (Meehan et al., 2001; O'Connor et al., 2001; Farnham et al., 2003; Kim et al., 2004b; Mikami et al., 2005; Brunborg et al., 2007). Naturally occurring PCV2-associated reproductive failure have mainly been reported in newly established production facilities with first parity gilts (Ladekjær-Mikkelsen et al., 2001; O'Connor et al., 2001; Farnham et al., 2003; Brunborg et al., 2007; Pittman, 2008). PCV2-associated reproductive failure in breeding herds is principally characterised by increased numbers of mummified and dead piglets at birth (Madson and Opriessnig, 2011) (Fig. 5). Nonsuppurative to necrotising or fibrosing myocarditis associated with high amount of PCV2 antigen is the most characteristic lesion of PCV2-associated reproductive failure in the field (Mikami et al., 2005) (Fig. 5). However, it has also been indicated that PCV2-associated reproductive failure is a relatively rare event (Bogdan et al., 2001; Maldonado et al., 2005) because of the high seroprevalence of PCV2 at animal and herd level, which probably prevents the vertical transmission of PCV2 (Labarque et al., 2000; Sanchez et al., 2001b). Therefore, it might be hypothesized that foetal infection in a PCV2 immune sow might also occur through the presence of PCV2 in the semen of infected boars (Hamel et al.,

2000; Larochelle et al., 2000; Kim et al., 2001; Kim et al., 2003c; McIntosh et al., 2006; Wallgren et al., 2008). PCV2 can be shed in the semen up to 6 months after PCV2 infection without clinical signs or changes in the semen quality parameters (McIntosh et al., 2006; Madson et al., 2008). However, only a limited percentage (0 – 6%) of semen samples from boars used for artificial insemination contain PCV2 DNA (Hamel et al., 2000; McIntosh et al., 2006; Wallgren et al., 2008) and usually the PCV2 DNA load in the semen is loo low to transmit a PCV2 infection to PCV2 negative sows (Grasland et al., 2008). Nevertheless, it is not known whether the amount of PCV2 naturally shed in the boar semen is sufficient to infect the sows or their foetuses under field conditions.

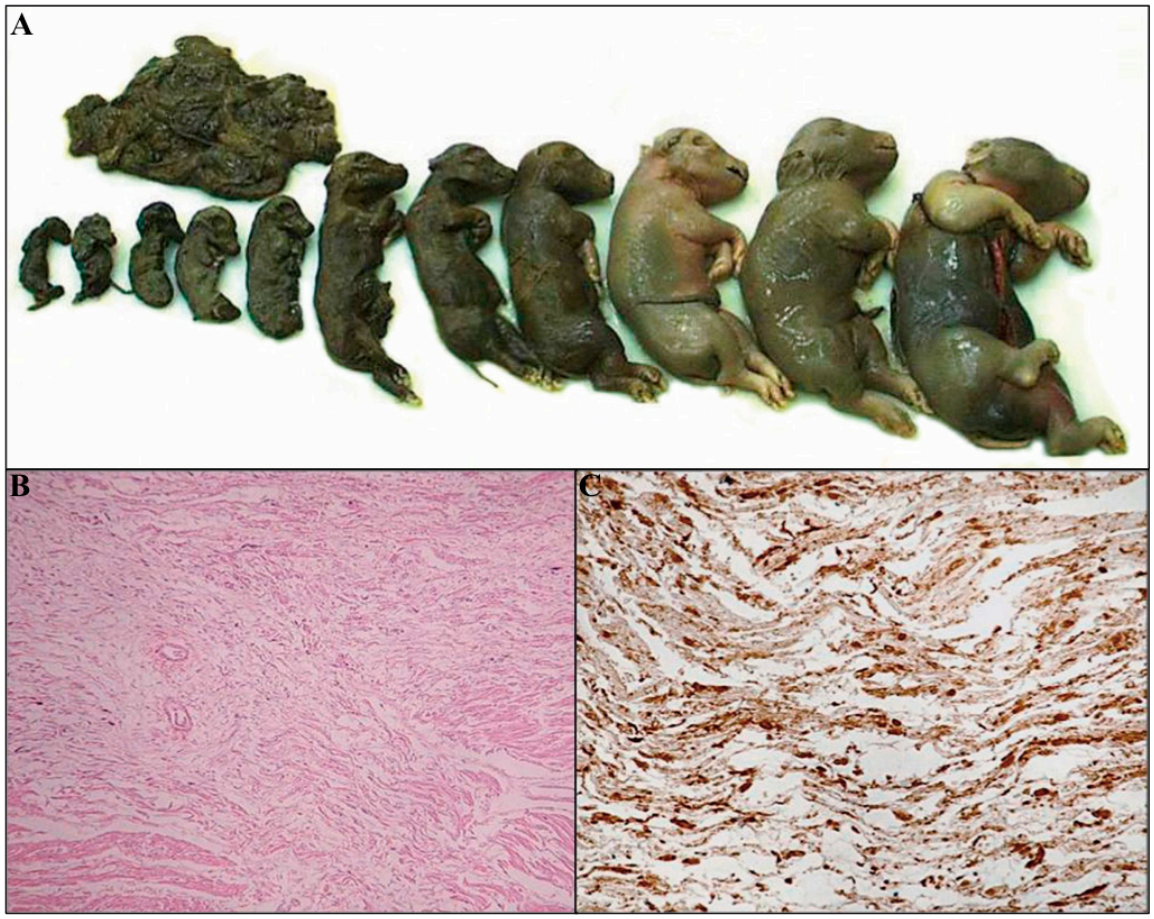

Fig. 5. PCV2-associated reproductive failure. (A) Affected litter showing foetuses at different stages of mummification and maceration. (B) Separations of cardiomyocytes by oedema and low numbers of inflammatory cells in the foetal myocardium. (C) PCV2 antigens (brown staining) within cytoplasm of cardiomyocytes. Figure was adapted from Opriessnig et al. (2007).

1.6.2.2. Experimental observations

PCV2-associated reproductive failure can be reproduced experimentally showing that porcine

embryos and foetuses are susceptible to PCV2 infection (Sanchez et al., 2001a; Johnson et al., 2002; Sanchez et al., 2003; Mateusen et al., 2004; Sanchez et al., 2004; Yoon et al., 2004; Mateusen et al., 2007; Madson et al., 2009a). Exposure of porcine embryos to PCV2 for 48 hours presented that 15% ZP-free morulae, 50% ZP-free early blastocysts and 100% hatched blastocysts become infected with PCV2. All embryos with an intact ZP remain negative for PCV2 infection (Mateusen et al., 2004). In another study, Mateusen et al. (2004) demonstrated that PCV2 could replicate in embryos, which may lead to embryonic death since the survival rate (6.4%) of the PCV2-exposed embryos was singinificantly lower than the survival rate (65.4%) of the PCV2-negative embryos. In the later study, hatched blastocysts were exposed to PCV2 and subsequently transferred to the PCV2-immune receptor sows at the 7th day of the cycle followed by collection of the embryos at 14 days after transfer. Furthermore, it was also shown that sows artificially inseminated with semen spiked with PCV2 resulted in early embryonic death with subsequent resorption and return to oestrus (Madson et al., 2009a).

In a series of surgical, trans-uterine, intra-foetal experimental inoculation of PCV2 in porcine foetuses at 57, 75, 92 or 104 days of gestation (Sanchez et al., 2001a; Sanchez et al., 2003; Pensaert et al., 2004; Sanchez et al., 2004), it was demonstrated that porcine foetuses are highly susceptible to PCV2 replication and this susceptibility decreases with increasing age of the foetuses. Foetuses inoculated at 57 or 75 days of age results in the birth of mummified or stillborn or weakborn piglets (57 days) and stillborn or autolysed foetuses (75 days). Other gross lesions due to experimental PCV2 infections include subcutaneous oedema, abdominal distension, haemorrhages and congestion in internal organs and liver enlargement (Sanchez et al., 2001a; Yoon et al., 2004; Madson et al., 2009a). These gross lesions appear because of the extensive PCV2 replication in the heart, which is the main target organ, leading to heart failure and subsequent congestion of internal organs, followed by autolysis and mummification. Besides the heart, PCV2 efficiently replicates in the liver, spleen and other lymphoid organs of mid-gestational porcine foetuses (Sanchez et al., 2001a; Sanchez et al., 2003). On the other hand, inoculation of foetuses at 92 or 104 days of gestation results in the birth of apparently normal, PCV2-immune piglets. PCV2 inoculation at the later stages of gestation resulted in a limited range of PCV2 replications in different organs, which include mainly heart, and to a lesser extent in the lymphoid (spleen and lymph nodes) and non-lymphoid organs (lungs and liver) (Sanchez et al., 2003). The restricted replication of PCV2 is correlated with the development of an adaptive humoral immune response starting in pigs

from 70 days of gestation and reduction of mitosis rate during the progress of gestation (Salmon, 1984; Nauwynck et al., 2012). Foetal death, mummification, abortion and premature farrowing have also been experimentally reproduced by intranasal inoculation of PCV-seronegative specified pathogen free (SPF) sows with PCV2 during last third of gestation (Park et al., 2005), by intra-uterine inoculation of SPF sows with PCV2 at insemination (Rose et al., 2007; Madson et al., 2009a) and by trans-uterine, intra-foetal PCV2 inoculation during mid-gestation (58-76 days) or during the last third of gestation (84-94 days) (Johnson et al., 2002; Yoon et al., 2004). Foetal death due to PCV2 infection can occur at different stages of gestation resulting in variably sized mummified foetuses (crown-to-rump length) and stillborns at parturition (Madson et al., 2009a). Mummified foetuses can vary in crown-to-rump length with the smallest foetuses around 6-7 cm (O'Connor et al., 2001; Madson et al., 2009a). Following the experimental intra-foetal PCV2 inoculation, the intra-uterine spread of PCV2 does not occur rapidly from one foetus to another (Sanchez et al., 2001a; Pensaert et al., 2004; Yoon et al., 2004) and if it happens, it remains limited only from PCV2-inoculated foetuses to adjacent foetuses at the end of gestation (Pensaert et al., 2004). Intra-uterine spread of PCV2 between foetuses has also been suggested by Madson et al. (2009a); however, in that study it could not be ruled out that foetuses were infected through vertical transmission. The experimental vertical transmission of PCV2 has been reported by several research groups (Park et al., 2005; Rose et al., 2007; Madson et al., 2009b).

Both PCV2a (Meehan et al., 2001; Farnham et al., 2003) and PCV2b (Pittman, 2008; Hansen et al., 2010) have been isolated from naturally occurring reproductive failure and thus, apparently no association with a specific genotype is found (Hansen et al., 2010). However, the more recent studies indicate that naturally occurring PCV2-associated reproductive failure are currently more associated with genotype PCV2b than PCV2a. In addition, a higher prevalence of PCV2b than PCV2a has been reported in pre-suckle piglets (Shen et al., 2010b). The majority of experimental studies that have been performed up till now, used PCV2a strains to reproduce PCV2-associated reproductive failure (Sanchez et al., 2001a; Johnson et al., 2002; Sanchez et al., 2003; Pensaert et al., 2004; Yoon et al., 2004; Mateusen et al., 2007), but not all experimentally used PCV2 sequences are available in GenBank (Park et al., 2005; Rose et al., 2007). Consequently, only little is known about the outcome of experimental PCV2b infection during gestation or at insemination (Madson et al., 2009a). In this thesis, both PCV2a and PCV2b, with special preference to PCV2b, have been used to

explore the PCV2-associated reproductive failure.

Piglets derived from sows with low PCV2 antibody titres or with viraemia had higher morbidity and mortality rates associated with PMWS (Calsamiglia et al., 2007), which indicates the possible *in utero* infection of foetuses that eventually ended up with the development of PMWS. In an experimental study, PCV2 naïve sows were oro-nasally inoculated with PCV2 at late gestation (93 days of gestation) and subsequently PCV2-infected live-born piglets at 3 days of age were challenged with porcine epidemic diarrhea virus (PEDV). It resulted in a clinical form of PEDV (Jung et al., 2006). Similarly, Ha et al. (2008) inoculated PCV2 oro-nasally in naïve sows three weeks prior to the expected date of farrowing, which resulted in the birth of live-born PCV2 infected piglets. The live-born PCV2 infected piglets, challenged with porcine parvovirus (PPV) or immunostimulated with KLH/ICFA at 28 days of age, developed PMWS at 35 days post-challenge or immunostimulation. Non-stimulated PCV2-infected live-born piglets did not develop PMWS (Ha et al., 2008). These two studies clearly indicate that the *in utero* PCV2 infection in porcine foetuses at late gestation could cause birth of live-born PCV2-infected piglets and then these piglets are highly susceptible to the development of disease upon co-infections with other pathogens or immunostimulation. Recently, it was also shown that sows inseminated with semen spiked with PCV2 resulted in approximately 22% live-born piglets. Presuckle serum samples from all live-born piglets were positive for PCV2 DNA and approximately 50% of them had detectable anti-PCV2 antibodies (Madson et al., 2009a). It has been suggested that the piglets that had anti-PCV2 antibodies were infected with PCV2 after the foetuses reached immunocompetence. And the remaining piglets that did not have anti-PCV2 antibodies may have been infected with PCV2 either just prior to parturition and therefore did not have time to develop antibodies or prior to the development of foetal immunocompetence. The live-born piglets that were negative for anti-PCV2 antibodies all had PCV2 antigens in their heart tissues, which indicate that these piglets were persistently infected with PCV2. Later on, the same research group using similar approach in vaccinated sows reported the birth of approximately 86% live-born piglets and 58% of the live-born piglets were persistently infected without showing any PCV2-associated gross or microscopic lesions (Madson et al., 2009c). However, it was not investigated whether these persistently infected piglets were immunologically tolerant for a postnatal super-infection with an antigenically related PCV2 strain and if this would lead to the subsequent development of PMWS.

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Chapter 2. Aims of the thesis

Porcine circoviruses (PCV1 and PCV2) are widely distributed around the world. PCV1 is considered to be apathogenic. However, it has been isolated from cases of reproductive failure and congenital tremor in newborn pigs. Nonetheless, no experimental inoculations have been performed to analyse the foeto-virulence/pathogenicity of PCV1 isolates/strains. PCV2 is associated with Postweaning Multisystemic Wasting Syndrome (PMWS) and can cause reproductive failure. The pathogenesis of PMWS is not fully understood and needs further research. The absence or presence of low levels of neutralising antibodies in PMWS pigs has been reported and it may be hypothesized that this could be due to a certain immunotolerance upon an intra-uterine infection with a less virulent PCV2 strain. The general aim of the present thesis was to obtain a better understanding of antibody-PCV2 interaction with emphasis on neutralising antibodies and to determine foeto-virulence/pathogenicity for different PCV1/PCV2 strains.

Despite the existence of eight different PCV2 genetic clusters/subtypes (PCV2a-2A to PCV2a-2E and PCV2b-1A to PCV2b-1C), the putative existence of antigenic or pathogenetic differences among PCV2 isolates was initially not investigated. Later on, pathogenetic differences were found and distinct antigenic differences were demonstrated using mouse monoclonal antibodies (mAbs). However, no antigenic subtyping has been performed yet enclosing PCV2 strains of all eight genetic subtypes. It has also been shown that mAbs directed against the PCV2 capsid protein react with PCV2 in immuno-peroxidase monolayer assay (IPMA+); however, some isolates are neutralised (N+) and some isolates are not neutralised (N-) by the mAbs. This points to the existence of two distinct PCV2 neutralisation phenotypes: phenotype α (mAb recognition with neutralisation; IPMA+N+) and phenotype β (mAb recognition without neutralisation; IPMA+N-). Since only few amino acid differences exist among the PCV2 strains, it is now questioned which change(s) in the PCV2 capsid could cause a switch from phenotype α (IPMA+N+) to phenotype β (IPMA+N-) and vice-versa. Accordingly, this thesis aims to antigenically subtype PCV2 strains of all eight genetic subtypes and to identify the amino acids in the PCV2 capsid that are important in determining the PCV2 neutralisation phenotype.

Despite the isolation of PCV1 and PCV2 from aborted/stillborn piglets and from cases of congenital tremors in newborn piglets, nothing is known about the outcome of experimental PCV1 infections in porcine foetuses and the outcome of experimental PCV2 (PCV2a or PCV2b) infections in porcine foetuses has only been examined with a restricted number of

strains. Therefore, this thesis aims to examine the outcomes of experimental intra-foetal inoculations with PCV1, PCV2a or PCV2b. When a less virulent PCV2 strain can be found, it will be examined if this strain can induce immunotolerance and subsequently PMWS upon super-infection with a second, more virulent PCV2 strain. The presence of different PCV2 strains with a different virulence within single isolates from pigs with PMWS in the field supports this hypothesis.

Chapter 3. Antigenic characterisation of porcine circovirus 2 strains

Chapter 3.1.

Antigenic subtyping and epitopes' competition analysis of porcine circovirus type 2 using monoclonal antibodies

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Abstract

Porcine circovirus type 2 (PCV2) strains have been classified into two major genotypes (PCV2a and PCV2b) and 8 genetic clusters: PCV2b-1A to PCV2b-1C and PCV2a-2A to PCV2a-2E. To date, no studies have been performed to antigenically subtype PCV2 strains enclosing eight PCV2 clusters. The present study aimed to antigenically subtype PCV2 and perform epitopes' competition analysis using monoclonal antibodies (mAbs). Fourteen PCV2 strains representative for eight clusters were tested with 20 mAbs (fifteen of them were generated against PCV2a strain Stoon-1010 and 5 of them against PCV2b strain 1147) in immuno-peroxidase monolayer assays. Four mAbs reacted to all 14 PCV2 strains and one mAb reacted with all strains except for a PCV2a-2C strain. One mAb reacted with all PCV2a strains, except for a PCV2a-2C strain and one mAb reacted with all PCV2b strains, except for a PCV2b-1C strain. Nine mAbs reacted with the strains of PCV2b-1A/1B, PCV2a-2A and PCV2a-2E. Three mAbs only reacted with the strains of PCV2a-2A and PCV2a-2E. One mAb reacted specifically with the strains of PCV2b-1A/1B. This suggests that discrete antigenic differences exist between different PCV2 genetic clusters and that these clusters can be discriminated by the use of a panel of universal and cluster-specific mAbs. Six mAbs were selected for cross-competition analysis by a competitive ELISA using PCV2 strain Stoon-1010. Six overlapping epitopes were identified on the PCV2 capsid protein. The universal mAbs recognised larger epitopes than the cluster-specific mAbs. These findings are helpful in the development of diagnostic tests and new generation vaccines against PCV2.

1. Introduction

Porcine circoviruses (PCVs) belong to the genus *Circovirus* of the family *Circoviridae*. They are non-enveloped and contain circular single stranded DNA. Porcine circovirus type 1 (PCV1) was characterised as a non-cytopathic contaminant of the PK-15 cell line ATCC-CCL33 and non-pathogenic to pigs (Tischer et al., 1982; Tischer et al., 1986; Allan et al., 1995). Porcine circovirus type 2 (PCV2) is associated with several clinical manifestations collectively known as porcine circovirus-associated diseases (PCVAD) with postweaning multisystemic wasting syndrome (PMWS) as the most significant manifestation (Gillespie et al., 2009).

The circular PCV2 genome consists of 1766-1769 nucleotides and contains three major open reading frames (ORFs). ORF1 encodes for the non-structural replicase proteins Rep and Rep' and they have a size of 314 and 178 amino acids (aa), respectively (Cheung, 2003; Hamel et al., 1998). The structural capsid protein is encoded by ORF2 and consists of 233-235 aa (Hamel et al., 1998; Nawagitgul et al., 2000; Lefebvre et al., 2008; Lefebvre et al., 2009; Huang et al., 2011a; Huang et al., 2011b). The non-structural ORF3 protein consists of 102-104 aa (Hamel et al., 1998). PCV2 ORF3 protein has been associated with apoptosis *in vitro* and viral pathogenesis *in vivo* (Liu et al., 2005; Liu et al., 2006), but these results are highly controversial because they could not be reproduced by other laboratories (as described and reviewed by Juhan et al., 2010).

Among various strains of PCV2, the Rep and Cap have 97-100% and 91-100% identity, respectively at the nucleotide level and 97-100% and 89-100% identity, respectively at protein level (Larochelle et al., 2002). The EU consortium on porcine circovirus diseases proposed a classification system that divides the PCV2 strains into two major genotypes: PCV2a and PCV2b and 8 genetic subtypes/clusters: PCV2b-1A to PCV2b-1C and PCV2a-2A to PCV2a-2E based on their genomic sequences (Olvera et al., 2007; Grau-Roma et al., 2008; Segalés et al., 2008). Later on, PCV2 strains were also divided into three different phenotypes (^{1766}PCV2, ^{1767}PCV2 and ^{1768}PCV2) on the basis of the number of the nucleotides present in the PCV2 genome. Antigenic differences between these three PCV2 phenotypes were shown by Shang et al. (2009) using monoclonal antibodies (mAbs).

Allan et al. (1999) and McNeilly et al. (2001) did not find any major differences in the reactivity of mAbs with different PCV2 strains. Lefebvre et al. (2008) demonstrated that

antigenic differences exist among different PCV2 strains. Mouse monoclonal antibodies (mAbs) raised against PCV2a, were used to study their reactivity with a limited number of PCV2a and PCV2b strains enclosing only 3 out of the 8 genetic subtypes. To date, no studies have been performed to antigenically subtype PCV2 strains enclosing all eight PCV2 clusters by applying a large panel of mAbs raised against both PCV2a or PCV2b. Lekcharoensuk et al. (2004) identified at least five overlapping conformational epitopes within residues 47-85, 165-200 and 230-233 of the PCV2 capsid protein using chimeric PCV1 and PCV2. PEPSCAN analysis using anti-PCV2 swine polyclonal serum revealed 6 linear epitopes within the residues 25-43, 69-83, 113-127, 117-131, 169-183 and 193-207 of the PCV2 capsid protein that were targeted by the immune system (Mahe´ et al., 2000). However, epitopes' competition analysis and the spatial relationships among the epitopes of the capsid protein have not been studied yet.

The present study aimed to antigenically subtype PCV2 strains of all 8 genetic clusters and to identify universal mAbs that could be reactive with all PCV2 strains by applying a large panel of mAbs raised against PCV2a and PCV2b. This study also aimed for epitopes' competition analysis and to identify spatial relationships among different epitopes of the capsid protein of PCV2 by a mAb-based competitive ELISA. These could be useful to develop diagnostic tests and new generation vaccines against PCV2.

2. Materials and methods

2.1. Viruses

Lefebvre et al. (2008) previously demonstrated antigenic differences between PCV2 strains of cluster PCV2b-1A/1B and cluster PCV2a-2E, using mAbs produced against cluster PCV2a-2E strain Stoon-1010. In order to perform antigenic subtyping with a large panel of mAbs, a collection of 14 different PCV2 strains from all 8 PCV2 genetic clusters was established. These strains are shown in Table 1. The PCV2 strains Stoon-1010, 1121, 1103 (PCV2a-2E), 48285, 1147, 1206 (PCV2b-1A/1B) have been described in Lefebvre et al. (2008) and strains II11A (PCV2b-1C) and II9F (PCV2b-1A/1B) have been described in Lefebvre et al. (2009). Three other PCV2 strains were selected from the different clusters from Olvera et al. (2007): NL_Control_4 (PCV2b-1C) (Grierson et al., 2004), Pingtung-1 and Pingtung-4 (PCV2a-2B) (Liao et al., unpublished data). The PCV2 strain Aust-10 (Muhling et al., 2006) was clustered in PCV2a-2A according to the classification system of Olvera et

al. (2007). The PCV2 strains 390 and Slovak were sequenced in our lab and classified as PCV2a-2C and PCV2a-2D, respectively. These 14 strains were cultured on PK-15 cells and their capsid sequence was determined as previously described (Lefebvre et al., 2008; Lefebvre et al., 2009). The nucleotide sequences that were obtained were 100% identical with the sequences available in GenBank. The sequences of 390 and Slovak were deposited in GenBank (GenBank: JN133304 and JN133305). Subsequently, the genotype and genetic cluster of the 14 PCV2 strains were confirmed by phylogenetic analysis as adapted from Tripathi and Sowdhamini (2006) and as previously described by Lefebvre et al. (2008). This is shown in Fig. 1.

The PCV1 PK-15 cell culture contaminant strain ATCC-CCL33 (Tischer et al., 1982) and the PCV1 field isolate 3384 (Allan et al., 1995) were enclosed as controls.

Table 1. Porcine circovirus type 2 (PCV2) strains used in this study.

Accession no.	Strain	Genotype[a]	Cluster[b]	Clinical origin	Geographical origin
AF055394	48285	PCV2b	1A/1B	[c]PMWS-affected piglet	France
AJ293869	1147	PCV2b	1A/1B	[d]PDNS-affected piglet	UK
EU909688	II9F	PCV2b	1A/1B	[c]PMWS-affected piglet	Belgium
EF990644	1206	PCV2b	1A/1B	[c]PMWS-affected piglet	Belgium
AY484410	NL_Control_4	PCV2b	1C	Healthy pigs	The Netherlands
EU909686	II11A	PCV2b	1C	[c]PMWS-affected piglet	Belgium
AY754021	Aust 10	PCV2a	2A	Dead weaned pig	Australia
AY146991	Pingtung-1	PCV2a	2B	Unknown	Taiwan
AY180396	Pingtung-4	PCV2a	2B	Unknown	Taiwan
JN133304	390	PCV2a	2C	[c]PMWS-affected piglet	Spain
JN133305	Slovak	PCV2a	2D	[c]PMWS-affected piglet	Slovakia
AF055392	Stoon-1010	PCV2a	2E	[c]PMWS-affected piglet	Canada
AJ293868	1121	PCV2a	2E	Aborted foetuses	Canada
AJ293867	1103	PCV2a	2E	Aborted foetuses	Canada

[a] According to the nomenclature of Segalés et al. (2008)
[b] According to the classification system of Olvera et al. (2007)
[c] PMWS: postweaning multisystemic wasting syndrome
[d] PDNS: porcine dermatitis and nephropathy syndrome

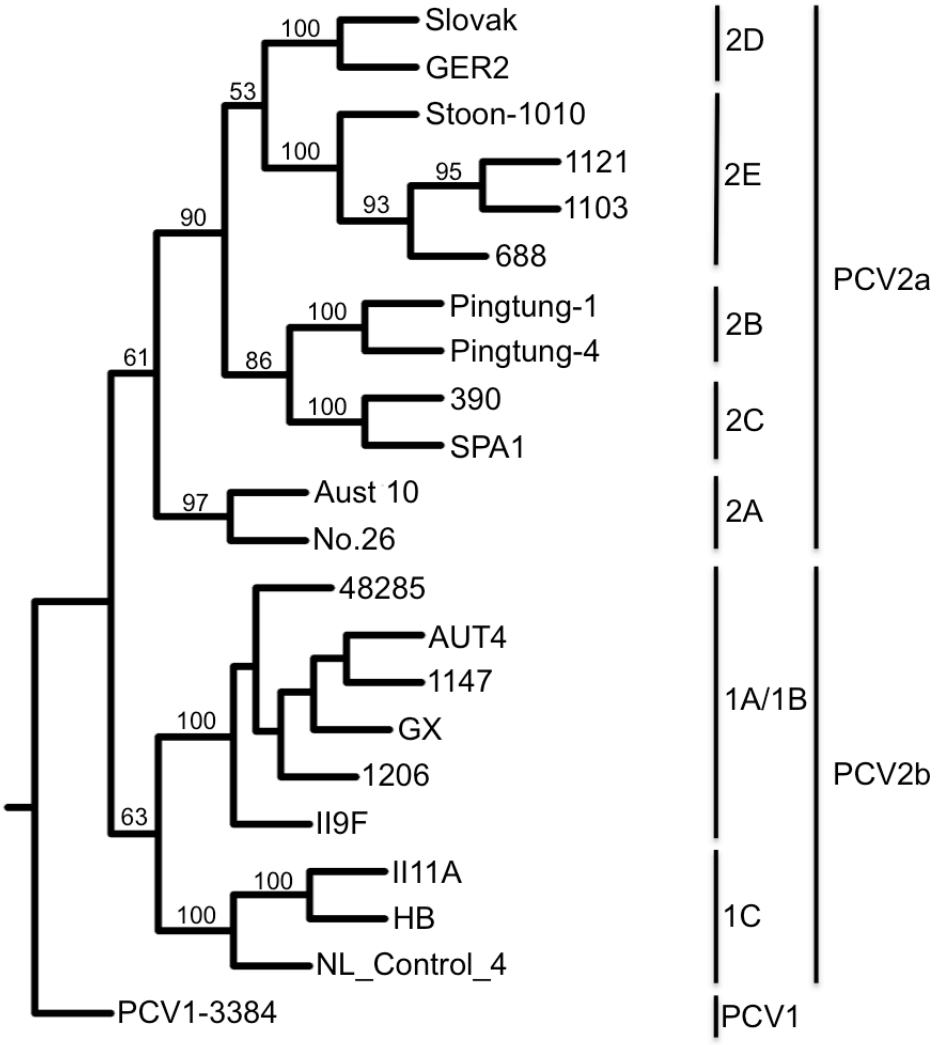

Fig. 1. Unrooted phylogenetic tree constructed by the Neighbour-Joining Method. Bootstrap values are indicated along the branches. This tree is based on the cap sequences of the PCV2 strains that were used in the present study. In addition, the following PCV2 sequences were selected from Olvera et al. (2007) and used for the construction of the tree: AUT4 (AY424404; PCV2b-1A), GX (AY556475; PCV2b-1B), HB (AY291317; PCV2b-1C), No. 26 (AB072302; PCV2a-2A), SPA1 (AF201308; PCV2a-2C), GER2 (AF201306; PCV2a-2D), 688 (AY094619; PCV2a-2E). PCV1-3384 (JN133302) was used as an outgroup. The accession numbers of the PCV2 strains used in this study are given in Table 1.

2.2. Cells

PCV-negative PK-15 cells were grown in minimal essential medium (MEM) containing Earle's salts and glutamine (Gibco, Grand Island, USA), supplemented with 5% foetal calf serum (FCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. Cell cultures were maintained at 37°C in the presence of 5% CO2.

2.3. Mouse monoclonal antibodies

Twenty mAbs were enclosed. Fifteen mAbs (9C3, 16G12, 21C12, 31D5, 38C1, 43E10, 48B5, 55B1, 59C6, 63H3, 70A7, 94H8, 103H7, 108E8 and 114C8) were generated against genotype PCV2a strain Stoon-1010 (Lefebvre et al., 2008). Five mAbs (6E9, 12E12, 14G2, 19G10 and 22C1) were generated against genotype PCV2b strain 1147 in our laboratory following similar procedures as for the PCV2a mAbs (Lefebvre et al., 2008).

2.4. Reactivity of monoclonal antibodies to different PCV2 strains

In order to check the reactivity of mAbs to the 14 PCV2 strains, all PCV2 strains were used to make 96-well IPMA plates as previously described by Labarque et al. (2000). PCV-negative PK-15 cells and PCV1-infected PK-15 cells were used as control IPMA plates. The staining procedure was identical to the IPMA technique described by Lefebvre et al. (2008) with ten-fold serial dilutions (started from 1:10) of hybridoma supernatants in PBS used as primary Abs. The IPMA antibody titres of a hybridoma supernatant were expressed as the reciprocal of the last dilution that resulted in a positive reaction. These assays were performed independently 3 times for each strain.

2.5. Purification and biotinylation of monoclonal antibodies

Six mAbs (31D5, 38C1, 59C6, 94H8, 108E8 and 114C8) generated against genotype PCV2a strain Stoon-1010 were chosen in order to perform cross-competition analysis by a competitive ELISA. To produce the competitor and detector antibody, 300 ml of supernatants for each of the 6 hybridomas was collected. The hybridoma supernatants were then purified by protein G SepharoseTM CL-4B (GE Healthcare, Uppsala, Sweden), followed by biotinylation using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

2.6. Cross-competition analysis for mAbs by a competitive ELISA

In order to perform a cross-competition analysis for mAbs, a competitive enzyme-linked immunosorbent assay (ELISA) was used. Briefly, MaxiSorp ELISA plates (Nunc, Glostrup, Denmark) were coated with purified mAb 38C1 in 0.05 M carbonate buffer (pH 9.6) with a final concentration of 5 mg/ml and the plates were incubated overnight at 4°C. After three washings with PBS containing 0.05% Tween 20 (PBS-T), the plates were blocked with dilution buffer (PBS-T containing 10% negative goat serum) for 1 h at 37°C. Then, PCV2

strain Stoon-1010 diluted in dilution buffer was distributed in each well with a final concentration of $10^{5.0}$ $TCID_{50}$/ml, followed by incubation for 1 h at 37°C. Afterwards, the plates were washed three times and 5 mg of purified mAbs diluted in the dilution buffer was added in each well and incubated for 1 h at 37°C. The mAb concentration was already predetermined to be saturating. An appropriate dilution of a biotin-labeled mAb in dilution buffer was added to the virus-mAb mixture. After 1 h incubation at 37°C, unbound reagents were washed away and bound biotin-labeled mAb was detected by using an appropriate dilution of streptavidin-biotinylated horseradish peroxidase complex (GE Healthcare, United Kingdom) and a substrate solution of tetramethylbenzidine and H2O2 (R&D systems, Minneapolis, USA). After 20 min, the reaction was stopped with 1 M H2SO4, and the optical density (OD) at 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). The amount of biotin-labeled mAb used was determined from a prior titration of the reagent against the PCV2 strain stoon-1010 in the absence of competitor. The competitive ELISA conditions for each step were investigated by varying the conditions in each step while maintaining conditions for all the other steps were constant, except for the steps of colorimetric reaction and stopping reaction. Each test mAb was tested in triplicate, and the control tests using a PCV2 irrelevant mAb 1C11 (Nauwynck and Pensaert, 1995) were carried out at least six times. Each test plate also contained background wells (no virus and no competitor) and the mean OD450 nm values derived from these wells were subtracted from all other mean test OD450 nm values of mAbs. After background correction, the OD450 nm value was converted to a percent inhibition (PI) value using the formula: PI (%) = 100× [1 - (Test mAb $OD_{450\ nm}$ / Irrelevant mAb $OD_{450\ nm}$)].

Each biotin-labeled and unlabeled mAb combination was tested for 3 times. The average PI for all the experiments was then calculated for each combination of biotin-labeled and non-biotin labeled mAb.

2.7. Construction of a competition map in the capsid protein

A competition map was made on one capsid protein of PCV2 to explain the general spatial relationship among competition mAbs.

3. Results

3.1. Reactivity of mAbs to different PCV2 strains

An IPMA experiment was performed to determine the reactivity of mAbs to all different PCV2 strains. The reactivity of mAbs to 14 PCV2 strains is shown in Table 2. Four out of 20 mAbs (12E12, 21C12, 38C1 and 114C8) reacted with all PCV2 strains with titres ranging from 10 to 10,000. One mAb, 19G10 stained all PCV2 strains except the strain 390 which belongs to subtype PCV2a-2C. MAbs 6E9, 9C3, 16G12, 43E10, 55B1, 63H3, 70A7, 94H8 and 103H7 did not react with the PCV2 strains of subtypes PCV2b-1C, PCV2a-2B, PCV2a-2C and PCV2a-2D and reacted with the PCV2 strains of subtypes PCV2b-1A/1B, PCV2a-2A and PCV2a-2E. Only one mAb 14G2 was found to be specific for PCV2b-1A/1B strains. MAbs 31D5, 59C6 and 108E8 had titres ranging from 1000 to 10,000 for PCV2a-2A and PCV2a-2E strains and did not react with the other strains of other subtypes. MAb 22C1 reacted with all PCV2b strains (except PCV2b-1C strain NL_Control_4) and did not react with PCV2a strains. On the other hand, mAb 48B5 reacted with all PCV2a strains (except PCV2a-2C strain 390) and with one PCV2b-1A/1B strain 1206 and did not show reaction to other PCV2b strains. None of the 20 mAbs reacted with PCV1-infected cells or PCV-negative PK-15 cells (Table 2).

Table 2. IPMA antibody titres of mAbs and identification of aa that are crucial for binding of mAbs.

mAbs		IPMA antibody titres[a]															
name	raised against	48285 PCV2b 1A/1B	1147 PCV2b 1A/1B	II9F PCV2b 1A/1B	1206[b] PCV2b 1A/1B	NL4[d] PCV2b 1C	II11A PCV2b 1C	Aust 10 PCV2a 2A	P1[e] PCV2a 2B	P4[f] PCV2a 2B	390 PCV2a 2C	Sv[g] PCV2a 2D	1010[h] PCV2a 2E	1121 PCV2a 2E	1103 PCV2a 2E	specificity	aa positions that are responsible for the differential reactivity of mAbs to different PCV2 strains (see Fig. 2)
12 E12	PCV2b	100	100	1000	100	100	100	100	100	100	100	100	100	100	100	Universal	-
21C12	PCV2a	100	100	10	100	100	10	1000	100	100	100	100	1000	1000	1000	Universal	-
38C1	PCV2a	10000	1000	1000	1000	100	10	1000	1000	1000	1000	100	1000	1000	1000	Universal	-
114C8	PCV2a	10000	1000	1000	1000	100	100	1000	100	1000	1000	1000	10000	1000	10000	Universal	-
19G10	PCV2b	1000	100	1000	100	100	100	100	100	100	-ve	100	100	100	100	Universal (except 2C)	30, 130 and 133
6 E9	PCV2b	1000	1000	1000	1000	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	1000	1A/1B, 2A, 2E	59
9C3	PCV2a	10000	1000	1000	1000	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	1000	1A/1B, 2A, 2E	59
16G12	PCV2a	10000	1000	1000	1000	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	10000	1000	1A/1B, 2A, 2E	59
43E 10	PCV2a	10000	1000	1000	1000	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	1000	1A/1B, 2A, 2E	59
55B1	PCV2a	10000	1000	1000	1000	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	1000	1A/1B, 2A, 2E	59
63H3	PCV2a	1000	1000	1000	1000	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	1000	1A/1B, 2A, 2E	59
70A7	PCV2a	10000	1000	1000	1000	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	1000	1A/1B, 2A, 2E	59
94H8	PCV2a	10000	10000	1000	1000	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	1000	1A/1B, 2A, 2E	59
103H7	PCV2a	10000	1000	1000	1000	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	1000	1A/1B, 2A, 2E	59
14G2	PCV2b	1000	1000	1000	1000	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	1A/1B	89, 206 and 210
31D5	PCV2a	-ve	-ve	-ve	-ve	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	10000	2A, 2E	63
59C6	PCV2a	-ve	-ve	-ve	-ve	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	1000	2A, 2E	63
108 E8	PCV2a	-ve	-ve	-ve	-ve	-ve	-ve	1000	-ve	-ve	-ve	-ve	1000	1000	1000	2A, 2E	63
22C1	PCV2b	100	100	100	100	-ve	100	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	PCV2b (except NL4[d])	not conclusive
48B5	PCV2a	-ve	-ve	-ve	100[c]	-ve	-ve	100	100	100	-ve	100	100	100	100	PCV2a (except 2C, 1206[b])	not conclusive

[a] IPMA titres of mAbs were expressed as the reciprocal of the last dilution that resulted in a positive reaction. –ve: negative.

[b] Strain 1206 contains two different subpopulations of viruses, where 99% of the virus behave as a PCV2b strain and 1% of the virus behaves as a PCV2a strain (Lefebvre et al., 2008).

[c] MAb 48B5 stained only PCV2a subpopulation of the strain 1206 as described previously by Lefebvre et al. (2008).

[d] NL_Control_4, [e] Pingtung-1, [f] Pingtung-4, [g] Slovak, [h] Stoon-1010

-: no specific aa position could be identified as universal mAbs react to all PCV2 strains.

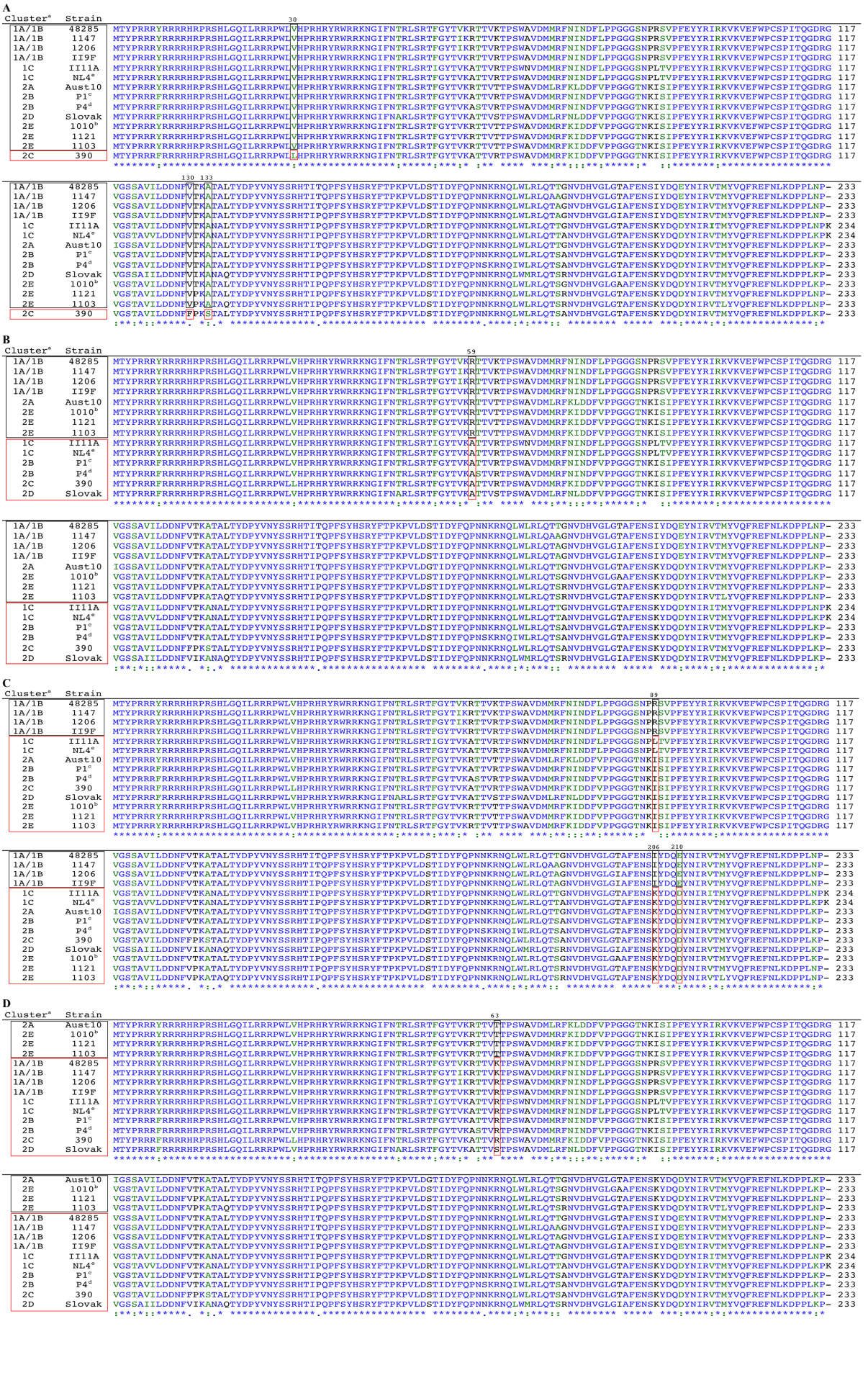
A
B
C
D
Cluster[a]
Strain

Fig. 2. ORF2 amino acid alignment of the PCV2 strains used in this study. The aa residue(s) that discriminate the different PCV2 strains have been marked (in box) and are indicated by the numbering on the top of each box. (A) The aa positions 30, 130 and 133 differentiated the strain 390 (PCV2a-2C) from the rest of the PCV2 strains by the mAb 19G10; (B) position 59 discriminated the strains of PCV2b-1A/1B, PCV2a-2A and PCV2a-2E from the strains of PCV2b-1C, PCV2a-2B, PCV2a-2C and PCV2a-2D by the mAbs 6E9, 9C3, 16G12, 43E10, 55B1, 63H3, 70A7, 94H8 and 103H7; (C) positions 89, 206 and 210 differentiated the strains of PCV2b-1A/1B from the rest of the PCV2 strains by the mAb 14G2; and (D) position 63 distinguished the strains of PCV2a-2A and PCV2a-2E from the remaining PCV2 strains of PCV2b-1A/1B, PCV2b-1C, PCV2a-2B, PCV2a-2C and PCV2a-2D by the mAbs 31D5, 59C6 and 108E8. Consensus key: (*) single, fully conserved residue; (:) conservation of strong groups; (.) conservation of weak groups; (–) no consensus. [a]According to the classification system of Olvera et al. (2007); [b]Stoon-1010; [c]Pingtung-1; [d]Pingtung-4; [e]NL_Control_4.

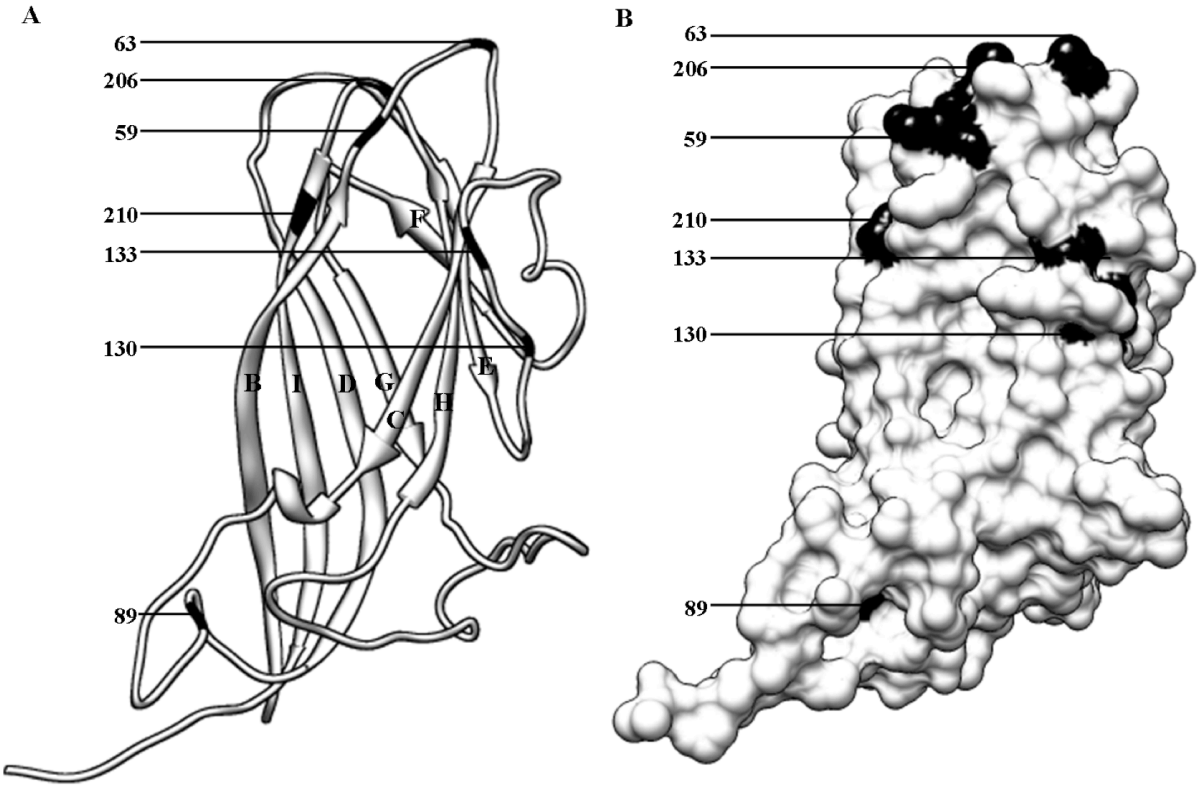

Fig. 3. Location of aa 59, 63, 89, 130, 133, 206 and 210 on PCV2 capsid protein. A homology model of the PCV2 (Stoon-1010) capsid protein was generated using the $PCV2^{CS}$ crystal structure (PDB accession no. 3R0R) (Khayat et al., 2011) as a template with SWISS-MODEL Workspace (Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006). Figures were made using University of California, San Francisco Chimera 1.5.3 (Pei and Grishin, 2001). (A) Ribbon diagram of the PCV2 (Stoon-1010) capsid protein with the secondary structures. Eight β-strands labeled with letters B to I. The aa positions crucial for binding of the different mAbs (as mentioned in Table 2 and Fig. 2) have been shown (black bar) on the capsid protein (positions 59 and 63 in loop BC; position 89 in loop CD; positions 130 and 133 in loop EF; position 206 in loop HI and position 210 in β-strand I). (B) Surface diagram of the PCV2 (Stoon-1010) capsid protein. The aa positions 59, 63, 89, 130, 133, 206 and 210 are located on the exterior surface of a capsid protein (in black).

3.2. Identification of amino acid (aa) residues that are crucial for the differential reactivity of mAbs to different PCV2 strains

Since mAbs directed against the capsid protein of PCV2 and some mAbs showed differential reactivity to different PCV2 strains, capsid protein sequences of all the PCV2 strains were compared and the aa residues that are responsible for the differential reactivity of mAbs to the PCV2 strains have been identified and are shown in Table 2 and Fig. 2. The aa positions 30, 130 and 133 differentiated the strain 390 (PCV2a-2C) from the rest of the PCV2 strains by the mAb 19G10 (Fig. 2A); position 59 discriminated the strains of PCV2b-1A/1B, PCV2a-2A and PCV2a-2E from the strains of PCV2b-1C, PCV2a-2B, PCV2a-2C and PCV2a-2D by the mAbs 6E9, 9C3, 16G12, 43E10, 55B1, 63H3, 70A7, 94H8 and 103H7 (Fig. 2B); positions 89, 206 and 210 differentiated the strains of PCV2b-1A/1B from the rest of the PCV2 strains by the mAb 14G2 (Fig. 2C); and position 63 distinguished the strains of PCV2a-2A, PCV2a-2E from the remaining PCV2 strains of PCV2b-1A/1B, PCV2b-1C, PCV2a-2B, PCV2a-2C and PCV2a-2D by the mAbs 31D5, 59C6 and 108E8 (Fig. 2D). Furthermore, these identified aa residues (except aa position 30) have been positioned on a crystal structure of the PCV2 (PCV2a-2E strain Stoon-1010) capsid protein (Fig. 3A) and they are located on the exterior surface of the PCV2 capsid protein (Fig. 3B).

3.3. Cross-competition analysis for mAbs by competitive ELISA

Six mAbs, mAbs 38C1 and 114C8 from the universal group; mAb 94H8 from PCV2b-1A/1B, PCV2a-2A, PCV2a-2E group; mAbs 31D5, 59C6 and 108E8 from PCV2a-2A, PCV2a-2E group (Table 2), were selected for the antibody cross-competition analysis. All the six mAbs were purified and labeled with biotin. The cross-competition matrix therefore comprised 36 individual mAb combinations (one non-biotin labeled mAb and one biotin-labeled mAb). The mean values derived from multiple experiments are presented in Fig. 4. Each biotin-labeled mAb was strongly inhibited by its homologous mAb, which confirms that this competitive ELISA is a valid method that could be used in mAbs cross-competition analysis (Moore and Sodroski, 1996).

Both mAbs 38C1 and 114C8 completely inhibited the other 5 mAbs from reacting with the PCV2 strain Stoon-1010 with a PI of more than 98%. MAbs 31D5 and 94H8 strongly inhibited the other 4 mAbs, except mAb 38C1 (41.43% and 42.17% for mAbs 31D5 and 94H8, respectively) from reacting with Stoon-1010. The inhibition extent of mAb 108E8 to other mAbs was 85.14% (31D5), 47.58% (38C1), 77.8% (59C6), 43.94% (94H8) and 48.21%

(114C8). MAb 59C6 strongly blocked mAbs 31D5 (86.48%), 94H8 (88.61%) and 114C8 (84.17%) and showed weak inhibition to mAb 108E8 (69.94%) and a very weak inhibition to mAb 38C1 (31.09%) (Fig. 4).

81-100%
61-80%
41-60%
21-40%

mAbs	Antigenic subtype	38C1-Biotin	114C8-Biotin	94H8-Biotin	108E8-Biotin	59C6-Biotin	31D5-Biotin
38C1	Universal	99.47 ±0.72	99.66 ±1.07	98.37 ±0.65	99.24 ±1.28	100.44 ±0.44	99.31 ±0.74
114C8	Universal	98.98 ±0.41	99.46 ±0.39	99.32 ±0.76	99.64 ±0.32	99.02 ±0.60	99.08 ±0.22
94H8	1A/1B, 2A, 2E	42.17 ±2.98	96.39 ±1.01	98.37 ±0.79	86.22 ±2.54	99.41 ±0.39	98.35 ±0.84
108E8	2A, 2E	47.58 ±1.99	48.21 ±3.80	43.94 ±2.69	94.76 ±1.94	77.80 ±5.86	85.14 ±0.56
59C6	2A, 2E	31.09 ±4.32	84.17 ±3.76	88.61 ±1.32	69.94 ±3.38	98.30 ±1.16	86.48 ±2.34
31D5	2A, 2E	41.43 ±2.08	87.46 ±2.84	87.92 ±1.03	91.15 ±2.92	96.03 ±1.78	90.07 ±1.75

Fig. 4. Competition matrix of mAbs binding to PCV2 (Stoon-1010) capsid protein. The competitor mAbs and their antigenic subtypes are listed at the left and the biotin-labeled mAbs are listed on the top. The extent of competition is depicted by the colour and intensity of shading: red indicates inhibition of 81–100%; reddish yellow indicates inhibition of 61–80%; yellow indicates inhibition of 41–60%; white indicates inhibition of 20–40%. The values recorded within each box represent the percent inhibition of the labeled mAb as compared with the control (0%).

The epitopes' competition map of the selected 6 mAbs were placed on a capsid protein surface (as shown in Fig. 5) based on the mAbs' competition data (Fig. 4), aa positions crucial for the binding of mAbs (Table 2; Fig. 2; Fig. 3) and an assumed biological molecule of PCV2 (PDB accession number: 3R0R) (Khayat et al., 2011). Generally, the interaction surface of mAb 38C1 and its epitope was bigger than that of other mAbs and their epitopes. The interaction surface of mAb 114C8 and its epitope was smaller than that of mAb 38C1 and its epitope, but bigger than that of other 4 mAbs (31D5, 59C6, 94H8, 108E8) and their epitopes. The interaction surface of mAb 108E8 and its epitope partly overlapped with other five mAbs and their epitopes while interaction surfaces of other five mAbs and their epitopes fully overlapped. Epitopes of mAbs 31D5, 59C6 and 94H8 overlapped each other and were

located inside of the epitopes of mAbs 38C1 and 114C8 (Fig. 5). As aa position 59 has been identified as crucial for the binding of the mAb 94H8 and position 63 for the binding of mAbs 31D5, 59C6 and 108E8 (Table 2), the aa position 59 was positioned in the interaction surface of mAb 94H8 and its epitopes and aa position 63 was placed in the interaction surfaces of mAbs 31D5, 59C6 and 108E8 and their epitopes. The other 5 residues (aa positions 89, 130, 133, 206 and 210) were located within the interaction surfaces of the universal mAbs 38C1 and 114C8 (Fig. 5).

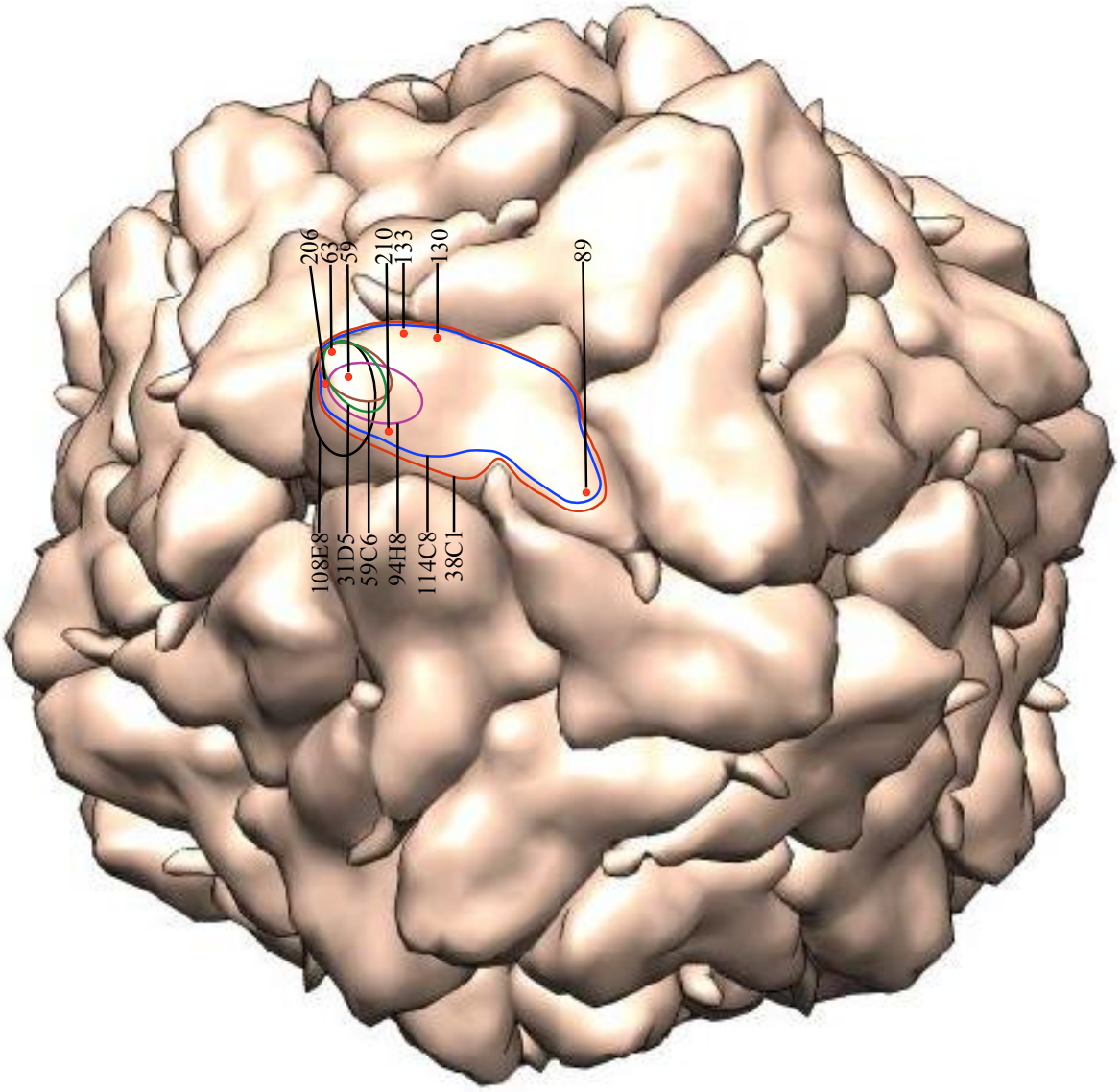

Fig. 5. Epitopes competition map of different mAbs on a capsid protein of PCV2. The diagram of PCV2 particle is an assumed biological molecule (biological assembly) (taken from PDB accession no. 3R0R). The epitopes of different mAbs are shown on a PCV2 capsid protein with different circles based on the competition data (Fig. 4). The aa positions that have been identified as crucial for binding of the mAbs (Table 2) are positioned and marked with red circles on the surface of a PCV2 capsid protein.

4. Discussion

The reactivity pattern of 15 mAbs generated against cluster PCV2a-2E strain Stoon-1010 and 5 mAbs produced against cluster PCV2b-1A/1B strain 1147 was determined against representative strains of all PCV2 genetic clusters. Four universal mAbs (12E12, 21C12, 38C1 and 114C8) were identified that reacted to all clusters of both genotypes (PCV2a and PCV2b). It shows that PCV2 strains of both genotypes, PCV2a and PCV2b have common epitopes in the capsid protein. Sequence comparison of the capsid protein of 14 PCV2 strains revealed that there were more than 180 aa residues (out of 233/234/235) in common among the different PCV2 strains which are dispersed throughout the capsid protein (Fig. 2). The above-mentioned mAbs will be reactive to parts of these common aa residues. Lekcharoensuk et al. (2004) showed that the first 47 aa (out of 233 aa) of the capsid protein are most probably not involved in epitopes on the exterior surface of the PCV2 capsid. These 47 aa residues make the interior surface of the capsid which most probably remains unexposed to react with antibodies (Lekcharoensuk et al., 2004; Khayat et al., 2011). The epitopes that are common between PCV2a and PCV2b will be present in between the aa 48 and 233/234, which form the exterior surface of the PCV2 capsid protein and reacts with the antibodies (Lekcharoensuk et al., 2004; Khayat et al., 2011). Our results not only indicate the presence of common epitopes between PCV2a and PCV2b but also the presence of epitope(s) that are specific to genotype PCV2a or PCV2b. Indeed, mAbs 31D5, 48B5, 59C6 and 108E8 raised against PCV2a recognised only PCV2a strains and mAbs 14G2 and 22C1 raised against PCV2b recognised only PCV2b strains. Further in depth investigations are necessary to identify the common epitopes or genotype-specific epitopes to PCV2a and PCV2b.

Some mAbs were reactive or not reactive to a small number of PCV2 strains suggesting the existence of unique recognition sites. We compared the capsid protein sequences and observed few aa differences between PCV2 strains of which some strains reacted with mAbs and others did not. This different reactivity between different PCV2 strains was associated with the low number of differentiating aa of the capsid protein as shown in Table 2 and Fig. 2. The aa positions 30, 130, 133 (for mAb 19G10); 59 (for mAbs 6E9, 9C3, 16G12, 31D5, 43E10, 55B1, 59C6, 63H3, 70A7, 94H8, 103H7 and 108E8); 89, 206 and 210 (for mAb 14G2); and 63 (for mAbs 31D5, 59C6 and 108E8) that have been shown as crucial for binding of the different mAbs are located in the inner and outer border of the earlier described linear epitopes or immunoreactive regions: 25-43, 47-84, 185-202, 113-147, 193-

207 (Mahe´ et al., 2000; Lekcharoensuk et al., 2004; Shang et al., 2009). More research is needed for further confirmation of these findings.

MAbs against PCV2 could be used for diagnosis of PCVAD. PCV2-specific and mAb-based ELISA has been developed in the past (McNeilly et al., 2001; McNeilly et al., 2002; Lekcharoensuk et al., 2004; Huang et al., 2011a; Huang et al., 2011b). To our knowledge, mAb-based diagnostic tests have not been validated yet to detect all different clusters of PCV2. Four universal mAbs (12E12, 21C12, 38C1 and 114C8) that were reactive to all PCV2 clusters could be used to develop a universal mAb-based IFA, IHC, IPMA or ELISA in diagnostic assays to detect all types of PCV2 strains irrespective of their genotypes, clusters, and clinical and geographical origin. Further research should be carried out to deal with these issues.

Although antigenic differences between PCV2a and PCV2b strains exist based on the use of monoclonal antibodies, they still share common epitopes in the capsid protein. The existence of common epitopes could explain why the immunity induced by a PCV2a infection can cross-protect pigs upon a subsequent infection with PCV2b and vice-versa and why currently available PCV2a-based vaccines can protect pigs against subsequent challenges with PCV2b strains and vice-versa (Fort et al., 2008; Fort et al., 2009; Beach et al., 2011; Beach and Meng, 2012). Differences in some genotypes may be the reason for somewhat better results in homologous situations. When PCV2b-vaccinated animals were challenged with PCV2a, then they had higher viral DNA loads at 21 days post-challenge than when they were challenged with PCV2b (Beach et al., 2011). When PCV2a-vaccinated animals were challenged with PCV2b, then a higher percentage of animals were viral DNA positive in nasal and faecal swabs at 20 days post-challenge than when they were challenged with PCV2a (Fort et al., 2008).

Reciprocal inhibitions were observed between mAbs 38C1 and 114C8, mAbs 114C8 and 94H8, mAbs 114C8 and 59C6, mAbs 114C8 and 31D5, mAbs 94H8 and 59C6, mAbs 94H8 and 31D5, mAbs 108E8 and 59C6, mAbs 108E8 and 31D5 and mAbs 59C6 and 31D5. Several mAbs exhibited a unidirectional inhibitory effect. These include mAbs 38C1 and 94H8, mAbs 38C1 and 108E8, mAbs 38C1 and 59C6, mAbs 38C1 and 31D5, mAbs 114C8 and 108E8, mAbs 94H8 and 108E8. Reciprocal and unidirectional inhibitory effects of mAbs 38C1 and 114C8 indicate that the epitope of 38C1 was different from that of 114C8. However, they did indeed recognise very similar and overlapping antigenic sites or the

epitopes that are spatially close to each other within the same antigenic site and therefore these two mAbs (38C1 and 114C8) could inhibit each other. Although mAb 114C8 and 94H8 could inhibit each other and mAb 114C8 could completely inhibit mAb 38C1 from reacting with virus, mAb 94H8 could not completely inhibit 38C1. Therefore, the epitope of 94H8 was different from that of 114C8. MAb 108E8 epitope was different from that of 38C1, 114C8 and 94H8 as three unidirectional inhibitory effects were found in the competition analysis of its and other 3 mAbs (38C1, 114C8 and 94H8). The epitope of mAb 59C6 seemed the same as that of mAb 31D5. In fact, there were some differences present between them as a weak inhibition was found from mAb 59C6 to biotin-108E8 while strong inhibition was found from mAb 31D5 to biotin-108E8. From this discussion, we could conclude that there were at least 6 overlapping epitopes present on the surface of PCV2 capsid protein. This is in agreement with previous findings (Lekcharoensuk et al., 2004). The epitopes recognised by the universal mAbs 38C1 and 114C8 were bigger than those recognised by the other 4 non-universal mAbs (31D5, 59C6, 94H8 and 108E8). It indicates that the universal mAbs could completely inhibit the binding of the non-universal mAbs with PCV2.

This study demonstrates that some genetic clusters can be discriminated by the use of a large panel of mAbs. The mAbs that have been identified as universal could be used for the development of diagnostic tests. Six overlapping epitopes were identified on the surface of the PCV2 capsid protein by epitopes' competition analysis. The epitopes' competition map that has been designed to explain the antibody competition data, should not be taken as a model to determine the exact spatial relationship among the different epitopes of the capsid protein.

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Chapter 3.2.

Single amino acid mutations in the capsid switch the neutralisation phenotype of porcine circovirus 2

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Abstract

Porcine circovirus 2 (PCV2) is the causative agent of Porcine Circovirus Associated Diseases in pigs. Previously, it was demonstrated that monoclonal antibodies (mAbs) 16G12, 38C1, 63H3 and 94H8 directed against the PCV2 capsid protein recognise PCV2 strains Stoon-1010 (PCV2a), 48285 (PCV2b), 1121 (PCV2a), 1147 (PCV2b) and II9F (PCV2b) but only neutralise Stoon-1010 and 48285. This points to the existence of two distinct PCV2 neutralisation phenotypes: phenotype α (mAb recognition with neutralisation; Stoon-1010 and 48285) and phenotype β (mAb recognition without neutralisation; 1121, 1147 and II9F). In the present study, amino acids that are important in determining the neutralisation phenotype were identified in the capsid. Mutation of T at position 190 to A in 48285 (phenotype α) resulted in a capsid that resembles that of 1147 (phenotype β) and caused a loss of neutralisation (switch from α to β). Mutations of P at position 151 to T and A at position 190 to T in II9F (phenotype β) resulted in a capsid that resembles that of 48285 (phenotype α) and gave a gain of neutralisation (switch from β to α). Mutations of T at position 131 to P and E at position 191 to R in Stoon-1010 (phenotype α) changed the capsid into that of 1121 (phenotype β) and reduced neutralisation (switch from α to β). This study demonstrated that single amino acid changes in the capsid result in a phenotypic switch from α to β or β to α.

1. Introduction

Porcine circovirus type 2 (PCV2) is a non-enveloped, single-stranded DNA virus. It is the causative agent of several economically important Porcine Circovirus Associated Diseases (PCVAD) (Gillespie et al., 2009), of which the Postweaning Multisystemic Wasting Syndrome (PMWS) in weaned pigs (Kennedy et al., 2000; Albina et al., 2001; Bolin et al., 2001) and reproductive failure in sows (Park et al., 2005; Rose et al., 2007; Madson et al., 2009; Saha et al., 2010) have been experimentally reproduced with PCV2 alone.

The covalently closed circular PCV2 genome consists of 1,766-1,769 nucleotides (Meehan et al., 1998; Huang et al., 2011) and contains 11 putative open reading frames (ORFs) (Hamel et al., 1998). Protein expression has been described for 3 of these ORFs. ORF1 codes for the non-structural replication-associated protein Rep and its spliced frame-shifted variant Rep' (Cheung, 2003; Mankertz et al., 2003). ORF2 codes for the structural capsid protein (Nawagitgul et al., 2000). The non-structural ORF3 protein has been associated with apoptosis *in vitro* and viral pathogenesis *in vivo* (Liu et al., 2005; Liu et al., 2006), but these results are somewhat controversial because they could not yet be reproduced by other laboratories (as described and reviewed by Juhan et al., 2010).

The PCV2 capsid protein consists of 233-235 amino acids (aa) (Nawagitgul et al., 2000; Olvera et al., 2007; Huang et al., 2011) and there are three major regions of amino acid heterogenecity (57-91, 121-136 and 180-191), of which two have a high immunogenic index (Larochelle et al., 2002; de Boisséson et al., 2004). Several antigenic domains have been identified on the PCV2 capsid protein by using porcine polyclonal antibodies (Mahé et al., 2000; Truong et al., 2001) or mouse monoclonal antibodies (mAbs) (Lekcharoensuk et al., 2004; Shang et al., 2009). Recently, PCV2 strains have been divided into two major genotypes: PCV2a and PCV2b and 8 genetic clusters: PCV2b-1A to PCV2b-1C and PCV2a-2A to PCV2a-2E based on their genomic sequences (Olvera et al., 2007; Grau-Roma et al., 2008; Segalés et al., 2008). Genotype- and cluster-specific domains on the capsid protein have also been identified using mouse mAbs generated against genotype PCV2a or PCV2b (Cheung et al., 2007; Cheung and Greenlee, 2011; Saha et al., 2012a).

PMWS-affected pigs have significantly lower levels of PCV2-neutralising antibodies when compared to sub-clinically infected animals (Meerts et al., 2006; Fort et al., 2007), suggesting a crucial role for neutralising antibodies in the prevention of PMWS. Several authors have

described monoclonal antibodies (mAbs) with neutralising activity to the PCV2 capsid protein (McNeilly et al., 2001; Lekcharoensuk et al., 2004; Zhou et al., 2005; Lefebvre et al., 2008a; Shang et al., 2009) and Lefebvre et al. (2008a) demonstrated that although PCV2 isolates Stoon-1010, 48285, 1121, 1147 and II9F were recognised by mAbs 16G12, 38C1, 63H3 and 94H8 using the immuno-peroxidase monolayer assays (IPMA), only Stoon-1010 and 48285 were neutralised (N+). This points to the existence of two distinct PCV2 neutralisation phenotypes: phenotype α (mAb recognition with neutralisation; IPMA+N+) and phenotype β (mAb recognition without neutralisation; IPMA+N-). It was now questioned which change(s) in the capsid of PCV2 cause a switch from phenotype α (IPMA+N+) to β (IPMA+N-) and vice-versa.

The aim of the present study was to demonstrate which change(s) in the capsid switches the phenotype (α to β or β to α) of PCV2.

2. Materials and methods

2.1. Viruses and cells

Five different PK-15 adapted PCV2 strains Stoon-1010 (AF055392), 48285 (AF055394), 1121 (AJ293868), 1147 (AJ293869), and II9F (EU909688) were enclosed in this study.

PCV-negative PK-15 cells were grown in minimal essential medium (MEM) containing Earle's salts and GlutaMAX^{TM}-I (MEM+GlutaMAX^{TM}-I, Gibco, Grand Island, USA) and supplemented with 5 % foetal calf serum (FCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. Cell cultures were maintained at 37°C in the presence of 5 % CO2.

2.2. PCV2 Pairs

Previously, alignment of the capsid proteins (Lefebvre et al., 2008a) has shown a very limited number of non-conserved aa differences between the neutralised strain 48285 (phenotype α) and the non-neutralised strains 1147 and II9F (both phenotype β), and between the neutralised strain Stoon-1010 (phenotype α) and the non-neutralised strain 1121 (phenotype β). Therefore, 48285 (α)/1147 (β), II9F (β)/48285 (α) and Stoon-1010 (α)/1121 (β) were chosen as PCV2 pairs and these non-conserved aa residues were selected for site-directed mutagenesis in order to switch the neutralisation phenotype.

2.3. Construction of PCV2 infectious clones

The full-length genome of strain II9F has previously been cloned in pCR-BluntII-TOPO, resulting in II9F-13 (Van Doorsselaere et al., 2010). The strain II9F corresponds with the strain VC2002-K39 (Lefebvre et al., 2009). Amplification of the genomes of Stoon-1010 and 48285 was performed (as described above for II9F) with primers INFCL-PCV2-FW (5'-ggaagcttcagtaatttatttcatatggaa-3') and INFCL-PCV2-REV (5'-ggaagctttttatcacttcgtaatggtt-3'), hereby introducing a HindIII site (underlined in the primer sequence) after the stop codon of the *Rep gene*. Infectious clones produced from Stoon-1010 and 48285 were Stoon-1010-8 and 48285-24, respectively.

2.4. Mutagenesis of the *capsid gene*

Mutagenesis of the *capsid gene* (Fig. 1) was performed using a Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, USA). The *capsid gene* of 48285-24 was mutated using primers 48285TtoA1147FW (5'-ctgagactacaaactgctggaaatgtagaccac-3') and 48285TtoA1147REV (5'-gtggtctacatttccagcagtttgtagtctcag-3') introducing a T to A change at aa position 190.

The *capsid gene* of II9F-13 was mutated at aa position 190 (from A to T) using primers II9FAtoT48285FW (5'-ctgagactacaaactactggaaatgtagaccac-3') and II9FAtoT48285REV (5'-gtggtctacatttccagtagtttgtagtctcag-3') and at aa position 151 (from P to T) using II9FPtoT48285FW (5'-tcccgccataccataactcagcccttctcctac-3') and II9FPtoTREV48285 (5'-gtaggagaagggctgagttatggtatggcggga-3'). Mutagenesis at both positions was performed with all four primers.

The *capsid gene* of Stoon-1010-8 was mutated at position 191 (from E to R) using primers StoonEtoR1121FW (5'-agactacaaacctctagaaatgtggaccacgta-3') and StoonEtoR1121REV (5'-tacgtggtccacatttctagaggtttgtagtct-3') and at aa position 131 (from T to P) using StoonTtoP1121FW (5'gatgataactttgtaccaaaggccacagcccta-3') and stoonTtoP1121REV (5'-tagggctgtggcctttggtacaaagttatcatc-3'). Mutagenesis at both positions was performed with the four primers simultaneously.

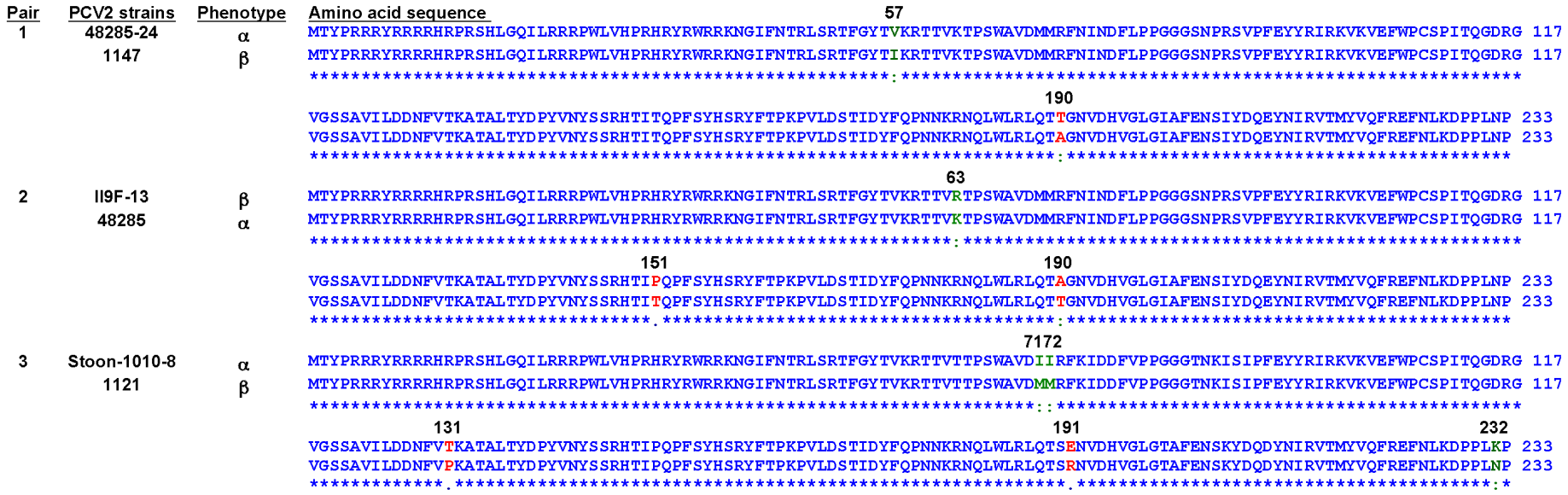

Fig. 1. ORF2 amino acid alignment of three pairs of PCV2 strains with small aa differences (mutated aa in red) and a different phenotype within each pair.

Consensus key: * fully conserved residue : conservation of strong groups . conservation of weak groups.

2.5. Transfection of PK-15 cells and production of PCV2 virus

The HindIII-digested (48285-24, II9F-13 and Stoon-1010-8 and their mutants) plasmids were transfected onto PCV-negative PK-15 cells with lipofectamine (Invitrogen, Merelbeke, Belgium), according to the manufacturer's instructions. Seventy-two hours post transfection, PCV2-transfected cells and supernatant fluids were collected. PCV2-transfected cells were smeared onto glass slides, fixed in methanol at -20°C for 10 min and stored at -20°C until use. Thereafter, cell smears were air dried at room temperature for 10 min and stained with an indirect immunofluorescence staining adapted from Saha et al. (2012b). Monoclonal antibody F190 (McNeilly et al., 2001) or biotinylated purified porcine polyclonal antibodies (pAbs) were used as primary antibodies and fluorescein isothiocyanate (FITC)-labelled goat-anti-mouse pAbs (Molecular Probes, Eugene, USA) or Texas Red-labelled streptavidin (Molecular Probes) were used as secondary antibodies, respectively. Cells were visualised with a fluorescent microscope as described before (Lefebvre et al., 2008b). Supernatant fluids were passed several times on PCV-negative PK-15 cells and the resulting supernatant fluids were titrated on PCV-negative PK-15 cells as described by Meerts et al. (2005).

2.6. Sequence analysis

The sequences of the PCV2 genomes (48285-24, II9F-13 and Stoon-1010-8 and their mutants) in the pCR-bluntII-TOPO vector were determined using M13FW, M13REV and PCV2-specific primers (Lefebvre et al., 2008a; Lefebvre et al., 2009) and PCV2 viruses produced from these plasmids were sequenced as described previously (Lefebvre et al., 2008a; Lefebvre et al., 2009).

2.7. Reactivity of monoclonal antibodies to different PCV2 strains in IPMA

The reactivity of the mAbs to different PCV2 strains was described previously (Lefebvre et al., 2008a; Saha et al., 2012a). In the present study, a serial four-fold dilution, starting from 1:10, of the four neutralising mAbs 16G12, 38C1, 63H3 and 94H8 were made in PBS and used to test their reactivity to different PCV2 strains (original PCV2 viruses, the viruses derived from infectious clones and the mutant viruses) in an immuno-peroxidase monolayer assay (IPMA), similar to the technique described before (Lefebvre et al., 2008a; Saha et al., 2012a). These assays were performed 3 times for each of the PCV2 strains.

2.8. Sensitive neutralisation assays

The neutralising activity of mAbs 16G12, 38C1, 63H3 and 94H8 to different PCV2 strains was determined in a sensitive neutralisation assay, identical to the technique described by Lefebvre et al. (2008a). MAbs 1C11 and 13D12 (Nauwynck and Pensaert, 1995) were used as negative controls. The original PCV2 strains 48285 and Stoon-1010 (neutralised by the mAbs) and 1147, 1121 and II9F (not neutralised by the mAbs) (Lefebvre et al., 2008a), were enclosed as controls. These experiments were performed 3 times for each of the PCV2 strains.

2.9. Statistical analysis

The neutralisation percentages of the mAbs to the PCV2 viruses derived from infectious clones and the mutants were compared by applying unpaired t-test with Welch's correction e.g. the neutralisation percentages of mAbs to 48285-24 were compared with that of 48285(T190A). Similarly, the neutralisation percentages of mAbs to Stoon-1010-8 were compared with those of 1010(E191R), 1010(T131P) and 1010(E191R/T131P). The neutralisation percentages of mAbs to II9F-13 were compared with those of II9F(A190T), II9F(P151T) and II9F(A190T/P151T). Statistical analyses were performed using GraphPad Prism Software version 5.0b (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered significant when $P<0.05$.

3. Results

3.1. Construction of infectious clones from the PCV2 strains Stoon-1010 and 48285

In order to produce small change(s) in the PCV2 capsid, infectious clones were constructed from Stoon-1010 and 48285 and the resulting clones are Stoon-1010-8 and 48285-24, respectively. Sequencing of Stoon-1010-8 revealed four aa differences with the GenBank sequence (AF055392) at positions 71 (MtoI), 72 (MtoI), 191 (GtoE) and 200 (AtoT), respectively. Sequencing of 48285-24 showed 1 aa difference at position 200 (TtoI) with the GenBank sequence (AF055394). Van Doorsselaere et al. (2010) previously constructed an infectious clone from II9F (II9F-13).

In order to produce infectious virus, the HindIII-digested plasmids (48285-24, Stoon-1010-8 and II9F-13) were transfected onto PCV-negative PK-15 cells and subsequently, PCV2 was passed several times on PK-15 cells. Indirect immunofluorescence staining of PCV2-infected

PK-15 cells and titration of supernatant fluids from PCV2-infected PK-15 cells demonstrated that PCV2 virus was produced. PCV2 genomic sequences were obtained after 5 passages on PK-15 cells and the nucleotide sequences were 100% identical with the genomes in the pCR-BluntII-TOPO vectors.

3.2. Mutagenesis of the *cap* from 48285-24, Stoon-1010-8 and II9F-13

As shown in Fig. 1, alignments of the capsid proteins of 48285-24 (α; IPMA+N+) and 1147 (β; IPMA+N-) demonstrated 2 aa differences at positions 57 and 190. Alignment of the capsid proteins of II9F-13 (β; IPMA+N-) and 48285 (α; IPMA+N+) showed 3 aa differences at positions 63, 151 and 190. Alignment of the capsid proteins of Stoon-1010-8 (α; IPMA+N+) and the 1121 (β; IPMA+N-) revealed five aa differences at positions 71, 72, 131, 191 and 232. Since strains Stoon-1010 and 48285 are neutralised with the mAbs 16G12, 38C1, 63H3 and 94H8 and strains 1121, 1147 and II9F are not, we hypothesised that these particular aa could be of importance for a phenotypic switch (α to β or β to α).

The *capsid gene* of 48285-24 (α; IPMA+N+) was mutated from T to A at position 190, resulting in mutant 48285(T190A), which resembles the capsid of 1147 (β; IPMA+N-). The hydrophobic aa valine (V) at position 57 was not mutated since in strain 1147 another hydrophobic aa isoleucine (I) is present at this position (Fig. 1).

The capsid of II9F-13 (β; IPMA+N-) was mutated from A to T at position 190 and/or from P to T at position 151. This resulted in mutants II9F(A190T), II9F(P151T) and II9F(A190T/P151T) and the capsid of the double mutant looks like that of 48285 (α; IPMA+N+). The basic aa arginine (R) at position 63 was not mutated since in strain 48285 a conserved substitution (lysine, K) is present at this position (Fig. 1).

The capsid of Stoon-1010-8 (α; IPMA+N+) was mutated from E to R at position 191 and/or from T to P at position 131. These mutations of Stoon-1010-8 resulted in mutants 1010(E191R), 1010(T131P) and 1010(E191R/T131P) and the capsid of the double mutant resembles that of 1121 (β; IPMA+N-). The isoleucine (I) residues at position 71 and 72 of Stoon-1010-8 were not mutated into methionine (M) (Fig. 1) since both aa residues have hydrophobic characteristics.

The HindIII-digested plasmids of all the mutants were transfected onto PCV-negative PK-15 cells and the resulting PCV2 viruses were passed on PK-15 cells as described above. After 5

passages, the mutant viruses were verified by sequencing and were found to be 100% identical with the mutants in the plasmid.

3.3. Reactivity of mAbs to different PCV2 strains in IPMA

The reactivity of mAbs 16G12, 38C1, 63H3 and 94H8 with the viruses 48285-24, Stoon-1010-8 and II9F-13 obtained from infectious clones and their mutant viruses was assessed by IPMA and compared to the reactivity of the mAbs with their original PCV2 strains (48285, Stoon-1010, II9F, 1121 and 1147) (Table 1). All four mAbs reacted to 48285, Stoon-1010, II9F, 1121, 1147 and the viruses obtained from infectious clones and the mutants in a similar manner, although a small difference between the strain II9F-13 and its mutants were observed.

3.4. Sensitive neutralisation assays (SN)

Sensitive neutralisation assays were performed to determine the neutralising activity of the mAbs with the 48285, Stoon-1010, II9F, 1121, 1147 and the viruses obtained from infectious clones and the mutant viruses. The neutralising activities of mAbs 13D12 and 1C11 were -2 ± 15 % and 13 ± 19 %, respectively. Because the mean neutralising activity of mAb 1C11 ± its standard deviation was 13 + 19 = 32 %, a mAb was arbitrarily considered as neutralising when its mean neutralising activity was higher than 32 %.

PCV2 48285 and 48285-24 (α; IPMA+N+) were neutralised by mAbs 16G12, 38C1, 63H3 and 94H8, whereas 1147 (β; IPMA+N-) and the mutant 48285(T190A) were not neutralised. The mutant had the β phenotype (Table 2).

PCV2 II9F and II9F-13 (β; IPMA+N-) were not neutralised by mAbs 16G12, 38C1, 63H3 and 94H8, whereas 48285 (α; IPMA+N+) and the mutants [II9F(A191T), II9F(P151T) and II9F(A191T/P151T)] were. The mutants had the α phenotype (Table 2).

PCV2 Stoon-1010 and Stoon-1010-8 (α; IPMA+N+) were equally neutralised by the mAbs 16G12, 38C1, 63H3 and 94H8, whereas 1121 (β; IPMA+N-) and the double mutant 1010(E191R/T131P) were not neutralised (except with mAb 38C1). This double mutant had the β phenotype. Single mutants 1010(E191R) and 1010(T131P) remained neutralised by mAbs 16G12, 38C1, 63H3 and 94H8; however, the neutralisation was slightly reduced in the

mutant 1010(E191R) in comparison to that of Stoon-1010-8 and a significant drop of neutralisation (except with mAb 94H8) was observed in the mutant 1010(T131P). The single mutants had the α phenotype (Table 2).

Table 1. Recognition of the different PCV2 strains and their mutants by mAbs 16G12, 38C1, 63H3 and 94H8 directed against the PCV2 capsid protein.

		IPMA antibody titres with mAb			
PCV2 pair	Strains/mutants	16G12	38C1	63H3	94H8
48285 (α)/ 1147 (β)	48285	2560	2560	640	2560
	48285-24^{IC}	640	640	2560	2560
	48285(T190A)	2560	2560	2560	2560
	1147	2560	2560	2560	2560
II9F (β)/ 48285 (α)	II9F	640	640	640	640
	II9F-13^{IC}	640	640	640	640
	II9F(A190T)	2560	2560	2560	2560
	II9F(P151T)	2560	2560	2560	2560
	II9F(A190T/P151T)	2560	2560	2560	2560
	48285	2560	2560	2560	2560
Stoon-1010 (α)/ 1121 (β)	Stoon-1010	640	640	640	640
	Stoon-1010-8^{IC}	2560	640	640	2560
	1010(E191R)	2560	2560	640	2560
	1010(T131P)	640	2560	2560	2560
	1010(E191R/T131P)	2560	2560	2560	2560
	1121	2560	2560	2560	2560

^{IC}derived from infectious clone; phenotype α: mAb recognition with neutralisation (IPMA+N+); phenotype β: mAb recognition without neutralisation (IPMA+N-).

Table 2. Neutralisation of the different PCV2 strains and their mutants by mAbs 16G12, 38C1, 63H3 and 94H8 directed against the PCV2 capsid protein.

		Neutralising activity of mAbs (% neutralisation ± standard deviation)				
PCV2 pair	Strains/mutants	16G12	38C1	63H3	94H8	Phenotype
48285 (α)/ 1147 (β)	48285	**77** ± 14	**77** ± 10	**60** ± 4	**59** ± 6	α
	48285-24^{IC}	**79** ± 8	**75** ± 12	**59** ± 5	**71** ± 6	α
	48285(T190A)	22 ± 18*	23 ± 25	-7 ± 11*	8 ± 11*	β
	1147	29 ± 1	31 ± 1	-2 ± 11	6 ± 10	β
II9F (β)/ 48285 (α)	II9F	19 ± 8	6 ± 7	2 ± 5	7 ± 5	β
	II9F-13^{IC}	14 ± 14	5 ± 7	-1 ± 3	7 ± 4	β
	II9F(A190T)	**60** ± 5*	**62** ± 8*	**43** ± 14*	**58** ± 6*	α
	II9F(P151T)	**60** ± 7*	**61** ± 7*	**53** ± 13*	**55** ± 16*	α
	II9F(A190T/P151T)	**60** ± 4*	**55** ± 8*	**53** ± 5*	**58** ± 2*	α
	48285	**77** ± 14	**77** ± 10	**60** ± 4	**59** ± 6	α
Stoon-1010 (α)/ 1121 (β)	Stoon-1010	**90** ± 1	**91** ± 2	**76** ± 2	**76** ± 4	α
	Stoon-1010-8^{IC}	**90** ± 1	**91** ± 1	**77** ± 2	**82** ± 5	α
	1010(E191R)	**85** ± 4	**83** ± 4	**65** ± 9	**66** ± 12	α
	1010(T131P)	**69** ± 4*	**74** ± 4*	**53** ± 1*	**56** ± 17	α
	1010(E191R/T131P)	13 ± 6*	**56** ± 7*	28 ± 1*	31 ± 1*	β
	1121	0 ± 8	0 ± 10	-3 ± 4	6 ± 0	β

A mean neutralising activity of higher than 32 % was considered as neutralisation (indicated in bold).
^{IC}derived from infectious clone; *significantly different ($P<0.05$) when the value of the mutant virus was compared with the value of the corresponding virus derived from the infectious clone. phenotype α: mAb recognition with neutralisation (IPMA+N+); phenotype β: mAb recognition without neutralisation (IPMA+N-).

4. Discussion

In this study, we demonstrated that single amino acid mutations in the PCV2 capsid protein result in a switch from phenotype α (IPMA+N+) to β (IPMA+N-) and vice-versa.

This study confirms the previous work of Lefebvre et al. (2008a) stating that strains 48285 and Stoon-1010 are recognised as well as neutralised by mAbs 16G12, 38C1, 63H3 and 94H8 (α; IPMA+N+), whereas strains 1147, 1121 and II9F are recognised but not neutralised (β; IPMA+N-).

For the four mAbs tested, a mutation of T at position 190 to A of 48285-24 resulted in a complete loss of neutralisation. This clearly shows that one single aa change in the capsid may switch the neutralisation phenotype of PCV2. The aa at position 190 seems to play a critical role in the neutralisation capacity of a mAb. The major difference between T and A consists of a hydroxyl group (on T) and therefore this hydroxyl group might be important as a part of the binding site of the mAbs. Another PCV2 strain 1206 has also A at position 190 and is also not neutralised or partially neutralised by mAbs 16G12, 38C1, 63H3 and 94H8 (Lefebvre et al., 2008a) and this is in agreement with the above findings. And it looks like that the disappearance of a small part of the recognition area of a mAb does not change the binding of the mAb to the virus but does no longer allow the mAb to block infection. Most probably the aa at position 190 is close to the viral ligand that interacts with the cellular receptors, such as heparan sulphate and dermatan sulphate (Misinzo et al., 2006).

Mutation of A at position 190 to T or mutation of P at position 151 to T or mutations at both positions of II9F-13 resulted in a gain of neutralisation. This clearly demonstrates that aa change(s) at position 190 or 151 or both in the capsid could switch the neutralisation phenotype of PCV2. This suggests that amino acids at position 190 or 151 appear to play an important role to determine the neutralisation capacity of a mAb. As described above, presence of a hydroxyl group on T could be critical as a part of the binding site of the mAbs, since A or P does not have this hydroxyl group. Previously, it was demonstrated that the aa residues 145-162, 175-192 (Shang et al., 2009) and 165-200 (Lekcharoensuk et al., 2004) in the capsid were recognised by neutralising mAbs. In our case, the aa at positions 151 and 190 in the capsid have been identified as critical that determine the neutralisation capacity of the mAbs. No additive effect was observed in the double mutant II9F(A190T/P151T). The neutralisation values of the double mutant II9F(A190T/P151T) were not different from the

values of 48285, since their capsids are identical except at position 63 where a basic aa is present in both II9F(A190T/P151T) and 48285 (R and K, respectively). The aa residue 190 is situated on the exterior surface of the PCV2 capsid protein, whereas aa position 151 is located in the interior surface of the capsid protein (as shown in Fig. 2). Khayat et al. (2011) indicated that the interior surface of the PCV2 capsid can be antigenic if the capsid is transiently externalised for 'breathing' described in a number of viruses, such as flock house virus (Bothner et al., 1998), human rhinovirus 14 (Lewis et al., 1998) and poliovirus (Li et al., 1994). Exposition of internal polypeptides was shown to be essential for the infectivity of these viruses. In addition, it was shown that poliovirus was only neutralised when the internal peptides of this virus were externally exposed (Li et al., 1994).

A mutation of E at position 191 to R of Stoon-1010-8 showed a slight drop in neutralisation but this drop was not statistically significant. A mutation of T at position 131 to P resulted in a significant loss of neutralisation ($P<0.05$). Mutations at both positions 191 (from E to R) and 131 (from T to P) had a clear additive effect on the neutralisation and the neutralisation was similar to that of 1121 (β; IPMA+N-) (except mAb 38C1 showed partial neutralisation with the double mutant). This suggests that changes of both positions are necessary to switch the neutralisation phenotype. The residual neutralisation activities of the mAb 38C1 on the double mutant may be associated with the aa at position 232 (K in Stoon-1010-8 and N in 1121). Further mutagenesis at position 232 (from K to N) is needed. The neutralisation results clearly demonstrated that position 131 (from T to P) has a greater impact in determining the neutralisation capacity of the mAb than position 191 (from E to R). Since both E and R have polar and hydrophilic characteristics, changes from E to R did not show any significant effect on the neutralisation. The T at position 131 might be critical as a part of the binding site of the mAb. On the other hand, E at position 191 may not be a part of the binding site of the mAb; however, we can designate it as a 'helper' because an additive effect on the neutralisation was produced when aa at position 191 was mutated together with aa at position 131. The position 191 is situated close to the mAbs' binding site (position 190 as shown above) and this could be the reason why this position has a 'helper' effect on the neutralisation. Like aa residue 190, the aa residues 131 and 191 are also located on the exterior surface of the PCV2 capsid protein (Fig. 2).

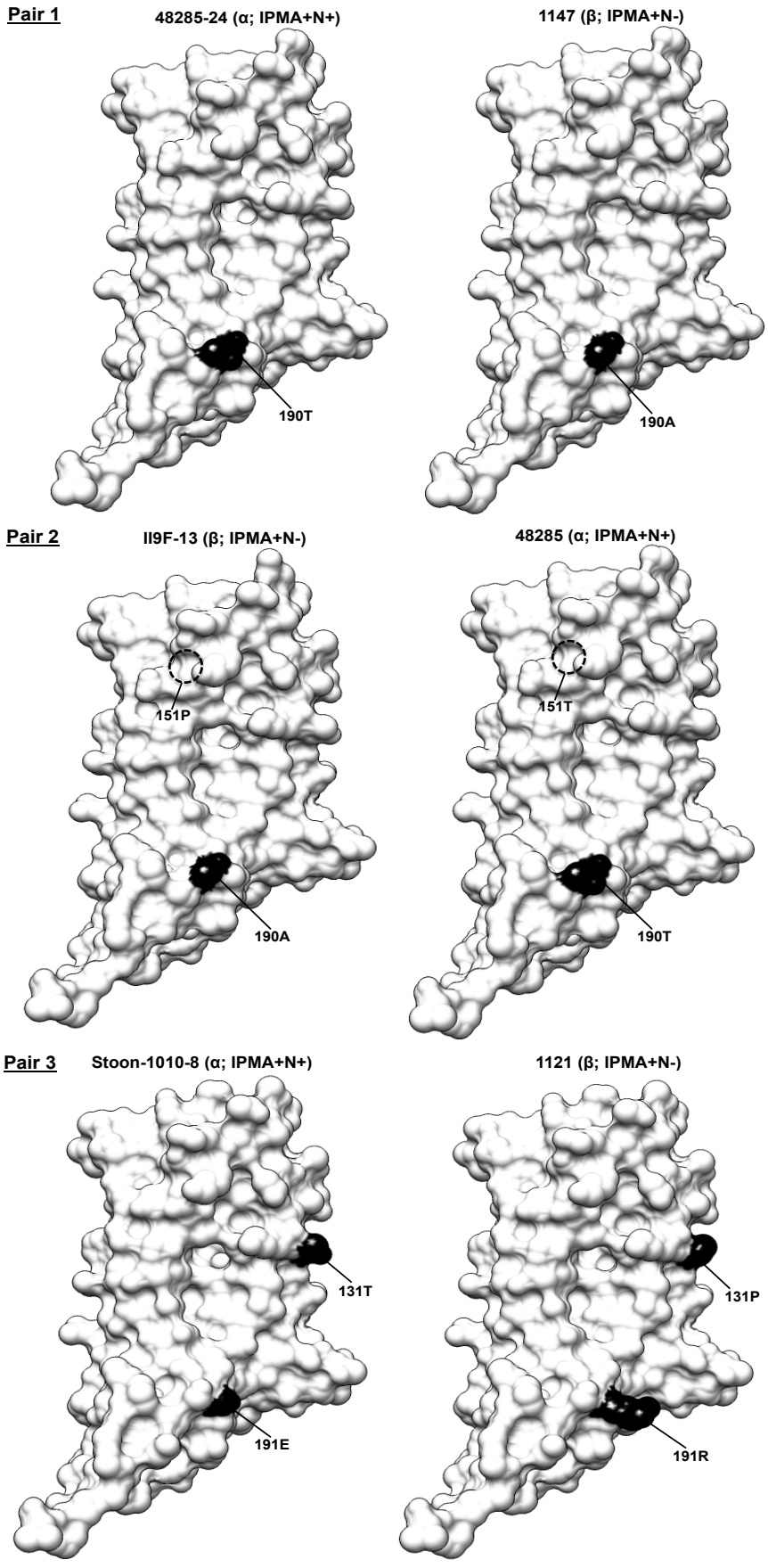
Pair 1
48285-24 (α; IPMA+N+)
190T
1147 (β; IPMA+N-)
190A
Pair 2
II9F-13 (β; IPMA+N-)
151P
190A
48285 (α; IPMA+N+)
151T
190T
Pair 3
Stoon-1010-8 (α; IPMA+N+)
131T
191E
1121 (β; IPMA+N-)
131P
191R

Fig. 2. Location of aa residues on the capsid proteins that are different in between the two strains of each PCV2 pair with different phenotype (α; IPMA+N+ and β; IPMA+N-).
Surface diagrams of the PCV2 capsid proteins of different PCV2 strains (Stoon-1010, 48285, 1121, 1147 and II9F) of two phenotypes were generated using the PCV2 crystal structure (PDB accession no. 3R0R) (Khayat et al., 2011) as a template with SWISS-MODEL Workspace (Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006). Figures were made using University of California, San Francisco Chimera 1.5.3 (Pei and Grishin, 2001).

Still it remains to be determined why the phenotype α strains were recognised as well as neutralised by the mAbs and phenotype β strains were only recognised but not neutralised. Glaser et al. (1995) also pointed to the existence of two neutralisation phenotypes in case of equine arteritis virus (EAV). MAbs recognised all field isolates of EAV and all of the isolates were neutralised by mAbs except one isolate DL11, which remained non-neutralised. Absence of neutralising activity of mAbs to phenotype β strains might be related with the low binding strength or avidity of the mAbs. The changes of aa at positions 131, 151 and 190 in phenotype β strains might lead to a modification of the viral capsid conformation, which could be responsible for the lower avidity of mAbs. Further research is required to solve these issues.

This study demonstrates that single amino acid mutations in the PCV2 capsid protein switch the neutralisation phenotypes of PCV2.

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Chapter 4. Outcome of experimental porcine circovirus 1 and 2 infections in porcine foetuses and in pigs

Chapter 4.1.

Outcome of experimental porcine circovirus type 1 infections in mid-gestational porcine foetuses

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BMC Veterinary Research (2011), 7:64

Abstract

Porcine circovirus type 1 (PCV1) has been described as a non-cytopathic contaminant of the PK-15 cell line. Several experimental infections with PCV1 failed to reproduce disease in pigs. Therefore, PCV1 is generally accepted as non-pathogenic to pigs. To our knowledge, nothing is known about the outcome of PCV1 infections in porcine foetuses. This was examined in the present study. Nine foetuses from three sows were inoculated at 55 days of gestation: three with $10^{4.3}$ $TCID_{50}$ of the PCV1 cell culture strain ATCC-CCL33, three with $10^{4.3}$ $TCID_{50}$ of the PCV1 field strain 3384 and three with cell culture medium (mock-inoculated). At 21 days post inoculation, all 6 PCV1-inoculated and all 3 mock-inoculated foetuses had a normal external appearance. Microscopic lesions characterised by severe haemorrhages were observed in the lungs of two foetuses inoculated with CCL33. High PCV1 titres (up to $10^{4.7}$ $TCID_{50}$/g tissue) were found in the lungs of the CCL33-inoculated foetuses. All other organs of the CCL33-inoculated foetuses and all the organs of the 3384-inoculated foetuses were negative ($<10^{1.7}$ $TCID_{50}$/g tissue) by virus titration. PCV1-positive cells (up to 121 cells/10 mm^2 in CCL33-inoculated foetuses and up to 13 cells/10 mm^2 in 3384-inoculated foetuses) were found in the heart, lungs, spleen, liver, thymus and tonsils. PCR and DNA sequencing of Rep recovered CCL33 or 3384 sequences from CCL33- or 3384-inoculated foetuses, respectively. From this study, it can be concluded that cell culture PCV1 can replicate efficiently and produce pathology in the lungs of porcine foetuses inoculated at 55 days of foetal life.

1. Introduction

Porcine circovirus type 1 (PCV1) is a small, non-enveloped circular single-stranded DNA virus of the family *Circoviridae*. PCV1 was originally detected as a non-cytopathic contaminant of the PK-15 cell line, ATCC-CCL33 (Tischer et al., 1974). PCV1 infections are widely distributed around the world as described before (Allan et al., 1998; Allan and Ellis, 2000; Fenaux et al., 2000). Seroprevalence of PCV1 at herd level varies between 10% (Puvanendiran et al., 2011) and 100% (Labarque et al., 2000). Although PCV1 DNA has been isolated from lymph nodes of a piglet in France with a wasting condition (LeCann et al., 1997), it is generally accepted that PCV1 is non-pathogenic to pigs (Tischer et al., 1986; Allan et al., 1995; Tischer et al., 1995; Allan et al., 2000; Finsterbusch and Mankertz, 2009; Beach et al., 2010). Experimental infections with PCV1 failed to reproduce disease in pigs (Tischer et al., 1986; Allan et al., 1995; Fenaux et al., 2003). The distribution of PCV1 in different pig tissues after experimental infections has been demonstrated (Allan et al., 1995). PCV1 has been detected in cases of congenital tremors in newborn pigs and aborted/stillborn piglets, indicating the possible occurrence of vertical transmission of PCV1 (Hines and Lukert, 1994; Allan et al., 1995; Stevenson et al., 2001; Choi et al., 2002). In contrast, no evidence of PCV1 infection was found in piglets affected with congenital tremors in an 11 years retro-prospective study (Kennedy et al., 2003). To our knowledge, nothing is known about the outcome of PCV1 infections in porcine foetuses.

In the present study, the virological and pathological outcomes were examined in porcine foetuses that were experimentally inoculated with PCV1 at 55 days of gestation.

2. Materials and methods

2.1. Viruses

Two different PCV1 strains were used in this study. The PCV1 cell culture strain CCL33, was originally detected as a non-cytopathic contaminant of the PK-15 cell line (Tischer et al., 1974; Tischer et al., 1982). The PCV1 field strain 3384 was isolated from stillborn piglets (Allan et al., 1995). Both PCV1 strains have been sequenced and their full genomic sequences have been deposited in GenBank (GenBank: JN133302 and JN133303).

2.2. Experimental design

Due to the high seroprevalence of PCV1 in Flemish sows (Labarque et al., 2000), viral

replication and pathology cannot be studied by (oro)nasal inoculation of sows during gestation or by intra-uterine inoculation of sows at insemination. Therefore, experimental PCV1 infections in foetuses have to be performed by direct *in utero* inoculation. Three conventional PCV1 seropositive Landrace sows were submitted to laparatomy at 55 days of gestation. Laparotomy of the sows was performed under anaesthesia as described previously (Saha et al., 2010). In each of the three sows, three foetuses were inoculated: one foetus with the PCV1 cell culture strain CCL33; one with the PCV1 field isolate 3384 and one foetus with cell culture medium. The position in the uterus of the PCV1- and mock-inoculated foetuses, and their adjacent foetuses, is shown in Table 1. The inoculations were performed as described previously (Saha et al., 2010). Briefly, the foetuses were inoculated by trans-uterine injection with 200 µL, containing $10^{4.3}$ $TCID_{50}$ of PCV1, into the peritoneal (100 µL) and amniotic (100 µL) cavities. For mock-inoculated foetuses, PK-15 cell culture medium (200 µL) was inoculated by trans-uterine injection with 200 µL into the peritoneal (100 µL) and amniotic (100 µL) cavities. The inoculated foetuses were marked with a synthetic, non-absorbable, superficial suture (Prolene 2-0, Ethicon, Inc., Somerville, New Jersey, U.S.A.) on the exterior uterine wall. Antibiotics were administered to the sows before closure of the operation wound (Duphapen Strep, Fort Dodge Animal Health Benelux, Netherlands), 10 mL intraperitoneally and 10 mL in the operation wound.

The sows were housed individually in A2 experimental units. The sows were observed daily for clinical signs and their rectal temperature was monitored daily during the first week after surgery. Twenty-one days post inoculation (dpi), the sows were humanely euthanised with an overdose of pentobarbitalum natricum [Natriumpentobarbital 20% 40 mg/kg iv in the V. jugularis externa] (Kela Laboratoria, Hoogstraten, Belgium). Hysterectomy was performed and all foetuses were collected. The specific length of the tail ends of the sutures was used to determine the PCV1 strain the foetus was inoculated with.

All inoculated and non-inoculated foetuses were examined for gross lesions and tissue samples were collected from the heart, lungs, spleen, liver, kidneys, thymus, tonsils, ileum and cerebrum for histopathological examinations (haematoxylin and eosin staining), for virus titrations and for staining of infected cells by indirect immunofluorescence. Serum and abdominal fluid were collected as well. The serum samples of the sows were collected prior to surgery (pre-serum) and at the time of euthanasia (post-serum).

The animal experiments described in this study were authorised and supervised by the Ethical

and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University.

Table 1. PCV1-inoculated, mock-inoculated and their adjacent foetuses and result of PCR amplications of the *Rep gene*.

Sow no.	Foetus no.[a]	Inoculated with	PCR result
S1	L5	NI[b]	-
	L6[c]	Mock	-
	R1	CCL33	+
	R2	NI[b]	-
	R3	NI[b]	-
	R4	3384	+
	R5[c]	NI[b]	-
S2	L1	Mock	-
	L2[d]	NI[b]	-
	R1	NI[b]	-
	R2	CCL33	+
	R3	3384	+
	R4[d]	NI[b]	-
S3	L1[e]	Mock	-
	R1	CCL33	+
	R2	3384	+
	R3[e]	NI[b]	-

[a]Foetuses were identified by their position in the uterus. L = left horn; R = right horn; Numbering is in sequence from ovary to cervix. [b]NI = not inoculated; [c]L6 and R5 were adjacent to each other; [d]L2 and R4 were adjacent to each other; [e]L1 and R3 were adjacent to each other

2.3. PCV1 isolation and titrations

Ten% (wt/vol) tissue suspensions (spleen, thymus, tonsils, ileum) and 20% (wt/vol) tissue suspensions (heart, lungs, liver, kidneys and cerebrum) were prepared in phosphate-buffered saline (PBS). For the PCV1-inoculated foetuses, the PCV1 titres in the above-mentioned organs were determined by virus titration in PK-15 cells, as described before for PCV2 titration (Sanchez et al., 2001; Saha et al., 2010). Briefly, PCV1-infected PK-15 cells were revealed by immuno-peroxidase staining with using an optimal dilution of mono-specific anti-PCV1 swine polyclonal serum (produced in our laboratory) and peroxidase-labelled goat-anti-swine IgG (Jackson ImmunoReasearch, UK) as primary and secondary antibodies, respectively. For the mock-inoculated and non-inoculated foetuses, PCV1 titres were determined in the heart, lungs and spleen. The titration experiments were repeated independently for 3 times. For 10% suspensions, the detection limit of this technique was $10^{2.0}$ $TCID_{50}$/g tissue and for 20% suspensions, the detection limit was $10^{1.7}$ $TCID_{50}$/g tissue.

2.4. Single immunofluorescence staining

The number of PCV1-positive cells in all of the collected organs of PCV1-inoculated foetuses and in the lungs of mock-inoculated and non-inoculated foetuses was determined by an indirect immunofluorescence staining (IIF), adapted from the technique described by Sanchez et al. (2001). Methanol-fixed cryostat sections were incubated with an optimal dilution of biotin-conjugated porcine anti-PCV1 polyclonal antibodies (pAbs). Subsequently, a 1:200 dilution of fluorescein isothiocyanate (FITC)-labelled streptavidin (Molecular Probes, Eugene, Oregon, USA) in PBS was applied. Both incubations were performed for 1 h at 37°C and sections were washed three times with PBS between the incubations. Finally, sections were incubated with Hoechst (Molecular Probes, Eugene, Oregon, USA) for 10 min followed by three washings with PBS. The specificity of the staining was confirmed by the deletion of primary antibody (anti-PCV1 pAb) and by the complete absence of fluorescence in the tissue sections of non-inoculated, age-matched foetuses. Stained tissue sections were mounted with a glycerol solution containing 1,4-diazobicyclo-2.2.2-octane (DABCO) anti-fading agent (Janssen Chimica, Beerse, Belgium). The number of PCV1-positive cells was determined in an area of 10 mm^2 of tissue by a LEICA DM/RBE fluorescence microscope (Leica Microsystems GmbH, Heidelberg, Germany) as described by Sanchez et al. (2001). Representative digital images of the stained tissue sections were made using the Olympus IX81 microscope connected with a Cell-M Live-Cell imaging module.

2.5. Double immunofluorescence staining

To our knowledge, the target cells of PCV1 have not been characterised with cell markers yet. A cytokeratin marker monoclonal antibody (mAb) AE1/AE3 (Neomarkers, Fremont, CA) was used to identify epithelial cells (Tseng et al., 1982). Polyclonal rabbit antibodies against the human Von Willebrand Factor (pAb anti-human VWF) (Dako- Cytomation) were used to detect endothelial cells. Since PCV1 has previously been associated with cells showing macrophage morphology in pigs (Allan et al., 1995), the macrophage marker mAb 41D3, detecting porcine sialoadhesin, was used (Duan et al., 1998; Vanderheijden et al., 2003). Since mAb 41D3 is specific for macrophages, a mAb 74.22.15 (directed against SWC3) was used which detects macrophages but also monocytes, dendritic cells and granulocytes (Pescovitz et al., 1984; Sanchez et al., 2003). MAb 28.4.1 directed against IgM (Van Zaane and Hulst, 1987) was used as marker for B-lymphocytes. MAbs BB23-8E6 (Costers et al., 2009), 74.12.4 (Saalmuller et al., 1989; Pescovitz et al., 1994) and 76.2.11

(Saalmuller et al., 1989; Summerfield et al., 1996; Zuckerman et al., 1998) directed against CD3, CD4 and CD8, respectively, were used as markers for T-lymphocytes.

A double immunofluorescence staining for epithelial cells/endothelial cells/macrophages/B-lymphocytes/T-lymphocytes and PCV1 was performed in the lungs of PCV1-inoculated foetuses, adapted from the technique described by Sanchez et al. (2003; 2004). Briefly, methanol-fixed cryostat sections were first incubated with mAb AE1/AE3 (1:100), pAb VWF (1:25), mAb 41D3 (1:5), mAb anti-SWC3 (1:10), mAb anti-IgM (1:50), mAb anti-CD3 (1:50), mAb anti-CD4 (1:50) or mAb anti-CD8 (1:50) and then with FITC-labelled goat-anti-mouse IgG (1:200) (Molecular Probes, Eugene, Oregon, USA) or FITC-labelled goat-anti-rabbit IgG (1:200) (Molecular Probes). Afterwards, the sections were stained for PCV1 antigens by incubation with an optimal dilution of biotin-conjugated porcine anti-PCV1 pAbs. Subsequently, a 1:200 dilution of Texas Red-labelled streptavidin (Molecular Probes) in PBS was added. All antibodies were diluted in PBS and all incubations were performed for 1 h at 37°C. The sections were washed three times with PBS after each incubation with primary and secondary antibodies. Specificity of the staining for different cell markers (except for the endothelial marker) was demonstrated using an irrelevant, isotype-matched mAb 1C11, 3H12 and 13D12 (Nauwynck and Pensaert, 1995) and by the deletion of primary antibodies (pAb anti-human VWF and anti-PCV1 pAb), and by the complete absence of PCV1-specific fluorescence in tissue sections of non-inoculated, age-matched foetuses. The stained tissue sections were mounted as described above and PCV1-positive cells and double positive cells (cell marker and PCV1-positive cells) were quantitated as described previously (Sanchez et al., 2003; Sancehz et al., 2004) by using a LEICA DM/RBE fluorescence microscope (Leica Microsystems GmbH, Heidelberg, Germany). Representative digital images of stained preparations were made using the Olympus IX81 microscope connected with a Cell-M Live-Cell imaging module.

2.6. Amplification of PCV1 *rep gene* and sequencing

DNA was extracted from the heart and lung tissues of PCV1-inoculated, mock-inoculated and their adjacent foetuses by using a NucleospinR tissue kit (Macherey- Nagel). A set of primers (PF2: 5'-TTGCTGAGCCTAGCGACACC-3'; PR2: 5'-TCCACTGCTTCAAATCGGCC-3') was used to amplify a PCV1 349 bp *replicase gene* (Rep) fragment following the same methods as described by Larochelle et al. (1999). PCR products (Rep) were treated with Exonuclease I and Antarctic Phosphatase (New England

Biolabs, Ipswich, USA) and used directly for cycle sequencing with a Big Dye Terminator Cycle sequencing kit V1.1 (Applied Biosystems, Foster City, USA). The cycle sequencing reaction products were purified using ethanol precipitation and separated on an ABI Genetic Analyzer 310 (Applied Biosystems, Foster City, USA). Sequence alignments were performed using bl2seq at http://blast.ncbi.nlm.nih.gov. To confirm the absence of porcine circovirus type 2 (PCV2) in PCV1-inoculated foetuses, amplification of PCV2 capsid was performed with the heart and lung tissues as described previously in Saha et al. (2010).

2.7. Serology

The PCV1-specific antibody titres in serum were determined by an immuno-peroxidase monolayer assay (IPMA) as described previously (Labarque et al., 2000). The PCV1 cell culture strain CCL33 was used as antigen. The foetuses were also checked for PCV2-specific antibodies by IPMA as described above but with PCV2 strain 1121 (Meehan et al., 2001) as antigen. These assays were independently repeated 3 times.

The sow antibody titres against PCV1, PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV) were determined by an IPMA (as described above) and the sow antibody titres against porcine parvovirus (PPV) were determined by a haemagglutination inhibition (HI) test as described elsewhere (Joo et al., 1976a; Wensvoort et al., 1991).

3. Results

3.1. Evaluation of the sows

The three sows were clinically healthy during the whole study period. No rise of rectal temperature was noticed in any of the three sows. The operation wounds were slightly swollen until 48 hours after the operations and somewhat painful at palpation. The PCV1- and PCV2-specific IPMA Ab titres of the three sows ranged from 40 to 640 and from 20,480 to 163,840, respectively in pre-serum and were identical in post-serum. The PRRSV-specific IPMA Ab titres and PPV-specific HI Ab titres in the pre-serum ranged from <10 to 640 and from <8 to 512, respectively. Seroconversion against PRRSV and PPV was not observed in any of the three sows.

3.2. Gross examinations

All PCV1-inoculated foetuses were normal in appearance and no evidence of gross

pathological lesions was observed in any of the PCV1-inoculated foetuses (Fig. 1a). The mock-inoculated and non-inoculated foetuses were also normal in appearance and no gross pathology was observed.

3.3. Microscopic examinations

A haematoxylin and eosin (HE) staining was performed for different organs (heart, lungs, spleen, liver, kidney, thymus, cerebrum) of PCV1-inoculated and mock-inoculated foetuses. Microscopic lesions including severe haemorrhages in the interlobular regions were observed in the lung tissue of two foetuses (S1R1 and S2R2) inoculated with the cell culture PCV1 strain, CCL33 (Fig. 1b). Microscopic lesions were not present in the other organs of these two foetuses. Microscopic lesions could not be observed in the third CCL33-inoculated foetus, the three 3384-inoculated foetuses and the mock-inoculated foetuses.

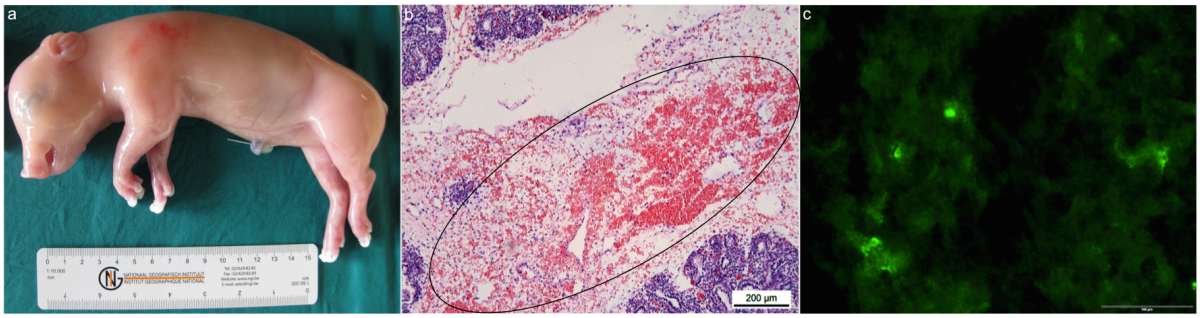

Fig. 1. Different aspects of PCV1-CCL33 replication after inoculation of a 55-day old foetus. a) CCL33-inoculated foetus (S1R1) with a normal external appearance. b) Haematoxylin and eosin staining of the lungs of a CCL33-inoculated foetus (S1R1). Haemorrhages (indicated by a circle) in interlobular regions (magnification 10X). Bar = 200 µm. c) PCV1-positive cells in the lungs of CCL33-inoculated foetus (S1R1). Bar = 100 µm.

3.4. PCV1 isolation and titrations

High PCV1 titres were found in the lungs of the foetuses S1R1 ($10^{4.7}$ $TCID_{50}$/g tissue), S2R2 ($10^{4.6}$ $TCID_{50}$/g tissue) and S3R1 ($10^{2.9}$ $TCID_{50}$/g tissue) inoculated with the cell culture strain CCL33. All other organs of CCL33-inoculated foetuses were negative ($<10^{1.7}$ $TCID_{50}$/g tissue) by virus titration. All collected organs from the 3384-inoculated foetuses were negative ($<10^{1.7}$ $TCID_{50}$/g tissue) by virus titration. Mock-inoculated and non-inoculated foetuses were negative for PCV1.

3.5. Single immunofluorescence staining

The number of PCV1-positive cells in the lungs of the three CCL33-inoculated foetuses, i.e.

S1R1, S2R2 and S3R1, were 121, 31 and 28 cells/10 mm^2, respectively (Fig. 1c.). A low number of PCV1-positive cells/10 mm^2 was observed in the lungs of the three foetuses inoculated with the field strain 3384 (4 (S1R4), 13 (S2R3) and 1 (S3R2) cells/10 mm^2 respectively). PCV1-positive cells were also observed in several other organs such as spleen, liver (S1R1); spleen, liver and tonsils (S2R2); and heart and thymus (S2R3) and the numbers varied between 1 and 6 cells/10 mm^2 of tissues (Table 2). PCV1-positive cells were not observed in the lungs of the mock-inoculated and non-inoculated foetuses.

Table 2. Quantification of PCV1 positive cells in different foetal organs collected at 21 days post-PCV1 inocualtion.

Strain	Sow no.	Inoculated foetus[a]	Number of PCV1 positive cells/10 mm^2 tissue								
			Heart	Lungs	Spleen	Liver	Kidney	Thymus	Tonsils	Ileum	Cerebrum
CCL33	S1	R1	-	121	6	4	-	-	-	-	-
CCL33	S2	R2	-	31	3	2	-	-	1	-	-
CCL33	S3	R1	-	28	-	NA	NA	NA	-	-	NA
3384	S1	R4	-	4	-	-	-	-	NA	NA	-
3384	S2	R3	4	13	-	-	-	3	-	-	-
3384	S3	R2	-	1	-	-	-	-	-	NA	-

[a] Foetuses were identified by their position in the uterus. R = right horn. Numbering is in sequence from ovary to cervix. NA: not available; -: no positive cells detected

3.6. Double immunofluorescence staining

The PCV1 antigens were mainly (97%) localised in the epithelial cells of the lungs (Fig. 2) of the PCV1-inoculated foetuses. The other 3% of the PCV1 antigens were localised in SWC3+ cells (cells of the monocytic lineage) of the lungs (Fig. 3). No co-localisation of PCV1 antigens was observed in endothelial cells, 41D3+ macrophages, B-lymphocytes and T-lymphocytes (data not shown).

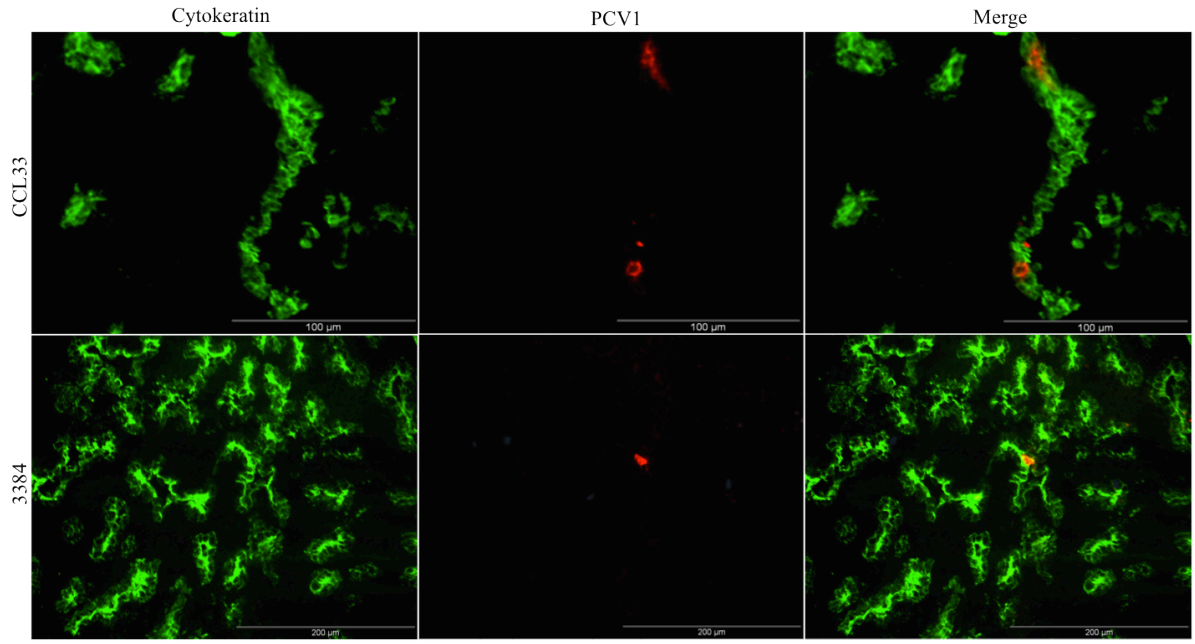

Fig. 2. Co-localisation of PCV1 in the epithelial cells.
Co-localisation of PCV1 in the epithelial cells of the lungs of a CCL33-inoculated foetus (Bar = 100 µm) and a 3384-inoculated foetus (Bar = 200 µm) collected at 21 days post inoculation (76 days of gestation). Methanol-fixed cryostat sections were incubated (as described in Materials and Methods) with a cytokeratin marker monoclonal antibody AE1/AE3 to stain epithelial cells (green fluorescence). Cells containing PCV1 antigens were localised using biotin-labelled mono-specific porcine anti-PCV1 polyclonal antibodies (red fluorescence).

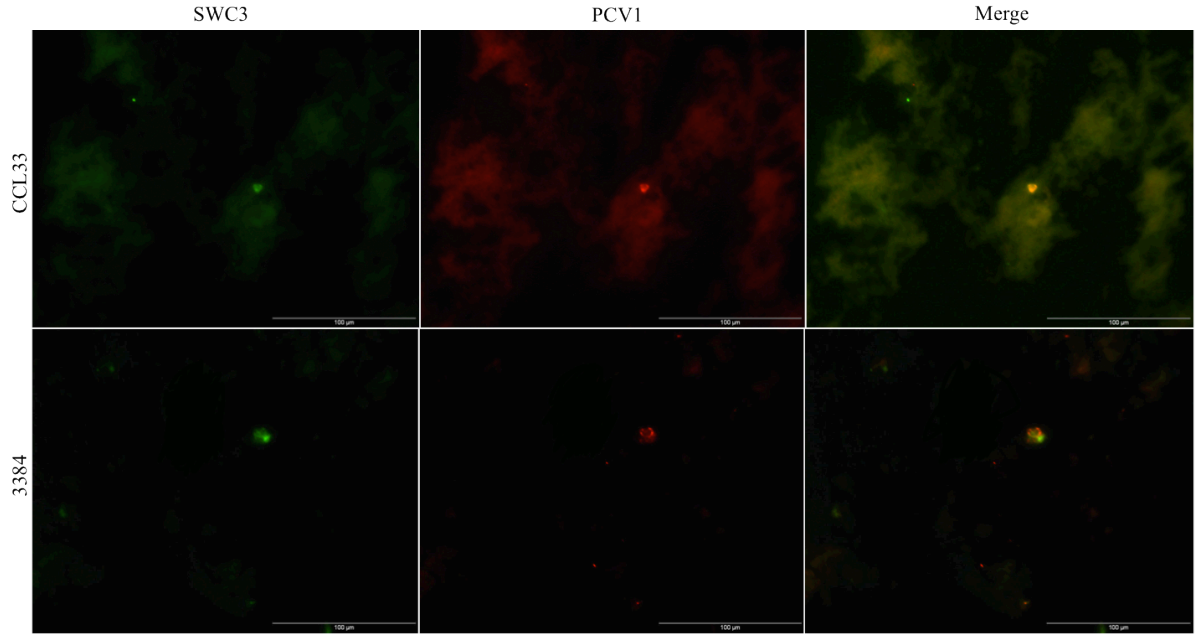

Fig. 3. Co-localisation of PCV1 in monocytes (SWC3+ cells).
Co-localisation of PCV1 in monocytes (SWC3+ cells) of the lungs of a CCL33-inoculated foetus (Bar = 100 µm) and a 3384-inoculated foetus (Bar = 100 µm) collected at 21 days post inoculation (76 days of gestation). Methanol-fixed cryostat sections were incubated (as described in Materials and Methods) with a mAb anti-SWC3 (74.22.15) (green fluorescence).

Cells containing PCV1 antigens were localised using biotin-labelled mono-specific porcine anti-PCV1 polyclonal antibodies (red fluorescence).

3.7. Amplification of PCV1 *rep gene* and sequencing

In order to confirm that the foetuses had been inoculated with the specific PCV1 strain and were not contaminated with PCV1 from neighbouring foetuses (as mentioned in Table 1), a PCV1 Rep fragment was amplified using heart and/or lung tissue from the infected foetuses (21 days after inoculation) followed by sequencing. PCR and DNA sequencing recovered CCL33 and 3384 sequences from CCL33- and 3384-inoculated foetuses, respectively. No evidence for mixed samples (e.g. containing more than one PCV1 strain) was found. All the adjacent foetuses of PCV1-inoculated foetuses were negative in PCR assays. All the mock-inoculated foetuses and their adjacent foetuses were also negative in PCR assays (Table 1). All the PCV1-inoculated foetuses were also negative for PCV2 in PCR assays.

3.8. Serology

All the PCV1-inoculated foetuses had a very low anti-PCV1 IPMA Ab titre of 10 to 40, except one foetus (S2R2) inoculated with CCL33, which had a titre of 160. The mock-inoculated and non-inoculated foetuses were negative (<10) for PCV1-specific IPMA Ab. All the PCV1-inoculated and mock-inoculated foetuses were negative (<10) for PCV2-specific IPMA Ab.

4. Discussion

In this study, the virological and pathological outcomes were examined in porcine foetuses after inoculation with PCV1 at 55 days of gestation.

The PCV1 cell culture strain CCL33 was found to be pathogenic to porcine foetuses inoculated at 55-days of foetal life. Severe haemorrhages were present in the lungs of two out of three CCL33-inoculated foetuses. These lesions correlated well with the highest PCV1 titres ($10^{4.7}$ $TCID_{50}$/g and $10^{4.6}$ $TCID_{50}$/g) in the lungs. The lungs with the lower level of virus replication ($10^{2.9}$ $TCID_{50}$/g) did not have histopathological changes. Haemorrhages in the lungs can be explained by the fact that due to the high PCV1 replication in the epithelial cells, there might be the release of inflammatory mediators, which may ultimately lead to the increased permeability of the blood vessels followed by leakage of blood or haemorrhage. Under the conditions of the present study, the PCV1 field strain 3384 was non-pathogenic to

porcine foetuses. This suggests that a high PCV1 load could be essential to induce pathology in porcine foetuses. Several experimental studies had been performed in the past to study the pathogenesis of PCV1 infections in 1 day old, 2 days old, 1 month old and 9 months old pigs (Tischer et al., 1986; Allan et al., 1995; Fenaux et al., 2003) but these experimental studies failed to reproduce disease in pigs. However, under the conditions of the present study, it was demonstrated that PCV1 is pathogenic for porcine foetuses. More research is needed to determine the pathogenicity of PCV1.

Previously, Allan et al. (1995) showed the distribution of PCV1 antigens by virus isolation and IIF staining in different organs of pigs, to be predominantly in the lungs, although the level of PCV1 replication was not quantitatively determined in that study. Our study showed that the lung tissue was the main target organ of replication of CCL33 strain, as this PCV1 strain could only be isolated with high titres (up to $10^{4.7}$ $TCID_{50}$/g) from the lungs. The field strain 3384 could not be isolated from any of the inoculated foetuses, although they were clearly PCR positive. These findings show that the replication kinetics of the strains CCL33 and 3384 are different from each other. Sequence comparison of the Rep and capsid of CCL33 and 3384 revealed one synonymous nucleotide substitution in the Rep and 4 amino acid (aa) differences (at aa positions 69, 72, 74 and 116) in the capsid protein (data not shown), suggesting that these aa differences in the capsid protein could be responsible for the different replication kinetics of these two strains in porcine foetuses. Further research may clarify this issue.

Single immunofluorescence staining revealed that moderate (up to 121 cells/10 mm^2 of tissues) and low numbers (up to 13 cells/10 mm^2) of PCV1-positive cells were present in the lungs of the CCL33-inoculated and the 3384-inoculated foetuses, respectively, although no PCV1 could be isolated from the lungs of the 3384-inoculated foetuses. Moreover, all the other organs of the PCV1-inoculated foetuses were negative in virus isolation, whereas PCV1 antigens were found at a low level (up to 6 cells/10 mm^2) in the heart, liver, spleen, thymus and tonsils. These results suggest that immunostaining of PCV1 is a more sensitive technique than virus isolation and titration for the detection of PCV1. Comparably, a previous study of McNeilly et al. (2002) with PCV2 indicated that immunostaining of PCV2 is a more sensitive technique than virus isolation for the detection of PCV2 in porcine tissues.

The double staining of PCV1 and different cell markers established that the PCV1 antigens were mainly localised in the lung epithelial cells and not in endothelial cells, macrophages or

lymphocytes of the lungs of PCV1-inoculated foetuses. This is in contradiction with previous observations in newborn piglets (Allan et al., 1995; Stevenson et al., 2001), where it was shown that PCV1 is mainly present in non-epithelial cells, morphologically resembling macrophages. It could be possible that the target cells for PCV1 in foetal life might be different from the target cells for PCV1 in newborn pigs, as previously shown for PCV2 by Sanchez et al. (2003). PCV1 needs cellular DNA polymerases of actively dividing cells to replicate (Tischer et al., 1987) and presumably epithelial cell types possess more mitotic activity in immuno-incompetent porcine foetuses than in newborn pigs. The remaining 3% of the PCV1 antigens were localised in SWC3+ cells, which were 41D3-. It could be possible that due to high PCV1 replication in the lungs, there were newly infiltrating monocytes (SWC3+, 41D3-). This study also indicates that PCV1 and PCV2 have different cell tropism during foetal life, since PCV2 replicates mainly in the cardiomyocytes and macrophages of the heart tissue (Sanchez et al., 2003) and PCV1 targets mainly the epithelial cells of the lungs.

In previous studies with PCV2 (Sanchez et al., 2001; Pensaert et al., 2004; Yoon et al., 2004; Saha et al., 2010), it was shown that PCV2 does not spread rapidly from one foetus to another. Under the conditions of the present study, no intra-uterine spread of PCV1 from PCV1-inoculated to non-inoculated foetuses was observed. Immuno-competency in porcine foetuses develops at around 80 days of gestation (Salmon, 1984). More specifically, porcine foetuses are able to mount a protective immune response against small, non-enveloped, single-stranded DNA viruses like PCV1, PCV2 or PPV when they are infected after 70 days of gestation (Joo et al., 1976b; Sanchez et al., 2001; Saha et al., 2010). Foetuses were inoculated with PCV1 strains at 55 days of foetal life and their immune response to PCV1 was confirmed by determining the PCV1-specific IPMA Ab titre. All PCV1-inoculated foetuses developed very low anti-PCV1 antibody titres (10 to 40), except one foetus, which had a titre of 160 that was inoculated with CCL33 strain.

From this study, it can be concluded that PCV1 can replicate and may produce pathology in the lungs of porcine foetuses inoculated at 55-days of foetal life. More research is needed to confirm the pathogenic character of PCV1 for porcine foetuses.

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Chapter 4.2.

Pathologic and virologic findings in mid-gestational porcine foetuses after experimental inoculation with PCV2a or PCV2b

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Abstract

Two major genotypes of porcine circovirus type 2 (PCV2) have been described: PCV2a and PCV2b. Previous studies mainly used PCV2a to experimentally reproduce reproductive failure in sows. This study aims to determine the clinical and virological outcome of surgical inoculation of 55-day-old immuno-incompetent porcine foetuses with PCV2a or PCV2b. Twelve foetuses were inoculated with PCV2: three with the postweaning multisystemic wasting syndrome (PMWS)-associated PCV2a strain Stoon-1010, three with the reproductive failure-associated PCV2a strain 1121, three with the PMWS-associated PCV2b strain 48285 and three with the porcine dermatitis and nephropathy syndrome-associated PCV2b strain 1147. Four foetuses were mock-inoculated with cell culture medium. At 21 days post inoculation eleven out of twelve PCV2-inoculated foetuses were oedematous and had distended abdomens, whereas one had a normal external appearance. All PCV2-inoculated foetuses had haemorrhages and congestion in internal organs and an enlarged liver. High PCV2 titres (>$10^{4.5}$ $TCID_{50}$/g tissue) were found in all PCV2-inoculated foetuses, especially in the heart, spleen and liver. High numbers of PCV2-infected cells (>1000 infected cells/10 mm^2 tissue) were observed in the hearts. PCR and DNA sequencing of the *capsid gene* recovered pure PCV2a and pure PCV2b sequences from PCV2a- and PCV2b-inoculated foetuses, respectively. All mock-inoculated and the remaining foetuses were normal in appearance and were PCV2 negative in virus titrations and indirect immunofluorescence stainings. The present study shows that PCV2a and PCV2b induce similar gross pathological lesions and replicate to similar high titres in organs of 55-day-old immuno-incompetent porcine foetuses.

1. Introduction

Porcine circovirus type 2 (PCV2) is a small, non-enveloped virus with a circular single-stranded DNA. PCV2 is the causal agent of postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease that causes severe weight loss and increased mortality in weaned pigs (Allan and Ellis, 2000; Segales and Domingo, 2002). PCV2 infections may also cause foetal death, mummification and abortion, especially in swine herds that were recently primary infected with PCV2 (West et al., 1999; O'Connor et al., 2001; Brunborg et al., 2007).

Foetal death, mummification, abortion and premature farrowing have been experimentally reproduced by intranasal inoculation of PCV-seronegative specified pathogen free (SPF) sows with PCV2 during last third of gestation (Park et al., 2005), by intra-uterine inoculation of SPF sows with PCV2 at insemination (Rose et al., 2007; Madson et al., 2009) and by trans-uterine, intra-foetal PCV2 inoculation during mid-gestation or during the last third of gestation (Johnson et al., 2002; Pensaert et al., 2004; Yoon et al., 2004). Gross lesions due to experimental PCV2 infections in foetuses include subcutaneous oedema, abdominal distension, haemorrhages and congestion in internal organs and liver enlargement (Sanchez et al., 2001; Yoon et al., 2004; Madson et al., 2009). The heart is the main target organ for PCV2 infection in foetuses (West et al., 1999; O'Connor et al., 2001; Sanchez et al., 2001; Sanchez et al., 2003) and there is a strong correlation between PCV2 replication levels and the severity of the observed histopathological lesions (O'Connor et al., 2001; Sanchez et al., 2003; Yoon et al., 2004; Brunborg et al., 2007; Rose et al., 2007). Furthermore, it has been demonstrated that hatched blastocysts are susceptible to PCV2 infection (Mateusen et al., 2004) and that PCV2 replication in embryos may lead to embryonic death, subsequent resorption and return to oestrus (Mateusen et al., 2007).

A recently proposed scientific classification system divides PCV2 strains into two major genotypes - PCV2a and PCV2b - based on their genomic sequences (Olvera et al., 2007; Grau-Roma et al., 2008; Segales et al., 2008). The majority of experimental studies used PCV2a strains to reproduce PCV2-associated reproductive failure in sows (Sanchez et al., 2001; Sanchez et al., 2003; Johnson et al., 2002; Pensaert et al., 2004; Yoon et al., 2004; Mateusen et al., 2007), but not all experimentally used PCV2 sequences are available in GenBank (Park et al., 2005; Rose et al., 2007). Consequently, only little is known on the effect of experimental PCV2b infection during gestation or at insemination (Madson et al.,

2009).

The present study aims to determine the clinical and virological outcome of surgical inoculation of 55-day-old immuno-incompetent porcine foetuses with PCV2a or PCV2b.

2. Materials and methods

2.1. Viruses and cells

Four different PK-15 cell adapted PCV2 strains were used in this study; PCV2a strain Stoon-1010 and PCV2b strain 48285, both originating from cases of postweaning multisystemic wasting syndrome (PMWS) (Meehan et al., 1998), PCV2a strain 1121 originating from a case of reproductive failure (Meehan et al., 2001) and PCV2b strain 1147 originating from a case of porcine dermatitis and nephropathy syndrome (PDNS) (Meehan et al., 1998; Meehan et al., 2001). The replication kinetics of these strains have been described in PK-15 cells by Meerts et al., 2005). The antigenic and phylogenetic relationships of these strains were described by Lefebvre et al. (2008). PCV2 strains may have different replication kinetics *in vitro* and *in vivo*, even when PCV2 strains belong to the same genotype (Meerts et al., 2005; Opriessnig et al., 2006). In order to obtain more reliable results, not one but two strains were used per genotype.

PCV-negative PK-15 cells were grown in minimal essential medium (MEM) containing Earle's salts (Gibco, Grand Island, USA), supplemented with 5% foetal bovine serum (FBS), 0.3 mg/mL glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.1 mg/mL kanamycin. Cell cultures were maintained at 37°C in the presence of 5% CO2.

2.2. Experimental design

Four conventional PCV2 seropositive Landrace sows were submitted to laparatomy at 55 days of gestation. Sows were pre-medicated with azaperonum [Stresnil1 2 mg/kg im] (Janssen Animal Health, Beerse, Belgium) and general anaesthesia was induced with thiopentalum natricum [Pentothal 1.5 g iv in an ear vein] (Hospira enterprises BV, Hoofddorp, The Netherlands). The left flank was locally anaesthetised with procainii chloridum 4% with adrenalinum [60 mL sc and im] (Kela Laboratoria, Hoogstraten, Belgium). The anaesthetic state was maintained by repeated iv administration of thiopentalum natricum in doses of 0.25 g.

In the first sow, two foetuses were inoculated with PCV2a strain Stoon-1010, one with PCV2a strain 1121 and one was mock-inoculated with PK-15 cell culture medium. In the second sow, two foetuses were inoculated with PCV2b strain 48285, one with PCV2b strain 1147 and one with PK-15 cell culture medium. In the third sow, one foetus was inoculated with PCV2a strain Stoon-1010, one with PCV2a strain 1121, one with PCV2b strain 1147 and one with PK-15 cell culture medium. In the fourth sow, one foetus was inoculated with PCV2a strain 1121, one with PCV2b strain 48285, one with PCV2b strain 1147 and one with PK-15 cell culture medium. The position in the uterus of the PCV2- and mock-inoculated foetuses, and their adjacent foetuses is shown in Table 1. Inoculations were performed as described by Sanchez et al. (2001). Briefly, foetuses were inoculated by trans-uterine injection with 200mL, containing $10^{4.3}$ $TCID_{50}$ of PCV2, into the peritoneal (100mL) and amniotic (100mL) cavities. For mock-inoculated foetuses, PK-15 cell culture medium (200mL) was inoculated by trans-uterine injection with 200mL into the peritoneal (100mL) and amniotic (100mL) cavities. Inoculated foetuses were marked with a synthetic, non-absorbable, superficial suture (Prolene 2-0, Ethicon, Inc., Somerville, New Jersey, USA) on the exterior uterine wall. Antibiotics [Pen-Strep 20/20 10 mL ip and 10mL in the operation wound] (V.M.D., Arendonk, Belgium) were administered to the sows before closure of the operation wound.

The sows were housed individually in A2 experimental facility units. The sows were observed daily for clinical signs and their rectal temperature was monitored daily during the first week after surgery. Twenty-one days post inoculation (dpi), the sows were humanely euthanised with an overdose of pentobarbitalum natricum [Natriumpentobarbital 20% 40 mg/kg iv in the V. jugularis externa] (Kela Laboratoria, Hoogstraten, Belgium). Hysterectomy was performed and all foetuses were collected. The specific length of the tail ends of the sutures was used to determine the PCV2 strain a foetus was inoculated with.

All inoculated and non-inoculated foetuses were examined for gross lesions and tissue samples were collected from the heart, lungs, spleen, liver, kidneys, thymus, tonsils, ileum and cerebrum. Serum and abdominal fluid were collected as well. Serum samples of the sows were collected prior to surgery (pre-serum) and at the time of euthanasia (post-serum).

The animal experiments described in this study were authorised and supervised by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University.

Table 1. List of PCV2-inoculated, mock-inoculated foetuses and their adjacent foetuses and the result of PCR amplification of the *capsid gene*.

Sow no.	Foetus no.[a]	Inoculated with	PCR result
1	L3	NI[b]	-
	L4	mock	-
	L5	1121	+
	L6	NI[b]	-
	R3	NI[b]	+
	R4	Stoon-1010	+
	R5	Stoon-1010	+
	R6	NI[b]	-
2	L1	48285	+
	L2	48285	+
	L3	NI[b]	-
	R1	NI[b]	-
	R2	1147	+
	R3	NI[b]	-
	R10	NI[b]	-
	R11	mock	-
	R12	NI[b]	-
3	L5	NI[b]	-
	L6[c]	1121	+
	R1	mock	-
	R2	NI[b]	-
	R3	NI[b]	-
	R4	Stoon-1010	+
	R5[c]	1147	+
4	L1	1147	+
	L2[d]	48285	+
	R2	NI[b]	-
	R3	1121	+
	R4[d]	mock	-

[a] Foetuses were identified by their position in the uterus. L = left horn; R = right horn. Numbering is in sequence from ovary to cervix. [b]NI = Not inoculated; [c]L6 and R5 were adjacent to each other; [d]L2 and R4 were adjacent to each other.

2.3. PCV2 replication

Ten % (w/v) tissue suspensions of spleen, thymus, tonsils and ileum and 20% (w/v) tissue suspensions of heart, lungs, liver, kidneys and cerebrum were prepared in phosphate-buffered saline (PBS). For PCV2-inoculated foetuses, the PCV2 titres in each of the aforementioned tissues were determined by virus titration on PK-15 cells as described before (Sanchez et al., 2001). For mock-inoculated and non-inoculated foetuses, the PCV2 titres were determined in heart, lungs and spleen. Titration experiments were repeated three times. For 10% suspensions, the detection limit of this technique was $10^{2.0}$ $TCID_{50}$/g tissue and for 20% suspensions, the detection limit was $10^{1.7}$ $TCID_{50}$/g tissue.

The number of PCV2 antigen positive cells in the hearts of PCV2-inoculated, mock-inoculated and non-inoculated foetuses was determined by an indirect immunofluorescence staining, adapted from the technique described by Sanchez et al. (2001). Methanol-fixed cryostat sections were incubated with an optimal dilution of anti-PCV2 monoclonal antibody (Ab) F190 (McNeilly et al., 2001) in PBS. This monoclonal Ab is directed against the PCV2 capsid protein. Subsequently, a 1:500 dilution of fluorescein isothiocyanate (FITC)-labelled goat-anti-mouse polyclonal Ab (Molecular Probes, Eugene, Oregon, USA) in PBS was applied. Both incubations were performed for 1 h at 37°C and sections were washed three times with PBS between the incubations. Stained tissue sections were mounted with a glycerol solution containing 1,4-diazobicyclo-2.2.2-octane (DABCO) anti-fading agent (Janssen Chimica, Beerse, Belgium). The number of PCV2-positive cells was determined in an area of 10 mm^2 of tissue by using a LEICA DM/RBE fluorescence microscope (Leica Microsystems GmbH, Heidelberg, Germany).

2.4. Amplification of PCV2 *capsid genes* and sequencing

DNA was extracted from heart and lung tissue suspensions of all PCV2- and mock-inoculated and their adjacent foetuses, by using a NucleoSpin tissue kit (Macherey-Nagel, GmbH & Co. KG, Duren, Germany). Amplification of the PCV2 *capsid gene* was performed as described previously (Lefebvre et al., 2008). PCR products (capsid) were treated with Exonuclease I and Antarctic Phosphatase (New England Biolabs, Ipswich, USA) and used directly for cycle sequencing with a Big Dye Terminator Cycle sequencing kit V1.1 (Applied Biosystems, Foster City, USA). Cycle sequencing reaction products were purified using ethanol precipitation and separated on an ABI Genetic Analyzer 310 (Applied Biosystems, Foster City, USA). Sequence alignments were performed using bl2seq at blast.ncbi.nlm.nih.gov.

2.5. Serology

PCV2-specific Ab titres in serum or abdominal fluids were determined by an immuno-peroxidase monolayer assay (IPMA) as described previously (Labarque et al., 2000). PCV2 strain 1121 was used as antigen. These assays were repeated three times.

Sow Ab titres against porcine reproductive and respiratory syndrome virus (PRRSV) and porcine parvovirus (PPV) were, respectively determined by an IPMA and a hemagglutination inhibition (HI) test as described previously (Joo et al., 1976a; Wensvoort et al., 1991).

2.6. Statistical analysis

Virus titres were compared between the groups of foetuses inoculated with PCV2a strains and foetuses inoculated with PCV2b strains by applying a two-sided, non-parametric Mann-Whitney test. Virus titres were similarly compared between the groups of foetuses inoculated with PCV2 strains originating from PMWS cases and foetuses inoculated with PCV2 strains originating from cases of reproductive failure or PDNS, and between the groups of foetuses from the four sows. Differences were considered significant when $P<0.05$. Statistical analyses were performed using GraphPad Software version 5.0a (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Evaluation of sows

All four sows remained clinically healthy for the entire duration of the study. A rise in their rectal temperatures was not recorded. Only at the first days after laparatomy, the operation wounds were slightly swollen and somewhat painful at palpation. PCV2-specific IPMA Ab titres in pre-serum ranged from 20,480 to 81,920 and were equal to those in post-serum. PRRSV-specific IPMA Ab titres in preserum ranged from <10 to 640 and pre-sera had a PPV-specific HI Ab titre ranging between <8 and 512. Seroconversion against PRRSV or PPV was not observed.

3.2. Gross examinations

All PCV2-inoculated foetuses, except one strain 1147-inoculated foetus, were oedematous and had distended abdomens (Fig. 1a). Ascites, hydrothorax, hydropericardium, haemorrhages and congestion in internal organs and liver enlargement were observed (Fig. 1b). Enlarged livers were fragile and easily damaged when manipulated. One strain 1147-inoculated foetus had a normal external appearance, but oedema of the lungs, liver enlargement and generalised lymph node enlargement were observed. Mock-inoculated and non-inoculated foetuses were normal in appearance and gross pathological lesions were not observed.

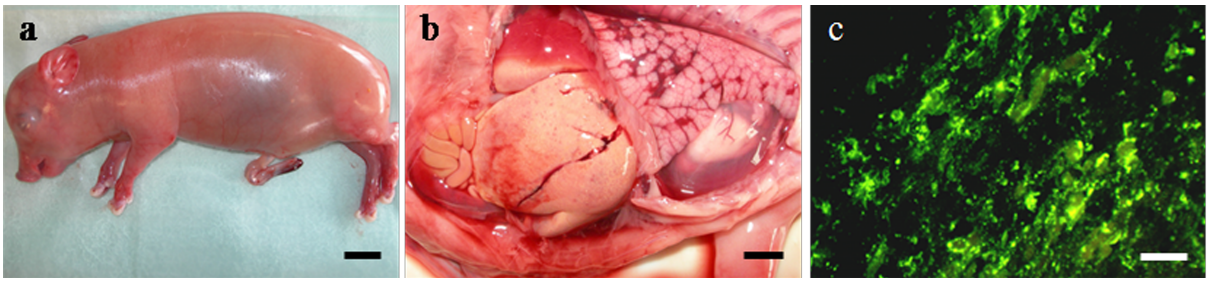

Fig. 1. Effects of PCV2 replication after inoculation of a 55-day-old foetus with PCV2. a) Subcutaneous oedema and abdominal distension. Bar = 2 cm. b) Haemorrhages and congestion in internal organs and liver enlargement. Bar = 1 cm. c) Immunofluorescence staining for the PCV2 capsid protein in the heart. More than 10,000 PCV2 positive cells were present per 10 mm^2 tissue. Bar = 50 μm.

3.3. PCV2 replication

Table 2 shows the virus titres (expressed as log_{10} $TCID_{50}$/g of tissue) in different organs of PCV2-inoculated foetuses. The highest mean PCV2 titres (±SD) were found in the heart (PCV2a: 6.2 ± 0.7; PCV2b: 6.1 ± 0.4), spleen (PCV2a: 5.5 ± 0.3; PCV2b: 5.1 ± 0.4), liver (PCV2a: 5.3 ± 0.4; PCV2b: 4.4 ± 1.0) and thymus (PCV2a: 4.4 ± 0.8; PCV2b: 4.8 ± 1.0) (Table 3). Mean virus titres in other organs ranged from 1.9 ± 0.2 (PCV2a) or <1.7 (PCV2b) in cerebrum to 4.0 ± 1.0 (PCV2a) or 3.5 ± 1.0 (PCV2b) in the lungs (Table 3). Mock-inoculated and non-inoculated foetuses were negative for PCV2. Virus titres were significantly different ($P<0.05$) in the kidneys of PCV2a-inoculated foetuses (3.4 ± 0.8) when compared to PCV2b-inoculated foetuses (2.2 ± 0.7). Statistically significant differences were not observed in any of the other organs.

Very high numbers of PCV2-positive cells (>10,000 PCV2-positive cells/10 mm^2 tissue) were observed in the hearts (Fig. 1c) of all inoculated foetuses, except for the two foetuses inoculated with PCV2-48285 strain and one foetus inoculated with PCV2-1147 strain. In these foetuses, high numbers of PCV2-positive cells (1000 to 10,000 PCV2-positive cells/10 mm^2 tissue) were observed in the heart. PCV2-positive cells were mainly seen in large to very large homogeneously distributed coalescent foci. Viral antigens were localised predominantly in the cytoplasm of cells with morphological characteristics typical of cardiomyocytes or macrophages, respectively. PCV2 antigens were occasionally (<5%) observed in the nucleus of these cells. PCV2-positive cells were not observed in the hearts of mock-inoculated and non-inoculated foetuses.

Table 2. Virus titres in different foetal organs after intra-foetal inoculation with PCV2 at 55 days of gestation and collected at 21 days post inoculation

Strain	Genotype[a]	Sow no.	Inoculated foetus[b]	Virus titre (log_{10} $TCID_{50}$ / g tissue)								
				Heart	Lungs	Spleen	Liver	Kidneys	Thymus	Tonsils	Ileum	Cerebrum
1010	PCV2a	1	R4	7.0	4.4	5.3	5.5	3.3	3.2	3.2	2.3	1.9
1010	PCV2a	1	R5	6.7	4.9	5.1	5.6	4.8	4.2	3.0	3.5	1.9
1010	PCV2a	3	R4	6.6	4.7	5.5	5.6	2.6	5.3	3.7	4.0	2.2
1121	PCV2a	1	L5	5.2	3.3	5.7	4.7	3.5	4.8	2.7	3.0	1.7
1121	PCV2a	4	R3	5.5	2.4	5.3	5.3	2.9	4.0	<3.0[c]	<3.0[c]	1.9
1121	PCV2a	3	L6	6.3	4.2	5.8	5.1	3.4	5.1	4.2	2.9	<1.7
48285	PCV2b	2	L1	6.0	2.4	5.6	5.6	2.6	5.6	2.2	2.8	1.7
48285	PCV2b	2	L2	6.4	2.3	5.1	4.2	2.5	4.4	2.1	2.4	1.7
48285	PCV2b	4	L2	5.6	4.0	4.8	3.5	<1.7	5.3	2.8	<2.0	<1.7
1147	PCV2b	2	R2	6.2	4.6	5.3	5.3	1.7	3.0	<3.0[c]	<3.0[c]	<1.7
1147	PCV2b	3	R5	6.6	4.3	4.6	4.4	2.9	5.0	2.8	2.8	<1.7
1147	PCV2b	4	L1	6.0	3.3	5.1	3.2	<1.7	5.3	4.7	4.8	<1.7

[a]According to the nomenclature of Segales et al. (2008).
[b]Inoculated foetuses were identified by their position in the uterus; L = left horn; R = right horn. Numbering is in sequence from ovary to cervix.
[c]Very limited amounts of tissue material were available. In order to determine the PCV2 titre, the detection limit of the assay was $10^{3.0}$ $TCID_{50}$/g tissue instead of $10^{2.0}$ $TCID_{50}$/g tissue.

Table 3. Mean virus titres in different foetal organs of PCV2a- and PCV2b-inoculated foetuses

Foetal organs	PCV2a	PCV2b
Heart	6.2 ± 0.7	6.1 ± 0.4
Spleen	5.5 ± 0.3	5.1 ± 0.4
Liver	5.3 ± 0.4	4.4 ± 1.0
Thymus	4.4 ± 0.8	4.8 ± 1.0
Lungs	4.0 ± 1.0	3.5 ± 1.0
Kidney	3.4 ± 0.8*	2.2 ± 0.7*
Tonsils	3.3 ± 0.6	2.9 ± 0.9
Ileum	3.1 ± 0.6	2.8 ± 1.1
Cerebrum	1.9 ± 0.2	<1.7

Data presented as mean value ± standard deviation and expressed as $\log_{10}$ $TCID_{50}$/g tissue
*Significant differences ($P<0.05$) between PCV2a- and PCV2b-inoculated foetuses were determined with a two-sided, non-parametric Mann-Whitney test.

3.4. Amplification of PCV2 *capsid genes* and sequencing

In order to verify that the foetuses had been inoculated with the specific PCV2 strain (as mentioned in Table 1), the *capsid gene* was amplified using heart and/or lung tissue from the infected foetuses (21 days after inoculation) followed by sequencing. Pure PCV2a sequences were obtained from PCV2a-inoculated foetuses and pure PCV2b sequences were obtained from PCV2b-inoculated foetuses. No evidence for mixed samples (e.g. containing more than one PCV2 strain) was seen. All adjacent foetuses of PCV2-inoculated foetuses, with the exception of foetus R3 of sow one that was adjacent to foetus R4 that was inoculated with Stoon-1010 (PCV2a), were negative in PCR assays. Only heart tissue of foetus R3 was positive in PCR assays and a pure Stoon-1010 sequence was obtained after sequencing of the *capsid gene*. However, heart of this foetus was negative in virus isolation and indirect immunofluorescence staining. All mock-inoculated foetuses and their adjacent foetuses were negative in PCR assays (Table 1).

3.5. Serology

The strain 1121-inoculated foetus of the second sow had a low PCV2-specific IPMA Ab titre of 10 and the strain 1121- and strain 1147-inoculated foetuses of the third sow had low PCV2-specific IPMA Ab titre of 40 and 10, respectively. The nine other PCV2-inoculated foetuses, the four mock-inoculated and all non-inoculated foetuses were negative (<10) for PCV2-specific IPMA Ab.

4. Discussion

This study confirms previous work from Sanchez et al. (2001; 2003) stating that mid-gestational porcine foetuses are highly susceptible to PCV2 replication and that PCV2 replication causes foetal pathology. Furthermore, it confirms that the heart, liver and lymphoid organs are major sites of PCV2 replication in mid-gestational foetuses, with the heart being most important target organ of replication for PCV2 (West et al., 1999; Sanchez et al., 2001; Sanchez et al., 2003). The observed gross pathological lesions, such as ascites, hydrothorax, hydropericardium, oedema and congestion are suggestive for heart failure (Madson et al., 2009).

This study also confirms that PCV2 does not rapidly spread from one foetus to another (Sanchez et al., 2001; Pensaert et al., 2004; Yoon et al., 2004). Intra-uterine spread of PCV2 has previously been observed by Pensaert et al. (2004). It was limited to adjacent foetuses at the end of gestation. Intra-uterine spread between foetuses was also suggested by Madson et al. (2009), but in this study it could not be ruled out that foetuses were infected through vertical transmission. In this study, a non-inoculated foetus that was adjacent to a Stoon-1010-inoculated foetus, was PCV2 DNA positive in the heart and a pure Stoon-1010 genomic sequence was found in this non-inoculated foetus. Still, this foetus was negative for infectious virus and viral protein. This suggests a spread of PCV2 from the inoculated foetus to the adjacent foetus during the 3 weeks post inoculation. Although we did not perform qPCR to quantitatively measure the number of PCV2 copies, the PCR amplicon obtained from the non-inoculated foetus was visibly weaker in intensity compared to the amplicons obtained from inoculated foetuses, which suggests that the amount of PCV2 was low in the heart of this non-inoculated foetus (data not shown). It is very well possible that the level of viral replication in the adjacent foetus was still too low to be detected by virus titration and indirect immunofluorescence. A previous study of McNeilly et al. (2002) indicated that PCR was a more sensitive technique than virus titration and immunostaining of cryostat sections for the detection of PCV2 in porcine tissues.

In the present study, it was observed that different PCV2a and PCV2b strains, originating from different clinical presentations, induced similar gross pathological lesions and replicated to similar high titres in different foetal organs, without apparent differences between the 2 genotypes. To our knowledge, this is the first experimental study that describes and compares the pathogenic character of different strains of both PCV2 genotypes in porcine foetuses,

since previous studies predominantly used PCV2a strains (Sanchez et al., 2001; Sanchez et al., 2003; Johnson et al., 2002; Pensaert et al., 2004; Yoon et al., 2004) or only used a PCV2b strain (Madson et al., 2009). Recent field observations on the occurrence of PMWS suggest that some currently circulating PCV2b strains may be more virulent than currently circulating PCV2a strains, because PCV2b is more frequently isolated from PMWS-cases than PCV2a (Carman et al., 2008; Dupont et al., 2008; Grau-Roma et al., 2008). In the present study, PCV2b strains did not induce more severe gross pathological lesions in mid-gestational porcine foetuses than PCV2a strains nor did PCV2b strains replicate to higher titres in foetal organs. Previously, Madson et al. (2008) and Opriessnig et al. (2008) were not able to demonstrate differences in pathogenicity between PCV2a and PCV2b in boars or growing pigs.

PMWS is a disease that develops in weaned pigs that are primarily infected with PCV2 and that are not able to mount a protective immune response against PCV2 (Meerts et al., 2006). Porcine foetuses become immuno-competent at around 80 days of gestation (Salmon, 1984) and consequently, porcine foetuses are only able to mount a protective immune response against small, non-enveloped, single-stranded DNA viruses such as PCV2 or PPV when they are infected after day 70 of gestation (Joo et al., 1976b; Sanchez et al., 2001). In the present study, PCV2 was inoculated in 55-day-old, immuno-incompetent foetuses. Their immuno-incompetence was confirmed by the fact that at day 76 of gestation, only three out of twelve PCV2-inoculated foetuses had a very low PCV2-specific IPMA Ab titre, while the other PCV2-inoculated foetuses were negative for PCV2-specific IPMA Ab.

It can be concluded that different PCV2 strains of both genotypes and originating from different clinical presentations induced similar gross pathological lesions and replicated to similar high titres in different foetal organs of immuno-incompetent porcine foetuses experimentally inoculated at day 55 of foetal life.

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Chapter 4.3.

Outcome of an *in utero* infection with a less virulent PCV2 strain and postnatal super-infection with a highly virulent PCV2 strain

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Abstract

VC2002, isolated from a PMWS-affected pig, is a mixture of two PCV2 viruses: K2 and K39 and a preliminary experiment showed that K2 had no short-term adverse effects on foetuses, whereas K39 had. This led to the working hypothesis that infection of immuno-incompetent foetuses with a less virulent K2 could give a status of immunotolerance and postnatal super-infection with a highly virulent K39 may lead to PMWS. Firstly, nine 55-day-old foetuses were inoculated to test their foeto-pathogenicity: three with K2-$10^{4.3}$ $TCID_{50}$, three with K39-$10^{4.3}$ $TCID_{50}$ and three with medium. At 21 days post-inoculation (dpi), K2 did not induce any pathology, whereas K39 did. Afterwards, 24 45-day-old foetuses were inoculated to examine the long-term effect: six with K2-high dose ($10^{4.3}$ $TCID_{50}$), six with K2-low dose ($10^{2.3}$ $TCID_{50}$) and 12 mock-inoculated. Both doses resulted in five mummies and one live-born (69dpi). Twelve mock-inoculated foetuses were born alive. K2 was recovered from all mummies. In the two live-born K2-infected piglets, K2 and K2-specific antibodies were not detected in serum at birth. Most probably, these foetuses had fully controlled K2 infection. Among 14 live-born piglets, six mock-inoculated and K2-low dose-infected piglets were immunostimulated at day 2 and the rest (six mock-inoculated and K2-high dose-infected) were not immunostimulated. Six mock- (3 stimulated and 3 non-stimulated) and two K2-infected (stimulated and non-stimulated) piglets were super-inoculated with K39 at day 6 or 8 (0 days post super-inoculation (dpsi)). Other six mock-inoculated (3 stimulated and 3 non-stimulated) piglets were super-inoculated with medium (mock-inoculated). A low viral replication was observed in all six (3 stimulated and 3 non-stimulated) mock-K39 piglets and in non-stimulated K2-K39 piglet (up to $10^{3.3}$ $TCID_{50}$/g; identified as K39). The stimulated K2-K39 piglet had a high viral replication (up to $10^{5.6}$ $TCID_{50}$/g; identified as K2), which indicates that K2 infection was controlled during foetal life and emerged after birth upon immunostimulation. However, none of them had any sign of PMWS.

1. Introduction

Porcine circovirus type 2 (PCV2) is the causative agent of postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease that causes severe growth retardation, wasting and increased mortality in weaned pigs (Segalés and Domingo, 2002). Besides PMWS, PCV2 has been isolated from aborted, mummified foetuses and nonviable neonatal piglets (Meehan et al., 2001; O'Connor et al., 2001; Farnham et al., 2003; Kim et al., 2004; Mikami et al., 2005; Brunborg et al., 2007). The PCV genome contains two major open reading frames (ORFs): ORF1 and ORF2 (Hamel et al., 1998). ORF1 codes for the non-structural replication-associated protein Rep and its spliced frame-shifted variant Rep' (Cheung, 2003; Mankertz et al., 2003). ORF2 codes for the structural capsid protein (Nawagitgul et al., 2000). Among various strains of PCV2, the Rep (ORF1) and Cap (ORF2) have 97-100% and 91-100% identity, respectively, at nucleotide level and 97-100% and 89-100% identity, respectively, at protein level (Larochelle et al., 2002). Further, it has been shown that there is a close antigenic homology between different PCV2 strains (Allan et al., 1999; McNeilly et al., 2001). Nonetheless, Lefebvre et al. (2008) and Saha et al. (2012) demonstrated antigenic differences among PCV2 strains using monoclonal antibodies.

Several studies have tried to experimentally reproduce PMWS by inoculating pigs with PCV2, which mainly led to subclinical infections (reviewed by Segalés, 2012). Only in a restricted number of studies, symptoms comparable with those seen in naturally PMWS-affected pigs were observed in a low percentage of the animals. Some research groups were able to increase the chance to get PMWS affected animals by immunostimulation and co-infection with other viruses or bacteria. As other research groups had problems to reproduce these findings, it was generally accepted that some other factors are important as well. Both viral and host factors may be involved. An important host factor may be the host immune response. Despite the presence of anti-PCV2 antibodies (IPMA-Ab), neutralising antibodies could not be found or were present at low titres in experimentally and naturally PMWS-affected animals (Meerts et al., 2006; Fort et al., 2007). Absence of neutralising antibodies can be considered as the cause of high replication (genetically determined absence of recognition of the neutralising epitope) or as a consequence of the high replication (inhibition of the production of neutralising antibodies by replication of PCV2 in lymphoblasts). At present, researchers working in basic and applied research or vaccine development have

problems in studying the pathogenesis of PMWS and to test vaccines against PMWS due to absence of a highly reproducible PMWS model.

Innate immunity in porcine foetuses consists of (a) the recruitment and activation of cellular components including macrophages, neutrophils, natural killer (NK) cells, and dendritic cells (DCs) and (b) the release of a broad spectrum of extracellular mediators such as cytokines, chemokine, complement and antimicrobial proteins (AMPs) (Sinkora and Butler, 2009). Most cellular components of innate immunity appear together with the haematopoietic activity of the different foetal organs starting from 16, 18 and 40 days of gestation in yolk sac, foetal liver and bone marrow, respectively (Tlaskalova-Hogenova et al., 1994; Trebichavsky et al., 1996; Sinkora et al., 2003). Macrophages isolated from umbilical blood start their phagocytic activity already at 40 days of gestation and those isolated from the foetal spleen and liver at 57 days of gestation (Rehakova et al., 1998). NK cells are first found at 45 days of gestation in umbilical cord and spleen. The proportion of NK cells in various tissues is between 1 and 10% of the total lymphocyte populations and their number increases during the progress of the foetal life (Sinkora et al., 1998). The occurrence and the frequency of NK cells stabilize at about 70 days of gestation and remains approximately the same through birth into postnatal life. NK cells in adult conventional pigs represent maximum 15% of all lymphocytes. The killing activities of NK cells are not observed before birth and are delayed in germ-free piglets (Huh et al., 1981; Yang and Schultz, 1986), which indicates that NK cells require some sort of maturation and probably need stimulation by the microbial pathogens for the development of their full functional status (Sinkora and Butler, 2009). The extracellular mediators of innate immunity and inflammatory proteins are found very early during foetal development. For example, IFN-α and IFN-α secreting cells can be detected in the foetal liver as early as at 26 days of gestation (Splichal et al., 1994). At later stages of the foetal life, these cells are found in different foetal tissues like the blood, spleen or bone marrow. The possible effect that PCV2 replication in different foetal organs has on the functional development of the foetal innate immunity or vice-versa is not known.

The *in utero* PCV2 infection during insemination (Madson et al., 2009a; Madson et al., 2009b) or at late gestation (Jung et al., 2006; Ha et al., 2008) resulted in the birth of live-born PCV2-infected piglets, which are highly susceptible to the development of disease upon co-infections with other pathogens or immunostimulation (Jung et al., 2006; Ha et al., 2008). However, it is not investigated yet whether porcine foetuses of early stages of gestation

(before the development of immunocompetence) inoculated with a less virulent PCV2 strain could be viable and immunotolerant to PCV2 (unable to immunologically recognise PCV2) at birth and if postnatal super-infection of such PCV2-immunotolerant pigs with a highly virulent PCV2 strain could cause disease and no recognition by the immune system. This would resemble the pathogenesis of bovine viral diarrhoea (BVD) in cattle. Non-cytopathic BVD virus infection of immuno-incompetent bovine foetuses may result in the birth of calves that are immunotolerant (unable to immunologically recognise BVDV) (McClurkin et al., 1984). Mucosal disease occurs when immunotolerant cattle become super-infected with an antigenically similar cytopathic BVD virus or when the non-cytopathic BVD virus mutates into a cytopathic BVD virus.

The presence of two different PCV2 sequences in one pig has been reported previously (de Boisséson et al., 2004; Opriessnig et al., 2006; Cheung et al., 2007; Lefebvre et al., 2008). PCV2 strain VC2002 was isolated from a Belgian PMWS-affected pig (Meerts et al., 2004) and our group discovered that VC2002 is a mixture of two different PCV2 viruses: K2 and K39 (Lefebvre et al., 2008). The K2 and K39 viruses were cloned by limiting dilution and renamed as II11A and II9F, respectively (Lefebvre et al., 2009). Strain K2 has 94% identity with K39 at nucleotide and at amino acid level (Lefebvre et al., 2008). Further analysis of these two viruses revealed that K2 has different biological characteristics than K39 in porcine foetuses inoculated at 55 days of gestation (Saha et al., 2011a). The strain K2 did not induce any pathology in foetuses collected at 21 days post inoculation (76 days of gestation), whereas strain K39 did. This led to the hypothesis that the *in vivo* infection of embryos/foetuses with a less virulent K2 before the development of immunocompetence could give a status of immunotolerance at birth. Postnatal super-infection of such an immunotolerant animal with a highly virulent K39 could cause disease and no recognition by the immune system.

The aim of the present study was to develop PCV2-immunotolerant pigs by intra-uterine inoculation of a less virulent K2 strain in immuno-incompetent porcine foetuses and to analyse the outcome of a postnatal super-infection with a highly virulent K39 strain after birth.

2. Materials and methods

2.1. Viruses and cells

Two different PK-15 adapted PCV2b strains, K2 (EF990645) and K39 (EF990646) (Lefebvre et al., 2009) were used in this study. Both strains originated from strain VC2002, which was isolated from an inguinal lymph node from a Belgian-PMWS affected pig (Meerts et al., 2004).

PCV-negative PK-15 cells were grown in minimal essential medium (MEM) containing Earle's salts and GlutaMAX^{TM}-I (MEM+GlutaMAX^{TM}-I, Gibco, Grand Island, USA) and supplemented with 5 % foetal calf serum (FCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. Cell cultures were maintained at 37°C in the presence of 5 % CO2.

2.2. Experimental design

2.2.1. *Short-term outcome (21 days post inoculation/76 days of gestation)*

At 55 days of gestation, three conventional PCV2 seropositive Landrace sows (S1, S2 and S3) were submitted to laparotomy. Laparotomy was performed on the left side of the abdomen under full anaesthesia as described previously (Saha et al., 2010; Saha et al., 2011b). Three foetuses of each sow were inoculated: one foetus with strain K2, one foetus with strain K39 and one foetus with cell culture medium (mock-inoculated). The position in the uterus of the PCV2- and mock-inoculated foetuses, and their adjacent foetuses is shown in Table 1. Inoculations were performed as described before (Saha et al., 2010; Saha et al., 2011b). Briefly, foetuses were inoculated by trans-uterine injection with 200 μL, containing $10^{4.3}$ $TCID_{50}$ of PCV2 or PK-15 cell culture medium, into the peritoneal (100 μL) and amniotic (100 μL) cavities. Inoculated foetuses were marked with a synthetic, non-absorbable, superficial suture (Prolene 2-0, Ethicon, Inc., Somerville, New Jersey, U.S.A.) on the exterior uterine wall. Antibiotics [Duphapen Strep, 10 mL ip and 10 mL in the operation wound] (Fort Dodge, Naarden, The Nederlands) were administered to the sows before closure of the operation wound.

The sows were housed individually in A2 experimental units and they were observed daily for clinical signs and their rectal temperature was monitored daily during the first week after surgery. Twenty-one days post inoculation (dpi), the sows were humanely euthanised with an

overdose of pentobarbitalum natricum [Natriumpentobarbital 20% 40 mg / kg iv in the V. jugularis externa] (Kela Laboratoria, Hoogstraten, Belgium). All foetuses were collected by hysterectomy. The specific length of the suture ends was used to identify the inoculated foetuses.

All inoculated and non-inoculated foetuses were examined for gross lesions. Tissue samples were collected from the heart, lungs, spleen, liver, kidneys, thymus, tonsils, ileum and cerebrum for histopathological examinations, virus titrations and indirect immunofluorescence. Serum and abdominal fluid were also collected from all foetuses. Pre-serum (before laparotomy) and post-serum (at the time of euthanasia) were taken from the sows.

2.2.2. *Long-term outcome (69 days post inoculation/114 days of gestation)*

Four conventional PCV2 seropositive Landrace sows were submitted to laparotomy on the left side of the abdomen at 45 days of gestation (Table 2). In this experiment, only K2 strain was used to inoculate foetuses. Six foetuses of each sow were inoculated: three with the strain K2 and three with medium (mock-inoculated). In the first two sows (S4 and S5), six foetuses (3 for each sow) were inoculated with a high dose of K2 ($10^{4.3}$ $TCID_{50}$/foetus), whereas six foetuses (3 for each sow) from the other two sows (S6 and S7) were inoculated with a low dose of K2 ($10^{2.3}$ $TCID_{50}$/foetus) (Table 2). The procedures of laparotomy, intra-foetal inoculation of virus or medium were identical to the technique described above. The inoculated foetuses were marked with non-absorbable sutures on the exterior uterine wall and clinical monitoring of the sows was performed as explained above. Sixty-nine days post inoculation (114 days of gestation), a second laparotomy was carried out in all four sows on the right side of the abdomen under full anaesthesia and all foetuses including 12 K2-inoculated and 12 mock-inoculated foetuses were collected (Table 2). The specific length of the tail ends of the sutures was used to identify the foetuses inoculated with K2 or medium. After collection of foetuses, the sows were humanely euthanised as described above.

Blood was collected at birth from the umbilical cord of K2-inoculated and mock-inoculated piglets. Serum samples of the sows were collected as well prior to the first laparotomy (pre-serum) at 45 days of gestation and prior to the second laparotomy (post-serum) at 114 days of gestation.

2.2.3. *Super-inoculation of in utero K2- or mock-inoculated piglets with PCV2 (K39)*

At day 6, live-born K2- and mock-inoculated colostrum-deprived and caesarean-derived (CD/CD) piglets from the sows S4 and S5 were oronasally super-inoculated either with K39 strain ($10^{4.3}$ $TCID_{50}$/pig) or medium (mock-inoculated).

Live-born K2- and mock-inoculated CD/CD piglets from the other two sows S6 and S7 were vaccinated (2 ml intramuscularly, 1 ml at each side of the neck) against parvovirus (Parvoject, Merial Belgium NV/SA, Brussels, Belgium) at day 2 to induce immunostimulation. At day 8, they were oronasally super-inoculated with K39 strain ($10^{4.3}$ $TCID_{50}$/pig) or medium (mock-inoculated).

All piglets were monitored daily for the appearance of clinical signs that are typically associated with PMWS. These clinical signs include cachexia, depression, respiratory distress and jaundice (Segalés and Domingo, 2002). In order to follow the degree of PCV2 replication, half of the left and right inguinal lymph nodes were biopsied at 14 and 21 days post super-inoculation (dpsi), respectively, as described before (Meerts et al., 2005). The remaining parts of the left and right inguinal lymph nodes were collected at euthanasia (28 dpsi) for virus titration and indirect immunofluorescence. All pigs were bled at 0, 7, 14, 21 and 28 dpsi.

2.3. Virus titration

Depending on the size of the collected tissues, 10 % or 20 % (wt/vol) tissue suspensions were prepared in phosphate-buffered saline (PBS). The PCV2 titres in different organs (organs collected during short-term, long-term and super-infection experiment) were determined by virus titration on PK-15 cells, adapted from the technique described before (Labarque et al., 2000). The immuno-peroxidase staining for PCV2 antigens was performed using an optimal dilution of mono-specific anti-PCV2 swine polyclonal serum (produced at our laboratory) and peroxidase-labelled goat-anti-swine IgG (Jackson ImmunoReasearch, UK) as primary and secondary antibodies, respectively. The detection limit of this technique was $10^{1.7}$ $TCID_{50}$ / g tissue.

2.4. Quantification of viral antigens by immunofluorescence

2.4.1. *Single immunofluorescence staining*

The number of PCV2-positive cells in different organs collected during short-term and super-inoculation experiments was determined by an indirect immunofluorescence staining (IIF), adapted from the technique described elsewhere (Sanchez et al., 2001; Saha et al., 2010; Saha et al., 2011b). Briefly, tissue samples were embedded in methylcellulose medium and rapidly frozen at -70°C. Depending on the size of the tissue samples, 3 to 7 cryostat sections (8 µm) were made 100 µm apart from each other in the tissue block. Afterwards, tissue sections were fixed in methanol at -20°C for 20 minutes and stored at -20°C until use. Then, the sections were air-dried at room temperature for 5 minutes, followed by rinsing in PBS for 5 minutes. Thereafter, cryostat sections were incubated with anti-PCV2 monoclonal antibodies (mAbs) F190 (1:500) or F217 (1:100) (McNeilly et al., 2001) or biotin-conjugated anti-PCV2 polyclonal antibodies (pAbs) (1:50) in PBS. Subsequently, a 1:200 dilution of fluorescein isothiocyanate (FITC)-labelled goat-anti-mouse pAbs (Molecular Probes, Eugene, Oregon, USA) or FITC-labelled streptavidin (Molecular Probes) in PBS was applied. Both incubations were performed for 1 h at 37 °C and sections were washed three times with PBS between the incubations. Finally, sections were incubated with Hoechst (Molecular Probes) during 10 min followed by three washings with PBS. Specificity of the staining was confirmed by using irrelevant, isotype-matched anti-PRV mAbs, 3H12 and 13D12 (Nauwynck and Pensaert, 1995) or by deletion of the primary Abs (biotin anti-PCV2) and by the complete absence of fluorescence in the corresponding tissue sections of non-inoculated, age-matched foetuses or pigs. Stained tissue sections were mounted with a glycerol solution containing 1,4-diazobicyclo-2.2.2-octane (DABCO) anti-fading agent (Janssen Chimica, Beerse, Belgium). The number of PCV2-positive cells was determined in an area of 10 mm^2 of tissue by using a LEICA DM/RBE fluorescence microscope (Leica Microsystems GmbH, Heidelberg, Germany) as described by Sanchez et al. (2001b).

2.4.2. *Double immunofluorescence staining*

In order to differentiate PCV2 strain K2 from strain K39 in pigs that were inoculated with K2 *in utero* and postnatally super-inoculated with K39, a double immunofluorescence staining was performed. Briefly, methanol-fixed cryostat sections of inguinal lymph nodes of dual-infected pigs collected at 14, 21 and 28 dpsi were incubated with 1:10 dilution of mAbs 114C8 (isotype: IgG1; which reacts with both strains K2 and K39) and 16G12 (isotype:

IgG2a; which reacts with strain K39 only) (Lefebvre et al., 2008; Saha et al., 2012). Subsequently, a 1:200 dilution of mAbs FITC-labelled goat-anti-mouse IgG1 (Molecular Probes) and goat-anti-mouse IgG2a Alexa-Flour 594 (Molecular Probes) in PBS were applied. Finally, sections were incubated with Hoechst (1:100 in PBS) (Molecular Probes) and specificity of the staining was confirmed by using irrevant, isotype-matched anti-PRV mAbs, 1C11 (IgG2a isotype) and 13D12 (IgG1 isotype) (Nauwynck and Pensaert, 1995) as explained above. Stained tissue sections were mounted with a glycerol solution and PCV2-positive cells were determined by using a LEICA DM/RBE fluorescence microscope as mentioned above.

2.5. PCR and DNA sequencing of the PCV2 *capsid gene*

DNA was extracted from the heart and lung tissue suspensions of PCV2- and mock-inoculated and their adjacent foetuses collected at 21 dpi (76 days of gestation; short-term experiment) and from the serum samples of all newborn piglets collected at 69 dpi (114 days of gestation; long-term experiment), by using a NucleoSpin tissue kit (Macherey-Nagel, GmbH & Co KG, Duren, Germany). Amplification of the PCV2 *capsid gene* was performed as described previously (Lefebvre et al., 2008; Lefebvre et al., 2009; Saha et al., 2010). PCR products (capsid) were treated with Exonuclease I and Antarctic Phosphatase (New England Biolabs, Ipswich, USA) and used directly for cycle sequencing with a Big Dye Terminator Cycle sequencing kit V1.1 (Applied Biosystems, Foster City, USA). Cycle sequencing reaction products were purified using ethanol precipitation and separated on an ABI Genetic Analyzer 310 (Applied Biosystems, Foster City, USA). Sequence alignments were performed using bl2seq at blast.ncbi.nlm.nih.gov.

2.6. Serology

PCV2-specific Ab titres in serum or abdominal fluids of foetuses from short-term experiment were determined by an immuno-peroxidase monolayer assay (IPMA) as described previously (Labarque et al., 2000). PCV2 strain K2 or K39 was used as antigen.

Sow antibody titres in pre- and post-serum samples against PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV) were assessed by an IPMA (Labarque et al., 2000) and sow Ab titres against porcine parvovirus (PPV) were investigated by a haemagglutination inhibition (HI) test as described elsewhere (Joo et al., 1976; Wensvoort et al., 1991).

PCV2-specific antibody titres were checked in serum samples of piglets collected at birth by IPMA as described above. At 0, 7, 14, 21 and 28 days post K39 or mock-inoculation, blood was taken from the jugular vein in order to monitor total anti-PCV2 Ab titres by an IPMA. PCV2 strain K2 or K39 was used as antigen.

The animal experiments described in this study were authorised and supervised by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University.

3. Results

3.1. Short-term outcome

As strains K2 and K39 showed different biological characteristics *in vivo* in foetuses (Saha et al., 2011a), they were characterised in detail *in vivo* in mid-gestational porcine foetuses.

3.1.1. *Evaluation of sows*

All three sows used in this experiment remained clinically healthy during the whole study period. The operation wounds were slightly swollen until 72 hours after surgery and somewhat painful on palpation. No rise of rectal temperature was noticed during the first week after surgery.

PCV2-specific IPMA antibody titre was 40960 in pre-serum and post-serum of the three sows. PRRSV-specific IPMA antibody titres and PPV-specific HI antibody titres were <10 and ≤8, respectively in pre-serum and were the same in post-serum.

3.1.2. *Macroscopic lesions*

At 21 days post inoculation (76 days of gestation), the K2-inoculated foetuses had a normal external appearance (Fig. 1a). No gross pathological lesions were observed in the internal organs of three foetuses inoculated with K2, except one foetus, which had an enlarged spleen (S2R3). On the other hand, all K39-inoculated foetuses showed macroscopic lesions. Two were oedematous, had a hydrothorax, ascites and distended abdomens (Fig. 1b), haemorrhages and congestion in internal organs and an enlarged heart. The third K39-inoculated foetus had a hydroperitonium. Mock-inoculated and non-inoculated foetuses had no gross pathological lesions.

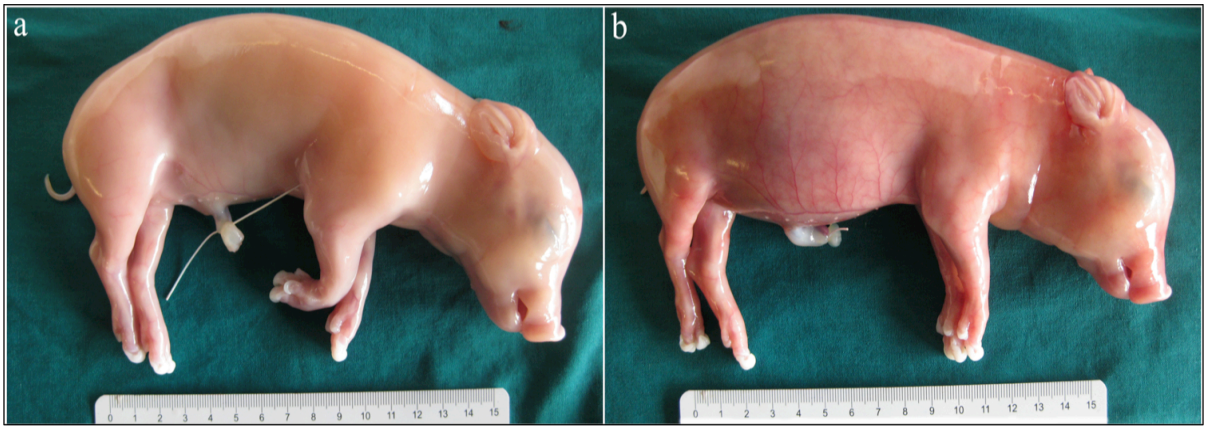

Fig. 1. Effects of PCV2 replication after inoculation of 55-day-old foetuses and collected at 21 days post-inoculation (76-day-old foetuses). a) K2-inoculated foetus with normal external appearance. b) Subcutaneous oedema and abdominal distension in K39-inoculated foetus.

3.1.3. *Microscopic lesions*

No histopathological lesions were observed in different organs of K2-inoculated foetuses, except one foetus, which had an infiltration of neutrophils in the spleen (S2R3). On the other hand, histopathological lesions were seen in several organs of K39-inoculated foetuses: haemorrhages and infiltration of mononuclear cells in heart and lungs; vacuolar degeneration of hepatocytes and neutrophil infiltration in liver; neutrophil infiltration in spleen; interstitial haemorrhage and tubular necrosis in kidney. No histopathology was seen in thymus and cerebrum of K39-inoculated foetuses. Mock-inoculated and non-inoculated foetuses had no microscopic lesions.

3.1.4. *PCV2 replication*

The virus titres ($log_{10}TCID_{50}/g$) in different organs of K2- and K39-inoculated foetuses are shown in Table 3. In K2-inoculated foetuses, the highest PCV2 titres (mean ± standard deviation) were found in the heart (5.3 ± 1.2), spleen (5.9 ± 0.4), liver (5.2 ± 0.2) and thymus (5.4 ± 0.5). In K39-inoculated fetuses, high mean virus titres were found only in the heart (5.9 ± 0.1) and the mean titres were low in spleen (3.9 ± 0.5), liver (3.7 ± 0.8) and thymus (3.4 ± 0.8) in comparison to that of K2-inoculated foetuses. The mean virus titres in the lungs (3.7 ± 0.9), kidneys (3.4 ± 1.4), tonsils (4.2 ± 0.6), ileum (4.4 ± 0.4) and cerebrum (2.3 ± 0.3) of K2-inoculated foetuses were higher than that of lungs (2.6 ± 0.4), kidneys (2.1 ± 0.6), tonsils (3.1 ± 0.6), ileum (2.2 ± 0.7) and cerebrum (1.8 ± 0.6) of K39-inoculated foetuses. The mock-inoculated and the non-inoculated foetuses were PCV2-negative in virus titrations.

Moderate to high numbers of PCV2-infected cells/10 mm^2 tissue (900, 976 and 580 in K2-

and 4027, 4350 and 3550 in K39-inoculated foetuses) were observed in the hearts (Table 3). The number of PCV2-positive cells in the heart tissues of K2-inoculated foetuses was 4.9 times lower than that of K39-inoculated foetuses. On the other hand, like the virus titres in different foetal organs, considerably high amount of PCV2-positive cells were observed in different organs of K2-inoculated foetuses than that of the K39-inoculated foetuses (Table 3). The strain K2 produced small to large focal areas of infection, whereas strain K39 was dispersed throughout the whole tissue. Mock-inoculated and the remaining foetuses were PCV2 negative in indirect immunofluorescence.

3.1.5. *PCR and DNA sequencing of the capsid gene*

PCR and DNA sequencing of the *capsid gene* recovered K2 and K39 sequences from the K2- and K39-inoculated foetuses, respectively (Table 1). The mock-inoculated and their adjacent foetuses and all adjacent foetuses of PCV2-inoculated foetuses were negative in PCR assays (Table 1).

3.1.6. *Serology*

Both K2- and K39-inoculated foetuses were negative (<10) for PCV2-specific IPMA Ab, except one foetus inoculated with strain K39 that developed very low PCV2-specific IPMA Ab titre of 40. Mock-inoculated and non-inoculated foetuses were negative (<10).

Table 1. List of foetuses inoculated with K2 or K39 (or mock-/non-inoculated) at 55 days of gestation and collected 21 days later and the results of PCR amplification of the *capsid gene*.

Sow no	Foetus no.*	Inoculated with	PCR results
S1	L1	K2	+
	L2	K39	+
	R1	mock	-
	R2	NI	-
S2	L1	NI	-
	L2	K39	+
	R1	NI	-
	R2	NI	-
	R3	K2	+
	R4	mock	-
S3	L1	NI	-
	L2	K2	+
	L3	K39	+
	L4	NI	-
	L5	NI	-
	L6	NI	-
	R1	NI	-
	R2	NI	-
	R3	NI	-
	R4	NI	-
	R5	mock	-
	R6	NI	-
	R7	NI	-

*Inoculated foetuses were identified by their position in the uterus; L = left horn; R = right horn. Numbering is in sequence from ovary to cervix. NI = not inoculated.

Table 2. Clinical outcome of pigs inoculated intra-foetally with the less virulent PCV2 strain K2 or mock-inoculated at 45 days of gestation and collected at 69 days post-inoculation (at birth).

Sow no.	Numbering of pigs at birth	Intra-foetal inoculation with	Dose ($log_{10}TCID_{50}$)	Clinical outcome at birth (69 dpi)
S4	F1	K2	4.3	live
	F2	K2	4.3	mummified
	F3	K2	4.3	mummified
	F4	mock	-	live
	F5	mock	-	live
	F6	mock	-	live
S5	F1	K2	4.3	mummified
	F2	K2	4.3	mummified
	F3	K2	4.3	mummified
	F4	mock	-	live
	F5	mock	-	live
	F6	mock	-	live
S6*	F1	K2	2.3	live
	F2	K2	2.3	mummified
	F3	K2	2.3	mummified
	F4	mock	-	live
	F5	mock	-	live
	F6	mock	-	live
S7*	F1	K2	2.3	mummified
	F2	K2	2.3	mummified
	F3	K2	2.3	mummified
	F4	mock	-	live
	F5	mock	-	live
	F6	mock	-	live

*live-born pigs from sows S6 and S7 that were *in utero* inoculated with K2-low dose ($10^{2.3}$ $TCID_{50}$) or medium (mock-inoculated) were immunostimulated by vaccination against parvovirus at day 2 after birth

Table 3. Virus replication in different foetal organs after intra-foetal inoculation with K2 or K39 at 55 days of gestation and collected at 21 days post-inoculation.

Sow no.	Foetus no.*	PCV2 strain	PCV2 replication																	
			Heart		Lungs		Spleen		Liver		Kidneys		Thymus		Tonsils		Ileum		Cerebrum	
			VT	IIF	VT	IIF	VT	IIF	VT	IIF	VT	IIF	VT	IIF	VT	IIF	VT	IIF	VT	IIF
S1	L1	K2	6.3	900	3.0	253	5.7	NA	5.2	10353	2.7	587	5.6	NA	3.7	NA	4.1	NA	2.0	10
S 2	R3	K2	5.5	976	4.7	2363	6.3	23803	5.3	12110	5.0	2890	5.8	7073	4.8	3416	4.3	3850	2.5	13
S 3	L2	K2	4.0	580	3.5	1260	5.6	8320	5.0	2337	2.5	880	4.8	10383	4.1	1297	4.8	670	2.5	17
S 1	L2	K39	6.0	3550	2.5	223	4.5	1160	2.8	1337	<1.7	10	3.0	11883	3.8	13	1.8	63	<1.7	20
S 2	L2	K39	6.0	4027	2.2	3	3.5	80	3.9	1297	2.1	27	3.0	30	2.8	37	3.0	10	<1.7	0
S 3	L3	K39	5.8	4350	3.0	20	3.8	163	4.3	1137	2.7	3	4.3	NA	2.8	NA	1.8	NA	2.5	0

*Inoculated foetuses were identified by their position in the uterus; L = left horn; R = right horn. Numbering is in sequence from ovary to cervix.
VT = Virus titres ($log_{10}TCID_{50}/g$). IIF = Indirect immunofluorescence staining (number of PCV2-positive cells / 10 mm^2); NA = not available

3.2. Long-term outcome

PCV2 strain K2 did not induce pathology in porcine foetuses (inoculated at 55 days of gestation and collected at 21 dpi), whereas strain K39 did. This brought us to the hypothesis that *in vivo* infection of foetuses with the low virulent strain K2 before the development of immuno-competence could give a status of immunotolerance at birth.

3.2.1. *Evaluation of the sows*

All four sows remained clinically healthy during the study period between the first and second laparotomy. The wounds after the first laparotomy were slightly swollen until 2 days after surgery and somewhat painful on palpation. No rise of rectal temperature was noticed during the first week after surgery.

PCV2-specific IPMA antibody titre was 40960 in pre-serum and post-serum of the four sows. PRRSV-specific IPMA antibody titre and PPV-specific HI antibody titre were <10 and ≤8, respectively in pre-serum and were the same in post-serum.

3.2.2. *Evaluation of piglets at birth*

Since K2 did not induce pathology and did not raise an immune response in mid-gestational foetuses, six foetuses from two sows were inoculated at 45 days of gestation with the less virulent PCV2 strain K2 ($10^{4.3}$ $TCID_{50}$ /foetus), which resulted in the birth of five mummies and one live-born piglet (Table 2). Six foetuses from the other two sows were inoculated with K2 but with a lower dose ($10^{2.3}$ $TCID_{50}$ /foetus), which also resulted in five mummies and one live-born piglet. Twelve mock-inoculated foetuses from four sows were born alive (Table 2). The live-born *in utero* K2-inoculated and mock-inoculated piglets appeared clinically normal at birth.

3.2.3. *Virus titration in mummies*

PCV2 virus was recovered from all the mummies and the titres varied from $10^{3.7\text{-}5.7}$ $TCID_{50}$ /g tissue.

3.2.4. *PCV2-PCR of serum samples collected at birth*

In order to determine the PCV2-status in pigs at birth, the *capsid gene* was amplified in serum samples collected from their umbilical cord. The live-born piglets including two live-born K2-inoculated piglets were negative in the PCV2-PCR at birth.

3.2.5. *Serology*

Live-born K2-inoculated and mock-inoculated piglets were negative (<10) for PCV2-specific IPMA Abs at birth (Table 4).

3.3. Super-inoculation of *in utero* K2- or mock-inoculated pigs with PCV2 (K39)

Since *in utero* inoculation of K2 resulted in the birth of living piglets, the outcome of a super-inoculation with a highly virulent PCV2 strain K39 was investigated.

3.3.1. *Evaluation of piglets*

All piglets (two K2-K39, six mock-K39 and six mock-mock inoculated) were clinically healthy during the entire study period. No disease was observed.

3.3.2. *PCV2 replication*

High dose K2 *in utero* - PCV2 titres in the inguinal lymph nodes (ILNs) (21 and 28 dpsi) varied from 2.3 to 3.3 in mock-K39 inoculated piglets (S4F4, S4F5 and S4F6). The piglet (S4F1) that was inoculated *in utero* with the less virulent K2 (4.3) and postnatally with a highly virulent K39 (4.3) had a low level of PCV2 replication (up to 2.3) in the ILNs collected at 21 and 28 dpsi. No PCV2 was detected at 14 dpsi (Table 4). Mock-mock inoculated piglets were negative (<1.7) in virus titration (Table 4). PCV2-positive cells could not be detected in the ILNs (collected at 14, 21 and 28 dpsi) of mock-K39, K2-K39 and mock-mock inoculated piglets, except two PCV2-positive cells/10 mm^2 were found in the ILNs of the K2-K39 inoculated piglet (S4F1) collected at 14 dpsi (Table 4). Double immunofluorescence staining showed that these two PCV2-positive cells were K39+K2-.

Low dose K2 *in utero* - In mock-K39 inoculated piglets (S6F4, S6F5 and S6F6), no PCV2 was detected in ILNs collected at 14 dpsi. PCV2 titres in inguinal lymph nodes at 21 and 28 dpsi varied from 2.0 to 3.0 and. The piglet (S6F1) that received a lower dose of K2 (2.3) at 45 days of gestation, K39 inoculation (4.3) after birth produced a high level of viral titres in the ILNs collected at 14 dpsi (5.6) and 21 dpsi (5.1) and a significant reduction of viral titres were observed at 28 dpsi (1.9) (Table 4). The mock-mock inoculated piglets were negative (<1.7) in virus titration (Table 4). PCV2-positive cells were not seen in different organs of mock-K39 and mock-mock inoculated piglets, except only two positive cells/10 mm^2 were seen in ILN (21 dpsi) of a mock-K39 inoculated piglet (S6F4) (Table 4). In the K2-K39 inoculated piglet (S6F1), low to moderate numbers of PCV2-positive cells/10 mm^2 were

found in ILNs (8 to 86 positive cells/10 mm^2) (Table 4). In order to differentiate strain K2 from strain K39 in the ILNs of the K2-K39 inoculated piglet, a double immunofluorescence staining was performed and only strain K2 was detected. In addition, no K39 replication was observed in this piglet.

3.3.3. *Serology*

Super-inoculation of piglets with K39 during their postnatal life resulted in a seroconversion against PCV2 at 21 dpsi, except pig no. S6F1 (seroconverted already at 0 dpsi) and pig no. S6F5 (seroconverted at 14 dpsi), and the highest IPMA Ab titres were observed at 28 dpsi in all PCV2-inoculated piglets (Table 4). In all mock-K39 piglets, PCV2-specific IPMA Ab titres against K39 were 4 to 64 folds higher than that of K2 at 21 and 28 dpsi. In K2-K39 piglet (S4F1), the Abs titres against K39 were also 4 to 16 folds higher than that of K2 at 21 and 28 dspi. Another K2-K39 piglet (S6F1) produced different results. This piglet developed PCV2-specific Abs (IPMA titres of 640) against K2 and K39 already at the day of K39 inoculation (0 dpsi) and the Ab titres remained the same until 14 dpsi. At 21 and 28 dpsi, Ab titres increased and the anti-K2 Ab titres were 4 folds higher than the anti-K39 titres (Table 4).

Table 4. PCV2 replication and IPMA antibody titres in pigs that were inoculated with the less virulent PCV2 strain K2 (and mock-inoculated) during prenatal life (at 45 days of gestation) followed by oronasal inoculation with a highly virulent PCV2 strain K39 (or mock-inoculated) during postnatal life.

	Prenatal life		Postnatal life																	
	Intra-uterine inoculation	Inoculated dose of K2	Oronasal inoculation at 6 or 8 days after birth	PCV2 replication						IPMA antibody titres											
				14 dpsi		21 dpsi		28 dpsi		at birth		0 dpsi		7 dpsi		14 dpsi		21 dpsi		28 dpsi	
				ILN		ILN		ILN													
Pig no.	at 45 days of gestation			VT	IIF	VT	IIF	VT	IIF	K2	K39	K2	K39	K2	K39	K2	K39	K2	K39	K2	K39
S4F1	K2	$10^{4.3}$	K39	<1.7	2	2.3	0	2.3	0	<10	<10	<10	<10	<10	<10	<10	<10	10	160	160	640
S4F4	mock		K39	<1.7	0	<1.7	0	3.3	0	<10	<10	<10	<10	<10	<10	<10	<10	160	640	160	10240
S4F5	mock		K39	<1.7	0	2.3	0	2.3	0	<10	<10	<10	<10	<10	<10	<10	<10	640	2560	640	10240
S4F6	mock		K39	<1.7	0	2.3	0	2.3	0	<10	<10	<10	<10	<10	<10	<10	<10	160	2560	640	10240
S5F4	mock		mock	<1.7	0	<1.7	0	<1.7	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
S5F5	mock		mock	<1.7	0	<1.7	0	<1.7	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
S5F6	mock		mock	<1.7	0	<1.7	0	<1.7	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
S6F1	K2	$10^{2.3}$	K39	5.6	76	5.1	86	1.9	8	<10	<10	640	640	640	640	640	640	10240	2560	40960	10240
S6F4	mock		K39	<1.7	0	3.0	2	2.3	0	<10	<10	<10	<10	<10	<10	<10	<10	160	2560	640	10240
S6F5	mock		K39	<1.7	0	2.0	0	<1.7	0	<10	<10	<10	<10	<10	<10	40	40	640	2560	2560	40960
S6F6	mock		K39	<1.7	0	2.7	0	<1.7	0	<10	<10	<10	<10	<10	<10	<10	<10	160	2560	2560	10240
S7F4	mock		mock	<1.7	0	<1.7	0	<1.7	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
S7F5	mock		mock	<1.7	0	<1.7	0	<1.7	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
S7F6	mock		mock	<1.7	0	<1.7	0	<1.7	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

IPMA = Immuno-peroxidase monolayer assay. PCV2-specific antibody titres in pig serum during postnatal life were determined by an IPMA; dpsi = days post super-inoculation; ILN = inguinal lymph node; VT = Virus titres ($log_{10}TCID_{50}/g$); IIF = Indirect immunofluorescence staining (number of PCV2-positive cells / 10 mm^2)

Pigs that were immunostimulated by vaccination against parvovirus at day 2 after birth have been indicated in bold

4. Discussion

In the first part of this study, the foeto-pathogenicity of the two PCV2 viruses was examined in porcine foetuses by intra-foetal inoculation of PCV2 at 55 days of their foetal life. At 21 dpi (76 days of gestation), although viral titres in different organs of K2-inoculated foetuses (except heart) were higher than that of K39-inoculated foetuses, strain K2 did not produce pathology in foetuses. On the other hand, K39 induced pathological lesions in different foetal organs, which confirms the earlier descriptions with the other PCV2a and PCV2b infections in porcine foetuses causing distended abdomens, ascites, hydrothorax, haemorrhages and congestion in internal organs, which are all suggestive of heart failure (Madson et al., 2009a; Saha et al., 2010). It clearly indicates that K2 induces less lesions in mid-gestational porcine foetuses than K39 despite the fact that K2 titres were higher than K39 titres. The number of PCV2-positive cells in different organs of K2- and K39-inoculated foetuses reflect the K2 and K39 titres in the corresponding foetal organs since the number of K2-positive cells in the foetal organs of K2-inoculated foetuses were considerably higher than that of K39-inoculated foetuses; however, the heart tissue was a clear exception. Despite the presence of similar PCV2 titres in hearts of K2- and K39-inoculated foetuses, the number K2-positive cells in the hearts of K2-inoculated foetuses were 4.9 times lower than those of K39-inoculated foetuses. It could be due to the small focal distribution of K2 infection in the heart while K39 infection was dispersed throughout the heart tissue.

Since K2 did not induce pathology in mid-gestational foetuses for a short-term period, we hypothesized that intra-foetal inoculation of foetuses at the early stages of gestation with the less virulent K2 strain could give a status of immunotolerance at birth and postnatal super-inoculation of immunotolerant pigs with a highly virulent strain K39 could cause disease. In this study, intra-foetal inoculation of a high or low dose of less virulent K2 at 45 days of gestation resulted in the birth of five mummies and one live-born piglet in both cases. The K2 and K2-specific antibodies were not detected in the *in utero* K2-inoculated live-born piglets at birth. It indicates that young foetuses had fully controlled the K2 infection upon intra-foetal inoculation of K2 in these two foetuses, most probably by the raise of an innate immune response. Wrong inoculations can be excluded because in the past we were 100% successful in performing trans-uterine, intra-foetal inoculation of either PCV1 or PCV2 in 54 foetuses from 23 sows (Sanchez et al., 2001; Sanchez et al., 2003; Pensaert et al., 2004; Sanchez et al., 2004; Saha et al., 2010; Saha et al., 2011b) following the same procedure as

described above. The accuracy of intra-foetal inoculation of K2 is further confirmed by the recovery of viruses from the mummies.

Super-inoculation of *in utero* mock-inoculated piglets with a highly virulent K39 strain did not result in disease since a very low level of K39 replication was observed in these piglets. It clearly shows that PCV2 strain K39 has different replication kinetics from foetal life to early postnatal life. Super-inoculation of *in utero* K2-inoculated live-born piglet (S4F1) with K39 did not result in disease either, since a low level of viral replication was observed. High PCV2 replication is the prerequisite for the successful development of disease or PMWS (Meerts et al., 2006; Fort et al., 2007). The inefficient viral replication corresponds with the late onset of humoral immune response. Serology (IPMA) indicates that the viral replication in this piglet was associated with K39, not K2 since anti-K39 Ab titres were four to 16 folds higher than the anti-K2 Ab titres. Immunofluorescence staining confirmed that the viral replication in this *in utero* K2-inoculated piglet was associated with K39, not K2 and this was further established by sequencing (data not shown). These results obviously indicate that K2 infection was fully controlled by the young foetus without priming the immune response. On the other hand, piglet (S6F1) that was *in utero* inoculated with a low dose of K2 developed a PCV2-specific immune response early after birth (IPMA titer 640 at 8 days after birth or at 0 dpsi of K39), which is suggestive for a re-activation of K2. This might be associated with the immunostimulation at 2 days after birth. Super-inoculation of this piglet with K39 did not cause any disease, despite an extremely high level of viral replication that was noticed in the inguinal lymph nodes ($10^{5.6}$ $TCID_{50}$/g at 14 dpsi and $10^{5.1}$ $TCID_{50}$/g at 21 dpsi). Serological analysis (IPMA) suggests that the viral replication in this piglet was associated with K2, not K39 as anti-K2 IPMA Ab titres were four folds higher than anti-K39 Ab titres. The K2 replication was further confirmed by immunofluorescence staining as well as by sequencing (data not shown). It suggests that K2 infection was silent during foetal life but broke silence after birth upon vaccination against parvovirus. It could also be possible that K39 replication was present but at a very low level in the earlier period before the sampling of the lymph nodes (14 dpsi). Another possibility could be the presence of neutralising Abs against K39 already at 0 or 7 dpsi, which might inhibit the replication of K39. A cross seroneutralisation experiment using PCV2 strains K2 and K39 may solve this issue.

This study demonstrated that PCV2 K2 was not pathogenic to porcine foetuses for a short-term period (21 dpi), whereas K39 was; however, long-term infection with K2 (69 dpi)

resulted in the birth of mummies or viable piglets. Super-inoculation of the live-born K2-infected piglets with a highly virulent K39 did not result in disease.

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Chapter 5. General discussion

Porcine circoviruses (PCV1 and PCV2) are ubiquitious around the world. PCV1 is generally considered as being not pathogenic (Tisher et al., 1986; Tischer et al., 1995; Allan et al., 2000; Fenaux et al., 2003; Finsterbusch and Mankertz, 2009; Beach et al., 2010). However, PCV1 has been isolated from cases of reproductive failure and congenital tremor in newborn pigs (Hines and Lukert, 1994; Allan et al., 1995; Stevenson et al., 2001; Choi et al., 2002). PCV2 is associated with Postweaning Multisystemic Wasting Syndrome (PMWS) (Segalés and Domingo, 2002) and can cause reproductive failure (West et al., 1999). The pathogenesis of PMWS is not fully understood. In experimentally and naturally PMWS-affected pigs, PCV2-neutralising antibodies could not be found or were present at low titres (Meerts et al., 2005; Meerts et al., 2006; Fort et al., 2007). It indicates that neutralising antibodies play a crucial role in the pathogenesis of PMWS. It can be hypothesized that absence or presence of low levels of PCV2-neutralising antibodies in PMWS-affected pig could be due to a certain immunotolerance upon an intra-uterine infection with a less virulent PCV2 strain. The studies included in the present thesis were to obtain a better understanding of antibody-PCV2 interaction with emphasis on neutralising antibodies and to determine foeto-virulence/pathogenicity for different PCV1/PCV2 strains.

The circular PCV2 genome has two major open reading frames (ORFs). ORF1 codes for the non-structural replicase proteins, Rep and Rep' (Mankertz and Hillenbrand, 2001; Cheung, 2003; Mankertz et al., 2003) and ORF2 codes for the structural capsid protein (Nawagitgul et al., 2000). The capsid protein consists of 233-236 amino acids (Nawagitgul et al., 2000; Lefebvre et al., 2008; Huang et al., 2011). Since the capsid is the most variable protein, a link between the capsid protein variation and the pathogenicity of PCV2 has been proposed (Larochelle et al., 2002). Due to the presence of considerable genetic diversity among the different PCV2 strains (Larochelle et al., 2002), PCV2 strains have been divided into two major genotypes (PCV2a and PCV2b) and eight genetic clusters/subtypes (PCV2a-2A to 2E and PCV2b-1A to 1C) (Olvera et al., 2007; Grau-Roma et al., 2008; Segalés et al., 2008). In the past, it was thought that only genetic and no antigenic differences exist among the different PCV2 isolates since monoclonal antibodies (mAbs) directed against the PCV2 capsid protein reacted in a similar pattern with all of the PCV2 strains originating from different geographic regions (Allan et al., 1999; McNeilly et al., 2001). Afterwards, antigenic diversity among the PCV2 isolates enclosing some but not all genetic clusters was demonstrated using mAbs generated against the PCV2 capsid protein (Lefebvre et al., 2008; Shang et al., 2009; Guo et al., 2011; Huang et al., 2011). In this thesis, antigenic subtyping

was performed enclosing PCV2 strains of the eight genetic clusters using a large panel of mouse mAbs generated against PCV2a or PCV2b. Four mAbs were identified that reacted to all PCV2 clusters of both genotypes (PCV2a and PCV2b) and they were defined as universal mAbs. It means that PCV2 strains of both genotypes, PCV2a and PCV2b, have (a) common universal epitope(s) in the capsid protein that were recognised by the four universal mAbs. The existence of common universal epitope(s) in the capsid protein could explain why the immunity induced by PCV2a infection largely cross-protects pigs upon a subsequent infection with PCV2b and vice-versa and why currently available PCV2a-based vaccines can protect pigs against subsequent challenges with PCV2b strains and vice-versa (Fort et al., 2008; Fort et al., 2009; Beach et al., 2011; Beach and Meng, 2012). Besides the presence of common universal epitopes, the existence of genotype-specific epitope(s) was also suggested since four mAbs raised against PCV2a recognised only PCV2a strains and two mAbs raised against PCV2b recognised only PCV2b strains. The existence of genotype-specific epitope(s) may explain why a somewhat better immunity and protection can be obtained upon a homologous challenge than upon a heterologous challenge. When PCV2b-vaccinated animals were challenged with a PCV2a strain, then they had higher viral DNA loads at 21 days post-challenge than when they were challenged with a PCV2b strain (Beach et al., 2011). When PCV2a-vaccinated animals were challenged with a PCV2b strain, then a higher percentage of animals were viral DNA positive in nasal and faecal swabs at 20 days post-challenge than when they were challenged with a PCV2a strain (Fort et al., 2008). Besides universal or genotype-specific mAbs, several mAbs were identified as cluster-specific recognising only a certain PCV2 cluster(s) of both genotypes or within a genotype. The differential reactivity of the mAbs with the different PCV2 clusters could be associated with few amino acid differences of the capsid protein at positions 30, 59, 63, 89, 130, 133, 206 and 210. More research is needed for further confirmation of these findings. Site-directed mutagenesis by targeting above-suggested aa positions in the PCV2 capsid protein, followed by checking the reactivity of the resulting mutant viruses with mAbs would confirm the mAbs' recognition site(s). Identification of the mAbs' binding site(s) in the capsid might form a basis towards the development of a more broadly active PCV2 vaccine. It would also be necessary to investigate if such antigenic diversity associated with certain domains on the capsomeres could reflect any pathogenetic diversity *in vivo* in pigs.

PMWS-affected pigs have significantly lower levels of PCV2-neutralising antibodies when compared to sub-clinically infected animals (Meerts et al., 2006; Fort et al., 2007), suggesting

a crucial role for neutralising antibodies in the prevention of PMWS. Therefore, further characterisation of neutralising antibodies could shed new light on this important issue. Previously, it was demonstrated that several neutralising mAbs directed against the PCV2 capsid protein were able to recognise different PCV2 isolates of both PCV2a and PCV2b in immuno-peroxidase monolayer assay (IPMA+); however, some of them were neutralised (N+) and some isolates were not neutralised (N-) by mAbs in a sensitive neutralisation assay irrespective of their genotype (Lefebvre et al., 2008). Accordingly, in this thesis, two distinct PCV2 neutralisation phenotypes were described: phenotype α (mAb recognition with neutralisation; IPMA+N+) and phenotype β (mAb recognition without neutralisation; IPMA+N-). Several authors have described monoclonal antibodies (mAbs) with neutralising activity to the PCV2 capsid protein (McNeilly et al., 2001; Lekcharoensuk et al., 2004; Zhou et al., 2005; Lefebvre et al., 2008; Shang et al., 2009). Alignment of the capsid proteins (Lefebvre et al., 2008) has shown a very limited number of aa differences between the phenotype α and β strains. Therefore, it was hypothesized that these aa residues were responsible in determining the PCV2 neutralisation phenotype and they were selected for site-directed mutagenesis in order to perform a phenotypic switch. The aa at positions 131, 151 and 190 of the PCV2 capsid were identified as critical for the reaction with different neutralising mAbs. In addition, aa at position 191 was identified as a 'helper' in determining the PCV2 neutralisation phenotype since it induces an additive effect on the neutralisation when aa at position 191 was mutated together with the aa at position 131. However, it still remains to be determined why the phenotype α strains were recognised as well as neutralised by the mAbs and phenotype β strains were only recognised but not neutralised. Glaser et al. (1995) also pointed to the existence of two neutralisation phenotypes in case of equine arteritis virus (EAV). MAbs recognised all field isolates of EAV and all of the isolates were neutralised by mAbs except one isolate DL11, which remained non-neutralised. Absence of neutralising activity of mAbs to phenotype β strains might be related with the low binding strength or avidity of the mAbs. The changes of aa at positions 131, 151, 190 and 191 in phenotype β strains might lead to a modification of the viral capsid conformation, which could be responsible for the lower affinity of mAbs. Further research is needed to solve these issues. In this thesis, several aa at positions 30, 59, 63, 89, 130, 131, 133, 151, 190, 191, 206 and 210 were suggested or identified as critical as a part of the binding site(s) of different mAbs. Several linear epitopes or immunoreactive regions identified in the PCV2 capsid protein using mouse mAbs or porcine polyclonal antibodies (pAbs) and they are located

within aa residues 25-43, 69-83, 113-127, 117-131, 169-183 and 193-207 (Mahé et al., 2000; Truong et al., 2001); 156-162, 195-202 and 231-233 (Shang et al., 2009); 47-85, 165-200 and 230-233 (Lekcharoensuk et al., 2004). Amino acid sequence alignment of the PCV2 capsid proteins revealed three major regions of amino acid heterogeneity at aa residues 57-91, 121-136 and 180-191 and three constant substitutions at positions 190, 210 and 232 (Larochelle et al., 2002; de Boisséson et al., 2004; Grau-Roma et al., 2008). The aa residues that have been suggested or identified as a part of the binding site(s) of different mAbs in this thesis are located in the inner and outer border of the above mentioned regions. In addition, according to Lekcharoensuk et al. (2004) and Khayat et al. (2011), these aa positions (except positions 30 and 151) are located on the exterior surface of the PCV2 capsid and react with antibodies (as shown in Fig. 1). On the other hand, the aa positions 30 and 151 are located on the interior surface of the PCV2 capsid protein (Lekcharoensuk et al., 2004; Khayat et al., 2011). There are two possibilities where the interior surface of the capsid can be externalised and react with antibodies. The first possibility is the breathing phenomenon as described for other viruses, such as flock house virus (Bothner et al., 1998), human rhinovirus 14 (Lewis et al., 1998) and poliovirus (Li et al., 1994). Exposition of internal polypeptides was shown to be essential for the infectivity of these viruses. In addition, it was shown that poliovirus was only neutralised when the internal peptides of this virus were externally exposed (Li et al., 1994). The second possibility is the modification of the viral capsid conformation due to single aa mutation.

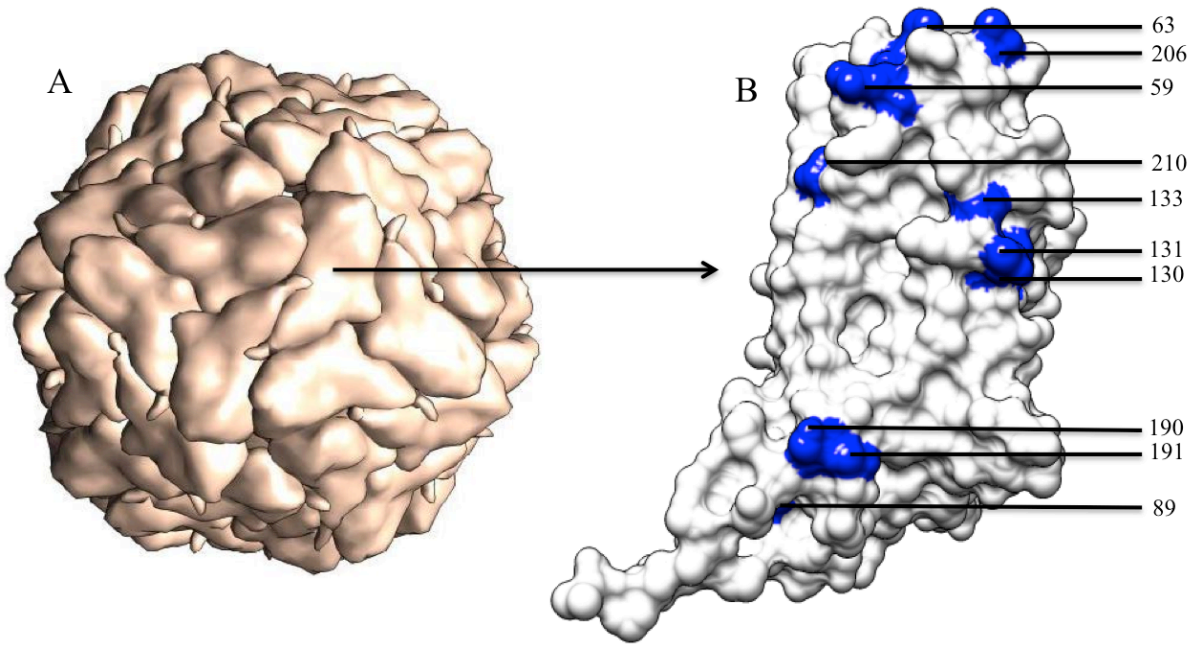

Fig. 1. Location of different aa positions on the PCV2 capsid protein. A homology model of the PCV2 (Stoon-1010) capsid protein was generated using the PCV2 crystal structure (PDB accession no. 3R0R) (Khayat et al., 2011) as a template with SWISS-MODEL Workspace (Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006). Figures were made using University of California, San Francisco Chimera 1.5.3 (Pei and Grishin, 2001). (A) An assumed biological PCV2 molecule (biological assembly). (B) Surface diagram of the PCV2 (Stoon-1010) capsid protein. The aa positions 59, 63, 89, 130, 131, 133, 190, 191, 206 and 210 are located on the exterior surface of a PCV2 capsid protein (in blue).

PCV1 has been isolated from aborted/stillborn piglets and from cases of congenital tremors in newborn piglets (Allan et al., 1995; Stevenson et al., 2001; Choi et al., 2002), suggesting the occurrence of a vertical transmission and possible clinical outcome of an infection. PCV2 is associated with PMWS (Segalés and Domingo, 2002) and has been isolated from cases of reproductive failure in pigs (West et al., 1999; Meehan et al., 2001; O'Connor et al., 2001; Farnham et al., 2003; Kim et al., 2004; Mikami et al., 2005; Brunborg et al., 2007; Pittman, 2008). In the second part of this thesis, the outcomes of experimental PCV1, PCV2a or PCV2b infections in immuno-incompetent porcine foetuses were described. In addition, it was examined if a less virulent PCV2 strain can induce immunotolerance in porcine foetuses and subsequently PMWS upon super-infection with a second, more virulent PCV2 strain. In order to determine the clinical outcomes of PCV1 infections during gestation, immuno-incompetent porcine foetuses were inoculated with PCV1 strains at 55 days of gestation. At 21 days post inoculation (21 dpi), cell culture PCV1 strain (Tischer et al., 1974; Tischer et al., 1982) produced severe lung haemorrhages without inducing any gross pathology. The lung haemorrhages correlated well with the presence of high amounts of PCV1 virus (up to $10^{4.7}$

$TCID_{50}$/g) since the lungs with the low level of PCV1 virus replication (up to $10^{2.9}$ $TCID_{50}$/g) did not have histopathological changes. This is the unique finding in the field of PCV research since several other experimental studies failed to reproduce any disease in pigs after PCV1 infections (Tischer et al., 1986; Allan et al., 1995; Fenaux et al., 2003). This study also showed that the lungs were the main target of PCV1 replication and PCV1 antigens were mainly localised in epithelial cells of the lungs of PCV1-inoculated foetuses. This is in contradiction with the previous observations in newborn pigs (Allan et al., 1995; Stevenson et al., 2001) in which it was shown that PCV1 antigens are mainly localised in non-epithelial cells, morphologically resembling macrophages (Allan et al., 1995). It could be possible that the target cells for PCV1 in foetal life might be different from the target cells for PCV1 in newborn pigs, as previously shown for PCV2 by Sanchez et al. (2003). This study showed that PCV1 replicated efficiently with producing lesions in the lungs of porcine foetuses inoculated at 55 days of foetal life; however, more research is needed in order to get a full picture on the pathogenic character of PCV1 in porcine foetuses. The pathogenicity of PCV1 in porcine foetuses can be examined if intra-foetal inoculation of PCV1 would be performed at different stages of gestation such as 55, 75 or 92 days and if the foetuses are kept until birth (114 days of gestation) after PCV1 inoculation. It is not known whether intra-foetal inoculation of PCV1 at very early stages of gestation could give a status of immunotolerance at birth. It is also not known what would be the outcome if PCV1 infection occurs at the time of conception.

Both PCV2a (Meehan et al., 2001; Farnham et al., 2003) and PCV2b (Pittman, 2008; Hansen et al., 2010) have been isolated from naturally occurring reproductive failure and thus, apparently no association with a specific genotype is found (Hansen et al., 2010). Recent field observations on the occurrence of PCVAD have indicated that some currently circulating PCV2b strains may be more virulent than currently circulating PCV2a strains, because PCV2b is more frequently isolated from the cases of reproductive failure or PMWS than PCV2a (Carman et al., 2008; Dupont et al., 2008; Grau-Roma et al., 2008; Pittman, 2008; Hansen et al., 2010). In addition, a higher prevalence of PCV2b than PCV2a has been reported in pre-suckling piglets (Shen et al., 2010). In order to determine the clinical outcomes of PCV2 infections in porcine foetuses, an infection experiment was performed in 55-day-old porcine foetuses. PCV2 strains of both genotypes, with special preference to PCV2b (PCV2a strains: Stoon-1010 and 1121; PCV2b strains: 48285, 1147, VC2002-K2 and VC2002-K39), originating from different clinical presentation were used (Meehan et al.,

1998; Meehan et al., 2001; Lefebvre et al., 2008). At 21 dpi, it was observed that different PCV2a and PCV2b strains induced similar gross pathological lesions and replicated to similar high titres in different foetal organs without apparent differences between the two genotypes. Previously, Madson et al. (2008) and Opriessnig et al. (2008) were not able to demonstrate differences in pathogenicity between PCV2a and PCV2b in boars or growing pigs. However, PCV2b strain VC2002-K2 was a clear exception. VC2002-K2 did not induce any pathology for a short-term period (21 dpi). This is the first experimental study that describes and compares the pathogenic character of different strains of both PCV2 genotypes in porcine foetuses, since previous studies predominantly used PCV2a strains (Sanchez et al., 2001; Johnson et al., 2002; Sanchez et al., 2003; Pensaert et al., 2004; Yoon et al., 2004) or only used a PCV2b strain (Madson et al., 2009).

PCV2 strain VC2002 was originally isolated from a Belgian PMWS-affected pig (Meerts et al., 2004) and it has been demonstrated that VC2002 is a mixture of two different PCV2b viruses: VC2002-K2 and VC2002-K39 (Lefebvre et al., 2008). Since K2 did not induce any pathology in porcine foetuses for a short-term period (21 dpi), whereas K39 did, it was hypothesized that intra-foetal inoculation of foetuses at the early stages of gestation with this less virulent K2 could give a status of immunotolerance at birth and super-infection of immunotolerant pigs with the highly virulent K39 could cause disease. Previously, it has been demonstrated that the *in utero* PCV2 infection in porcine foetuses at late gestation could cause birth of live-born PCV2-infected piglets and then, these piglets are highly susceptible to the development of disease upon co-infections with other pathogens or immunostimulation (Jung et al., 2006; Ha et al., 2008). In this thesis, it was shown that intra-foetal inoculation of a high ($10^{4.3}$ $TCID_{50}$) or low dose ($10^{2.3}$ $TCID_{50}$) of less virulent K2 in porcine foetuses at 45 days of gestation resulted in the birth of five mummies and only one live-born piglet in both cases. The K2 and K2-specific antibodies were not detected in the *in utero* K2-inoculated live-born piglets at birth. It indicates that young foetuses may fully control the K2 infection, most probably by the raise of an innate immune response. Super-inoculation of these two viable piglets with a highly virulent K39 after birth did not cause any disease.

In the first part of this thesis, PCV2 strains of the eight genetic clusters were antigenically subtyped using a large panel of mouse mAbs and several aa positions in the PCV2 capsid were suggested to be critical as a part of the binding site(s) of the different mAbs. In this thesis, few amino acids in the capsid were also identified that determine the PCV2

neutralisation phenotype. In the second part of the thesis, it was shown that PCV1 could replicate efficiently and produce pathology in the lungs of immuno-incompetent porcine foetuses inoculated at 55 days of foetal life. PCV2 strains from both PCV2a and PCV2b genotypes were all found equally pathogenic in mid-gestational porcine foetuses, with the exception of one PCV2b strain, which was not pathogenic in porcine foetuses for a short-term infection but long-term infection resulted in the birth of mummies or viable piglets. Super-infection of a PCV2-infected live-born piglet with a highly virulent PCV2 strain did not result in disease.

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Summary / Samenvatting

Porcine circoviruses (PCV1 and PCV2) are ubiquitious around the world. Although the genetic diversity among the PCV2 strains has been extensively studied, the antigenic diversity has been much less explored. PCV1 has been isolated from cases of reproductive failure and congenital tremor in newborn pigs. PCV2 is associated with Postweaning Multisystemic Wasting Syndrome (PMWS) and can cause reproductive failure. Despite the isolation of PCV1 and PCV2 from aborted/stillborn piglets and from cases of congenital tremors in newborn piglets, nothing is known about the outcome of experimental PCV1 infections in porcine foetuses and the outcome of experimental PCV2 (PCV2a or PCV2b) infections in porcine foetuses has only been examined with a restricted number of strains. The pathogenesis of PMWS is not fully understood yet and further research is needed. The absence or presence of low levels of neutralising antibodies in PMWS pigs has been reported and it may be hypothesized that this could be due to a certain immunotolerance upon an intra-uterine infection with a less virulent PCV2 strain.

In Chapter 1, an introduction was given on the history, taxonomy and genome organisation of PCVs, the phylogeny and antigenic features of PCV2 and on the current knowledge on the pathogenesis of PCVs and PCV2-associated diseases (PCVAD).

In Chapter 2, the aims of this thesis were given. The general aim of this thesis was to achieve a better understanding of antibody-PCV2 interaction with emphasis on neutralising antibodies and to determine foeto-virulence/pathogenicity for different PCV1/PCV2 strains. The aims were divided into two major parts: (1) to antigenically characterise the PCV2 strains based on the reactivity and neutralising activity of monoclonal antibodies (mAbs) and (2) to examine the outcomes of experimental intra-foetal inoculations with PCV1, PCV2a or PCV2b and to evaluate the outcomes of experimental prenatal infection with a less virulent PCV2 strain, followed by postnatal super-infection with a highly virulent PCV2 strain.

In Chapter 3, PCV2 strains were antigenically characterised using mouse mAbs. In Chapter 3.1, a study was carried out using mAbs (directed against the PCV2 capsid protein) generated against genotype PCV2a or PCV2b to antigenically subtype the PCV2 strains of the 8 different genetic clusters: PCV2b-1A to PCV2b-1C and PCV2a-2A to PCV2a-2E and to perform competition analysis of the different epitopes. Fourteen PCV2 strains representative for the eight different genetic clusters were enclosed to test their reactivity with 20 mAbs (fifteen of them were generated against PCV2a strain Stoon-1010 and 5 of them against PCV2b strain 1147) in immunoperoxidase monolayer assays (IPMA). Four mAbs 12E12,

21C12, 38C1 and 114C8 were defined as universal mAbs because they reacted with all 14 PCV2 strains. Few mAbs were identified as genotype-specific such as, mAbs 14G2 and 22C1 were specific for genotype PCV2b and mAbs 31D5, 48B5, 59C6 and 108E8 were specific for genotype PCV2a. Several mAbs such as, 6E9, 9C3, 16G12, 43E10, 55B1, 63H3, 70A7, 94H8 and 103H7 were identified as cluster-specific. Based on the reactivity of mAbs to the capsid of different PCV2 strains, few aa positions were suggested as crucial for the binding site(s) of the different mAbs. In addition, a cross-competition analysis by a competitive ELISA was performed using PCV2 strain Stoon-1010 and two universal (38C1 and 114C8) and four genotype- or cluster-specific mAbs (31D5, 59C6, 94H8 and 108E8). MAbs recognised six overlapping epitopes on the PCV2 capsid protein based on their cross-competition data and their reactivity in IPMA with different PCV2 strains. The universal mAbs recognised larger epitopes than the genotype- or cluster-specific mAbs. In this study, it was shown that discrete antigenic differences exist between different PCV2 genetic clusters and that these clusters can be discriminated by the use of a large panel of mAbs. In Chapter 3.2, PCV2 strains were further characterised using neutralising mAbs 16G12, 38C1, 63H3 and 94H8. These mAbs are directed against the PCV2 capsid protein and recognised PCV2 strains Stoon-1010 (PCV2a), 48285 (PCV2b), 1121 (PCV2a), 1147 (PCV2b) and II9F (PCV2b) but only neutralised Stoon-1010 and 48285. Accordingly in the present study, two distinct PCV2 neutralisation phenotypes were defined: phenotype α (mAb recognition with neutralisation; Stoon-1010 and 48285) and phenotype β (mAb recognition without neutralisation; 1121, 1147 and II9F) and amino acids that are important in determining the PCV2 neutralisation phenotypes were identified in the capsid. A comparison of the capsid protein sequences of the PCV2 strains (48285 vs 1147/II9F; Stoon-1010 vs 1121) from the two phenotypes showed only few major aa differences at positions 131, 151, 190 and 191 and it was hypothesized that these aa positions could be important in determining the neutralising activity of mAbs. And they were selected for site-directed mutagenesis in order to perform phenotypic switch. Mutation of T at position 190 to A in 48285 (phenotype α) resulted in a capsid that resembles that of 1147 (phenotype β) and caused a loss of neutralisation (switch from α to β). Mutations of P at position 151 to T and A at position 190 to T in II9F (phenotype β) resulted in a capsid that resembles that of 48285 (phenotype α) and gave a gain of neutralisation (switch from β to α). Mutations of T at position 131 to P and E at position 191 to R in Stoon-1010 (phenotype α) changed the capsid into one that resembles to that of 1121 (phenotype β)

and reduced neutralisation (switch from α to β). From this study, it was concluded that single amino acid changes in the capsid result in a phenotypic switch from α to β or β to α.

In Chapter 4, experimental PCV1, PCV2a or PCV2b infections in mid-gestational porcine foetuses were examined and the outcomes of prenatal infection with a less virulent PCV2 strain, followed by postnatal super-infection with a highly virulent PCV2 strain were evaluated. In Chapter 4.1, it was examined if PCV1 strains could replicate and induce pathology in porcine foetuses. The clinical and virological outcomes of experimental PCV1 infections in 55-day-old immuno-incompetent porcine foetuses were studied. Nine foetuses from three sows were inoculated at 55 days of gestation: three with the PCV1 cell culture contaminant ATCC-CCL33, three with the PCV1 field strain 3384 isolated from aborted foetuses and three with cell culture medium (mock-inoculated). At 21 days post inoculation, all 6 PCV1-inoculated and all 3 mock-inoculated foetuses had a normal external appearance. Microscopic lesions characterised by severe haemorrhages were observed in the lungs of two foetuses inoculated with CCL33. High PCV1 titres (up to $10^{4.7}$ $TCID_{50}$/g tissue) were found in the lungs of the CCL33-inoculated foetuses. All other organs of the CCL33-inoculated foetuses and all the organs of the 3384-inoculated foetuses were negative ($<10^{1.7}$ $TCID_{50}$/g tissue) by virus titration. PCV1-positive cells (up to 121 cells/10 mm^2 in CCL33-inoculated foetuses and up to 13 cells/10 mm^2 in 3384-inoculated foetuses) were found in the heart, lungs, spleen, liver, thymus and tonsils. It was concluded that cell culture PCV1 can replicate efficiently and produce pathology in the lungs of porcine foetuses inoculated at 55 days of foetal life. In Chapter 4.2, the clinical and virological outcomes of surgical inoculation of 55-day-old immuno-incompetent porcine foetuses with PCV2a or PCV2b were examined. Twelve foetuses were inoculated with PCV2: three with the postweaning multisystemic wasting syndrome (PMWS) associated PCV2a strain Stoon-1010, three with the reproductive failure associated PCV2a strain 1121, three with the PMWS associated PCV2b strain 48285 and three with the porcine dermatitis and nephropathy syndrome associated PCV2b strain 1147. Four foetuses were mock-inoculated with the cell culture medium. At 21 days post inoculation, eleven out of twelve PCV2-inoculated foetuses were oedematous and had distended abdomens, whereas one foetus that was inoculated with a PCV2b strain 1147 had a normal external appearance. All PCV2-inoculated foetuses had haemorrhages and congestion in internal organs and an enlarged liver. High PCV2 titres ($>10^{4.5}$ $TCID_{50}$/g tissue) were found in all PCV2-inoculated foetuses, especially in the heart, spleen and liver. High numbers of PCV2-infected cells (>1000 infected cells/10 mm^2 tissue) were observed in the

hearts. Two 1121- and one 1147-inoculated foetuses developed low PCV2-specific Ab titres of 10 to 40; the other PCV2-inoculated foetuses were negative for PCV2-specific antibodies. This study showed that PCV2a and PCV2b strains originating from different clinical presentations induce similar gross pathological lesions and replicate to similar high titres in different organs of 55-day-old immuno-incompetent porcine foetuses. In Chapter 4.3, it was examined if immuno-incompetent porcine foetuses of early stages of gestation inoculated with a less virulent PCV2 strain could give a status of immunotolerance at birth. And if postnatal super-infection of such immunotolerant pigs with a highly virulent PCV2 strain could cause disease and no recognition by the immune system. VC2002, isolated from a PMWS-affected pig, is a mixture of two different PCV2 viruses: K2 and K39 and a preliminary experiment showed that K2 had no adverse effects on foetuses in short-term infection (21 dpi), whereas K39 had. Firstly, the foeto-pathogenicity of both strains was tested and nine foetuses of three sows were inoculated at 55 days of gestation: three with K2, three with K39 and three with medium. At 21 dpi, K2 did not induce any pathology in porcine foetuses, whereas K39 did. Afterwards, the long-term effect was examined using only less virulent PCV2 strain K2 and 24 foetuses from four sows were inoculated at 45 days of gestation: six with a high dose of K2, six with a low dose of K2 and 12 with the cell culture medium. At 69 dpi (114 days of gestation), both high and low doses of K2 inoculation resulted in the birth of five mummies and one live-born piglet. All 12 mock-inoculated foetuses were born alive. PCV2 K2 was recovered from all of the mummies. The K2 and K2-specific antibodies were not detected in the two live-born K2-infected piglets at birth. It indicates that the young foetuses had fully controlled the K2 infection. Among 14 live-born piglets, six mock-inoculated and K2-low dose-infected piglets were immunostimulated by vaccination against parvovirus at day 2 and the rest (six mock-inoculated and K2-high dose-infected) were not immunostimulated. Six mock- (3 stimulated and 3 non-stimulated) and two K2-infected (stimulated and non-stimulated) piglets were super-inoculated with a highly virulent K39 at day 6 or 8 after birth (0 days post super-inoculation (dpsi)). Other six mock-inoculated (3 stimulated and 3 non-stimulated) piglets were super-inoculated with medium (mock-inoculated). A low viral replication (up to $10^{3.3}$ $TCID_{50}$ /g) was observed in all mock-K39 inoculated piglets at 21 and 28 dpsi. The non-stimulated K2-K39 piglet had also a low viral replication at 21 and 28 dpsi ($10^{2.3}$ $TCID_{50}$ /g; identified as K39), whereas the stimulated K2-K39 piglet had an exceptionally high viral replication at 14 and 21 dpsi (up to $10^{5.6}$ $TCID_{50}$/g; identified as K2). The former one showed a late immune response (at 21 dpsi of

K39) and the later one had already antibodies at 8 days of age (at the time of K39 inoculation), and this indicates that K2 infection was controlled during foetal life and emerged after birth upon immunostimulation. In the stimulated K2-K39 piglet, only K2, no K39 replication was observed in the inguinal lymph nodes collected at 14, 21 and 28 dpsi. It could be possible that K39 replication was present but at a very low level early before the sampling of the lymph nodes. None of the piglets had any signs of disease. It was concluded that, PCV2 strain K2 was not pathogenic to porcine foetuses for a short-term period (21 dpi), whereas K39 was; the long-term infection with the less virulent K2 (69 dpi) resulted in the birth of mummies or viable piglets. Postnatal super-infection of the mock- or live-born K2-infected piglets with a highly virulent PCV2 strain K39 did not result in disease.

From the first part of this thesis, it can be concluded that high antigenic diversity exists among the different PCV2 strains of the eight genetic clusters and the different PCV2 clusters can be antigenically subtyped using mAbs. In addition, two distinct PCV2 neutralisation phenotypes have been defined in this thesis based on the neutralising activity of mAbs to different PCV2 strains and the aa residue(s) that are important in determining the PCV2 neutralisation phenotypes have been identified in the PCV2 capsid protein. From the second part of this thesis, it can be stated that both PCV1 and PCV2 can be pathogenic in mid-gestational porcine foetuses. The inoculation of porcine foetuses with a less virulent PCV2 strain at the early stages of gestation may cause in the birth of viable piglets. However, the super-infection of such PCV2-infected live-born piglets with a highly virulent PCV2 strain did not cause any disease. Further research is necessary to investigate the pathogenic characters of PCV1 in porcine foetuses and to find out a less pathogenic PCV2 strain that could be used to infect foetuses at early stages of gestation in order to make PCV2-immunotolerant pigs.

Porciene circovirussen (PCV1 en PCV2) zijn wijdverspreid over de hele wereld. Hoewel de genetische diversiteit onder de PCV2 stammen uitgebreid bestudeerd is, is de antigene diversiteit veel minder onderzocht. PCV1 is geïsoleerd uit gevallen van voortplantingsproblemen en congenitale tremor bij pasgeboren varkens. PCV2 wordt geassocieerd met het Postweaning Multisystemic Wasting Syndrome (PMWS) of wegkwijnsyndroom en kan voortplantingsproblemen veroorzaken. Ondanks de isolatie van PCV1 en PCV2 uit geaborteerde/doodgeboren biggen en uit gevallen van congenitale tremor bij pasgeboren biggen, is niets bekend over de uitkomst van experimentele PCV1 infecties bij varkens foetussen en de uitkomst van experimentele PCV2 (PCV2a of PCV2b) infecties bij varkens foetussen is slechts onderzocht met een beperkt aantal stammen. De pathogenese van het wegkwijnsyndroom is nog niet volledig begrepen en verder onderzoek is nodig. De af- of aanwezigheid van lage hoeveelheden neutraliserende antilichamen bij PMWS varkens is gerapporteerd en er wordt verondersteld dat dit te wijten kan zijn aan een zekere immunotolerantie bij een intra-uteriene infectie met een laag virulente PCV2 stam.

In Hoofdstuk 1 werd een inleiding gegeven over de geschiedenis, taxonomie en genoom organisatie van PCVs, over de fylogenie en de antigene kenmerken van PCV2 en over de huidige kennis van de pathogenese van PCVs en PCV2-geassocieerde aandoeningen (PCVAD).

In Hoofdstuk 2 werden de doelstellingen van deze thesis uiteengezet. De voornaamste doelstelling van deze thesis was om tot een beter begrip te komen van antilichaam-PCV2 interactie met nadruk op neutraliserende antilichamen en om foeto-virulentie/pathogeniciteit te bepalen voor verschillende PCV1/PCV2 stammen. De doelstellingen werden verdeeld in twee grote delen: (1) om de PCV2 stammen antigenisch te karakteriseren op basis van de reactiviteit en neutraliserende activiteit van monoklonale antilichamen (mAbs) en (2) om de uitkomst van experimentele intra-foetale inoculaties met PCV1, PCV2a of PCV2b te onderzoeken en de uitkomst van experimentele prenatale infectie met een minder virulente PCV2 stam, gevolgd door een postnatale super-infectie met een zeer virulente PCV2 stam te beoordelen.

In Hoofdstuk 3 werden PCV2 stammen antigenisch gekarakteriseerd met behulp van muis mAbs. In Hoofdstuk 3.1 werd een onderzoek uitgevoerd met behulp van mAbs (gericht tegen het PCV2 capsied-eiwit) gegenereerd tegen genotype PCV2a of PCV2b om de PCV2 stammen van de 8 verschillende genetische clusters antigenisch te subtyperen: PCV2b-1A tot

PCV2b-1C en PCV2a-2A tot PCV2a-2E en om de competitie analyse van de verschillende epitopen uit te voeren. Veertien PCV2 stammen representatief voor de acht verschillende genetische clusters werden ingesloten om hun reactiviteit te testen met 20 mAbs (vijftien van hen werden gegenereerd tegen PCV2a stam Stoon-1010 en 5 van hen tegen PCV2b stam 1147) in immuno-peroxidase monolayer assays (IPMA). Vier mAbs 12E12, 21C12, 38C1 en 114C8 werden gedefinieerd als universele mAbs omdat ze reageerden met alle 14 PCV2 stammen. Weinig mAbs werden geïdentificeerd als genotype-specifiek zoals mAb 14G2 en 22C1 die specifiek waren voor genotype PCV2b en mAbs 31D5, 48B5, 59C6 en 108E8 die specifiek waren voor genotype PCV2a. Verschillende mAbs zoals 6E9, 9C3, 16G12, 43E10, 55B1, 63H3, 70A7, 94H8 en 103H7 werden geïdentificeerd als cluster-specifiek. Gebaseerd op de reactiviteit van de mAbs met het capsied van verschillende PCV2 stammen, werden slechts enkele aa posities beschouwd als cruciaal voor de bindingsplaats(en) van de verschillende mAbs. Daarnaast werd een kruis-competitie analyse met een competitieve ELISA uitgevoerd met behulp van PCV2 stam Stoon-1010 en twee universele (38C1 en 114C8) en vier genotype- of cluster-specifieke mAbs (31D5, 59C6, 94H8 en 108E8). MAbs herkenden zes overlappende epitopen op het PCV2 capsied-eiwit op basis van hun kruis-competitie gegevens en hun reactiviteit in IPMA met verschillende PCV2 stammen. De universele mAbs herkenden grotere epitopen dan de genotype- of cluster-specifieke mAbs. In deze studie werd aangetoond dat discrete antigene verschillen bestaan tussen verschillende PCV2 clusters en dat deze clusters onderscheiden kunnen worden door het gebruik van een grote groep van mAbs. In Hoofdstuk 3.2 werden PCV2 stammen verder gekarakteriseerd met behulp van neutraliserende mAbs 16G12, 38C1, 63H3 en 94H8. Deze mAbs zijn gericht tegen het PCV2 capsied-eiwit en ze herkenden PCV2 stammen Stoon-1010 (PCV2a), 48285 (PCV2b), 1121 (PCV2a), 1147 (PCV2b) en II9F (PCV2b), maar ze neutraliseerden enkel Stoon-1010 en 48285. Zo werden in deze studie twee verschillende PCV2 neutralisatie fenotypes gedefinieerd: fenotype α (mAb herkenning met neutralisatie; Stoon-1010 en 48285) en fenotype β (mAb herkenning zonder neutralisatie; 1121, 1147 en II9F) en aminozuren die belangrijk zijn bij het bepalen van de PCV2 neutralisatie fenotypes werden geïdentificeerd in het capsied. Een vergelijking van de capsied eiwitsequenties van de PCV2 stammen (48285 versus 1147/II9F; Stoon-1010 vs 1121) van de twee fenotypes toonde slechts enkele belangrijke aa verschillen op posities 131, 151, 190 en 191 en er wordt verondersteld dat deze aa posities van belang kunnen zijn bij het bepalen van de neutraliserende werking van mAbs. Deze aa verschillen werden geselecteerd voor site-

directed mutagenese om een fenotypische switch uit te voeren. Het muteren van T op positie 190 naar A bij 48285 (fenotype α) resulteerde in een capsied dat gelijkt op dat van 1147 (fenotype β) en veroorzaakte verlies van neutralisatie (omschakeling van α tot β). Mutaties van P op positie 151 naar T en van A op positie 190 naar T bij II9F (fenotype β) resulteerde in een capsied dat gelijkt op dat van 48285 (fenotype α) en veroorzaakte het bekomen van neutralisatie (omschakeling van β tot α). Mutaties van T op positie 131 naar P en van E op positie 191 naar R bij Stoon-1010 (fenotype α) leidde tot een capsied dat gelijkt op dat van 1121 (fenotype β) en daardoor verminderde de neutralisatie (omschakeling van α tot β). Uit dit onderzoek werd geconcludeerd dat afzonderlijke aminozuur-veranderingen in het capsied resulteren in een fenotypische omschakeling van α tot β of van β tot α.

In Hoofdstuk 4 werden experimentele PCV1, PCV2a of PCV2b infecties bij midden-dracht varkens foetussen onderzocht en de resultaten van prenatale infectie met een minder virulente PCV2 stam, gevolgd door een postnatale super-infectie met een zeer virulente PCV2 stam werden geëvalueerd. In Hoofdstuk 4.1 werd onderzocht of PCV1 stammen kunnen repliceren en pathologie kunnen induceren in varkens foetussen. De klinische en virologische gevolgen van experimentele PCV1 infecties bij 55-dagen-oude immuno-incompetente varkens foetussen werden bestudeerd. Negen foetussen van drie zeugen werden geïnoculeerd op 55 dagen dracht: drie met de PCV1 celcultuur contaminant ATCC-CCL33, drie met de PCV1 veldstam 3384 geïsoleerd uit geaborteerde foetussen en drie met celcultuur medium (mock-geïnoculeerd). Op 21 dagen na inoculatie hadden alle 6 PCV1-geïnoculeerde en alle 3 mock-geïnoculeerde foetussen een normaal uiterlijk. Microscopische letsels gekenmerkt door ernstige bloedingen werden waargenomen in de longen van twee foetussen geïnoculeerd met CCL33. Hoge PCV1 titers (tot $10^{4.7}$ $TCID_{50}$/g weefsel) werden gevonden in de longen van de CCL33-geïnoculeerde foetussen. Alle andere organen van de CCL33-geïnoculeerde foetussen en alle organen van de 3384-geïnoculeerde foetussen waren negatief (<$10^{1.7}$ $TCID_{50}$/g weefsel) in de virus titratie. PCV1-positieve cellen (tot 121 cellen/10 mm^2 bij CCL33-geïnoculeerde foetussen en tot 13 cellen/10 mm^2 bij 3384-geïnoculeerde foetussen) werden gevonden in het hart, de longen, milt, lever, thymus en tonsillen. Er werd geconcludeerd dat PCV1 gekweekt in celcultuur efficiënt kan repliceren en pathologie kan veroorzaken in de longen van varkens foetussen geïnoculeerd op 55 dagen van het foetale leven. In Hoofdstuk 4.2 werden de klinische en virologische gevolgen van chirurgische inoculatie van 55-dagen-oude immuno-incompetente varkens foetussen met PCV2a of PCV2b onderzocht. Twaalf

foetussen werden geïnoculeerd met PCV2: drie met de met het wegkwijnsyndroom (PMWS) geassocieerde PCV2a stam Stoon-1010, drie met reproductiestoornissen geassocieerde PCV2a stam 1121, drie met de PMWS geassocieerde PCV2b stam 48285 en drie met de met het porcien dermatitis en nefropathy syndroom geassocieerde PCV2b stam 1147. Vier foetussen werden mock-geïnoculeerd met celcultuur medium. Op 21 dagen na de inoculatie, waren elf van de twaalf PCV2-geïnoculeerde foetussen oedemateus en hadden een uitgezet abdomen, terwijl één foetus die geïnoculeerd was met PCV2b stam 1147 een normaal uiterlijk had. Alle PCV2-geïnoculeerde foetussen hadden bloedingen en stuwing in de interne organen en hadden een vergrote lever. Hoge PCV2 titers ($>10^{4.5}$ $TCID_{50}$/g weefsel) werden gevonden bij alle PCV2-geïnoculeerde foetussen, vooral in het hart, de milt en de lever. Hoge aantallen van PCV2-geïnfecteerde cellen (>1000 geïnfecteerde cellen/10 mm^2 weefsel) werden waargenomen in de harten. Twee 1121- en één 1147-geïnoculeerde foetussen ontwikkelden lage PCV2-specifieke antilichaam titers van 10 tot 40, de andere PCV2-geïnoculeerde foetussen waren negatief voor PCV2-specifieke antilichamen. Deze studie toonde aan dat PCV2a en PCV2b stammen afkomstig van verschillende klinische presentaties gelijkaardige macroscopische pathologische letsels induceren en dat hun virusvermeerdering even hoge titers induceren in verschillende organen van 55-dagen-oude immuno-incompetente varkens foetussen. In Hoofdstuk 4.3 werd onderzocht of inoculatie van immuno-incompetente varkens foetussen uit vroege stadia van de dracht met een minder virulente PCV2 stam een status van immunotolerantie kan geven bij de geboorte. Er werd ook onderzocht of postnatale super-infectie van dergelijke immunotolerante varkens met een zeer virulente PCV2 stam ziekte kan veroorzaken zonder herkend te worden door het immuunsysteem. VC2002, geïsoleerd uit een PMWS getroffen varken, is een combinatie van twee verschillende PCV2 virussen: K2 en K39 en een preliminair experiment toonde aan dat K2 geen nadelige effecten had op de foetussen bij korte termijn infectie (21 dpi), terwijl K39 wel nadelige effecten had. Eerst werd de foeto-pathogeniciteit van beide stammen getest en negen foetussen van drie zeugen werden geïnoculeerd op dagen dracht: drie met K2, drie met K39 en drie met medium. Op 21 dpi, induceerde K2 geen pathologie bij varkens foetussen, terwijl K39 dat wel deed. Daarna werd het lange termijn effect onderzocht enkel gebruik makend van de minder virulente PCV2 stam K2, 24 foetussen van vier zeugen werden geïnoculeerd op 45 dagen dracht: zes met een hoge dosis van K2, zes met een lage dosis van K2 en 12 met celcultuur medium. Op 69 dpi (114 dagen dracht), resulteerde inoculatie met zowel hoge als lage doses van K2 in de geboorte van vijf mummies en één levend geboren

big. Alle 12 mock-geïnoculeerde foetussen werden levend geboren. PCV2 K2 werd geïsoleerd uit alle mummies. De K2 en K2-specifieke antilichamen werden niet gedetecteerd bij de geboorte van de twee levend geboren K2-geïnfecteerde biggen. Het geeft aan dat de jonge foetussen de K2 infectie volledig onder controle hadden. Van 14 levend geboren biggen, werden zes mock-geïnoculeerde en één big dat geïnoculeerd werd met een lage dosis K2 immunogestimuleerd door door middel van parvovirus vaccinatie op dag 2. De overige biggen (zes mock-geïnoculeerde en één K2 big geïnoculeerd met een hoge dosis) werden niet gestimuleerd. Zes mock- (3 gestimuleerde en 3 niet-gestimuleerde) en twee K2-geïnfecteerde biggen (gestimuleerd en niet-gestimuleerd) werden super-geïnoculeerd met de zeer virulente PCV2 stam K39 op dag 6 of dag 8 na de geboorte (0 dagen post super-infectie (dpsi)). De andere zes mock-geïnoculeerde (3 gestimuleerde en 3 niet-gestimuleerde) biggen werder super-geïnoculeerd met medium (mock-geïnoculeerd). Een lage virale replicatie (tot $10^{3.3}$ $TCID_{50}$/g) werd waargenomen bij alle mock-K39 geïnoculeerde biggen op 21 en 28 dpsi. De niet-gestimuleerde K2-K39 big had ook een lage virale replicatie op 21 en 28 dpsi ($10^{2.3}$ $TCID_{50}$/g; geïdentificeerd als K39), terwijl de gestimuleerde K2-K39 big een uitzonderlijk hoge virale replicatie had op 14 en 21 dpsi (tot $10^{5.6}$ $TCID_{50}$/g; geïdentificeerd als K2). De eerstgenoemde vertoonde een late immuunrespons (op 21 dpsi met K39) en de laatstgenoemde had al antistoffen op 8 dagen ouderdom (op tijdstip van K39 inoculatie), dit geeft aan dat K2-infectie werd gecontroleerd tijdens het foetale leven en pas tot uiting kwam na geboorte als gevolg van immunostimulatie. Bij de gestimuleerde K2-K39 big werd alleen bij K2, en niet bij K39, replicatie waargenomen in de lies lymfeknopen verzameld op 14, 21 en 28 dpsi. Het is mogelijk dat K39 replicatie aanwezig was maar vroeg en op een zeer laag niveau vóór de staalname van de lymfeklieren. Geen enkele big vertoonde tekenen van ziekte. Er werd geconcludeerd dat, PCV2 stam K2 niet pathogeen was voor varkens foetussen op korte termijn (21 dpi), terwijl K39 dit wel was; de lange termijn infectie met de minder virulente K2 (69 dpi) resulteerde in de geboorte van mummies of levensvatbare biggen. Postnatale super-infectie van de mock- of levend geboren K2-geïnfecteerde biggen met een zeer virulente PCV2 stam K39 leidde niet tot ziekte.

Uit het eerste deel van deze thesis kan worden geconcludeerd dat een grote antigene diversiteit bestaat tussen de verschillende PCV2 stammen van de acht genetische clusters en de verschillende PCV2 clusters kunnen antigenisch worden gesubtypeerd met behulp van mAbs. Daarnaast zijn twee verschillende PCV2 neutralisatie fenotypes gedefinieerd in deze thesis op basis van de neutraliserende activiteit van mAbs tegenover verschillende PCV2

stammen en de aa residu(en) die belangrijk zijn bij het bepalen van de PCV2 neutralisatie fenotypes zijn geïdentificeerd in het PCV2 capsied eiwit. Uit het tweede deel van deze thesis, kan worden gesteld dat zowel PCV1 en PCV2 pathogeen kunnen zijn voor varkens foetussen in het midden van de dracht. De inoculatie van varkens foetussen met een minder virulente PCV2 stam in de vroege stadia van de dracht kan leiden tot de geboorte van levensvatbare biggen. Echter, de super-infectie van dergelijke PCV2-geïnfecteerde levend geboren biggen met een zeer virulente PCV2 stam leidde niet tot ziekte. Verder onderzoek is nodig om de pathogene karakters van PCV1 in varkens foetussen te onderzoeken en om een minder pathogene PCV2 stam te vinden die gebruikt kan worden om foetussen te infecteren in vroege stadia van de dracht om PCV2-immunotolerante varkens te maken.

Curriculum vitae

Dipongkor Saha was born in Mymensingh, Bangladesh, on January 4th, 1982. He completed his secondary and higher secondary education in 1997 and 1999, respectively in Mymensingh, Bangladesh. In 2005, he graduated with the degree of Doctor of Veterinary Medicine (DVM) from the Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh, Bangladesh. In the same year, he successfully finished a Field Internship Programme for six months on veterinary practice organised by the Faculty of Veterinary Science, Bangladesh Agricultural University. In 2007, he obtained the degree Master of Science (MS in Surgery) from the Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University. During the MS period, he received a 'National Science and Information & Communication Technology (NSICT) Fellowship' from the Ministry of Science and Technology, Government of the People's Republic of Bangladesh to finish his MS thesis. He was awarded with a 'Gold Medal' by the Education Minister, Government of the People's Republic of Bangladesh for his excellent results during DVM. Since June 9th, 2008 he has been working as a PhD student at the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University. In 2010, he was awarded with a PhD scholarship for candidates from developing countries from the Special Research Fund (BOF) of Ghent University. During his PhD period, he worked on pathogenesis of porcine circoviruses. He successfully completed the full curriculum (60 ECTS) of the Doctoral Training Programme, organised by the Ghent University Doctoral Schools.

He has published 8 papers in international scientific peer-reviewed journals. He has given 19 oral/poster presentations in national and international scientific congresses or symposia. He received a travel grant to attend the 6th International Symposium on Emerging and Re-emerging Pig Diseases, June 12 - June 15, 2011, Barcelona, Spain. He also received a 'Grant for participation in a conference abroad' from the Research Foundation Flanders (FWO) to attend the 22nd International Pig Veterinary Society Congress, June 10 - June 13, 2012, Jeju, South Korea.

Publications

I. Research articles as first author

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