



Genetic, antigenic and pathogenetic features of porcine circovirus type 2 strains

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List of abbreviations

aa	amino acid
Ab	antibody
AM	alveolar macrophages
ATCC	American Type Culture Collection
bp	base pairs
CD	cluster of differentiation
CD/CD	caesarean-derived colostrum-deprived
ConA	concanavalin A
CpG	cytosine-phosphate-guanine
DABCO	1,4-diazobicyclo-2.2.2-octane
DC	dendritic cells
DNA	deoxyribonucleic acid
dpi	days post inoculation
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ELS	endosomal-lysosomal system
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
GAG	glycosaminoglycan
hpi	hours post inoculation
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	intramuscular
IP	intraperitoneal
IPMA	immunoperoxidase monolayer assay
IV	intravenous
kb	kilobases
kDa	kilodalton

KLH	Keyhole limpet hemocyanin
LPS	lipopolysaccharide
mAb	monoclonal antibody
MEM	minimal essential medium
MHC	major histocompatibility class
ML	maximum likelihood
mRNA	messenger RNA
NGS	negative goat serum
NIPC	natural interferon producing cells
NJ	neighbor-joining
nt	nucleotide
ODN	oligodeoxynucleotides
ORF	open reading frame
Ori	origin of replication
P	probability
pAb	polyclonal antibody
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV	porcine circovirus, type 1 (PCV1) and type 2 (PCV2)
PDNS	porcine dermatitis and nephropathy syndrome
PK	porcine kidney
PMWS	postweaning multisystemic wasting syndrome
PPV	porcine parvovirus
PRRSV	porcine reproductive and respiratory syndrome virus
PVDF	polyvinylidene fluoride
RCR	rolling-circle replication
Rep	replicase protein
rIFN	recombinant interferon
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute

SC	subcutaneous
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPF	specified pathogen free
ss	single-stranded
SWC	swine workshop cluster
T	triangulation number
TCID ₅₀	50 % tissue culture infectious dose
TGF	transforming growth factor
TLMV	TTV-like mini virus
TNF	tumour necrosis factor
TRIS	thihydroxymethylaminomethane
TTV	torque teno virus
U	unit
VLP	virus-like particles
w/v	weight by volume

Chapter 1. Introduction

1.1. Introduction to porcine circoviruses

In 1974, Tischer et al. discovered a non-cytopathogenic, picornavirus-like contaminant in the continuous porcine kidney cell line PK-15 (ATCC-CCL33). This virus had a circular single-stranded DNA and was consequently called porcine circovirus (PCV) (Tischer et al., 1982). Although this virus was widespread in European and North-American pig populations (Tischer et al., 1986; Dulac and Afshar, 1989; Allan et al., 1994b; Edwards and Sands, 1994), experimental infections could not attribute any disease to PCV (Tischer et al., 1986; Allan et al., 1995).

In 1997 however, Nayar et al. detected PCV-like viruses in tissues from pigs suffering from a newly emerging disease called 'postweaning multisystemic wasting syndrome' (PMWS). This newly discovered virus was antigenically different from the original PK-15 cell culture PCV (Allan et al., 1998) and exhibited < 80 % nucleotide sequence identity with the original PCV (Meehan et al., 1998; Morozov et al., 1998). As a consequence, the original PK-15 cell culture PCV was called PCV1 and the newly isolated virus was called PCV2 (Meehan et al., 1998).

1.2. Characteristics of porcine circoviruses

1.2.1. Taxonomy of porcine circoviruses

PCVs have a single-stranded DNA (ssDNA) (Tischer et al., 1982). Viruses with a ssDNA are classified into 6 different families, based on morphological and genomic characteristics and host species. Viruses of the families *Inoviridae* and *Microviridae* infect bacteria; viruses of the families *Geminiviridae* and *Nanoviridae* are plant viruses. Two families of ssDNA viruses have been found in vertebrates: the *Parvoviridae* and the *Circoviridae*.

The *Circoviridae* family is divided into the genera *Circovirus*, *Anellovirus* and *Gyrovirus*, based on their morphology and genomic organisation. Besides PCV1 and PCV2, the genus *Circovirus* contains several recently characterized avian viral species: psittacine beak and feather disease virus (Ritchie et al., 1989), columbid or 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), raven circovirus (Stewart et al., 2006), starling

circovirus (Johne et al., 2006), gull circovirus (Smyth et al., 2007) and swan circovirus (Halami et al., 2008). The heterogenic genus *Anellovirus* includes species-specific torque teno viruses (TTV) of humans (Nishizawa et al., 1997), non-human primates and other vertebrate species including pigs, cats, dogs, cows, sheep and chickens (Okamoto et al., 2002; Hino and Miyata, 2007), as well as TTV-like mini virus (Takahashi et al., 2000), torque teno midi virus (Ninomiya et al., 2007) and small anelloviruses (Jones et al., 2005). Chicken anaemia virus (Yuasa et al., 1979) is the only member of the genus *Gyrovirus*.

Single-stranded DNA viruses

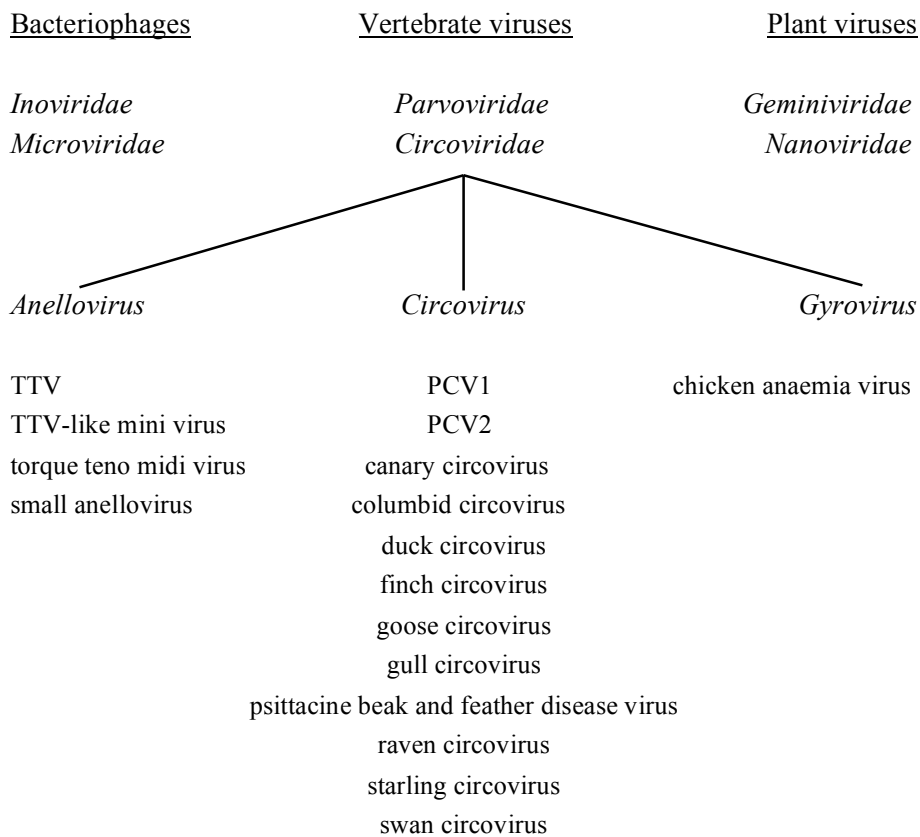


Fig. 1. Classification of the single-stranded DNA viruses.

1.2.2. Morphological and physical characteristics of porcine circoviruses

PCVs are small, non-enveloped viruses with a diameter of 17 ± 1.3 nm (Tischer et al., 1982). The virion has an icosahedral T=1 structure containing 60 capsid protein molecules arranged in 12 flat pentamer clustered units (Crowther et al., 2003), surrounding a covalently closed circular ssDNA (Tischer et al., 1982) of approximately 1.76 - 1.77 kilobases (Meehan et al., 1997; Meehan et al., 1998).

PCVs are resistant to inactivation by pH 3 (Allan et al., 1994b), thermal inactivation below 80° C (Allan et al., 1994b; Welch et al., 2006; O'Dea et al., 2008) and commercial disinfectants based on alcohol, iodine, chlorhexidine or phenol (Royer et al., 2001; Yilmaz and Kaleta, 2004; Martin et al., 2008). Incubation of PCV2-containing tissue homogenates at 37 °C only results in a $10^{0.5}$ TCID₅₀ reduction in virus titres per week (half life: 4 days) (P. Meerts, personal communication). PCVs can be inactivated by commercial disinfectants based on alkali (e.g. sodium hydroxide), oxidizing agents (e.g. sodium hypochlorite) or quaternary ammonium in combination or not with aldehydes (Royer et al., 2001; Yilmaz and Kaleta, 2004; Martin et al., 2008).

1.2.3. Genomic organization of porcine circoviruses

PCVs have a covalently closed circular ssDNA genome of 1,758 - 1,760 nucleotides for PCV1 (Fenaux et al., 2000) and 1,767 - 1,768 nucleotides for PCV2 (Meehan et al., 1998). The ambisense PCV genome (Fig. 2) contains 11 putative open reading frames (ORFs) (Hamel et al., 1998). At present, protein expression has only been described for 3 of these ORFs.

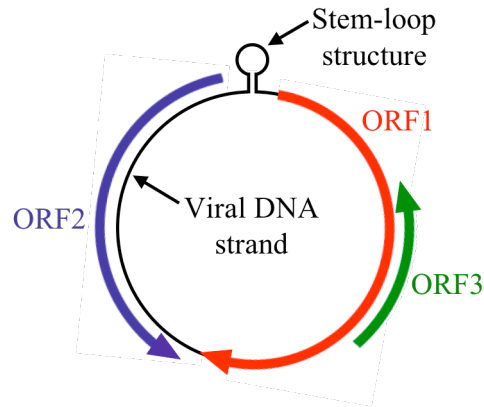


Fig. 2. Genome polarity of PCV. Clockwise arrows show transcription from the complementary anti-genomic DNA strand, counter-clockwise arrows show transcription from the genomic DNA strand. ORFs are reported below.

ORF1, located on the encapsidated genomic viral DNA strand and clockwise orientated (Fig. 2), codes for the conserved replication-associated protein Rep and its alternatively spliced frame-shifted variant Rep' (Mankertz and Hillenbrand, 2001; Cheung, 2003a; Mankertz et al., 2003). Rep and Rep' have a size of approximately 37 and 20 kDa. PCV1 Rep and Rep' proteins consist of 312 and 168 amino acids (aa), PCV2 Rep and Rep' proteins consist of 314 and 178 aa. Rep and Rep' proteins have 86 % and 80 % aa identity between PCV1 and PCV2, respectively (Meehan et al., 1998; Morozov et al., 1998; Mankertz et al., 2003). Despite these differences, the Rep and Rep' proteins are functionally exchangeable between PCV1 and PCV2 (Mankertz et al., 2003).

ORF2, located on the complementary anti-genomic DNA strand and counter-clockwise orientated (Fig. 2), encodes the less conserved structural capsid protein (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998; Mankertz et al., 2000; Nawagitgul et al., 2000). The ORF2 protein is the only structural protein and has a size of approximately 28 kDa. PCV1 and PCV2 capsid proteins consist of 230 - 233 aa and 233 - 234 aa, respectively. Capsid proteins have approximately 66 % aa identity between PCV1 and PCV2 (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998; Fenaux et al., 2000; Grierson et al., 2004b; Knell et al., 2005).

ORF3 is also located on the complementary strand and it overlaps with ORF1 in the opposite transcriptional orientation (Fig. 2) (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998). The PCV1 ORF3 protein has a molecular mass of approximately 23 kDa and consists of 206 aa. The PCV2 ORF3 protein is much smaller: 12 kDa and 104 aa. ORF3 proteins have 62 % aa identity between PCV1 and PCV2 (Hamel et al., 1998; Meehan et al., 1998). Recently, the PCV2 ORF3 protein has been associated with apoptosis *in vitro* and with viral pathogenesis *in vivo* (Liu et al., 2005; Liu et al., 2006), but these results are controversial because they could not be reproduced by other scientists (A. Mankertz and X.J. Meng, personal communication).

1.2.4. Replication of porcine circoviruses

PCVs replicate their genomes via the rolling-circle replication (RCR) mechanism (Gilbert and Dressler, 1968) in a manner similar to *Geminiviridae* (Saunders et al., 1991; Stenger et al., 1991) and *Nanoviridae* (Gronenborn, 2004). After infection of a host cell, cellular DNA polymerases convert the single-stranded viral DNA into a double-stranded DNA replication intermediate. After subsequent expression of Rep and Rep' in the cell, Rep and Rep' together bind to the double-stranded DNA replication intermediate at the origin of DNA replication (Ori) (Fig. 3, step 1). Rep and Rep' do not bind with the single-stranded viral DNA (Mankertz et al., 1997; Mankertz and Hillenbrand, 2001; Steinfeldt et al., 2001; Cheung, 2003b; Cheung, 2004a). The Ori comprises the intergenic region and the translation initiation points of the divergently transcribed Rep and capsid genes. Within the Ori, there is a stem-loop structure (Fig. 2, Fig. 4) with a nonanucleotide motif that is conserved among *Circoviridae*, *Geminiviridae* and *Nanoviridae*. This nonanucleotide motif is located at the apex of the stem-loop (Fig. 4) and consists of TAGTATTAC or AAGTATTAC for PCV1 and PCV2, respectively (Mankertz et al., 1997; Meehan et al., 1997; Meehan et al., 1998; Hamel et al., 1998). This conserved nonanucleotide motif is essential for DNA replication of both PCVs (Cheung, 2004b; Cheung, 2005). The binding site of replicase proteins Rep and Rep' is located at the right leg of the stem-loop and the hexamer repeats adjacent to it (Fig. 4) (Steinfeldt et al., 2001; Vega-Rocha et al., 2007). After binding of the replicase proteins to the double-stranded DNA replication intermediate, the replicase proteins expose the nonanucleotide motif of the single-stranded viral DNA part of the double-stranded

DNA replication intermediate. Subsequently, the replicase proteins cleave the viral DNA strand (Cheung, 2004c; Cheung, 2005; Steinfeldt et al., 2006), creating 2 free ends on the viral DNA strand. The replicase proteins do not cleave double-stranded DNA. One of the 2 free ends on the viral DNA strand serves as a starting point for DNA replication by cellular DNA polymerases (Fig. 3, step 2). During replication, the original viral DNA strand is displaced by the newly synthesized viral DNA strand (Fig. 3, step 3). After one round of DNA synthesis, Rep and Rep' join the 2 free ends of the displaced original viral DNA strand, as well as the 2 free ends of the newly synthesized viral DNA strand (Steinfeldt et al., 2006). This process releases the original single-stranded viral DNA (Fig. 3, step 4). The presence of a stem-loop structure is critical for termination of RCR (Cheung, 2007) and the palindromic sequences of the left and right leg of the stem-loop signal have an important role in the regulation of DNA replication termination (Cheung, 2004c; Cheung, 2004d). After releasing the original single-stranded viral DNA, the replicase proteins cleave the viral DNA strand of the newly formed double-stranded DNA replication intermediate (Fig. 3, step 4) and replication starts again.

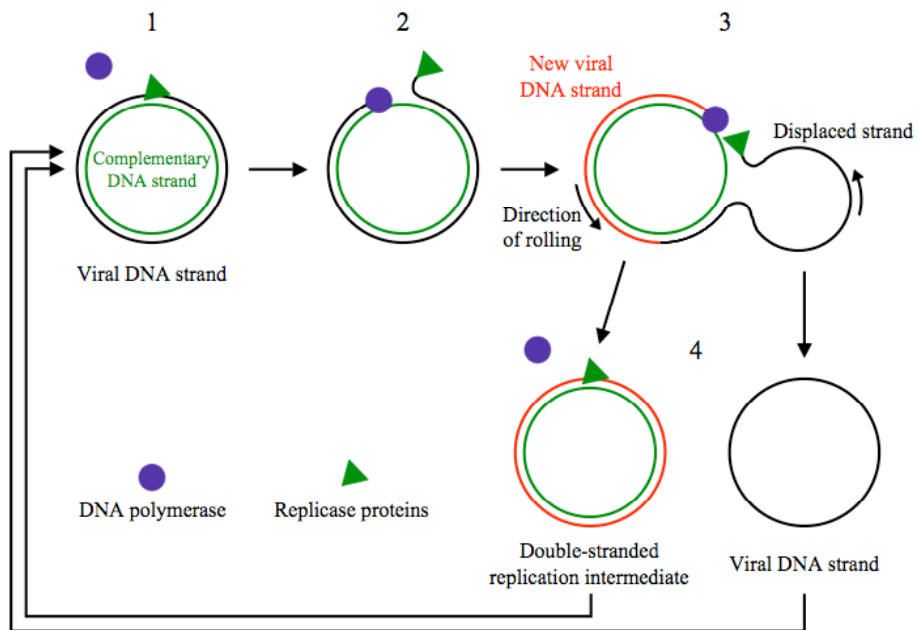


Fig. 3. Model of rolling-circle replication of PCV DNA. (1) Binding of the replicase proteins, Rep and Rep', to the double-stranded DNA replication intermediate at the origin of DNA replication. (2) Nicking of the viral DNA strand by the replicase proteins. (3) Displacement of the original viral DNA strand by the newly synthesized viral DNA strand. (4) Strand closure and release of the original single-stranded viral DNA. New nicking occurs and replication starts again.

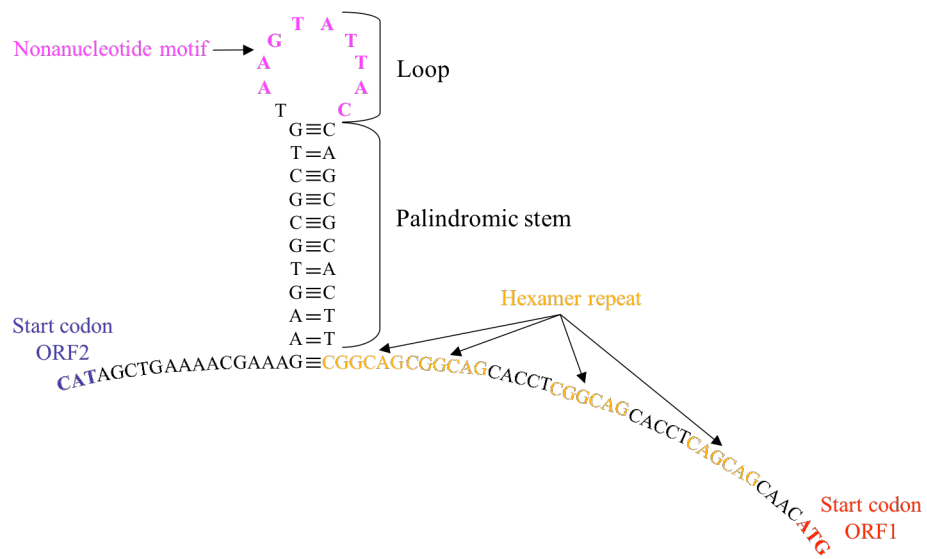


Fig. 4. The intergenic region of PCV2.

1.2.5. Phylogeny of PCV2

According to their genomic or amino acid sequences, or to restriction fragment length polymorphism, PCV2 strains can be divided into different genogroups, and different nomenclatures have been proposed (Hamel et al., 2000; Mankertz et al., 2000; Larochelle et al., 2002; de Boissésson et al., 2004; Wang et al., 2004; Knell et al., 2005; Wen et al., 2005; Carman et al., 2006; Gagnon et al., 2007; Cheung et al., 2007; Martins Gomes de Castro et al., 2007; Olvera et al., 2007; Dupont et al., 2008; Grau-Roma et al., 2008; Timmusk et al., 2008). A recently proposed classification system divides PCV2 into 2 genotypes (1 and 2) and 8 clusters (1A to 1C and 2A to 2E) (Fig. 5) (Cheung et al., 2007; Olvera et al., 2007; Grau-Roma et al., 2008). In general, genotype 1 genomes consist of 1,767 nucleotides and genotype 2 genomes consist of 1,768 nucleotides. The capsid protein consists of 233 aa, except for the capsid protein of cluster 1C PCV2 strains, that consists of 234 aa (Olvera et al., 2007). The capsid gene is a suitable phylogenetic marker for PCV2, because phylogenetic trees constructed with the whole PCV2 genome can be reconstructed with the capsid gene, but not with the Rep gene. This is merely due to recombination within the beginning of the Rep gene and because the capsid gene is more variable than the Rep gene and thus weighs more in phylogenetic tree construction (Cságola et al., 2006; Olvera et al., 2007). Recently, a third PCV2 genotype was discovered in archived serum samples from Denmark (Dupont et al., 2008). A methodology for a standardized PCV2 genotype definition was recently proposed and is based on the proportion of nucleotide differences between pairwise compared capsid gene sequences (Grau-Roma et al., 2008). In order to standardize PCV2 genotype nomenclature, the current European nomenclature of genotypes 1, 2 and 3 (Olvera et al., 2007; Grau-Roma et al., 2008; Dupont et al., 2008) will be adapted to the Canadian nomenclature (Gagnon et al., 2007). Genotype 1 will be called PCV2b, genotype 2 will be called PCV2a and genotype 3 will be called PCV2c (Segalés et al., 2008). Despite the existence of different genotypes, monoclonal and polyclonal antibodies react similarly with homologous and heterologous PCV2 isolates, indicating that there is only 1 PCV2 serotype (Allan et al., 1999; McNeilly et al., 2001; Duffy et al., 2007). Infection with PCV2a induces protective immunity against subsequent infection with PCV2b and vice versa (Opriessnig et al., 2007; Opriessnig et al., 2008b).

1.2.6. Interaction of PCV2 with host cells *in vitro*

PCV2 interacts with a wide variety of cell types *in vitro*, including primary cell types such as dendritic cells, monocytes, macrophages, lymphocytes and foetal cardiomyocytes and various continuous cell lines of epithelial or monocytic origin.

1.2.6.1. Attachment and internalization of PCV2

When PCV2 is added to porcine monocytic and epithelial cell lines *in vitro*, PCV2 randomly attaches to the entire cell surface of all cells of both cell types within 5 min and a plateau is reached starting from 15 min (Misinzio et al., 2005; Misinzio et al., 2008a). PCV2 uses the glycosaminoglycans heparan sulfate and chondroitin sulfate B as attachment receptors in monocytic cell lines (Misinzio et al., 2006). After attachment to the cell, PCV2 particles are slowly internalized in a time-dependent manner starting from 15 min and increasing till 6 h (Misinzio et al., 2005; Misinzio et al., 2008a). In monocytic cell lines, PCV2 is internalized via clathrin-mediated endocytosis and requires actin polymerization for infection (Misinzio et al., 2005). Acidification of the endosomal-lysosomal system (ELS) is required for cellular serine protease-mediated disassembly of the PCV2 (Misinzio et al., 2008c). In epithelial cell lines, PCV2 is internalized via clathrin-mediated endocytosis and a dynamin-independent clathrin- and caveolae-independent pathway. The former traps PCV2 while the latter is efficient for PCV2 replication. PCV2 infection of epithelial cell lines requires actin polymerization and depletion of membrane cholesterol enhances PCV2 infection (Misinzio et al., 2008a). Inhibition of ELS acidification enhances PCV2 infection of epithelial cell lines by increasing the disassembly of internalized PCV2 particles and a cellular serine protease is involved in this process (Misinzio et al., 2008c). In dendritic cells (DC), PCV2 replication is absent despite actin-dependent PCV2 internalization via clathrin-mediated endocytosis (Vincent et al., 2003; Vincent et al., 2005).

Treatment of porcine monocytic and epithelial cell lines with interferon-gamma (IFN- γ) before PCV2 inoculation or treatment with IFN- γ or interferon-alpha (IFN- α) after PCV2 inoculation increases PCV2 infection, probably due to enhanced PCV2 internalization (Meerts et al., 2005b). Combined treatment of epithelial cell lines with IFN- γ and inhibitors of ELS acidification induces an additional increase in PCV2 replication (Misinzio et al., 2008b).

1.2.6.2. The PCV2 replication cycle

PCV2 lacks its own DNA polymerases and therefore fully depends on cellular DNA polymerases to replicate its genome. As a result from this, PCV2 is a virus that needs replicating cells to complete its infectious cycle (Tischer et al., 1987). PCV2 is a slow growing virus. One single PCV2 replication cycle takes approximately 30 to 36 h in PK-15 cells (Cheung and Bolin, 2002; Meerts et al., 2005a).

After attachment to the cell surface and internalization into the cell, PCV2 particles co-localize with early endosomes and lysosomes and a cellular serine protease subsequently cleaves the PCV2 capsid (Misinzo et al., 2008c). How the PCV2 genome reaches the nucleus is still unknown. In PK-15 cells, the capsid protein is expressed in the cytoplasm between 6 and 12 h post inoculation (hpi) and it relocates to the nucleus between 12 and 24 hpi. Rep protein is detected in the nucleus from infected PK-15 cells between 12 and 24 hpi (Meerts et al., 2005a). Capsid (Liu et al., 2001) and Rep (Finsterbusch et al., 2005) proteins both have functional nuclear localization signals that direct them to the nucleus. During the infection cycle, capsid, Rep and Rep' proteins accumulate in the nucleus, where genome replication, capsid formation and encapsidation take place. Finally, assembled virions are translocated to the cytoplasm and progeny virus starts to be released from 30 to 36 hpi (Cheung and Bolin, 2002; Finsterbusch et al., 2005; Meerts et al., 2005a). It is believed that viral egress occurs through nuclear disintegration and cell lysis (Meerts et al., 2005a). In PK-15 cells, different replication kinetics are observed between PCV2 strains originating from cases of reproductive failure and PCV2 strains originating from cases of PMWS or porcine dermatitis and nephropathy syndrome (PDNS) (Meerts et al., 2005a).

Primary cells such as foetal cardiomyocytes, peripheral blood mononuclear cells (PBMC), alveolar macrophages (AM) and DC are much less susceptible to PCV2 replication than epithelial and monocytic cell lines. Despite the detection of capsid and Rep proteins in the nucleus in a limited number of cells in some of these primary cell types, infectious virus production could never be demonstrated in any of these primary cell types (Gilpin et al., 2003; Vincent et al., 2003; Meerts et al., 2005a; Chang et al., 2006; Fernandes et al., 2007; Yu et al., 2007b).

1.2.6.3. Interactions between PCV2 and immune cells *in vitro*

PCV2 does not seem to replicate in primary monocytic cells but it persists in these cells for an extended period of time, without losing its infectivity or without inducing cell death (Gilpin et al., 2003; Vincent et al., 2003; Chang et al., 2006). The persistence of PCV2 does not interfere with DC maturation, nor does it modulate the expression of major histocompatibility complex (MHC) class I and II, the expression of several clusters of differentiation (CD) associated with immune cell function or the ability to process and present antigens to T-lymphocytes. Silent PCV2 persistence in DC is a potential mechanism to escape the immune response of the host and because of their migratory capacity, DC are potential vehicles for PCV2 transportation throughout the host (Vincent et al., 2003; Vincent et al., 2005).

Recently, it was observed that PCV2 internalization in natural interferon producing cells (NIPC), a DC subpopulation, impairs the induction of IFN- α , tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) and IL-12, presumably through the presence of immunomodulatory oligodeoxynucleotides (ODN) with central cytosine-phosphate-guanine (CpG) motifs within the PCV2 genome, thereby preventing maturation of both NIPC and other DC populations (Vincent et al., 2007; Kekarainen et al., 2008a). One of these ODN completely inhibits IFN- α production induced in PBMC by viral or bacterial DNA, but the PCV2 genome as a whole induces IFN- α in PBMC (Hasslung et al., 2003; Wikström et al., 2007; Kekarainen et al., 2008a). In PK-15 cells however, PCV2 infection decreases IFN- α gene expression (Grasland et al., 2008b). The inhibitory ODN in the PCV2 genome also decreases the expression of IFN- γ , TNF- α , IL-1 β , IL-10, IL-12 and transforming growth factor-beta (TGF- β) in PBMC (Hasslung Wikström et al., 2008a). The presence of immunomodulatory sequences in the PCV2 genome is a potential mechanism to escape the immune response of the host and it may attribute to the development of clinical disease.

Furthermore, PCV2 increases the secretion of IL-10 in PBMC-derived monocytic cells, down regulating recall antigen responses through the inhibition of IFN- α , IFN- γ and IL-12 (Kekarainen et al., 2008a; Kekarainen et al., 2008b). PCV2 also inhibits IL-2 through an IL-10-independent mechanism (Kekarainen et al., 2008b). IL-10 directly inhibits T-lymphocyte proliferation and T-lymphocyte function, leading to impaired T-cell responses (Taga et al. 1993; Brooks et al., 2006). PCV2 also reduces phagocytosis, microbicidal capacity and production of O₂⁻ and H₂O₂ in alveolar

macrophages (Chang et al., 2006). In this way, pigs infected with PCV2 may be more vulnerable to opportunistic or secondary infections.

PCV2 does not replicate in PBMC-derived unstimulated lymphocytes *in vitro* (Gilpin et al., 2003; Yu et al., 2007b), neither does PCV1 (Allan et al., 1994a).

1.3. Porcine circovirus type 2-associated diseases

1.3.1. Pathogenesis of PCV2 infections

Pigs of all ages, including embryos and foetuses, are susceptible to PCV2 infections and the majority of natural infections presumably occur through ingestion and/or inhalation. Primary replication sites are still unknown, but there are some indications that early replication may take place in the distal part of the small intestine, colon, caecum and mesenteric lymph nodes, as well as in the tonsils (McNair et al., 2007). Starting from 7 days post inoculation (dpi), a long-lasting mainly cell-associated viraemia is observed (Pensaert et al., 2004), resulting in the spread of PCV2 to a large range of lymphoid and non-lymphoid organs, including the male and female reproductive tract (Ellis et al., 1999; Krakowka et al., 2000; Larochelle et al., 2000; Magar et al., 2000a; Pogranichniy et al., 2000; Bolin et al., 2001; Harms et al., 2001; Park et al., 2005).

Morphological analysis shows that PCV2 antigens are mainly detected in monocytic lineage cells and to a lesser extent in enterocytes, pneumocytes, bronchial, bronchiolar, biliary, pancreatic ductular and renal tubular epithelial cells, hepatocytes, lymphocytes, striated and smooth muscle cells, fibroblasts, neurons and vascular endothelial cells (Rosell et al., 1999; Kennedy et al., 2000; Stevenson et al., 2001).

In foetuses, the heart is the main target organ for PCV2 infection and PCV2 antigens are predominantly observed in cardiomyocytes and macrophages in the heart, but also in hepatocytes and macrophages in the liver. Monocytic lineage cells are the main target cells in other non-lymphoid organs and in lymphoid organs (Sanchez et al., 2001a; Sanchez et al., 2003; Yoon et al., 2004). In postnatal piglets, PCV2 positive cells are rarely observed in heart and liver and PCV2 antigens in lymphoid organs are found in monocytes, macrophages, CD4⁺ T-lymphocytes, CD8⁺ T-lymphocytes and B-lymphocytes (Sanchez et al., 2004; Yu et al., 2007a).

Starting from 14 dpi, the amount of PCV2 decreases in the majority of the inoculated pigs. This decrease coincides with the appearance of PCV2-specific antibodies (Bolin et al., 2001; Ladekjær-Mikkelsen et al., 2002; Meerts et al., 2005c), and more specifically, PCV2-neutralizing antibodies (Meerts et al., 2005c; Fort et al., 2007). There are strong indications that also the cellular immune response contributes to the control of PCV2 infection. (i) Pigs with high IFN- γ mRNA expression levels in PBMC are less susceptible to PCV2 replication (Meerts et al., 2005c). (ii) After immunization of pigs with a chimeric PCV1-2 infectious DNA clone, pigs do not develop a detectable humoral immune response against PCV2. Still, these pigs are clinically and virologically protected against subsequent challenge with PCV2 (Fenaux et al., 2004). (iii) Artificial immunosuppression with cyclosporin A dramatically increases PCV2 replication levels (Krakowka et al., 2002; Meerts et al., 2005c).

1.3.2. PCV2-associated diseases in the field

1.3.2.1. Postweaning multisystemic wasting syndrome

1.3.2.1.1. Epidemiology

Postweaning multisystemic wasting syndrome (PMWS) is a multifactorial disease affecting weaned pigs. PCV2 is the necessary agent to cause PMWS, but the vast majority of PCV2-infections are sub-clinical. Antibodies against PCV2 have been detected in archived sera from pigs from 1969 on (Magar et al., 2000b; Walker et al., 2000; Sanchez et al., 2001b) and PCV2 DNA and/or antigens have been detected in archived serum and tissue samples from 1977 on (Rodríguez-Arrijoja et al., 2003; Grierson et al., 2004a; Staebler et al., 2005; Dupont et al., 2008; Wiederkehr et al., 2008). However, the first cases of PMWS were reported in western Canada in 1991 (Clark, 1996; Harding, 1996). Since the late 90's, a PMWS epidemic has spread throughout the world and PMWS is nowadays reported to be an endemic disease in the majority of pig producing countries in North- and South-America, Europe and Asia. Recently, PMWS has also been diagnosed in South Africa (Drew et al., 2004) and New Zealand (Neumann et al., 2007), but not in Australia (Finlaison et al., 2007). Retrospective studies identified individual cases of PMWS as soon as 1986 in Spain and Switzerland (Rodríguez-Arrijoja et al., 2003; Staebler et al., 2005), long before the occurrence of the global PMWS epidemic.

1.3.2.1.2. Clinical presentation and pathology

PMWS usually appears in weaned pigs from 5 to 14 weeks of age and is characterized by severe growth retardation, weight loss and death. Other clinical symptoms include respiratory distress, diarrhoea, pallor, visibly enlarged lymph nodes and occasionally icterus. Figure 6 shows a group of Belgian PMWS-affected pigs. In PMWS-affected herds, morbidity usually ranges from 4 to 30 % and mortality rates are 70 - 80 % (Allan and Ellis, 2000; Segalés and Domingo, 2002). At necropsy, the most frequently observed macroscopic lesions are enlargement of lymph nodes and lungs that fail to collapse. White-spotted kidneys can sometimes be observed. The above-mentioned macroscopic lesions are not typical for PMWS. Interstitial lymphohistiocytic to granulomatous inflammation is microscopically observed in most affected organs (Allan and Ellis, 2000; Segalés and Domingo, 2002).

Microscopic lesions typically associated with PMWS can be found in lymphoid tissues. There is a moderate to severe depletion of lymphocytes combined with a moderate to intense histiocytic and/or multinucleated giant cell infiltration into the lymphocytic areas. Variably sized, sharply demarcated, basophilic or amphophilic cytoplasmic inclusion bodies are observed in monocytic lineage cells (Allan et al., 1998; Ellis et al., 1998; Kiupel et al., 1998; Morozov et al., 1998; Rosell et al. 1999). Lymphocytic depletion affects CD4⁺ T-lymphocytes, CD8⁺ T-lymphocytes and B-lymphocytes in lymphoid tissues (Shibahara et al., 2000; Sarli et al., 2001; Sanchez et al., 2004) and the number of circulating T- and B-lymphocytes is markedly reduced (Segalés et al., 2001; Darwich et al., 2002; Nielsen et al., 2003).



Fig. 6. A group of Belgian PMWS-affected pigs. PMWS is characterized by severe growth retardation, weight loss and death. Note the marked spine and hipbones of the emaciated pig in the centre of the picture (no. 1) and the icteric pig adjacent to it (no. 2).

1.3.2.1.3. Diagnosis

An individual pig is diagnosed with PMWS when 3 different criteria are fulfilled: i) clinical signs characterized by wasting or growth retardation, ii) histopathological lesions characterized by depletion of lymphocytes together with lymphohistiocytic to granulomatous inflammation and/or inclusion bodies and/or giant cells and iii) moderate to high amounts of PCV2 within characteristic histological lesions (Sorden, 2000; EU PCVD Consortium, 2005).

Serological analysis for PCV2-specific antibodies cannot be used for the diagnosis of PMWS due to the ubiquitous nature of the virus and due to the fact that the vast majority of PCV2-infections are sub-clinical. Combination of a viral neutralization test and a quantitative PCR in serum may be useful to exclude the diagnosis of PMWS in living animals (Fort et al., 2006), but a viral neutralization test is too complicated to be routinely applied. The combination viral neutralization test /

quantitative PCR in serum is not sensitive enough for the diagnosis of PMWS (Fort et al., 2006).

The detection of a PMWS-affected pig does not automatically imply that the corresponding farm has a PMWS problem. Individual PMWS-affected pigs can be found on farms with normal to good production and mortality rates and without economic losses due to PMWS (Meerts et al., 2004; Nielsen et al., 2008). A PMWS-affected farm is a farm in which individual pigs are diagnosed with PMWS and in which the current mortality due to PMWS is higher than the mean of historical mortality + 1.66 x SD or a farm in which statistical analysis via the chi-square test demonstrates that current mortality is higher than historical mortality. More specifically, current mortality is defined over a period of 1 or 2 months and historical mortality is defined over a period of at least 3 months. If mortality has not been recorded, a farm is considered to be a PMWS-affected farm when there is an increase in mortality exceeding the national or regional level by 50 % or more (EU PCVD Consortium, 2005).

1.3.2.1.4. Treatment and control

PMWS can be controlled by applying a 20-point plan that gives recommendations regarding housing and herd management (Madec et al., 2001). This plan is designed to reduce the PCV2 infection pressure and has 3 basic principles: i) reduce direct and indirect contact between piglets from different litters, ii) avoid all types of stress, including exposure to other pathogens and iii) optimize procedures for cleaning and disinfection. Reports show that this strategy successfully reduces production losses due to PMWS (Rose et al., 2003; Segalés et al., 2005; Allan and McNeilly, 2006; Wallgren et al., 2007; Madec et al., 2008).

Additional to Madec's 20-point plan, vaccination can be used as an extra measure to control PMWS. At this moment, only Circovac[®] (Merial) is broadly registered in Europe. This inactivated vaccine is administered to gilts and sows and enhances the levels of maternally derived antibodies in their offspring. This vaccine may also improve reproductive performance in gilts and sows (Delisle et al., 2008; Joisel et al., 2008). Three recombinant vaccines (Ingelvac CircoFLEX[®], Boehringer Ingelheim; Suvaxyn[®] PCV2 One Dose, Fort Dodge; Circumvent[®] PCV, Intervet/Schering-Plough) have been registered in a limited number of European countries. These single or dual injection vaccines are used for the immunization of piglets from 3 to 4 weeks of age. All PCV2 vaccines are based on PCV2a strains.

Vaccination against PCV2 confers virological protection against both PCV2a and PCV2b under experimental conditions (Charreyre et al., 2006a; Charreyre et al., 2006b; Fort et al., 2008; Opriessnig et al., 2008a; Reicks and Leuwerke, 2008) and reduces PCV2-associated productive losses under field conditions (Joisel et al., 2007a; Joisel et al., 2007b; Kixmüller et al., 2008; Thacker et al., 2008; Zizlavsky et al., 2008).

1.3.2.1.5. PMWS and the immune system

PMWS-affected pigs suffer from severe lymphocytic depletion (Kiupel et al., 1998; Morozov et al., 1998; Rosell et al. 1999), affecting T- and B-lymphocyte populations in lymphoid tissues and in the blood (Shibahara et al., 2000; Sarli et al., 2001; Segalés et al., 2001; Darwich et al., 2002; Nielsen et al., 2003; Sanchez et al., 2004). At present, it is not clear whether this lymphocytic depletion is primarily caused by viral replication (Sanchez et al., 2004; Yu et al., 2007a), apoptosis (Shibahara et al., 2000), decreased cellular proliferation (Mandrioli et al., 2004) or the disruption of cell signalling pathways. Still, this lymphocytic depletion is generally believed (Krakowka et al., 2002; Darwich et al., 2004; Segalés et al., 2004) to be the main cause of the slower and weaker PCV2-specific antibody response observed in PMWS-affected pigs when compared to sub-clinically PCV2-infected pigs (Pogranichniy et al., 2000; Bolin et al., 2001; Okuda et al., 2003; Meerts et al., 2006; Fort et al., 2007). More specifically, PMWS-affected animals lack an efficient neutralizing antibody response (Meerts et al., 2006; Fort et al., 2007) but it is not known whether this impaired humoral immune response is a cause or a consequence of the high PCV2 loads that are observed in PMWS-affected pigs. PMWS also affects the cellular immune response. Cytokine profiles in lymphoid tissues and in PBMC of PMWS-affected are severely altered (Darwich et al., 2003a; Darwich et al., 2003b) and several studies indicate that an increased expression of the immunosuppressive cytokine IL-10 (Taga et al. 1993; Brooks et al., 2006) may be involved in the development of PMWS (Darwich et al., 2003b; Sipos et al., 2004; Stevenson et al., 2006). Moreover, a correlation has been demonstrated between IL-10 overexpression and histopathological lesions (Darwich et al., 2003b) and PCV2 viraemia (Darwich et al., 2008). Again, it is not known whether this impaired cellular immune response is a cause or a consequence of the high PCV2 loads that are observed in PMWS-affected pigs.

Taken together, these data suggest an immunosuppressive state in PMWS-affected pigs, although the underlying mechanisms have not been revealed.

1.3.2.2. PCV2-associated reproductive failure

The first description of PCV2-associated reproductive failure was made in 1999 (West et al., 1999). PCV2 was isolated from a litter of aborted piglets from a newly established Canadian sow farm entirely stocked with first parity gilts and experiencing late-term abortions and stillbirths. In one foetus, severe diffuse myocarditis was associated with extensive immunohistochemical PCV2 staining. Other agents associated with reproductive disorders could not be isolated, suggesting that PCV2 was the cause of reproductive failure in this herd. A common feature for sow herds experiencing PCV2-induced mummification, stillbirth and abortion is that they house a high number of gilts or sows that were recently primary infected with PCV2 (Ladekjær-Mikkelsen et al., 2001; O'Connor et al., 2001; Farnham et al., 2003; Brunborg et al., 2007). High PCV2 seroprevalence at animal and herd level (Labarque et al., 2000; Sanchez et al., 2001b) presumably protects against PCV2-associated reproductive failure and field surveys indicate that reproductive failure due to vertical transmission of PCV2 is a rather rare event (Bogdan et al., 2001; Kim et al., 2004b; Maldonado et al., 2005).

Recently, it has been shown that hatched blastocysts are susceptible to PCV2 infection (Mateusen et al., 2004) and this infection induces embryonic death, subsequent resorption and regular return to oestrus in the majority of sows (Mateusen et al., 2007). In this way, the importance of PCV2-associated reproductive disorders may be underestimated under field conditions and it is hypothesized that PCV2-infected semen can be the principal route of PCV2 infection in hatched embryos. 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), despite the fact that PCV2 may be intermittently shed in the semen for a period up to 6 months after PCV2 infection. Especially boars less than 1 year of age are at risk to shed PCV2 in their semen (Larochelle et al., 2000; Le Tallec et al., 2001; McIntosh et al., 2006). However, the incidence of PCV2 DNA in semen samples seems to be a lot higher (20 - 47 %) when young boars are housed in production units with a high pig density instead of in low pig density centres for artificial insemination (Kim et al., 2001;

Kim et al., 2003b). Nevertheless, the PCV2 genomic load in semen generally seems to be too low to transmit a PCV2 infection to PCV2 negative sows (Grasland et al., 2008a). The question thus arises if the amount of infectious PCV2 that is generally present in boar semen is high enough to infect newly hatched embryos or not.

1.3.2.3. Other PCV2-associated diseases

PCV2 has been detected in pigs with porcine dermatitis and nephropathy syndrome (PDNS) (Allan et al., 2000b; Rosell et al., 2000; Saoulidis et al., 2002), porcine respiratory disease complex (Harms et al., 2002; Kim et al., 2003a; Fachinger et al., 2008), proliferative and necrotizing pneumonia (Grau-Roma and Segalés, 2007), diarrhoea (Kim et al., 2004a; Jensen et al., 2006), exudative epidermitis (Wattrang et al., 2002; Kim and Chae, 2004) and congenital tremors (Stevenson et al., 2001; Choi et al., 2002). However, PCV2 has never been experimentally shown to be the aetiological agent of any of these diseases.

1.3.3. Experimental models for PCV2-associated diseases

1.3.3.1. Postweaning multisystemic wasting syndrome

Several studies have tried to experimentally reproduce PMWS, but only a limited number has successfully reproduced PMWS with PCV2 alone (Kennedy et al., 2000; Albina et al., 2001; Bolin et al., 2001; Harms et al., 2001; Ladekjær-Mikkelsen et al., 2002; Allan et al., 2003; Okuda et al., 2003; Stockhofe-Zurwieden et al., 2003; Gauger et al., 2008). A more successful approach for the experimental reproduction of PMWS is co-infection with another infectious agent. Co-infections with porcine parvovirus (PPV) (Kennedy et al., 2000; Krakowka et al., 2000; Allan et al., 2003; Stockhofe-Zurwieden et al., 2003; Opriessnig et al., 2004a; Kim et al., 2006), porcine reproductive and respiratory syndrome virus (PRRSV) (Harms et al., 2001; Rovira et al., 2002; Stockhofe-Zurwieden et al., 2003) and *Mycoplasma hyopneumoniae* (Opriessnig et al., 2004b) enhance PCV2 replication and exacerbate the clinical outcome of a PCV2 infection. In the above-mentioned studies it was also demonstrated that PMWS always occurs with severe histopathological lesions in lymphoid organs and high PCV2 loads in these organs. Further, it was demonstrated that histopathological lesions in lymphoid organs are strongly

correlated with the PCV2 load in these organs. However, the above-mentioned studies have also shown that not all pigs with severe histopathological lesions and high PCV2 loads suffer from clinical PMWS. At present, specific co-factors responsible for the clinical expression of PMWS have not been identified yet.

The fact that co-infections with other infectious agents enhance PCV2 replication may find its origin in the fact that PCV2 may profit from the specific immune response mounted against other infectious agents. Injection of immune stimulating drugs (Krakowka et al., 2001; Kyriakis et al., 2002; Grasland et al., 2005; Wang et al., 2007; Krakowka et al., 2007) and vaccination (Opriessnig et al., 2003; Allan et al., 2007; Krakowka et al., 2007) enhance PCV2 replication as well, suggesting that the induction of an immune response might be a key factor in the induction of high levels of PCV2 replication. However, co-infections with other infectious agents, immune stimulating drugs or vaccination do not guarantee enhanced PCV2 replication and/or PMWS (Allan et al., 2000a; Allan et al., 2000c; Ladekjær-Mikkelsen et al., 2002; Stockhofe-Zurwieden et al., 2003; Opriessnig et al., 2004a; Resendes et al., 2004; Loizel et al., 2005; Ostanello et al., 2005; Haruna et al., 2006; Allan et al., 2007; Fernandes et al., 2007; Krakowka et al., 2007; Harding et al., 2008a; Harding et al., 2008b; Hasslung Wikström et al., 2008b).

Recently, a meta-analysis was performed on 44 experimental PCV2 inoculation studies in order to define which kind of experimental set-up has the highest chance to reproduce PMWS. It was found that inoculation of colostrum-deprived pigs younger than 3 weeks of age with a high dose ($> 10^{5.0}$ TCID₅₀ / pig) of a PCV2b (genotype 1) strain and co-infection with another porcine pathogen has the highest chance for success (Tomás et al., 2008). Still, it has to be taken into account that the vast majority of experimental PCV2 inoculation studies involved PCV2a (genotype 2) strains and that a substantial amount of these studies successfully reproduced PMWS, whereas the number of studies that reproduced PMWS with a PCV2b (genotype 1) strain is still limited (Stockhofe-Zurwieden et al., 2003; Grasland et al., 2005; Lager et al., 2007; Wang et al., 2007; Gauger et al., 2008). Despite the fact that some recent field observations suggest that PCV2b (genotype 1) may be more pathogenic than PCV2a (genotype 2), because PCV2b (genotype 1) strains are more frequently isolated from PMWS cases than PCV2a (genotype 2) strains (Carman et al., 2006; Cheung et al., 2007; Gagnon et al., 2007; Dupont et al., 2008; Carman et al., 2008; Grau-Roma et al., 2008; Timmusk et al., 2008; Wiederkehr et al., 2008), PMWS has been experimentally reproduced with both

genotypes and at present there is no experimental evidence that actually confirms that PCV2b (genotype 1) is more pathogenic than PCV2a (genotype 2) (Lager et al., 2007; Gauger et al., 2008; Opriessnig et al., 2008b).

Under field conditions, pigs can be infected with 2 or more PCV2 strains of the same or a different genotype at the same time (de Boissésion et al., 2004; Opriessnig et al., 2006; Cheung et al., 2007; Grau-Roma et al., 2008; Hesse et al., 2008), but it is not known which role multiple PCV2 infections may have in pathogenesis and development of PMWS. Recently, it has been suggested that dual heterologous PCV2a/PCV2b (genotype 2/ genotype 1) infections enhance PCV2 replication and PMWS expression under experimental conditions (Harding et al., 2008a), but more evidence is needed to support this hypothesis. At present, it is also not known if PCV2 may undergo recombination in multiple strain infected pigs *in vivo*, nor is it known to what extent putative recombination events *in vivo* may lead to the emergence of new PCV2 genotypes.

1.3.3.2. PCV2-associated reproductive failure

In a series of surgical, trans-uterine, intra-foetal PCV2 inoculation experiments 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 susceptible to PCV2 replication and that this susceptibility is negatively correlated with the foetal age at the time of inoculation. Gross lesions, merely due to heart failure and subsequent congestion of internal organs, were only observed in foetuses inoculated at 57 or 75 days of age, presumably due to foetal immuno-competence (Salmon, 1984) in foetuses inoculated at 92 or 104 days of age and due to decreased susceptibility of foetal tissues, especially cardiomyocytes, for PCV2 replication in older foetuses. Foetuses inoculated at 57 or 75 days of age were either mummified (57 days) or autolysed (75 days) at birth, or stillborn (75 days) (Sanchez et al., 2001b; Sanchez et al., 2003; Pensaert et al., 2004; Sanchez et al., 2004). Using similar experimental approaches, Johnson et al. (2002) and Yoon et al. (2004) described similar gross lesions and foetal death in foetuses inoculated during mid-gestation (58 - 76 days) or late gestation (84 - 94 days). Besides intra-foetal inoculation, reproductive failure has been experimentally reproduced by intranasal (Park et al., 2005) and intra-uterine inoculation (Rose et al., 2007) of sows with PCV2.

In vitro exposure of porcine embryos to PCV2 has demonstrated that hatched blastocysts (8 days post insemination) are susceptible to PCV2 infection and enzymatic removal of the zona pellucida makes morulae (6 days post insemination) and early blastocysts (7 days post insemination) susceptible to infection with PCV2 (Mateusen et al., 2004). In a following study (Mateusen et al., 2007), hatched blastocysts were exposed to PCV2 in vitro and subsequently surgically transferred to receptor sows. Two weeks after transfer, receptor sows were euthanized and embryos were collected. Only 15 % of the PCV2-inoculated embryos could be recovered and less than half of them were viable, and PCV2 antigens were found in embryonic tissues. In contrast, 69 % of non-inoculated embryos were recovered and 94 % of them were viable. In this way, it was demonstrated that PCV2 replicates in embryos and subsequently induces embryonic death.

At present, experimental studies have only used PCV2a (genotype 2) strains to reproduce PCV2-associated reproductive failure and it is not known if PCV2b (genotype 1) strains have a different virulence in foetuses or not.

References

- Albina, E., Truong, C., Hutet, E., Blanchard, P., Cariolet, R., L'Hospitalier, R., Mahé, D., Allée, C., Morvan, H., Amenna, N., Le Dimna, M., Madec, F., Jestin, A., 2001. An experimental model for post-weaning multisystemic wasting syndrome (PMWS) in growing piglets. *J Comp Pathol* 125, 292-303.
- Allan, G.M., Ellis, J.A., 2000. Porcine circoviruses: a review. *J Vet Diagn Invest* 12, 3-14.
- Allan, G.M., McNeilly, F., 2006. PMWS/PCVD: Diagnosis, disease, and control: What do we know? In: *Proceedings of the 19th International Pig Veterinary Society Congress*, Copenhagen, Denmark, Volume 1, pp. 1-9.
- Allan, G.M., McNeilly, F., Foster, J.C., Adair, B.M., 1994a. Infection of leucocyte cell cultures derived from different species with pig circovirus. *Vet Microbiol* 41, 267-279.
- Allan, G.M., Phenix, K.V., Todd, D., McNulty, M.S., 1994b. Some biological and physico-chemical properties of porcine circovirus. *Zentralbl Veterinarmed B* 41, 17-26.
- Allan, G.M., McNeilly, F., Cassidy, J.P., Reilly, G.A., Adair, B., Ellis, W.A., McNulty, M.S., 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. *Vet Microbiol* 44, 49-64.
- Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clark, E.G., Ellis, J.A., Haines, D.M., Meehan, B.M., Adair, B.M., 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. *J Vet Diagn Invest* 10, 3-10.
- Allan, G.M., McNeilly, F., Meehan, B.M., Kennedy, S., Mackie, D.P., Ellis, J.A., Clark, E.G., Espuna, E., Saubi, N., Riera, P., Bøtner, A., Charreyre, C.E., 1999. Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. *Vet Microbiol* 66, 115-123.

- Allan, G.M., McNeilly, F., Ellis, J., Krakowka, S., Meehan, B., McNair, I., Walker, I., Kennedy, S., 2000a. Experimental infection of colostrum deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. *Arch Virol* 145, 2421-2429.
- Allan, G.M., McNeilly, E., Kennedy, S., Meehan, B., Moffett, D., Malone, F., Ellis, J., Krakowka, S., 2000b. PCV-2-associated PDNS in Northern Ireland in 1990. Porcine dermatitis and nephropathy syndrome. *Vet Rec* 146, 711-712.
- Allan, G.M., McNeilly, F., Meehan, B.M., Ellis, J.A., Connor, T.J., McNair, I., Krakowka, S., Kennedy, S., 2000c. A sequential study of experimental infection of pigs with porcine circovirus and porcine parvovirus: immunostaining of cryostat sections and virus isolation. *J Vet Med B Infect Dis Vet Public Health* 47, 81-94.
- Allan, G., McNeilly, F., Meehan, B., McNair, I., Ellis, J., Krakowka, S., Fossum, C., Wattrang, E., Wallgren, P., Adair, B., 2003. Reproduction of postweaning multisystemic wasting syndrome in pigs experimentally inoculated with a Swedish porcine circovirus 2 isolate. *J Vet Diagn Invest* 15, 553-560.
- Allan, G.M., Caprioli, A., McNair, I., Lagan-Tregaskis, P., Ellis, J., Krakowka, S., McKillen, J., Ostanello, F., McNeilly, F., 2007. Porcine circovirus 2 replication in colostrum-deprived piglets following experimental infection and immune stimulation using a modified live vaccine against porcine respiratory and reproductive syndrome virus. *Zoonoses Public Health* 54, 214-222.
- Bogdan, J., West, K., Clark, E., Konoby, C., Haines, D., Allan, G., McNeilly, F., Meehan, B., Krakowka, S., Ellis, J.A., 2001. Association of porcine circovirus 2 with reproductive failure in pigs: a retrospective study, 1995-1998. *Can Vet J* 42, 548-550.
- Bolin, S.R., Stoffregen, W.C., Nayar, G.P., Hamel, A.L., 2001. Postweaning multisystemic wasting syndrome induced after experimental inoculation of cesarean-derived, colostrum-deprived piglets with type 2 porcine circovirus. *J Vet Diagn Invest* 13, 185-194.
- Brooks, D.G., Trifilo, M.J., Edelmann, K.H., Teyton, L., McGavern, D.B., Oldstone, M.B., 2006. Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* 12, 1301-1309.
- Brunborg, I.M., Jonassen, C.M., Moldal, T., Bratberg, B., Lium, B., Koenen, F., Schönheit, J., 2007. Association of myocarditis with high viral load of porcine circovirus type 2 in several tissues in cases of fetal death and high mortality in piglets. A case study. *J Vet Diagn Invest* 19, 368-375.
- Carman, S., McEwen, B., DeLay, J., van Dreumel, T., Lusi, P., Cai, H., Fairles, J., 2006. Porcine circovirus-2 associated disease in swine in Ontario (2004 to 2005). *Can Vet J* 47, 761-762.
- Carman, S., Cai, H.Y., DeLay, J., Youssef, S.A., McEwen, B.J., Gagnon, C.A., Tremblay, D., Hazlett, M., Lusi, P., Fairles, J., Alexander, H.S., van Dreumel, T., 2008. The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease--2004-2006. *Can J Vet Res* 72, 259-268.
- Chang, H.W., Jeng, C.R., Lin, T.L., Liu, J.J., Chiou, M.T., Tsai, Y.C., Chia, M.Y., Jan, T.R., Pang, V.F., 2006. Immunopathological effects of porcine circovirus type 2 (PCV2) on swine alveolar macrophages by in vitro inoculation. *Vet Immunol Immunopathol* 110, 207-219.
- Charreyre, C., Bésème, S., Brun, A., Bublot, M., Lapostolle, B., Sierra, P., Vaganay, A., 2006. Protection of piglets against a PCV2 experimental challenge by vaccinating gilts with an inactivated adjuvanted PCV2 vaccine, Circovac[®]: serological, clinical and pathological aspects. In: *Proceedings of the 19th International Pig Veterinary Society Congress, Copenhagen, Denmark, Volume 1*, p. 176.

- Charreyre, C., Bésème, S., Brun, A., Bublot, M., Lapostolle, B., Sierra, P., Vaganay, A., 2006. Virological protection of piglets against a PCV2 experimental challenge by vaccinating gilts with an inactivated adjuvanted PCV2 vaccine. In: Proceedings of the 19th International Pig Veterinary Society Congress, Copenhagen, Denmark, Volume 1, p. 177.
- Cheung, A.K., 2003a. Transcriptional analysis of porcine circovirus type 2. *Virology* 305, 168-180.
- Cheung, A.K., 2003b. The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. *Virology* 313, 452-459.
- Cheung, A.K., 2004a. Identification of the essential and non-essential transcription units for protein synthesis, DNA replication and infectious virus production of Porcine circovirus type 1. *Arch Virol* 149, 975-988.
- Cheung, A.K., 2004b. Identification of an octanucleotide motif sequence essential for viral protein, DNA, and progeny virus biosynthesis at the origin of DNA replication of porcine circovirus type 2. *Virology* 324, 28-36.
- Cheung, A.K., 2004c. Detection of template strand switching during initiation and termination of DNA replication of porcine circovirus. *J Virol* 78, 4268-4277.
- Cheung, A.K., 2004d. Palindrome regeneration by template strand-switching mechanism at the origin of DNA replication of porcine circovirus via the rolling-circle melting-pot replication model. *J Virol* 78, 9016-9029.
- Cheung, A.K., 2005. Detection of rampant nucleotide reversion at the origin of DNA replication of porcine circovirus type 1. *Virology* 333, 22-30.
- Cheung, A.K., 2007. A stem-loop structure, sequence non-specific, at the origin of DNA replication of porcine circovirus is essential for termination but not for initiation of rolling-circle DNA replication. *Virology* 363, 229-235.
- Cheung, A.K., Bolin, S.R., 2002. Kinetics of porcine circovirus type 2 replication. *Arch Virol* 147, 43-58.
- Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. *Arch Virol* 152, 1035-1044.
- Choi, J., Stevenson, G.W., Kiupel, M., Harrach, B., Anothayanontha, L., Kanitz, C.L., Mittal, S.K., 2002. Sequence analysis of old and new strains of porcine circovirus associated with congenital tremors in pigs and their comparison with strains involved with postweaning multisystemic wasting syndrome. *Can J Vet Res* 66, 217-224.
- Clark, E.G., 1996. Post-weaning multisystemic wasting syndrome. In: Proceedings of the 27th Annual Meeting of the Western Canadian Association of Swine Practitioners, Saskatoon, pp. 19-20.
- Crowther, R.A., Berriman, J.A., Curran, W.L., Allan, G.M., Todd, D., 2003. Comparison of the structures of three circoviruses: chicken anemia virus, porcine circovirus type 2, and beak and feather disease virus. *J Virol* 77, 13036-13041.
- Cságola, A., Kecskeméti, S., Kardos, G., Kiss, I., Tuboly, T., 2006. Genetic characterization of type 2 porcine circoviruses detected in Hungarian wild boars. *Arch Virol* 151, 495-507.
- Darwich, L., Segalés, J., Domingo, M., Mateu, E., 2002. Changes in CD4(+), CD8(+), CD4(+) CD8(+), and immunoglobulin M-positive peripheral blood mononuclear cells of postweaning multisystemic wasting syndrome-affected pigs and age-matched uninfected wasted and healthy pigs correlate with lesions and porcine circovirus type 2 load in lymphoid tissues. *Clin Diagn Lab Immunol* 9, 236-242.
- Darwich, L., Balasch, M., Plana-Durán, J., Segalés, J., Domingo, M., Mateu, E., 2003a. Cytokine profiles of peripheral blood mononuclear cells from pigs with postweaning

- multisystemic wasting syndrome in response to mitogen, superantigen or recall viral antigens. *J Gen Virol* 84, 3453-3457.
- Darwich, L., Pié, S., Rovira, A., Segalés, J., Domingo, M., Oswald, I.P., Mateu, E., 2003b. Cytokine mRNA expression profiles in lymphoid tissues of pigs naturally affected by postweaning multisystemic wasting syndrome. *J Gen Virol* 84, 2117-2125.
- Darwich, L., Segalés, J., Mateu, E., 2004. Pathogenesis of postweaning multisystemic wasting syndrome caused by Porcine circovirus 2: An immune riddle. *Arch Virol* 149, 857-874.
- Darwich, L., Segalés, J., Resendes, A., Balasch, M., Plana-Durán, J., Mateu, E., 2008. Transient correlation between viremia levels and IL-10 expression in pigs subclinically infected with porcine circovirus type 2 (PCV2). *Res Vet Sci* 84, 194-198.
- de Boissésou, C., Beven, V., Bigarre, L., Thiery, R., Rose, N., Eveno, E., Madec, F., Jestin, A., 2004. Molecular characterization of Porcine circovirus type 2 isolates from postweaning multisystemic wasting syndrome-affected and non-affected pigs. *J Gen Virol* 85, 293-304.
- Delisle, C., Delisle, G., Bridoux, N., Thibault, J.C., Longo, S., Joisel, F., 2008. Results of sow vaccination against PCV2 with Circovac[®] in France: improvement of reproduction parameters. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 1, p. 47.
- Drew, T.W., Grierson, S.S., King, D.P., Hicks, D., Done, S., Naser, J.A., Evans, D.P., Grimbeek, P., Banks, M., 2004. Genetic similarity between porcine circovirus type 2 isolated from the first reported case of PMWS in South Africa and North American isolates. *Vet Rec* 155, 149-11.
- Duffy, C., McNeilly, F., McNair, I., Tregaskis, P., Ellis, J., Krakowka, S., Adair, B.M., Allan, G.M., 2007. The serological characterisation of PCV2 isolates using an established virus neutralization test for PCV2 antibodies. In: Proceedings of the 5th International Symposium on Emerging and Re-emerging Pig Diseases, Krakow, Poland, p. 52.
- Dulac, G.C., Afshar, A., 1989. Porcine circovirus antigens in PK-15 cell line (ATCC CCL-33) and evidence of antibodies to circovirus in Canadian pigs. *Can J Vet Res* 53, 431-433.
- Dupont, K., Nielsen, E.O., Bækbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. *Vet Microbiol* 128, 56-64.
- Edwards, S., Sands, J.J., 1994. Evidence of circovirus infection in British pigs. *Vet Rec* 134, 680-681.
- Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin, K., McNeilly, F., Meehan, B., Todd, D., Haines, D., 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. *Can Vet J* 39, 44-51.
- Ellis, J., Krakowka, S., Lairmore, M., Haines, D., Bratanich, A., Clark, E., Allan, G., Konoby, C., Hassard, L., Meehan, B., Martin, K., Harding, J., Kennedy, S., McNeilly, F., 1999. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. *J Vet Diagn Invest* 11, 3-14.
- EU PCVD Consortium, 2005. PMWS Case Definition. <http://www.pcvd.org/news.php>, <http://www.thepigsite.com/articles/1/health-and-welfare/1514/pmws-case-definition>.
- Fachinger, V., Bischoff, R., Jedidia, S.B., Saalmüller, A., Elbers, K., 2008. The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. *Vaccine* 26, 1488-1499.
- Farnham, M.W., Choi, Y.K., Goyal, S.M., Joo, H.S., 2003. Isolation and characterization of porcine circovirus type-2 from sera of stillborn fetuses. *Can J Vet Res* 67, 108-113.

- Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.J., 2000. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. *J Clin Microbiol* 38, 2494-2503.
- Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. *J Virol* 78, 6297-6303.
- Fernandes, L.T., Mateu, E., Sibila, M., Fort, M., Andaluz, A., McNeilly, F., Allan, G., Sánchez, A., Segalés, J., Stevenson, L., 2007. Lack of in vitro and in vivo effects of lipopolysaccharide on porcine circovirus type 2 infection. *Viral Immunol* 20, 541-552.
- Finlaison, D., Kirkland, P., Luong, R., Ross, A., 2007. Survey of porcine circovirus 2 and postweaning multisystemic wasting syndrome in New South Wales piggeries. *Aust Vet J* 85, 304-310.
- Finsterbusch, T., Steinfeldt, T., Caliskan, R., Mankertz, A., 2005. Analysis of the subcellular localization of the proteins Rep, Rep' and Cap of porcine circovirus type 1. *Virology* 343, 36-46. Erratum in: *Virology* (2006) 349, 492.
- Fort, M., Olvera, A., Sibila, M., Segalés, J., Mateu, E., 2007. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. *Vet Microbiol* 125, 244-255.
- Fort, M., Sibila, M., Allepuz, A., Mateu, E., Roerink, F., Segalés, J., 2008. Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. *Vaccine* 26, 1063-1071.
- Gagnon, C.A., Tremblay, D., Tijssen, P., Venne, M.H., Houde, A., Elahi, S.M., 2007. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. *Can Vet J* 48, 811-819.
- Gauger, P.C., Lager, K.M., Vincent, A.L., Opriessnig, T., Kehrli, M.E.Jr., Cheung, A.K., 2008. Pathogenesis of PCV2a and PCV2b virus in germ-free pigs. In: *Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 1*, p. 28.
- Gilbert, W., Dressler, D., 1968. DNA replication: the rolling circle model. *Cold Spring Harb Symp Quant Biol* 33, 473-484.
- Gilpin, D.F., McCullough, K., Meehan, B.M., McNeilly, F., McNair, I., Stevenson, L.S., Foster, J.C., Ellis, J.A., Krakowka, S., Adair, B.M., Allan, G.M., 2003. In vitro studies on the infection and replication of porcine circovirus type 2 in cells of the porcine immune system. *Vet Immunol Immunopathol* 94, 149-161.
- Grasland, B., Loizel, C., Blanchard, P., Oger, A., Nignol, A.C., Bigarré, L., Morvan, H., Cariolet, R., Jestin, A., 2005. Reproduction of PMWS in immunostimulated SPF piglets transfected with infectious cloned genomic DNA of type 2 porcine circovirus. *Vet Res* 36, 685-697.
- Grasland, B., Blanchard, P., Jan, B., Oger, A., Rose, N., Madec, F., Jestin, A., Cariolet, R., 2008a. Transmission of porcine circovirus of type 2 (PCV2) with semen. In: *Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 2*, p. 56.
- Grasland, B., Blanchard, Y., Bigarre, L., Félix, H., Béven, V., Nignol, A.C., Jestin, A., 2008b. Transcriptomic analysis of PK15 cells infected by porcine circovirus of type 2. In: *Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 1*, p. 13.

- Grau-Roma, L., Segalés, J., 2007. Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky's disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain. *Vet Microbiol* 119, 144-151.
- Grau-Roma, L., Crisci, E., Sibila, M., López-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A., Segalés, J., 2008. A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. *Vet Microbiol* 128, 23-35.
- Grierson, S.S., King, D.P., Sandvik, T., Hicks, D., Spencer, Y., Drew, T.W., Banks, M., 2004a. Detection and genetic typing of type 2 porcine circoviruses in archived pig tissues from the UK. *Arch Virol* 149, 1171-1183.
- Grierson, S.S., King, D.P., Wellenberg, G.J., Banks, M., 2004b. Genome sequence analysis of 10 Dutch porcine circovirus type 2 (PCV-2) isolates from a PMWS case-control study. *Res Vet Sci* 77, 265-268.
- Gronenborn, B., 2004. Nanoviruses: genome organisation and protein function. *Vet Microbiol* 98, 103-109.
- Halami, M.Y., Nieper, H., Müller, H., Johne, R., 2008. Detection of a novel circovirus in mute swans (*Cygnus olor*) by using nested broad-spectrum PCR. *Virus Res* 132, 208-212.
- Hamel, A.L., Lin, L.L., Nayar, G.P., 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *J Virol* 72, 5262-5267.
- Hamel, A.L., Lin, L.L., Sachvie, C., Grudeski, E., Nayar, G.P., 2000. PCR detection and characterization of type-2 porcine circovirus. *Can J Vet Res* 64, 44-52.
- Harding, J.C.S., 1996. Postweaning multisystemic wasting syndrome (PMWS): Preliminary epidemiology and clinical presentation. In: Proceedings of the 27th Annual Meeting of the Western Canadian Association of Swine Practitioners, Saskatoon, p. 21.
- Harding, J.C., Ellis, J.A., Allan, G., Krakowka, S., 2008a. Dual heterologous PCV2a/2b infection induces PMWS in gnotobiotic pigs. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 1, p. 21.
- Harding, J.C., Ellis, J.A., Allan, G., Krakowka, S., 2008b. PCV2b (RFLP 321; genogroup 1) fails to induce PMWS in gnotobiotic pigs. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 2, p. 49.
- Harms, P.A., Sorden, S.D., Halbur, P.G., Bolin, S.R., Lager, K.M., Morozov, I., Paul, P.S., 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. *Vet Pathol* 38, 528-539.
- Harms, P.A., Halbur, P.G., Sorden, S.D., 2002. Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. *J Swine Health Prod* 10, 27-30.
- Haruna, J., Hanna, P., Hurmik, D., Ikede, B., Miller, L., Yason, C., 2006. The role of immunostimulation in the development of postweaning multisystemic wasting syndrome in pigs under field conditions. *Can J Vet Res* 70, 269-276.
- Hasslung, F.C., Berg, M., Allan, G.M., Meehan, B.M., McNeilly, F., Fossum, C., 2003. Identification of a sequence from the genome of porcine circovirus type 2 with an inhibitory effect on IFN-alpha production by porcine PBMCs. *J Gen Virol* 84, 2937-2945.
- Hasslung Wikström, F., Fossum, C., Fuxler, L., Lövgren, T., 2008a. DNA sequence motif in the genome of porcine circovirus type 2 inhibits production of cytokines by peripheral blood mononuclear cells in vitro. In: Interaction between porcine circovirus type 2 and the immune system of the pig with special reference to

- immunomodulatory sequences in the viral genome. Doctoral Thesis, Swedish University of Agricultural Sciences, Uppsala.
- Hasslung Wikström, F., Wallgren, P., Blomqvist, G., Belák, K., Tenk, M., Fuxler, L., Wolff, C., McNeilly, F., Krakowka, S., Allan, G.M., Fossum, C., 2008b. Experimental co-infection of 4-week-old colostrum fed pigs with PCV2 and *E. coli*. In: Interaction between porcine circovirus type 2 and the immune system of the pig with special reference to immunomodulatory sequences in the viral genome. Doctoral Thesis, Swedish University of Agricultural Sciences, Uppsala.
- Hattermann, K., Schmitt, C., Soike, D., Mankertz, A., 2003. Cloning and sequencing of Duck circovirus (DuCV). *Arch Virol* 148, 2471-2480.
- Hesse, R., Kerrigan, M., Rowland, R.R., 2008. Evidence for recombination between PCV2a and PCV2b in the field. *Virus Res* 132, 201-207.
- Hino, S., Miyata, H., 2007. Torque teno virus (TTV): current status. *Rev Med Virol* 17, 45-57.
- Jensen, T.K., Vigre, H., Svensmark, B., Bille-Hansen, V., 2006. Distinction between porcine circovirus type 2 enteritis and porcine proliferative enteropathy caused by *Lawsonia intracellularis*. *J Comp Pathol* 135, 176-182.
- Johne, R., Fernández-de-Luco, D., Höfle, U., Müller, H., 2006. Genome of a novel circovirus of starlings, amplified by multiply primed rolling-circle amplification. *J Gen Virol* 87, 1189-1195.
- Johnson, C.S., Joo, H.S., Direksin, K., Yoon, K.J., Choi, Y.K., 2002. Experimental in utero inoculation of late-term swine fetuses with porcine circovirus type 2. *J Vet Diagn Invest* 14, 507-512.
- Joisel, F., Brune, A., Schade, A., Longo, S., Charreyre, C., 2007a. Results of the vaccination against PCV2 diseases with Circovac[®] in 233 German sow herds: decrease in mortality. In: Proceedings of the 5th International Symposium on Emerging and Re-emerging Pig Diseases, Krakow, Poland, p. 126.
- Joisel, F., Brune, A., Schade, A., Longo, S., Charreyre, C., 2007b. Results of the vaccination against PCV2 diseases with Circovac[®] in 233 German sow herds: improvement of pig growth and decrease in antibiotic treatments. In: Proceedings of the 5th International Symposium on Emerging and Re-emerging Pig Diseases, Krakow, Poland, p. 127.
- Joisel, F., Brune, A., Schade, A., Longo, S., Charreyre, C., 2008. Improvement of reproduction performance induced by PCV2 vaccination of sows and gilts with Circovac[®] in 277 German sow farms. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 2, p. 72.
- Jones, M.S., Kapoor, A., Lukashov, V.V., Simmonds, P., Hecht, F., Delwart, E., 2005. New DNA viruses identified in patients with acute viral infection syndrome. *J Virol* 79, 8230-8236.
- Kennedy, S., Moffett, D., McNeilly, F., Meehan, B., Ellis, J., Krakowka, S., Allan, G.M., 2000. Reproduction of lesions of postweaning multisystemic wasting syndrome by infection of conventional pigs with porcine circovirus type 2 alone or in combination with porcine parvovirus. *J Comp Pathol* 122, 9-24.
- Kekarainen, T., Montoya, M., Dominguez, J., Mateu, E., Segalés, J., 2008a. Porcine circovirus type 2 (PCV2) viral components immunomodulate recall antigen responses. *Vet Immunol Immunopathol* 124, 41-49.
- Kekarainen, T., Montoya, M., Mateu, E., Segalés, J., 2008b. Porcine circovirus type 2-induced interleukin-10 modulates recall antigen responses. *J Gen Virol* 89, 760-765.
- Kim, J., Chae, C., 2004. Concurrent presence of porcine circovirus type 2 and porcine parvovirus in retrospective cases of exudative epidermitis in pigs. *Vet J* 167, 104-106.

- Kim, J., Han, D.U., Choi, C., Chae, C., 2001. Differentiation of porcine circovirus (PCV)-1 and PCV-2 in boar semen using a multiplex nested polymerase chain reaction. *J Virol Methods* 98, 25-31.
- Kim, J., Chung, H.K., Chae, C., 2003a. Association of porcine circovirus 2 with porcine respiratory disease complex. *Vet J* 166, 251-256.
- Kim, J., Han, D.U., Choi, C., Chae, C., 2003b. Simultaneous detection and differentiation between porcine circovirus and porcine parvovirus in boar semen by multiplex seminested polymerase chain reaction. *J Vet Med Sci* 65, 741-744.
- Kim, J., Ha, Y., Jung, K., Choi, C., Chae, C., 2004a. Enteritis associated with porcine circovirus 2 in pigs. *Can J Vet Res* 68, 218-221.
- Kim, J., Jung, K., Chae, C., 2004b. Prevalence of porcine circovirus type 2 in aborted fetuses and stillborn piglets. *Vet Rec* 155, 489-492.
- Kim, J., Ha, Y., Chae, C., 2006. Potentiation of porcine circovirus 2-induced postweaning multisystemic wasting syndrome by porcine parvovirus is associated with excessive production of tumor necrosis factor-alpha. *Vet Pathol* 43, 718-725.
- Kiupel, M., Stevenson, G.W., Mittal, S.K., Clark, E.G., Haines, D.M., 1998. Circovirus-like viral associated disease in weaned pigs in Indiana. *Vet Pathol* 35, 303-307.
- Kixmüller, M., Ritzmann, M., Eddicks, M., Saalmüller, A., Elbers, K., Fachinger, V., 2008. Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. *Vaccine* 26, 3443-3451.
- Knell, S., Willems, H., Hertrampf, B., Reiner, G., 2005. Comparative genetic characterization of Porcine Circovirus type 2 samples from German wild boar populations. *Vet Microbiol* 109, 169-177.
- Krakovka, S., Ellis, J.A., Meehan, B., Kennedy, S., McNeilly, F., Allan, G., 2000. Viral wasting syndrome of swine: experimental reproduction of postweaning multisystemic wasting syndrome in gnotobiotic swine by coinfection with porcine circovirus 2 and porcine parvovirus. *Vet Pathol* 37, 254-263.
- Krakovka, S., Ellis, J.A., McNeilly, F., Ringler, S., Rings, D.M., Allan, G., 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). *Vet Pathol* 38, 31-42.
- Krakovka, S., Ellis, J.A., McNeilly, F., Gilpin, D., Meehan, B., McCullough, K., Allan, G., 2002. Immunologic features of porcine circovirus type 2 infection. *Viral Immunol* 15, 567-582.
- Krakovka, S., Ellis, J., McNeilly, F., Waldner, C., Rings, D.M., Allan, G., 2007. *Mycoplasma hyopneumoniae* bacterins and porcine circovirus type 2 (PCV2) infection: induction of postweaning multisystemic wasting syndrome (PMWS) in the gnotobiotic swine model of PCV2-associated disease. *Can Vet J* 48, 716-24.
- Kyriakis, S.C., Saoulidis, K., Lekkas, S., Miliotis, Ch.C., Papoutsis, P.A., Kennedy, S., 2002. The effects of immuno-modulation on the clinical and pathological expression of postweaning multisystemic wasting syndrome. *J Comp Pathol* 126, 38-46.
- Labarque, G.G., Nauwynck, H.J., Mesu, A.P., Pensaert, M.B., 2000. Seroprevalence of porcine circovirus types 1 and 2 in the Belgian pig population. *Vet Q* 22, 234-236.
- Ladekjær-Mikkelsen, A.S., Nielsen, J., Storgaard, T., Bøtner, A., Allan, G., McNeilly, F., 2001. Transplacental infection with PCV-2 associated with reproductive failure in a gilt. *Vet Rec* 148, 759-760.
- Ladekjær-Mikkelsen, A.S., Nielsen, J., Stadjek, T., Storgaard, T., Krakowka, S., Ellis, J., McNeilly, F., Allan, G., Botner, A., 2002. Reproduction of postweaning multisystemic wasting syndrome (PMWS) in immunostimulated and non-immunostimulated 3-week-old piglets experimentally infected with porcine circovirus type 2 (PCV2). *Vet Microbiol* 89, 97-114.

- Lager, K.M., Gauger, P.C., Vincent, A.L., Opriessnig, T., Kehrl, M.E. Jr., Cheung, A.K., 2007. Mortality in pigs given porcine circovirus type 2 subgroup 1 and 2 viruses derived from DNA clones. *Vet Rec* 161, 428-429.
- Larochelle, R., Bielanski, A., Müller, P., Magar, R., 2000. PCR detection and evidence of shedding of porcine circovirus type 2 in boar semen. *J Clin Microbiol* 38, 4629-4632.
- Larochelle, R., Magar, M., D'Allaire, S., 2002. Genetic characterization and phylogenetic analysis of porcine circovirus type 2 (PCV2) strains from cases presenting various clinical conditions. *Virus Res* 90, 101-112.
- Le Tallec, B., Pozzi, N., Blanchard, P., Mahé, D., Jestin, A., Guérin, B., 2001. Longitudinal study of boars naturally infected by PCV2. In: *Proceedings: ssDNA Viruses of Plants, Birds, Pigs and Primates*, Saint-Malo, France, p. 120.
- Liu, Q., Tikoo, S.K., Babiuk, L.A., 2001. Nuclear localization of the ORF2 protein encoded by porcine circovirus type 2. *Virology* 285, 91-99.
- Liu, J., Chen, I., Kwang, J., 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. *J Virol* 79, 8262-8274.
- Liu, J., Chen, I., Du, Q., Chua, H., Kwang, J., 2006. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. *J Virol* 80, 5065-5073.
- Loizel, C., Blanchard, P., Grasland, B., Dory, D., Oger, A., Nignol, A.C., Cariolet, R., Jestin, A., 2005. Effect of granulocyte-macrophage colony-stimulating factor on post-weaning multisystemic wasting syndrome in porcine circovirus type-2-transfected piglets. *Int J Exp Pathol* 86, 33-43.
- Madec, F., Rose, N., Eveno, E., Morvan, P., Larour, G., Jolly, J.P., Le Digerher, G., Cariolet, R., Le Dimna, M., Blanchard, Ph., Jestin, A., 2001. PMWS: on-farm observations and preliminary analytic epidemiology. In: *Proceedings: ssDNA Viruses of Plants, Birds, Pigs and Primates*, Saint-Malo, France, pp. 86-88.
- Madec, F., Rose, N., Grasland, B., Cariolet, R., Jestin, A., 2008. Post-Weaning Multisystemic Wasting Syndrome and Other PCV2-Related Problems in Pigs: a 12-Year Experience. *Transbound Emerg Dis*, in press.
- Magar, R., Larochelle, R., Thibault, S., Lamontagne, L., 2000a. Experimental transmission of porcine circovirus type 2 (PCV2) in weaned pigs: a sequential study. *J Comp Pathol* 123, 258-269.
- Magar, R., Müller, P., Larochelle, R., 2000b. Retrospective serological survey of antibodies to porcine circovirus type 1 and type 2. *Can J Vet Res* 64, 184-186.
- Maldonado, J., Segalés, J., Martínez-Puig, D., Calsamiglia, M., Riera, P., Domingo, M., Artigas, C., 2005. Identification of viral pathogens in aborted fetuses and stillborn piglets from cases of swine reproductive failure in Spain. *Vet J* 169, 454-456.
- Mandrioli, L., Sarli, G., Panarese, S., Baldoni, S., Marcato, P.S., 2004. Apoptosis and proliferative activity in lymph node reaction in postweaning multisystemic wasting syndrome (PMWS). *Vet Immunol Immunopathol* 97, 25-37.
- Mankertz, A., Hillenbrand, B., 2001. Replication of porcine circovirus type 1 requires two proteins encoded by the viral rep gene. *Virology* 279, 429-438.
- Mankertz, A., Persson, F., Mankertz, J., Blaess, G., Buhk, H.J., 1997. Mapping and characterization of the origin of DNA replication of porcine circovirus. *J Virol* 71, 2562-2566.
- Mankertz, A., Domingo, M., Folch, J.M., Le Cann, P., Jestin, A., Segalés, J., Chmielewicz, B., Plana-Duran, J., Soike, D., 2000. Characterisation of PCV-2 isolates from Spain, Germany and France. *Virus Res* 66, 65-77.
- Mankertz, A., Mueller, B., Steinfeldt, T., Schmitt, C., Finsterbusch, T., 2003. New reporter gene-based replication assay reveals exchangeability of replication factors of porcine circovirus types 1 and 2. *J Virol* 77, 9885-9893.

- Martin, H., Le Potier, M.F., Maris, P., 2008. Virucidal efficacy of nine commercial disinfectants against porcine circovirus type 2. *Vet J* 177, 388-393.
- Martins Gomes de Castro, A.M., Cortez, A., Heinemann, M.B., Brandão, P.E., Richtzenhain, L.J., 2007. Genetic diversity of Brazilian strains of porcine circovirus type 2 (PCV-2) revealed by analysis of the cap gene (ORF-2). *Arch Virol* 152, 1435-1445.
- Mateusen, B., Sanchez, R.E., Van Soom, A., Meerts, P., Maes, D.G.D., Nauwynck, H.J., 2004. Susceptibility of pig embryos to porcine circovirus type 2 infection. *Theriogenology* 61, 91-101.
- Mateusen, B., Maes, D.G., Van Soom, A., Lefebvre, D., Nauwynck, H.J., 2007. Effect of a porcine circovirus type 2 infection on embryos during early pregnancy. *Theriogenology* 68, 896-901.
- McIntosh, K.A., Harding, J.C., Parker, S., Ellis, J.A., Appleyard, G.D., 2006. Nested polymerase chain reaction detection and duration of porcine circovirus type 2 in semen with sperm morphological analysis from naturally infected boars. *J Vet Diagn Invest* 18, 380-384.
- McNair, I., McNeilly, F., Duffy, C., Tregaskis, P., McKay, P., Fossum, C., Ellis, J., Krakowka, S., Allan, G.M., 2007. Early distribution of PCV2 in a PMWS disease model. In: *Proceedings of the 5th International Symposium on Emerging and Re-emerging Pig Diseases*, Krakow, Poland, p. 56.
- McNeilly, F., McNair, I., Mackie, D.P., Meehan, B.M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S., Allan, G.M., 2001. Production, characterization and applications of monoclonal antibodies to porcine circovirus 2. *Arch Virol* 146, 909-922.
- Meehan, B.M., Creelan, J.L., McNulty, M.S., Todd, D., 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. *J Gen Virol* 78, 221-227.
- Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *J Gen Virol* 79, 2171-2179.
- Meerts, P., Nauwynck, H., Sanchez, R., Mateusen, B., Pensaert, M., 2004. Prevalence of porcine circovirus 2 (PCV2)-related wasting on Belgian farms with or without a history of postweaning multisystemic wasting syndrome. *Vlaams Diergen Tijds* 73, 31-38.
- Meerts, P., Misinzo, G., McNeilly, F., Nauwynck, H.J., 2005a. Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, foetal cardiomyocytes and macrophages. *Arch Virol* 150, 427-441.
- Meerts, P., Misinzo, G., Nauwynck, H.J., 2005b. Enhancement of porcine circovirus 2 replication in porcine cell lines by IFN-gamma before and after treatment and by IFN-alpha after treatment. *J Interf Cytok Res* 25, 684-693.
- Meerts, P., Van Gucht, S., Cox, E., Vandebosch, A., Nauwynck, H.J., 2005c. Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. *Viral Immunol* 18, 333-341.
- Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Bøtner, A., Kristensen, C.S., Nauwynck, H.J., 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. *BMC Vet Res* 2, 6.
- Misinzo, G., Meerts, P., Bublot, M., Mast, J., Weingartl, H.M., Nauwynck, H.J., 2005. Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. *J Gen Virol* 86, 2057-2068.
- Misinzo, G., Delputte, P.L., Meerts, P., Lefebvre, D.J., Nauwynck, H.J., 2006. Porcine circovirus 2 uses heparan sulfate and chondroitin sulfate B glycosaminoglycans as receptors for its attachment to host cells. *J Virol* 80, 3487-3494.

- Misinzo, G., Delputte, P.L., Lefebvre, D.J., Nauwynck, H.J., 2008a. Porcine circovirus 2 infection of epithelial cells is clathrin-, caveolae- and dynamin-independent, actin and Rho-GTPase-mediated, and enhanced by cholesterol depletion. *Virus Res*, doi:10.1016/j.virusres.2008.09.005.
- Misinzo, G., Delputte, P.L., Lefebvre, D.J., Nauwynck, H.J., 2008b. Increased yield of porcine circovirus-2 by a combined treatment of PK-15 cells with interferon-gamma and inhibitors of endosomal-lysosomal system acidification. *Arch Virol* 153, 337-342.
- Misinzo, G., Delputte, P.L., Nauwynck, H.J., 2008c. Inhibition of endosome-lysosome system acidification enhances porcine circovirus 2 infection of porcine epithelial cells. *J Virol* 82, 1128-1135.
- Morozov, I., Sirinarumit, T., Sorden, S.D., Halbur, P.G., Morgan, M.K., Yoon, K.J., Paul, P.S., 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. *J Clin Microbiol* 36, 2535-2541.
- Nawagitgul, P., Morozov, I., Bolin, S.R., Harms, P.A., Sorden, S.D., Paul, P.S., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. *J Gen Virol* 81, 2281-2287.
- Nayar, G.P., Hamel, A., Lin, L., 1997. Detection and characterization of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *Can Vet J* 38, 385-386.
- Neumann, E.J., Dobbins, S.S., Welch, E.B., Morris, R.S., 2007. Descriptive summary of an outbreak of porcine post-weaning multisystemic wasting syndrome (PMWS) in New Zealand. *N Z Vet J* 55, 346-352.
- Nielsen, E.O., Enøe, C., Jorsal, S.E., Barfod, K., Svensmark, B., Bille-Hansen, V., Vigre, H., Bøtner, A., Bækbo, P., 2008. Postweaning multisystemic wasting syndrome in Danish pig herds: productivity, clinical signs and pathology. *Vet Rec* 162, 505-508.
- Nielsen, J., Vincent, I.E., Bøtner, A., Ladekaer-Mikkelsen, A.S., Allan, G., Summerfield, A., McCullough, K.C., 2003. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). *Vet Immunol Immunopathol* 92, 97-111.
- Ninomiya, M., Nishizawa, T., Takahashi, M., Lorenzo, F.R., Shimosegawa, T., Okamoto, H., 2007. Identification and genomic characterization of a novel human torque teno virus of 3.2 kb. *J Gen Virol* 88, 1939-1944.
- Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., Mayumi, M., 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem Biophys Res Commun* 241, 92-97.
- O'Connor, B., Gauvreau, H., West, K., Bogdan, J., Ayroud, M., Clark, E.G., Konoby, C., Allan, G., Ellis, J.A., 2001. Multiple porcine circovirus 2-associated abortions and reproductive failure in a multisite swine production unit. *Can Vet J* 42, 551-553.
- O'Dea, M.A., Hughes, A.P., Davies, L.J., Muhling, J., Buddle, R., Wilcox, G.E., 2008. Thermal stability of porcine circovirus type 2 in cell culture. *J Virol Methods* 147, 61-66.
- Okamoto, H., Takahashi, M., Nishizawa, T., Tawara, A., Fukai, K., Muramatsu, U., Naito, Y., Yoshikawa, A., 2002. Genomic characterization of TT viruses (TTVs) in pigs, cats and dogs and their relatedness with species-specific TTVs in primates and tupaia. *J Gen Virol* 83, 1291-1297.
- Okuda, Y., Ono, M., Yazawa, S., Shibata, I., 2003. Experimental reproduction of postweaning multisystemic wasting syndrome in cesarean-derived, colostrum-deprived piglets inoculated with porcine circovirus type 2 (PCV2): investigation of quantitative PCV2 distribution and antibody responses. *J Vet Diagn Invest* 15, 107-114.

- Olvera, A., Cortey, M., Segalés, J., 2007. Molecular evolution of porcine circovirus type 2 genomes: Phylogeny and clonality. *Virology* 357, 175-185.
- Opriessnig, T., Yu, S., Gallup, J.M., Evans, R.B., Fenaux, M., Pallares, F., Thacker, E.L., Brockus, C.W., Ackermann, M.R., Thomas, P., Meng, X.J., Halbur, P.G., 2003. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. *Vet Pathol* 40, 521-529.
- Opriessnig, T., Fenaux, M., Yu, S., Evans, R.B., Cavanaugh, D., Gallup, J.M., Pallares, F.J., Thacker, E.L., Lager, K.M., Meng, X.J., Halbur, P.G., 2004a. Effect of porcine parvovirus vaccination on the development of PMWS in segregated early weaned pigs coinfecting with type 2 porcine circovirus and porcine parvovirus. *Vet Microbiol* 98, 209-220.
- Opriessnig, T., Thacker, E.L., Yu, S., Fenaux, M., Meng, X.J., Halbur, P.G., 2004b. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. *Vet Pathol* 41, 624-640.
- Opriessnig, T., McKeown, N.E., Zhou, E.M., Meng, X.J., Halbur, P.G., 2006. Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. *J Gen Virol* 87, 2923-2932.
- Opriessnig, T., Madson, D.M., Prickett, J.R., Pogranichniy, R.M., Meng, X.J., Halbur, P.G., 2007. Long-term effect of PCV2-infection and reinfection on PCV2-antibody and DNA profiles in pigs over time. In: *Proceedings of the 5th International Symposium on Emerging and Re-emerging Pig Diseases*, Krakow, Poland, p. 45.
- Opriessnig, T., Patterson, A.R., Elsener, J., Meng, X.J., Halbur, P.G., 2008a. Influence of maternal antibodies on efficacy of porcine circovirus type 2 (PCV2) vaccination to protect pigs from experimental infection with PCV2. *Clin Vaccine Immunol* 15, 397-401.
- Opriessnig, T., Ramamoorthy, S., Madson, D.M., Patterson, A.R., Pal, N., Carman, S., Meng, X.J., Halbur, P.G., 2008b. Differences in virulence among porcine circovirus type 2 isolates are unrelated to cluster type 2a or 2b and prior infection provides heterologous protection. *J Gen Virol* 89, 2482-2491.
- Ostanello, F., Caprioli, A., Di Francesco, A., Battilani, M., Sala, G., Sarli, G., Mandrioli, L., McNeilly, F., Allan, G.M., Prospero, S., 2005. Experimental infection of 3-week-old conventional colostrum-fed pigs with porcine circovirus type 2 and porcine parvovirus. *Vet Microbiol* 108, 179-186.
- Park, J.S., Kim, J., Ha, Y., Jung, K., Choi, C., Lim, J.K., Kim, S.H., Chae, C., 2005. Birth abnormalities in pregnant sows infected intranasally with porcine circovirus 2. *J Comp Pathol* 132, 139-144.
- Pensaert, M.B., Sanchez, R.E., Ladekjær-Mikkelsen, A.S., Allan, G.M., Nauwynck, H.J., 2004. Viremia and effect of fetal infection with porcine viruses with special reference to porcine circovirus 2 infection. *Vet Microbiol* 98, 175-183.
- Phenix, K.V., Weston, J.H., Ypelaar, I., Lavazza, A., Smyth, J.A., Todd, D., Wilcox, G.E., Raidal, S.R., 2001. Nucleotide sequence analysis of a novel circovirus of canaries and its relationship to other members of the genus *Circovirus* of the family *Circoviridae*. *J Gen Virol* 82, 2805-2809.
- Pogranichniy, R.M., Yoon, K.J., Harms, P.A., Swenson, S.L., Zimmerman, J.J., Sorden, S.D., 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. *Viral Immunol* 13, 143-153.
- Reicks, D.L., Leuwerke, B.C., 2008. The effect of vaccination to porcine circovirus type 2 on detection in serum, blood swab and semen. In: *Proceedings of the 20th International Pig Veterinary Society Congress*, Durban, South Africa, Volume 1, p. 14.

- Resendes, A., Segalés, J., Balasch, M., Calsamiglia, M., Sibila, M., Ellerbrok, H., Mateu, E., Plana-Duran, J., Mankertz, A., Domingo M., 2004. Lack of an effect of a commercial vaccine adjuvant on the development of postweaning multisystemic wasting syndrome (PMWS) in porcine circovirus type 2 (PCV2) experimentally infected conventional pigs. *Vet Res* 35, 83-90.
- Ritchie, B.W., Niagro, F.D., Lukert, P.D., Steffens, W.L. 3rd, Latimer, K.S., 1989. Characterization of a new virus from cockatoos with psittacine beak and feather disease. *Virology* 171, 83-88.
- Rodríguez-Arrijoja, G.M., Segalés, J., Rosell, C., Rovira, A., Pujols, J., Plana-Durán, J., Domingo, M., 2003. Retrospective study on porcine circovirus type 2 infection in pigs from 1985 to 1997 in Spain. *J Vet Med B Infect Dis Vet Public Health* 50, 99-101.
- Rose, N., Larour, G., Le Diguerher, G., Eveno, E., Jolly, J.P., Blanchard, P., Oger, A., Le Dimna, M., Jestin, A., Madec, F., 2003. Risk factors for porcine post-weaning multisystemic wasting syndrome (PMWS) in 149 French farrow-to-finish herds. *Prev Vet Med* 61, 209-225.
- Rosell, C., Segalés, J., Plana-Durán, J., Balasch, M., Rodríguez-Arrijoja, G.M., Kennedy, S., Allan, G.M., McNeilly, F., Latimer, K.S., Domingo, M., 1999. Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. *J Comp Pathol* 120, 59-78.
- Rosell, C., Segalés, J., Ramos-Vara, J.A., Folch, J.M., Rodríguez-Arrijoja, G.M., Duran, C.O., Balasch, M., Plana-Durán, J., Domingo, M., 2000. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. *Vet Rec* 146, 40-43.
- Rovira, A., Balasch, M., Segalés, J., Garcia, L., Plana-Duran, J., Rosell, C., Ellerbrok, H., Mankertz, A., Domingo, M., 2002. Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. *J Virol* 76, 3232-3239.
- Royer, R.L., Nawagitgul, P., Halbur, P.G., Paul, P.S., 2001. Susceptibility of porcine circovirus type 2 to commercial and laboratory disinfectants. *J Swine Health Prod* 9, 281-284.
- Salmon, H., 1984. Immunité chez le fœtus et le nouveau-né: modèle porcin. *Reprod Nutr Dévelop* 24, 197-206.
- Sanchez, R.E., Nauwynck, H.J., McNeilly, F., Allan, G., Pensaert, M.B., 2001a. Porcine circovirus 2 infection in swine foetuses inoculated at different ages of gestation. *Vet Microbiol* 83, 169-176.
- Sanchez, R., Nauwynck, H., Pensaert, M., 2001b. Serological survey of porcine circovirus type 2 antibodies in domestic and feral pig populations in Belgium. In: *Proceedings: ssDNA Viruses of Plants, Birds, Pigs and Primates*, Saint-Malo, France, p. 122.
- Sanchez, R.E., Meerts, P., Nauwynck, H.J., Pensaert, M.B., 2003. Change of porcine circovirus 2 target cells in pigs during development from foetal to early postnatal life. *Vet Microbiol* 95, 15-25.
- Sanchez, R.E.Jr., Meerts, P., Nauwynck, H.J., Ellis, J.A., Pensaert M.B., 2004. Characteristics of porcine circovirus-2 replication in lymphoid organs of pigs inoculated in late gestation or postnatally and possible relation to clinical and pathological outcome of infection. *J Vet Diagn Invest* 16, 175-185.
- Saoulidis, K., Kyriakis, S.C., Kennedy, S., Lekkas, S., Miliotis, Ch.C., Allan, G., Balkamos, G.C., Papoutsis, P.A., 2002. First report of post-weaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome in pigs in Greece. *J Vet Med B Infect Dis Vet Public Health* 49, 202-205.

- Sarli, G., Mandrioli, L., Laurenti, M., Sidoli, L., Cerati, C., Rolla, G., Marcato, P.S., 2001. Immunohistochemical characterisation of the lymph node reaction in pig post-weaning multisystemic wasting syndrome (PMWS). *Vet Immunol Immunopathol* 83, 53-67.
- Saunders, K., Lucy, A., Stanley, J., 1991. DNA forms of the geminivirus African cassava mosaic virus consistent with a rolling circle mechanism of replication. *Nucleic Acids Res* 19, 2325-2330.
- Segalés, J., Domingo, M., 2002. Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. *Vet Q* 24, 109-124.
- Segalés, J., Alonso, F., Rosell, C., Pastor, J., Chianini, F., Campos, E., López-Fuertes, L., Quintana, J., Rodríguez-Arriola, G., Calsamiglia, M., Pujols, J., Domínguez, J., Domingo, M., 2001. Changes in peripheral blood leukocyte populations in pigs with natural postweaning multisystemic wasting syndrome (PMWS). *Vet Immunol Immunopathol* 81, 37-44.
- Segalés, J., Domingo, M., Chianini, F., Majó, N., Domínguez, J., Darwich, L., Mateu, E., 2004. Immunosuppression in postweaning multisystemic wasting syndrome affected pigs. *Vet Microbiol* 98, 151-158.
- Segalés, J., Allan, G.M., Domingo, M., 2005. Porcine circovirus diseases. *Anim Health Res Rev* 6, 119-142.
- Segalés, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynck, H., Larsen, L., Dupont, K., McCullough, K., Ellis, J., Krakowka, S., Mankertz, A., Fredholm, M., Fossum, C., Timmusk, S., Stockhofe-Zurwieden, N., Beattie, V., Armstrong, D., Grasland, B., Bækbo, P., Allan, G., 2008. PCV-2 genotype definition and nomenclature. *Vet Rec* 162, 867-868.
- Shibahara, T., Sato, K., Ishikawa, Y., Kadota, K., 2000. Porcine circovirus induces B lymphocyte depletion in pigs with wasting disease syndrome. *J Vet Med Sci* 62, 1125-1131.
- Shivaprasad, H.L., Hill, D., Todd, D., Smyth, J.A., 2004. Circovirus infection in a Gouldian finch (*Chloebia gouldiae*). *Avian Pathol* 33, 525-529.
- Sipos, W., Duvigneau, J.C., Willheim, M., Schilcher, F., Hartl, R.T., Hofbauer, G., Exel, B., Pietschmann, P., Schmoll, F., 2004. Systemic cytokine profile in feeder pigs suffering from natural postweaning multisystemic wasting syndrome (PMWS) as determined by semiquantitative RT-PCR and flow cytometric intracellular cytokine detection. *Vet Immunol Immunopathol* 99, 63-71.
- Smyth, J.A., Todd, D., Scott, A., Beckett, A., Twentyman, C.M., Brøjer, C., Uhlhorn, H., Gavier-Widen, D., 2006. Identification of circovirus infection in three species of gull. *Vet Rec* 159, 212-214.
- Sorden, S.D., 2000. Update on porcine circovirus and postweaning multisystemic wasting syndrome (PMWS). *Swine Health Prod* 8, 133-136.
- Staebler, S., Sydler, T., Buergi, E., McCullough, K., McNeilly, F., Allan, G., Pospischil, A., 2005. PMWS: an emerging disease identified in archived porcine tissues. *Vet J* 170, 132-134.
- Steinfeldt, T., Finsterbusch, T., Mankertz, A., 2001. Rep and Rep' protein of porcine circovirus type 1 bind to the origin of replication in vitro. *Virology* 291, 152-160.
- Steinfeldt, T., Finsterbusch, T., Mankertz, A., 2006. Demonstration of nicking/joining activity at the origin of DNA replication associated with the rep and rep' proteins of porcine circovirus type 1. *J Virol* 80, 6225-6234.
- Stenger, D.C., Revington, G.N., Stevenson, M.C., Bisaro, D.M., 1991. Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling-circle replication of a plant viral DNA. *Proc Natl Acad Sci USA* 88, 8029-8033.

- Stevenson, G.W., Kiupel, M., Mittal, S.K., Choi, J., Latimer, K.S., Kanitz, C.L., 2001. Tissue distribution and genetic typing of porcine circoviruses in pigs with naturally occurring congenital tremors. *J Vet Diagn Invest* 13, 57-62.
- Stevenson, L.S., McCullough, K., Vincent, I., Gilpin, D.F., Summerfield, A., Nielsen, J., McNeilly, F., Adair, B.M., Allan, G.M., 2006. Cytokine and C-reactive protein profiles induced by porcine circovirus type 2 experimental infection in 3-week-old piglets. *Viral Immunol* 19, 189-195.
- Stewart, M.E., Perry, R., Raidal, S.R., 2006. Identification of a novel circovirus in Australian ravens (*Corvus coronoides*) with feather disease. *Avian Pathol* 35, 86-92.
- Stockhofe-Zurwieden, N., Wellenberg, G.J., Schuurman, G., Zwart, R., 2003. Experimental inoculation of specified pathogen free pigs with porcine circovirus 2 only or in combination with other porcine viruses or immunostimulation, In: *Proceedings of the 4th International Symposium on Emerging and Re-emerging Pig Diseases*, Rome, Italy, pp. 164-165.
- Taga, K., Mostowski, H., Tosato, G., 1993. Human interleukin-10 can directly inhibit T-cell growth. *Blood* 81, 2964-2971.
- Takahashi, K., Iwasa, Y., Hijikata, M., Mishiro, S., 2000. Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. *Arch Virol* 145, 979-993.
- Thacker, B., Wilson, W., Johnson, J., Francisco, C., Schlueter, R., 2008. Circumvent™ PCV vaccine: performance and serology evaluation. In: *Proceedings of the 20th International Pig Veterinary Society Congress*, Durban, South Africa, Volume 2, p. 90.
- Timmusk, S., Wallgren, P., Brunborg, I.M., Wikström, F.H., Allan, G., Meehan, B., McMenamy, M., McNeilly, F., Fuxler, L., Belák, K., Pödersoo, D., Saar, T., Berg, M., Fossum, C., 2008. Phylogenetic analysis of porcine circovirus type 2 (PCV2) pre- and post-epizootic postweaning multisystemic wasting syndrome (PMWS). *Virus Genes* 36, 509-520.
- Tischer, I., Rasch, R., Tochtermann, G., 1974. Characterization of papovavirus- and picornavirus-like particles in permanent pig kidney cell lines. *Zentralbl Bakteriol Parasitenkd Infektionskr Hyg Abt 1 Orig* 26, 153-167.
- Tischer, I., Gelderblom, H., Vetterman, W., Koch, M.A., 1982. A very small porcine virus with circular single-stranded DNA. *Nature* 295, 64-66.
- Tischer, I., Miels, W., Wolff, D., Vagt, M., Griem, W., 1986. Studies on epidemiology and pathogenicity of porcine circovirus. *Arch Virol* 91, 271-276.
- Tischer, I., Peters, D., Rasch, R., Pociuli, S., 1987. Replication of porcine circovirus: induction by glucosamine and cell cycle dependence. *Arch Virol* 96, 39-57.
- Todd, D., Weston, J.H., Soike, D., Smyth, J.A., 2001. Genome sequence determinations and analyses of novel circoviruses from goose and pigeon. *Virology* 286, 354-362.
- Tomás, A., Fernandes, L.T., Valero, O., Segalés, J., 2008. A meta-analysis on experimental infections with porcine circovirus type 2 (PCV2). *Vet Microbiol* doi:10.1016/j.vetmic.2008.05.023.
- Vega-Rocha, S., Byeon, I.J., Gronenborn, B., Gronenborn, A.M., Campos-Olivas, R., 2007. Solution structure, divalent metal and DNA binding of the endonuclease domain from the replication initiation protein from porcine circovirus 2. *J Mol Biol* 367, 473-487.
- Vincent, I.E., Carrasco, C.P., Herrmann, B., Meehan, B.M., Allan, G.M., Summerfield, A., McCullough, K.C., 2003. Dendritic cells harbor infectious porcine circovirus type 2 in the absence of apparent cell modulation or replication of the virus. *J Virol* 77, 13288-13300.

- Vincent, I.E., Carrasco, C.P., Guzylack-Piriou, L., Herrmann, B., McNeilly, F., Allan, G.M., Summerfield, A., McCullough, K.C., 2005. Subset-dependent modulation of dendritic cell activity by circovirus type 2. *Immunology* 115, 388-398.
- Vincent, I.E., Balmelli, C., Meehan, B., Allan, G., Summerfield, A., McCullough, K.C., 2007. Silencing of natural interferon producing cell activation by porcine circovirus type 2 DNA. *Immunology* 120, 47-56.
- Walker, I.W., Konoby, C.A., Jewhurst, V.A., McNair, I., McNeilly, F., Meehan, B.M., Cottrell, T.S., Ellis, J.A., Allan, G.M., 2000. Development and application of a competitive enzyme-linked immunosorbent assay for the detection of serum antibodies to porcine circovirus type 2. *J Vet Diagn Invest* 12, 400-405.
- Wallgren, P., Belák, K., Ehlorsson, C.J., Bergström, G., Lindberg, M., Fossum, C., Allan, G.M., Robertsson, J.A., 2007. Postweaning multisystemic wasting syndrome (PMWS) in Sweden from an exotic to an endemic disease. *Vet Q* 29, 122-137.
- Wallgren, P., Blomqvist, G., Thorén, P., Elander, J., Wallgren, M., 2008. Incidence of PCV2 in semen collected at Swedish boar stations. In: *Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 2*, p. 62.
- Wang, C., Huang, T.S., Huang, C.C., Tu, C., Jong, M.H., Lin, S.Y., Lai, S.S., 2004. Characterization of porcine circovirus type 2 in Taiwan. *J Vet Med Sci* 66, 469-475.
- Wang, X., Jiang, P., Li, Y., Jiang, W., Dong, X., 2007. Protection of pigs against postweaning multisystemic wasting syndrome by a recombinant adenovirus expressing the capsid protein of porcine circovirus type 2. *Vet Microbiol* 121, 215-224.
- Wattrang, E., McNeilly, F., Allan, G.M., Greko, C., Fossum, C., Wallgren, P., 2002. Exudative epidermitis and porcine circovirus-2 infection in a Swedish SPF-herd. *Vet Microbiol* 86, 281-293.
- Welch, J., Bienek, C., Gomperts, E., Simmonds, P., 2006. Resistance of porcine circovirus and chicken anemia virus to virus inactivation procedures used for blood products. *Transfusion* 46, 1951-1958.
- Wen, L., Guo, X., Yang, H., 2005. Genotyping of porcine circovirus type 2 from a variety of clinical conditions in China. *Vet Microbiol* 110, 141-146.
- West, K.H., Bystrom, J.M., Wojnarowicz, C., Shantz, N., Jacobson, M., Allan, G.M., Haines, D.M., Clark, E.G., Krakowka, S., McNeilly, F., Konoby, C., Martin, K., Ellis, J.A., 1999. Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus 2. *J Vet Diagn Invest* 11, 530-532.
- Wiederkehr, D.D., Sydler, T., Brugnera, E., Pospischil, A., Buergi, E., Sidler, X., 2008. Pathogenic differences of porcine circovirus type 2 genotypes in Switzerland. In: *Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 1*, p. 26.
- Wikström, F.H., Meehan, B.M., Berg, M., Timmusk, S., Elving, J., Fuxler, L., Magnusson, M., Allan, G.M., McNeilly, F., Fossum, C., 2007. Structure-dependent modulation of alpha interferon production by porcine circovirus 2 oligodeoxyribonucleotide and CpG DNAs in porcine peripheral blood mononuclear cells. *J Virol* 81, 4919-27.
- Woods, L.W., Latimer, K.S., Barr, B.C., Niagro, F.D., Campagnoli, R.P., Nordhausen, R.W., Castro, A.E., 1993. Circovirus-like infection in a pigeon. *J Vet Diagn Invest* 5, 609-612.
- Yilmaz, A., Kaleta, E.F., 2004. Disinfectant tests at 20 and 10 degrees C to determine the virucidal activity against circoviruses. *Dtsch Tierarztl Wochenschr* 111, 248-251.
- Yoon, K.J., Jepsen, R.J., Pogranichniy, R.M., Sorden, S., Stammer, R., Evans, L.E., 2004. A novel approach to intrauterine viral inoculation of swine using PCV type 2 as a model. *Theriogenology* 61, 1025-1037.

- Yu, S., Opriessnig, T., Kitikoon, P., Nilubol, D., Halbur, P.G., Thacker, E., 2007a. Porcine circovirus type 2 (PCV2) distribution and replication in tissues and immune cells in early infected pigs. *Vet Immunol Immunopathol* 115, 261-272.
- Yu, S., Vincent, A., Opriessnig, T., Carpenter, S., Kitikoon, P., Halbur, P.G., Thacker, E., 2007b. Quantification of PCV2 capsid transcript in peripheral blood mononuclear cells (PBMCs) in vitro. *Vet Microbiol* 123, 34-42.
- Yuasa, N., Taniguchi, T., Yoshida, I., 1979. Isolation and some characteristics of an agent inducing anemia in chicks. *Avian Diseases* 23, 366-385.
- Zizlavsky, M., Czanderlova, L., Tydlit, D., Drabek, J., 2008. Field evaluation of Suvaxyn PCV2 One Dose vaccine. In: *Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 2*, p. 103.

Chapter 2. Aims of the thesis

Porcine circovirus type (PCV2) is a virus that has been present in pig populations worldwide for at least several decades. PCV2 is the causal agent of postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease syndrome in weaned pigs that was recognized for the first time in the early 90's. Besides PMWS, PCV2 also causes reproductive failure. Due to extensive research during the past decade, researchers have acquired substantial knowledge on the pathogenesis of PCV2 infection and its associated syndromes. A high level of PCV2 replication has been identified as a key factor in the development of PMWS, but not all factors that induce a high level of PCV2 replication have been identified yet. It has been shown that host-specific factors such as vaccination, co-infection with other pathogens, stimulation of the immune system as well as depression of the immune system induce a high level of PCV2 replication and the subsequent development of PMWS. Environmental factors such as overcrowding and inaccurate cleaning and disinfection contribute to increased PCV2 replication and PMWS as well. But virus-specific factors that affect PCV2 replication and the occurrence of PMWS are hardly known. Therefore, chapter 3 of this thesis aims to examine to what extent PCV2 strains originating from cases of PMWS, reproductive failure or porcine dermatitis and nephropathy syndrome (PDNS), may be different from antigenic, genetic and pathogenetic point of view. Furthermore, PMWS research is hampered because at present, a universally reproducible, experimental model for PMWS does not exist. Therefore, chapter 4 aims to establish a universally reproducible, experimental PMWS model.

Until recently, it was generally believed that only limited genetic variability existed among PCV2 isolates and that PCV2 isolates were antigenically, virologically and pathogenically very similar. However, recent field observations indicate that 2 distinct genotypes of PCV2, PCV2a (genotype 2) and PCV2b (genotype 1), are circulating in the pig population and that PCV2b (genotype 1) is more frequently associated with PMWS than PCV2a (genotype 2). At present, it is not known which virus-specific factors are responsible for this observation. Chapter 3.1 focuses on the antigenic structure of PCV2 by using monoclonal antibodies to determine the antigenic characteristics of PCV2 isolates from different genotypes and originating from different clinical presentations. Chapter 3.2 addresses the question whether recombination between 2 different PCV2 viruses may give rise to a new, genetically different, recombinant virus in PMWS-affected pigs. Chapter 3.3 aims to determine

if PCV2 isolates are equally able to replicate in porcine tissues. More specifically, by examining the clinical and virological outcome of experimental PCV2 infections in immuno-incompetent foetuses with PCV2 isolates from different genotypes and originating from different clinical presentations.

PMWS-affected pigs suffer from high levels of PCV2 replication and have an impaired humoral and cellular immune response. It is generally believed that specific interactions between PCV2 and the pig's immune system contribute to the induction of a high level of PCV2 replication and the subsequent development of PMWS. Chapter 4 aims to establish a universally reproducible, experimental PMWS model, based on the knowledge that stimulation of the immune system is one of the factors that enhance PCV2 replication and the development of PMWS. More specifically, chapter 4.1 focuses on PCV2 replication in immune cells *in vitro* and *in vivo* after stimulation with concanavalin A, a potent mitogen that causes proliferation and activation of various cell types of the immune system.

**Chapter 3. Antigenic, genetic and
pathogenetic differences between porcine
circovirus type 2 strains**

Chapter 3.1. Antigenic differences among porcine circovirus type 2 strains, as demonstrated by the use of monoclonal antibodies

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Abstract

It was examined if antigenic differences between porcine circovirus type 2 (PCV2) strains can be detected by the use of monoclonal antibodies (mAbs). A subtractive immunization protocol to the PCV2a (genotype 2) Post Weaning Multisystemic Wasting Syndrome (PMWS)-strain Stoon-1010 was used. Sixteen stable hybridomas that produced mAbs with an immunoperoxidase monolayer assay (IPMA) titre of 1,000 or more to Stoon-1010 were obtained. Staining of recombinant PCV2 virus-like particles demonstrated that all mAbs were directed against the PCV2 capsid protein. Cross-reactivity of mAbs was tested by IPMA and neutralization assay for PCV2b (genotype 1) strains 48285, 1206, VC2002 and 1147, and PCV2a (genotype 2) strains 1121 and 1103. Eleven mAbs (9C3, 16G12, 21C12, 38C1, 43E10, 55B1, 63H3, 70A7, 94H8, 103H7 and 114C8) recognized all strains in the IPMA and demonstrated neutralization to Stoon-1010, 48285, 1206 and 1103, but not to VC2002, 1147 and 1121. MAbs 31D5, 48B5, 59C6 and 108E8 did not react with PCV2b (genotype 1) strains or had a reduced affinity compared to PCV2a (genotype 2) strains in the IPMA and neutralization assay. MAb 13H4 reacted in the IPMA with PMWS-strains Stoon-1010, 48285, 1206 and VC2002, and the Porcine Dermatitis and Nephropathy Syndrome-strain 1147, but not with reproductive failure-strains 1121 and 1103. MAb 13H4 did not neutralize any of the tested strains. It can be concluded that despite the high amino acid identity of the capsid protein ($\geq 91\%$), antigenic differences at the capsid protein level are present between PCV2 strains with a different genetic and clinical background.

1. Introduction

Porcine circovirus type 2 (PCV2) is widespread in domestic and wild pigs. It belongs to the family of the *Circoviridae*. Another member of that family, porcine circovirus type 1 (PCV1) was discovered and characterized as a non-cytopathic contaminant of the continuous porcine kidney cell line PK-15 ATCC-CCL33 (Tischer et al., 1974; Tischer et al., 1982). PCV1 is not regarded as a pathogen for pigs (Tischer et al., 1986; Allan et al., 1995), whereas PCV2 is considered as the crucial pathogen in postweaning multisystemic wasting syndrome (PMWS), a multifactorial swine disease that causes wasting and death in weaned piglets (Harding, 1996; Nayar et al., 1997; Ellis et al., 1998; Allan and Ellis, 2000). Besides wasting, PCV2 may also cause reproductive failure (West et al., 1999; Ladekjær-Mikkelsen et al., 2001; Meehan et al., 2001; Sanchez et al., 2001; Sanchez et al., 2003; Mateusen et al., 2004). PCV2 has also been isolated from pigs with porcine dermatitis and nephropathy syndrome (PDNS) and a various number of other diseases, but neither PDNS nor these other diseases have been reproduced experimentally (Allan et al., 2000; Rosell et al., 2000; Segalés et al., 2005, Wellenberg et al., 2004).

The PCV2 virion measures approximately 17 nm in diameter, is non-enveloped and consists of a circular single-stranded DNA surrounded by an icosahedral capsid (Allan et al., 1998). The ambisense DNA molecule contains about 1.77 kilobases and 11 putative open reading frames (ORFs) (Hamel et al., 1998). Proteins encoded by 3 of these ORFs are considered to play a role in the pathogenesis of PCV2 infections. ORF1 codes for the replication associated proteins Rep and Rep' (Mankertz and Hillenbrand, 2001; Cheung, 2003; Mankertz et al., 2003). Rep and Rep' are 37.5 and 20.2 kDa, respectively. ORF2 encodes the 27.8 kDa capsid protein (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998; Mankertz et al., 2000; Nawagitgul et al., 2000). The ORF2 protein is the only structural protein. The ORF3 protein has a molecular mass of 11.8 kDa and has recently been associated with apoptosis *in vitro* and with viral pathogenesis in mice (Liu et al., 2005; Liu et al., 2006).

Meerts et al. (2005a) demonstrated biological differences between different PCV2 strains *in vitro*. Replication kinetics of PMWS- and PDNS-associated PCV2 strains were significantly different from reproductive failure-associated PCV2 strains. Recently, it was demonstrated that the virulence of a PCV2 isolate originating from

a PMWS-affected animal differed significantly from an isolate recovered from a sub-clinically infected animal. Important differences in serologic profile, virus replication and severity of lesions were shown after experimental inoculation of specific-pathogen-free pigs (Opriessnig et al., 2006).

Among various strains of PCV2, the identity at the nucleotide level of the Rep protein and the capsid protein is 97-100 % and 91-100 %, respectively. At protein level, identity is 97-100 % for Rep and 89-100 % for capsid (Larochelle et al., 2002). Several studies mentioned that genetic differences in PCV2 are associated with the geographic region from which the isolates originated (Fenaux et al., 2000; Hamel et al., 2000; Mankertz et al., 2000, Kim and Lyoo, 2002) and a recently proposed classification system (Olvera et al., 2007) divides PCV2 into two genotypes (1 and 2) and eight clusters (1A to 1C and 2A to 2E). Nowadays, genotype 1 is called PCV2b and genotype 2 is called PCV2a (Segalés et al., 2008). Although several antigenic domains have been discovered on the capsid protein (Mahé et al., 2000; Lekcharoensuk et al., 2004; Olvera et al., 2007), no association has been established so far between the sequence of the capsid and the pathogenicity of a PCV2 strain (Fenaux et al., 2000; Meehan et al., 2001; Larochelle et al., 2002; Pogranichniy et al., 2002; de Boissésou et al., 2004; Grierson et al., 2004). Until now, mouse monoclonal antibodies (mAbs) directed against PCV2 did not show major differences in reactivity to different PCV2 strains (Allan et al., 1999; McNeilly et al., 2001).

In this study, mAbs to PCV2 were produced, characterized and used to identify antigenic differences between PCV2 strains with a different genotype and originating from different clinical presentations.

2. Material and methods

2.1. Viruses

Seven different PK-15 adapted PCV2 strains were used in this study. Their origin and genotype (Cheung et al., 2007; Olvera et al., 2007) are shown in Table 1. The replication kinetics of these strains have been documented previously (Meerts et al., 2005a). PCV1 originated from the persistently infected PK-15 cell line ATCC-CCL33 (Tischer et al., 1974; Tischer et al., 1982). All used PCV2 strains were passaged 11 to 17 times, except for strain 1121, which was passaged 30 times.

Table 1. Origin of porcine circovirus type 2 (PCV2) strains used in this study.

Strain	Clinical origin	Geographical origin (reference)	Genotype ^a
Stoon-1010	PMWS-affected piglet ^b	Canada (Meehan et al., 1998)	PCV2a
48285	PMWS-affected piglet ^b	France (Meehan et al., 1998)	PCV2b
1206	PMWS-affected piglet ^b	Belgium (Meerts et al., 2005a)	PCV2b
VC2002	PMWS-affected piglet ^b	Belgium (Meerts et al., 2004)	PCV2b
1147	PDNS-affected piglet ^c	UK (Meehan et al., 2001)	PCV2b
1121	aborted fetuses	Canada (Meehan et al., 2001)	PCV2a
1103	aborted fetuses	Canada (Meehan et al., 2001)	PCV2a

^a According to the classification system of Segalés et al. (2008)

^b PMWS: postweaning multisystemic wasting syndrome

^c PDNS: porcine dermatitis and nephropathy syndrome

2.2. Recombinant PCV2 virus-like particles

PCV2 virus-like particles (VLPs) were obtained by infecting *Spodoptera frugiperda* 9 (Sf9) insect cells with a baculovirus recombinant P054 expressing the ORF2 of PCV2 strain Stoon-1010. Purification of VLPs was performed in a caesium chloride gradient as described by Nawagitgul et al. (2000).

2.3. Cells

PCV negative PK-15 cells and the persistently PCV1 infected PK-15 cell line ATCC -CCL33 were grown in minimal essential medium (MEM) containing Earle's salts (Gibco, Grand Island, USA), supplemented with 5 % or 10 % foetal bovine serum (FBS), 0.3 mg ml⁻¹ glutamine, 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin and 0.1 mg ml⁻¹ kanamycin. Cell cultures were maintained at 37 °C in the presence of 5 % CO₂.

2.4. Mouse immunization

Before immunization, mice were made immuno-tolerant to PK-15 cells as described by Matthew and Sandrock (1987). Four 6-weeks-old female Balb/c mice were injected intraperitoneally (IP) with 1.5 x 10⁷ PCV negative PK-15 cells in a volume of 300 µl phosphate-buffered saline (PBS). Ten minutes, 24 hours and 48 hours

later, cyclophosphamide (Sigma, Bornem, Belgium) was injected IP at a dose of 100 mg kg⁻¹ body weight in a total volume of 500 µl PBS. Three and six weeks later, injections with PK-15 cells and cyclophosphamide were repeated. Two weeks after the last treatment, 2.25 x 10⁷ Stoon-1010 inoculated PK-15 cells were injected IP in a volume of 300 µl PBS mixed with an equal amount of complete Freund's adjuvant (Sigma). At this time point and two weeks later, sera of mice were collected. Three weeks after the inoculation with PCV2, one mouse received an IP injection with 4.5 x 10⁷ Stoon-1010 inoculated PK-15 cells diluted in 600 µl PBS. Euthanasia was performed 4 days later and the spleen was collected.

2.5. Production and screening of hybridomas

Hybridoma cells were produced by fusion of spleen cells with SP 2/0 myeloma cells as described by Galfre and Milstein (1981). The resulting hybridoma cells were maintained in RPMI 1640 (Gibco, Grand Island, USA) supplemented with 10 % FBS. PCV2-specific mAbs in supernatant fluids were demonstrated on PCV negative and Stoon-1010 inoculated PK-15 cells by an IPMA adapted from Labarque et al. (2000). After incubation with undiluted supernatant fluids for 1 h at 37 °C, cells were washed twice with PBS. Subsequently, a 1:500 dilution of horseradish-peroxidase-labelled goat anti-mouse polyclonal antibodies (Abs) (Dako, Glostrup, Denmark) in PBS were added for 1 h at 37 °C. After washing twice in PBS, substrate solution was added and cell cultures were analyzed by light microscopy (Olympus Optical Co., Hamburg, Germany). Selected hybridoma cultures were cloned by limiting dilution.

2.6. Determination of monoclonal antibody class

The isotype of the produced mAbs was determined using a peroxidase-based commercial mouse mAb identification kit (Zymed, San Francisco, USA). This test identifies the IgG1, IgG2a, IgG2b, IgG3, IgA and IgM isotype classes and the κ and λ type of light chains by the use of mono-specific rabbit polyclonal Abs. Supernatant fluids of anti-PRV mAbs 13D12 (IgG1) and 1C11 (IgG2a) (Nauwynck and Pensaert, 1995) and anti-*E. coli* mAb E7G3 (IgG3) (Tiels et al., 2007) were used as positive controls.

2.7. Indirect immunofluorescence staining of recombinant PCV2 virus-like particles

The VLP staining technique was adapted from Misinzo et al. (2005). Briefly, purified VLPs were diluted 1:100 in PBS, smeared onto microscope slides, air-dried and fixed with 3 % (w/v) paraformaldehyde in PBS for 10 min at room temperature. Fixed VLPs were incubated with undiluted hybridoma supernatants for 1 h at 37 °C, followed by an incubation with a 1:500 dilution of FITC-labelled goat anti-mouse Abs (Molecular Probes, Eugene, USA) containing 10 % PCV2 negative goat serum (NGS) for 1 h at 37 °C. MAb F217 (McNeilly et al., 2001) diluted 1:50 in PBS was used as a positive control. MAbs 13D12 and 1C11 were included as negative controls. A Leica DM/RBE fluorescence microscope (Leica Microsystems GmbH, Heidelberg, Germany) was used for visualisation.

2.8. Western blot analysis

Stoon-1010 inoculated and mock-inoculated PCV negative PK-15 cells were harvested by scraping. Cells were pelleted by centrifugation at 15,700 x g for 20 min at 4 °C and subsequently lysed for 1 h at 37 °C in TNE (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA) containing 1% NP-40 (Roche, Mannheim, Germany), protease inhibitors (Complete; Roche, Mannheim, Germany) and 0.5 % SDS. Cells were centrifuged at 15,700 x g for 10 min at 4 °C and resuspended in a non-reducing Laemmli buffer. This mixture was boiled for 5 min and stored at -20 °C until use. Proteins were separated by standard SDS-PAGE (Laemmli, 1970) and transferred to a PVDF membrane (Amersham Biosciences, England). This membrane was then incubated for 1 h at room temperature in PBS containing 0.1 % Tween 20 (PBS-Tween), supplemented with 5 % bovine serum albumin (Sigma, Bornem, Belgium). After washing in PBS-Tween, membranes were incubated overnight at 4 °C with a 1:5 dilution of the mAbs in PBS-Tween. MAb F190 (McNeilly et al., 2001) and biotinylated purified porcine polyclonal Abs, originated from a PCV2 negative SPF pig inoculated with strain 1121 (Pensaert et al., 2004; Meerts et al., 2005a), were used as positive controls. MAbs 13D12 and 1C11 were included as negative controls. Afterwards, a 1:300 dilution of biotinylated polyclonal sheep anti-mouse Abs and a 1:300 solution of a streptavidin-biotinylated-horseradish peroxidase complex (Amersham Biosciences, England) were applied. Membranes were washed

twice with PBS-Tween in between the incubations. Antigen-Ab complexes were visualised by an enhanced chemiluminescence assay (Amersham Biosciences, England).

2.9. Reactivity of monoclonal antibodies to different PCV2 strains

PCV2 strains Stoon-1010, 48285, 1206, VC2002, 1147, 1121 and 1103 were used to make 96-well IPMA plates as described by Labarque et al. (2000). PCV negative PK-15 cells and the persistently PCV1 infected PK-15 cell line were used for control IPMA plates. The staining procedure was similar to the IPMA technique described above. Ten-fold dilutions of hybridoma supernatants were made in PBS and used as primary Abs. 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 3 times for each strain.

2.10. Sensitive neutralization assays

In order to detect the neutralizing activity of the mAbs, a sensitive neutralization assay was adapted from Meerts et al. (2005b). Briefly, $10^{4.3}$ TCID₅₀ PCV2 in a volume of 200 µl was incubated for 1 h at 37 °C with 200 µl of undiluted hybridoma supernatant. After incubation, this mixture was added to semi-confluent monolayers of PCV negative PK-15 cells in 4 wells of a 96-well plate. After 1 h at 37 °C, cell cultures were washed twice in MEM and fresh medium was added. Cell cultures were fixed 36 hours later. At this time point the first replication cycle of PCV2 was completed (Meerts et al., 2005a). PCV2 infected PK-15 cells were stained by an IPMA using porcine polyclonal PCV2-specific Abs, originating from a Stoon-1010 inoculated gnotobiotic pig. The number of infected cells per well was determined by light microscopy. The neutralizing activity of a hybridoma supernatant was expressed as the percentage of reduction in the number of infected cells in comparison with medium. Assays were performed with all 7 strains. Anti-PCV2 mAb F190 was used as a positive control. MAbs 13D12 and 1C11 were used as negative controls. A mAb was considered as neutralizing when its mean neutralizing activity was higher than the mean neutralizing activity + the standard deviation of the negative controls. Sensitive neutralization experiments were performed 3 times for each strain.

2.11. Sequencing of ORF2 from strains 1206 and VC2002

The Belgian PCV2 strains 1206 and VC2002 were purified by ultracentrifugation at 180,000 x g for 3 h through a 30 % sucrose gradient as described by Delputte et al. (2002). A set of PCR primers was designed based on the alignment of the genome sequences of strains Stoon-1010, 48285, 1147, 1121 and 1103. The primer set PCV2-FW (5'-AGCGCACTTCTTTTCGTTTTTCAG-3') and PCV2-REV: (5'-GAATGCGGCCGCTTATCACTTCGTAATGGTTTTTATTATTCA-3') amplifies the complete ORF2. Two internal oligonucleotides were synthesized: CV1 (5'-GGGCTGTGGCCTTTGKTAC-3') and CV2 (5'-TGTRGACCACGTAGGCCTCG-3'). These internal oligonucleotides were as in Fenaux et al. (2000) but with minor modifications and were used for sequencing. A 1/200 fraction of proteinase K treated ultra purified PCV2 was used as template in PCR reactions using Platinum *Pfx* DNA polymerase (Invitrogen, Merelbeke, Belgium) at 60°C annealing temperature and using the cycling conditions as described by the manufacturer. PCR products (approximately 800 bp) 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) and PCV2 primers. Cycle sequencing reaction products were purified using ethanol precipitation and separated on an ABI Genetic Analyzer 310 (Applied Biosystems, Foster City, USA). Additionally, PCR products (approximately 800 bp) were gel purified using a QiaQuick gel extraction kit (Qiagen Benelux, Venlo, The Netherlands) and cloned in pBluescript II SK(+) cut with EcoRV and treated with Antarctic Phosphatase. Clones containing the PCV2 ORF2 were sequenced using T7 and T3 primers as described above. The sequences were analyzed and compiled using Align, LAlign, ClustalW and Sixframe in the workbench (workbench.sdsc.edu) and Align2sequences, BlastN and BlastP at www.ncbi.nlm.nih.gov. Phylogenetic relationships among sequences were analyzed as described by Tripathi and Sowdhamini (2006). Briefly, phylogenetic trees were derived from multiple sequence alignments with PHYLIP version 3.67. Bootstrapping was performed 100 times using SEQBOOT. Pairwise distances between genomic sequences and protein sequences were determined with DNADIST and PROTDIST respectively. Neighbor-Joining (NJ) trees were calculated with NEIGHBOR and Maximum Likelihood (ML) trees with DNAML and PROML.

Majority rule consensus trees were obtained with CONSENSE and visualized with DRAWGRAM.

The ORF2 sequences (from ATG-stop: 702 nt) from strains 1206, VC2002-k2 and VC2002-k39 have been deposited in GenBank: accession numbers EF990644, EF990645 and EF990646 respectively.

3. Results

3.1. Mouse immunization

Prior to immunization, 4 Balb/c mice were made immuno-tolerant to PK-15 cells by repeated injection of PCV negative PK-15 cells and cyclophosphamide. After this treatment, no or little reaction to PK-15 cells was observed on IPMA. All serum samples taken before immunization were negative for anti-PCV2 antibodies as determined by IPMA. Two weeks after the first immunization, all mice had anti-Stoon-1010 Ab titres between 2,560 and 40,960. One mouse with an IPMA Ab titre of 10,240 and without reaction to PK-15 cells was selected. It received a boost injection one week later and its spleen was used for the production of hybridomas.

3.2. Production and screening of hybridomas

Forty-four hybridomas that produced mAbs against PCV2 infected PK-15 cells were frozen. Cloning by limiting dilution resulted in 16 stable hybridomas that produced mAbs with an IPMA titre of 1,000 or more to Stoon-1010.

3.3. Determination of monoclonal antibody class

A commercial identification kit was used to determine the isotypes of the mAbs. This is presented in Table 2. Six hybridomas produced IgG1 Abs and 8 hybridomas produced IgG2a Abs. MAb 21C12 had an IgG3 isotype. The isotype of mAb 48B5 could not be determined. All mAbs, including mAb 48B5 had a light chain of the κ -type.

3.4. Indirect immunofluorescence staining of recombinant PCV2 virus-like particles

The reactivity of the mAbs to VLPs was tested by performing an indirect immunofluorescence staining on VLPs that were smeared onto glass slides. All 16 mAbs reacted with the VLPs indicating that the mAbs were directed against the PCV2 capsid protein. No staining was observed with irrelevant mAbs.

3.5. Western blot analysis

The reactivity of the mAbs to Stoon-1010 inoculated PK-15 cells was determined in a Western blot assay. MAb 31D5, 38C1 and 108E8 gave a strong and specific reaction with a protein of approximately 28 kDa. This is demonstrated in Fig. 1. For mAb 21C12 a faint but specific band was observed at 28kDa. None of the other mAbs showed reactivity in the Western blot assay.

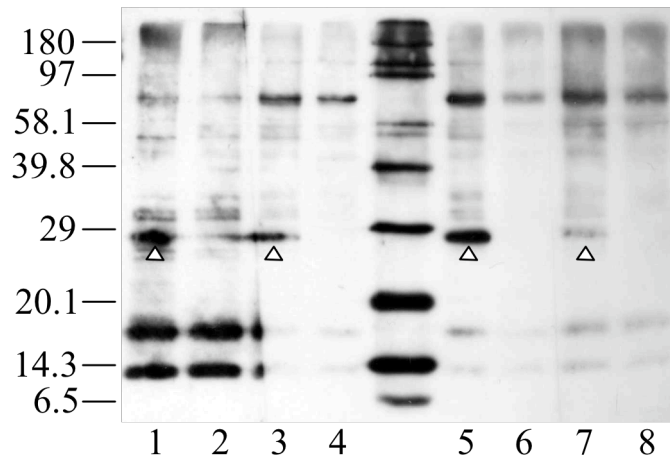


Fig. 1. Western blotting analysis of PCV2 (Stoon-1010) and mock inoculated PK-15 cells. Odd numbers represent PCV2 (Stoon-1010) inoculated cell lysates, even numbers show mock inoculated cell lysates. Lanes 1 and 2: mAb F190 (positive control). Lanes 3 and 4: mAb 31D5. Lanes 5 and 6: mAb 38C1. Lanes 7 and 8: mAb 108E8. All 4 mAbs reacted specifically with a 28 kDa protein (arrowhead).

3.6. Reactivity of monoclonal antibodies to different PCV2 strains

An IPMA was used to examine the reactivity of hybridoma supernatants to 7 different PCV2 strains (Table 2). Eleven out of 16 hybridomas stained all 7 strains with a maximum 10-fold variation in titres in between the strains (9C3, 16G12, 21C12, 38C1, 43E10, 55B1, 63H3, 70A7, 94H8, 103H7 and 114C8). MAbs 31D5, 48B5, 59C6 and 108E8 did not react with the PCV2b (genotype 1) strains 48285, VC2002 and 1147 or they had IPMA Ab titres to these strains that were at least 100 times lower than for the PCV2a (genotype 2) strains Stoon-1010, 1121 and 1103. These 4 mAbs stained 2 different populations of infected cells in strain 1206. IPMA Ab titres for the first population (approximately 99 % of the infected cells) were comparable to those of the other PCV2b (genotype 1) strains. IPMA Ab titres for the second population (approximately 1 % of the infected cells) were comparable to those of the PCV2a (genotype 2) strains. These populations were determined by counting the number of infected cells per well after staining with different dilutions of the mAbs. MAb 13H4 stained all 4 PMWS-associated (Stoon-1010, 48285, 1206 and VC2002) and the single PDNS-associated strain (1147) but did not react with the 2 reproductive failure-associated strains (1121 and 1103). None of the 16 mAbs reacted with PCV1 or PK-15 cells.

Table 2. Isotype and IPMA antibody titres of hybridoma supernatants.

MAb	Isotype	IPMA Ab titres ^a											
		1010 PMWS PCV2a	48285 PMWS PCV2b	1206 PMWS PCV2 b	VC2002 PMWS PCV2 b	1147 PDNS PCV2 b	1121 abortion PCV2 a	1103 abortion PCV2 a	PCV1				
9C3	IgG1	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	negative
13H4	IgG2a	1,000	1,000	1,000	100	100	100	100	100	100	100	100	negative
16G12	IgG2a	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	negative
21C12	IgG3	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	negative
31D5	IgG1	10,000	negative	negative / 1,000 ^b	negative	negative	negative	negative	negative	10,000	10,000	10,000	negative
38C1	IgG1	100,000	10,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000	negative
43E10	IgG1	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	negative
48B5	no reaction	1,000	negative	negative / 100 ^b	negative	negative	negative	negative	negative	1,000	1,000	100	negative
55B1	IgG2a	10,000	1,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	negative
59C6	IgG2a	10,000	10	100 / 10,000 ^b	100	100	100	100	100	10,000	10,000	10,000	negative
63H3	IgG2a	10,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	10,000	10,000	10,000	negative
70A7	IgG2a	10,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	10,000	10,000	1,000	negative
94H8	IgG2a	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	negative
103H7	IgG2a	10,000	1,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	negative
108E8	IgG1	1,000	10	negative / 1,000 ^b	1	1	1	1	1	1,000	1,000	1,000	negative
114C8	IgG1	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	negative

^a IPMA antibody titres of a hybridoma supernatant were expressed as the reciprocal of the last dilution that resulted in a positive reaction.

^b MAbs 31D5, 48B5, 59C6 and 108E8 stained 2 different populations of infected cells in strain 1206. IPMA Ab titres for the first population (approximately 99 % of the infected cells, at the left of the dash) were comparable to those of the PCV2b (genotype 1) strains 48285, VC2002 and 1147. IPMA Ab titres for the second population (approximately 1 % of the infected cells, at the right of the dash) were comparable to those of the PCV2a (genotype 2) strains 1010, 1121 and 1103. These populations were determined by counting the number of infected cells per well after staining with different dilutions of the mAbs.

3.7. Sensitive neutralization assays

A sensitive neutralization assay was used to determine the neutralizing activity of hybridoma supernatants. Table 3 shows the neutralization % with the standard deviations of the different mAbs. The neutralizing activities of mAbs 13D12 and 1C11 were 7 ± 19 % and -1 ± 14 % respectively. Because the mean neutralizing activity of mAb 13D12 + its standard deviation was $7 + 19 = 26$ %, a mAb was arbitrarily considered as neutralizing when its mean neutralizing activity was higher than 30 %. The 11 mAbs (9C3, 16G12, 21C12, 38C1, 43E10, 55B1, 63H3, 70A7, 94H8, 103H7 and 114C8) that reacted equally with all 7 PCV2 strains in the IPMA demonstrated neutralization to Stoon-1010 (up to 95 %), 48285 (up to 94 %), 1206 (up to 57 %) and 1103 (up to 61 %). The 4 mAbs (31D5, 48B5, 59C6 and 108E8) that had a higher affinity for PCV2a (genotype 2) strains than for PCV2b (genotype 1) strains in the IPMA demonstrated neutralization to the PCV2a (genotype 2) strains Stoon-1010 (up to 98 %) and 1103 (up to 67 %). For these 4 mAbs, neutralization of PCV2b (genotype 1) strains 48285 and 1206 was absent or very low (up to 35 %). MAb 13H4 did not neutralize any of the 7 tested strains. Only one mAb (21C12) demonstrated some neutralization (32 %) to strain VC2002 and only two mAbs (9C3 and 38C1) demonstrated some neutralization (34 % and 30 % respectively) to strain 1147. None of the 16 mAbs neutralized strain 1121.

Table 3. Neutralizing activity of hybridoma supernatants.

MAb	% neutralization \pm standard deviation						
	1010	48285	1206	VC2002	1147	1121	1103
	<i>PMWS</i> <i>PCV2a</i>	<i>PMWS</i> <i>PCV2b</i>	<i>PMWS</i> <i>PCV2b</i>	<i>PMWS</i> <i>PCV2b</i>	<i>PDNS</i> <i>PCV2b</i>	<i>abortion</i> <i>PCV2a</i>	<i>abortion</i> <i>PCV2a</i>
9C3	94 \pm 3	92 \pm 5	54 \pm 10	1 \pm 13	34 \pm 20	11 \pm 12	54 \pm 16
13H4	-3 \pm 14	8 \pm 15	10 \pm 14	12 \pm 12	-4 \pm 15	7 \pm 11	6 \pm 16
16G12	91 \pm 3	90 \pm 6	41 \pm 14	6 \pm 18	19 \pm 15	-4 \pm 9	26 \pm 11
21C12	94 \pm 1	82 \pm 8	44 \pm 14	32 \pm 16	0 \pm 19	-12 \pm 18	56 \pm 21
31D5	92 \pm 2	11 \pm 13	6 \pm 9	-4 \pm 8	6 \pm 20	3 \pm 12	54 \pm 20
38C1	95 \pm 1	94 \pm 2	42 \pm 17	20 \pm 10	30 \pm 20	-13 \pm 13	61 \pm 16
43E10	79 \pm 2	89 \pm 2	38 \pm 18	2 \pm 4	22 \pm 20	6 \pm 2	56 \pm 12
48B5	98 \pm 0	19 \pm 6	16 \pm 5	-5 \pm 3	-7 \pm 7	23 \pm 22	49 \pm 13
55B1	84 \pm 2	94 \pm 2	55 \pm 16	-2 \pm 3	25 \pm 12	6 \pm 4	32 \pm 15
59C6	72 \pm 3	30 \pm 9	17 \pm 13	4 \pm 9	4 \pm 10	6 \pm 16	28 \pm 20
63H3	72 \pm 4	77 \pm 3	30 \pm 16	0 \pm 5	10 \pm 8	-14 \pm 18	50 \pm 17
70A7	88 \pm 2	89 \pm 2	57 \pm 17	10 \pm 1	21 \pm 12	-9 \pm 10	39 \pm 16
94H8	62 \pm 14	82 \pm 4	49 \pm 14	6 \pm 5	16 \pm 12	2 \pm 2	56 \pm 8
103H7	51 \pm 6	62 \pm 9	32 \pm 12	5 \pm 12	8 \pm 22	3 \pm 14	55 \pm 19
108E8	88 \pm 2	35 \pm 11	9 \pm 9	8 \pm 3	2 \pm 9	-6 \pm 14	67 \pm 5
114C8	71 \pm 4	74 \pm 7	39 \pm 23	14 \pm 4	20 \pm 9	-10 \pm 17	51 \pm 5

The neutralizing activity of a hybridoma supernatant was expressed as the percentage of reduction in the number of infected cells in comparison with medium. A mean neutralizing activity of 30 % or more was considered as neutralization (indicated in bold).

3.8. Sequencing of ORF2 from strains 1206 and VC2002

The ORF2 of the Belgian PMWS-associated PCV2 strains 1206 and VC2002 was amplified by PCR and sequenced. Strain 1206 contained an ORF2 of 702 bp (starting from ATG including stop codon) encoding a 233 amino acid (aa) protein. Sequencing of the VC2002 ORF2 PCR product resulted in a sequence containing ambiguities at different positions. Therefore the VC2002 PCR fragment was cloned in pBluescript II SK(+) and 12 clones were sequenced. Clone VC2002-k39 contained an ORF of 702 bp (starting from ATG including stop codon) encoding a protein of 233 aa. Ten other VC2002 clones were almost 100% identical at nucleotide (nt) level with k39 with 1-3 nt differences. Clone VC2002-k2 contained an ORF of 705 bp encoding a protein of 234 aa. VC2002-k2 showed 94 % identity with VC2002-k39 at nt and aa level and 96-99% aa identity with strains from China (e.g. AAP44186, AAU87508, AAT97651), The Netherlands (AAS65982, Grierson et al., 2004) and a strain isolated from wild boars in Germany (AAU13781, Knell et al., 2005). Capsid protein similarity amongst the 7 different strains used in this study was determined using pairwise alignments and ClustalW (Fig. 2). The ORF2 aa identity of the strains that were used in this study is demonstrated in Table 4. Figure 3 shows a phylogenetic tree of the ORF2 protein based on the NJ method with the percentages of confidence along the branches. This figure was constructed with ORF2 protein sequences from this study and sequences chosen from the different clusters from Olvera et al. (2007). The latter sequences are shown in Table 5. PCV2b (genotype 1) strains 48285, 1206, VC2002-k39 and 1147 were assigned to cluster 1A/1B, VC2002-k2 to cluster 1C and PCV2a (genotype 2) strains Stoon-1010, 1121 and 1103 to cluster 2E. The same strain classification was obtained with the ML method and with ORF2 DNA sequences.

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1206     MTYPRRRYRRRRHRPRSHLGQILRRRPWLVPVPRHRYRWRKNGIFNTRLSRTFGYTIKRTTVKTPSWAVDMMRFNINDFL 80
1147     MTYPRRRYRRRRHRPRSHLGQILRRRPWLVPVPRHRYRWRKNGIFNTRLSRTFGYTIKRTTVKTPSWAVDMMRFNINDFL 80
48285    MTYPRRRYRRRRHRPRSHLGQILRRRPWLVPVPRHRYRWRKNGIFNTRLSRTFGYTIKRTTVKTPSWAVDMMRFNINDFL 80
VC2002-k39 MTYPRRRYRRRRHRPRSHLGQILRRRPWLVPVPRHRYRWRKNGIFNTRLSRTFGYTIKRTTVKTPSWAVDMMRFNINDFL 80
VC2002-k2  MTYPRRRYRRRRHRPRSHLGQILRRRPWLVPVPRHRYRWRKNGIFNTRLSRTIGYTIKATTVKTPSWAVDMMRFNINDFL 80
1121     MTYPRRRYRRRRHRPRSHLGQILRRRPWLVPVPRHRYRWRKNGIFNTRLSRTFGYTIKRTTVKTPSWAVDMMRFKIDDFV 80
1103     MTYPRRRYRRRRHRPRSHLGQILRRRPWLVPVPRHRYRWRKNGIFNTRLSRTFGYTIKRTTVKTPSWAVDMMRFKIDDFV 80
Stoon-1010 MTYPRRRYRRRRHRPRSHLGQILRRRPWLVPVPRHRYRWRKNGIFNTRLSRTFGYTIKRTTVKTPSWAVDMMRFKIDDFV 80
*****: * :*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:

1206     PPGGGSNPRSVPFYYRIRKVKVEFWPCSPITQDGRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFPSYHSRY 160
1147     PPGGGSNPRSVPFYYRIRKVKVEFWPCSPITQDGRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFPSYHSRY 160
48285    PPGGGSNPRSVPFYYRIRKVKVEFWPCSPITQDGRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFPSYHSRY 160
VC2002-k39 PPGGGSNPRSVPFYYRIRKVKVEFWPCSPITQDGRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFPSYHSRY 160
VC2002-k2  PPGGGSNPLTVPFYYRIRKVKVEFWPCSPITQDGRGVGSSAVILDDNFVTKANALTYDPYVNYSSRHTITQPFPSYHSRY 160
1121     PPGGGTNKISIPFYYRIRKVKVEFWPCSPITQDGRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFPSYHSRY 160
1103     PPGGGTNKISIPFYYRIRKVKVEFWPCSPITQDGRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFPSYHSRY 160
Stoon-1010 PPGGGTNKISIPFYYRIRKVKVEFWPCSPITQDGRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFPSYHSRY 160
*****: * :*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:

1206     FTPKPVLDSTIDYFQPNNKRNQLWLRLQTGNVDHVLGLGTAFENSIYDQEYNIRVTMVQFREFNLKDPPLN- 233
1147     FTPKPVLDSTIDYFQPNNKRNQLWLRLQAGNVDHVGLGTAFENSIYDQEYNIRVTMVQFREFNLKDPPLN- 233
48285    FTPKPVLDSTIDYFQPNNKRNQLWLRLQTTGNVDHVLGLGTAFENSIYDQEYNIRVTMVQFREFNLKDPPLN- 233
VC2002-k39 FTPKPVLDSTIDYFQPNNKRNQLWLRLQTAGNVDHVLGLGTAFENSIYDQEYNIRVTMVQFREFNLKDPPLN- 233
VC2002-k2  FTPKPVLDSTIDYFQPNNKRNQLWLRLQTTGNVDHVLGLGTAFENSKYDQDYNIRITMVQFREFNLKDPPLNK 234
1121     FTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVLGLGTAFENSKYDQDYNIRVTMVQFREFNLKDPPLN- 233
1103     FTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVLGLGTAFENSKYDQDYNIRVTLVQFREFNLKDPPLN- 233
Stoon-1010 FTPKPVLDSTIDYFQPNNKRNQLWLRLQTSGNVDHVLGLAAFENSKYDQDYNIRVTMVQFREFNLKDPPLK- 233
***** *****:***** ***** *****:*****:*****:*****:*****:*****:*****:

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Fig. 2. ORF2 amino acid alignment of the PCV2 strains used in this study. GenBank accession numbers are: EF990644 (1206), AJ293869 (1147), AF055394 (48285), EF990646 (VC2002-k39), EF990645 (VC2002-k2), AJ293868 (1121), AJ293867 (1103) and AF055392 (Stoon-1010). Consensus key: * - single, fully conserved residue; : - conservation of strong groups; . - conservation of weak groups; - no consensus (bold).

Table 4. ORF2 amino acid identity within PCV2 strains used in this study.

Strain	1206	1147	48285	VC2002-k39	1121	1103	1010	VC2002-k2
1206	100	98	98	98	92	91	93	<u>93</u>
1147		100	98	98	92	91	92	<u>93</u>
48285			100	98	93	92	93	<u>94</u>
VC2002-k39				100	93	93	93	<u>94</u>
1121					100	98	97	<u>91</u>
1103						100	97	<u>91</u>
1010							100	<u>92</u>
VC2002-k2								100

The percentage amino acid identity is given. This is the result of pairwise alignments of the ORF2 proteins. In bold, percentage identity between the PCV2b (genotype 1) strains (except VC2002-k2); in bold and italics, percentage identity between the PCV2a (genotype 2) strains; underlined, percentage identity between the VC2002-k2 strain and other strains.

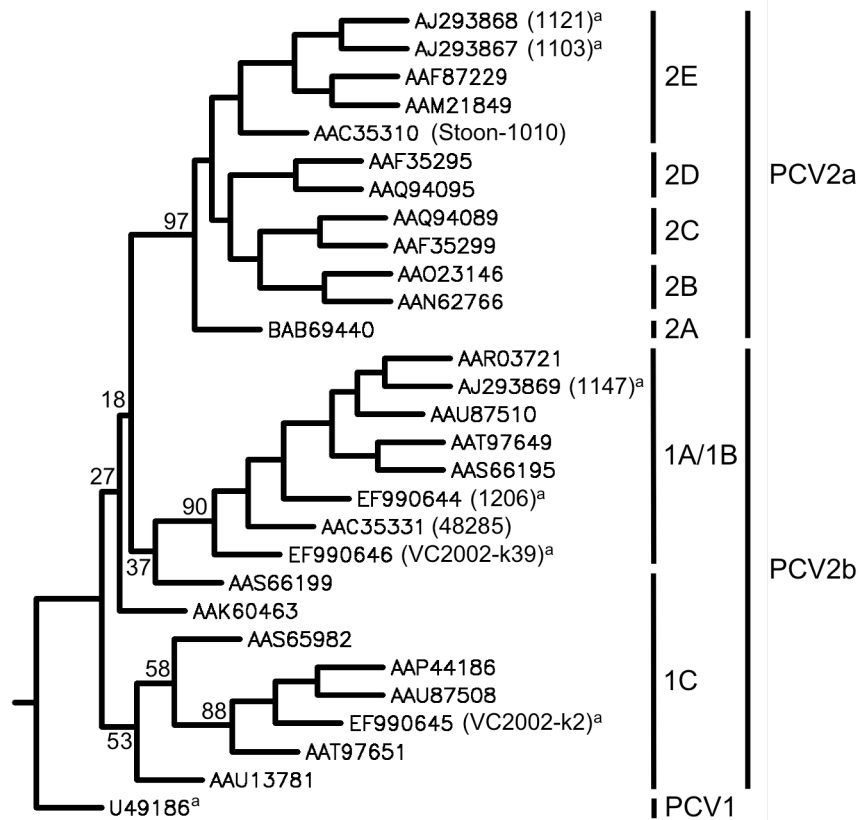


Fig. 3. Unrooted phylogenetic tree constructed by the Neighbor-Joining method. The percentages of confidence are indicated along the branches. This tree is based on the ORF2 protein sequences of the PCV2 strains that were used in the present study (strain name between parentheses), one PCV1 sequence (outgroup) and 20 PCV2 sequences that were obtained from Olvera et al. (2007). These sequences are listed in Table 5.

^a No NCBI protein accession number available for the ORF2 protein, so the GenBank nucleotide sequence accession number was used.

Table 5. Name, phylogenetic cluster (according to Olvera et al., 2007) and origin of the sequences used in Fig. 3.

NCBI protein accession no.	GenBank accession no.	Strain	Cluster	Clinical origin	Geographical origin (reference)
	U49186	pPCV-PS/II	PCV1	PK-15 cell line	UK (Meehan et al., 1997)
AA197649	AY686764	ZJ	1A	unknown	China
AAR03721	AY424404	AUT4	1A	unknown	Austria
AAU87510	AY682992	CHST	1B	unknown	China
AAS66195	AY556475	GX	1B	PMWS	China
AAK60463	AY035820		1C	PMWS	China (Song et al., 2007)
AAS66199	AY556477	HuNan	1C	PMWS	China
AAP44186	AY291317	HB	1C	PMWS	China
AAU87508	AY682991	CHL	1C	unknown	China
AA197651	AY686765	JXIII	1C	unknown	China
AAS65982	AY484410	NI-control-4	1C	subclinical	Netherlands (Grierson et al., 2004)
AAU13781	AY713470	wild boar	1C	subclinical	Germany (Knell et al., 2005)
BAB69440	AB072302	No.26	2A	PMWS	Japan
AAO23146	AY180396	Pingtung-4	2B	unknown	Taiwan
AAN62766	AY146991	Pingtung-1	2B	unknown	Taiwan
AAQ94089	AY256455	212	2C	PDNS	Hungary (Dán et al., 2003)
AAF35299	AF201308	SPA1	2C	PMWS	Spain (Mankertz et al., 2000)
AAQ94095	AY256458	326	2D	PMWS	Hungary (Dán et al., 2003)
AAF35295	AF201306	GER2	2D	PMWS	Germany (Mankertz et al., 2000)
AAM21849	AY094619	688	2E	PMWS	Canada (Cheung, 2003)
AAF87229	AF264039	26607	2E	PMWS	USA (Fenaux et al., 2000)

4. Discussion

This is the first study that demonstrates antigenic diversity among PCV2 strains. This was established by the production and characterization of mAbs directed to the PCV2 capsid protein. The cross-reactivity of the mAbs to 7 different PCV2 strains with a different genotype and originating from various clinical conditions was determined.

Eleven mAbs (9C3, 16G12, 21C12, 38C1, 43E10, 55B1, 63H3, 70A7, 94H8, 103H7 and 114C8) reacted equally with the 7 PCV2 strains that were enclosed in the IPMA. Four other mAbs (31D5, 48B5, 59C6 and 108E8) were able to differentiate the PCV2b (genotype 1) strains 48285, 1206, VC2002 and 1147 from the PCV2a (genotype 2) strains Stoon-1010, 1121 and 1103 by IPMA, since they did not react with PCV2b (genotype 1) strains or had a reduced affinity compared to PCV2a (genotype 2) strains. The IPMA results of the latter 4 mAbs were also reflected in the neutralization assays. Until now, mAbs did not allow to differentiate PCV2 strains (Allan et al., 1999; McNeilly et al., 2001). MAb 31D5, 48B5, 59C6 and 108E8 did also not react with or had a reduced affinity for tissue sections originating from the Belgian PMWS-affected pig from which the VC2002 strain was isolated. This was demonstrated by immunofluorescence staining and suggests that the results obtained by IPMA for mAbs 31D5, 48B5, 59C6 and 108E8 were not a consequence of PCV2 cell culture adaptation (data not shown).

Using the IPMA, mAbs 31D5, 48B5, 59C6 and 108E8 stained 2 different populations of infected cells in strain 1206. This suggests that the 1206 strain consists of 2 viral subpopulations, where 99 % of the virus behaves as a PCV2b (genotype 1) strain and 1 % of the virus behaves as a PCV2a (genotype 2) strain. No signs of the existence of subpopulations were detected by sequencing strain 1206. This may be explained by the fact that the putative PCV2a (genotype 2) subpopulation was present at a very low level (1 %). Sequencing of the VC2002 strain did reveal the existence of 2 PCV2 subpopulations in the virus stock. After cloning, 2 distinct sequences were derived from strain VC2002. Phylogenetic analysis assigned clone VC2002-k39 to cluster 1A/1B and demonstrated clustering of clone VC2002-k2 with strains from China, The Netherlands (Grierson et al., 2004) and a strain isolated from wild German boars (Knell et al., 2005), which documents the putative epidemiological link between PCV2 infections in domestic and wild pigs (Cságola et al., 2006). The identification of 2 different PCV2

sequences in one animal has been reported previously (de Boisséson et al., 2004; Opriessnig et al., 2006; Cheung et al., 2007), but the role of multiple PCV2 infections in the pathogenesis of PCV2-associated diseases is not clear.

Using protein sequences (NJ and ML), we were not able to differentiate between clusters 1A and 1B and not all sequences that were previously classified as 1C (Olvera et al., 2007) were found in the 1C cluster. Using the corresponding DNA sequences (NJ and ML), the same topology was obtained as Olvera et al. (2007), with the only difference that clusters 1A and 1B could not be differentiated in the present study (data not shown). We assume that these differences were a consequence of the reduced number of sequences that was used.

Putative amino acid substitutions that discriminate the PCV2b (genotype 1) strains 48285, 1206, VC2002-k39 and 1147 from the PCV2a (genotype 2) strains Stoon-1010, 1121 and 1103 are located at positions 63, 88, 89 and 206. At position 63, a threonine (T) was substituted for a lysine (K) or an arginine (R). At position 88 a lysine (K) was replaced by a proline (P) and at position 89 an isoleucine (I) was replaced by an arginine (R). These 3 substitutions all involve the basic aa K and R. Due to the differences in size, charge and hydrophobicity between K/R and T, P and I, this may have major consequences on the secondary and tertiary structure of the PCV2 capsid protein. The same comments can be made for position 206, where a lysine (K) was replaced by an isoleucine (I). Linear antigenic determinants of the PCV2 ORF2 protein, as determined by PEPSCAN, are located at positions 65-87, 113-139, 169-183 and 193-207 (Mahé et al., 2000). The positions 63, 88 and 89, where non-conserved mutations were found in the present study, are located at the outer borders of linear epitope 65-87, whereas position 206, where another non-conserved mutation was found, is located at the inner border of linear epitope 193-207. Therefore, we speculate that the aa substitutions that involve basic aa at positions 63, 88, 89 and 206 might be responsible for the fact that mAbs 31D5, 48B5, 59C6 and 108E8 did not react with the PCV2b (genotype 1) strains or that they had a reduced affinity for these strains in the IPMA and neutralization assay.

This study also demonstrated that mAb 13H4 did not react specifically with the reproductive failure-associated strains 1121 and 1103 in the IPMA. Strains 1121 and 1103 have a proline at position 131 instead of a threonine (T131P), and an arginine instead of a glycine at position 191 (G191R). Proline is known to be a helix-breaker and glycine has a great conformational flexibility. Apart from the changes in the primary structure of the protein, T131P and G191R may have important

consequences on the secondary and tertiary structure of a protein. Position 131 is located within and position 191 is located at the outer border of an antigenic domain (Mahé et al., 2000). Therefore, the substitutions at positions 131 and 191 might be involved in the absence of reaction of mAb 13H4 with strains 1121 and 1103. Previously, it was demonstrated by Meerts et al. (2005a) that the production of infectious virus in PK-15 cells is more efficient for Stoon-1010 than for strain 1121. Fenaux et al. (2004) demonstrated that PCV2 that was passaged 120 times in PK-15 cells (VP120) replicates more efficiently in PK-15 cells than wild type virus (VP1). Differences between VP1 and VP120 were a mutation from proline to alanine at position 110 (P110A) and a mutation from arginine to serine at position 191 (R191S). This may suggest that basic aa residues at position 191 influence not only mAb reactivity, but also the production of infectious virus.

Recently, it was demonstrated that PMWS-affected animals are not able to produce neutralizing Abs, whereas their ability to produce non-neutralizing Abs remains unaffected (Meerts et al., 2005b; Meerts et al., 2006; Fort et al., 2007). In these studies, it was suggested that PMWS-affected animals mount an immune response to non-neutralizing epitopes but not to neutralizing epitopes. In the present study, none of the tested mAbs was able to neutralize all 7 PCV2 strains, suggesting that a universal PCV2 neutralizing epitope does not exist. Neutralization was observed to Stoon-1010, 48285, 1206 and 1103, but not to VC2002, 1147 and 1121. No discriminative aa motifs that could explain these results were detected. The mAbs that neutralize Stoon-1010, 48285, 1206 and 1103 did not differentiate these strains from VC2002, 1147 and 1121 in the IPMA, indicating that 2 different groups of PCV2 strains have different neutralizing epitopes, and suggesting that these 2 different groups of PCV2 strains use different entry pathways in PK-15 cells. Recently, the glycosaminoglycans (GAGs) heparan sulfate and chondroitin sulfate B have both been described as attachment receptors for PCV2 (Misinzo et al., 2006). Protein binding to these 2 attachment receptors is restricted to the basic aa lysine (K) and arginine (R) (Esko, 1999), suggesting a crucial role of basic aa residues in the entry of PCV2 into the host cell. Positive aa charges of K and R interact three-dimensionally with negatively charged GAG sulfates and carboxylates (Esko, 1999), which indicates that three-dimensional conformation plays a crucial role in interactions between the PCV2 capsid protein and its receptors.

Until now, it was assumed that no distinct antigenic variation existed among PCV2 isolates. In this study, we clearly demonstrate the existence of major antigenic differences between the capsid proteins of PCV2 strains with a different genotype and isolated from different clinical presentations.

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References

- Allan, G.M., McNeilly, F., Cassidy, J.P., Reilly, G.A., Adair, B., Ellis, W.A., McNulty, M.S., 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. *Vet Microbiol* 44, 49-64.
- Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clark, E.G., Ellis, J.A., Haines, D.M., Meehan, B.M., Adair, B.M., 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. *J Vet Diagn Invest* 10, 3-10.
- Allan, G.M., McNeilly, F., Meehan, B.M., Kennedy, S., Mackie, D.P., Ellis, J.A., Clark, E.G., Espuna, E., Saubi, N., Riera, P., Bøtner, A., Charreyre, C.E., 1999. Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. *Vet Microbiol* 66, 115-123.
- Allan, G.M., Ellis, J.A., 2000. Porcine circoviruses: a review. *J Vet Diagn Invest* 12, 3-14.
- Allan, G.M., McNeilly, F., Kennedy, S., Meehan, B., Moffett, D., Malone, F., Ellis, J., Krakowka, S., 2000. PCV-2-associated PDNS in Northern Ireland in 1990. Porcine dermatitis and nephropathy syndrome. *Vet Rec* 146, 711-712.
- Cheung, A.K., 2003. Transcriptional analysis of porcine circovirus type 2. *Virology* 305, 168-180.
- Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. *Arch Virol* 152, 1035-1044.
- Csárgola, A., Kecskeméti, S., Kardos, G., Kiss, I., Tuboly, T., 2006. Genetic characterization of type 2 porcine circoviruses detected in Hungarian wild boars. *Arch Virol* 151, 495-507.

- Dán, A., Molnár, T., Biksi, I., Glávits, R., Shaheim, M., Harrach, B., 2003. Characterisation of Hungarian porcine circovirus 2 genomes associated with PMWS and PDNS cases. *Acta Vet Hung* 51, 551-562.
- de Boissésón, C., Beven, V., Bigarre, L., Thiery, R., Rose, N., Eveno, E., Madec, F., Jestin, A., 2004. Molecular characterization of Porcine circovirus type 2 isolates from postweaning multisystemic wasting syndrome-affected and non-affected pigs. *J Gen Virol* 85, 293-304.
- Delputte, P.L., Vanderheijden, N., Nauwynck, H.J., Pensaert, M.B., 2002. Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. *J Virol* 76, 4312-4320.
- Esko, J.D., 1999. Glycosaminoglycan-binding Proteins. In: *Essentials of Glycobiology*, pp. 441-453. Edited by A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart, J. Marth. New York: Cold Spring Harbor Laboratory Press.
- Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin, K., McNeilly, F., Meehan, B., Todd, D., Haines, D., 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. *Can Vet J* 39, 44-51.
- Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.J., 2000. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. *J Clin Microbiol* 38, 2494-2503.
- Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004. Two amino acid mutations in the capsid protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and attenuated the virus in vivo. *J Virol* 78, 13440-13446.
- Fort, M., Olvera, A., Sibila, M., Segalés, J., Mateu, E., 2007. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. *Vet Microbiol* 125, 244-255.
- Galfre, G., Milstein, C., 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol* 73, 1-46.
- Grierson, S.S., King, D.P., Wellenberg, G.J., Banks, M., 2004. Genome sequence analysis of 10 Dutch porcine circovirus type 2 (PCV-2) isolates from a PMWS case-control study. *Res Vet Sci* 77, 265-268.
- Hamel, A.L., Lin, L.L., Nayar, G.P., 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *J Virol* 72, 5262-5267.
- Hamel, A.L., Lin, L.L., Sachvie, C., Grudeski, E., Nayar, G.P., 2000. PCR detection and characterization of type-2 porcine circovirus. *Can J Vet Res* 64, 44-52.
- Harding J.C.S., 1996. Postweaning multisystemic wasting syndrome (PMWS): Preliminary epidemiology and clinical presentation. In: *Proceedings of the 27th Annual Meeting of the Western Canadian Association of Swine Practitioners, Saskatoon*, p. 21.
- Kim, J.H., Lyoo, Y.S., 2002. Genetic characterization of porcine-circovirus-2 field isolates from PMWS-pigs. *J Vet Sci* 3, 31-39.
- Knell, S., Willems, H., Hertrampf, B., Reiner, G., 2005. Comparative genetic characterization of Porcine Circovirus type 2 samples from German wild boar populations. *Vet Microbiol* 109, 169-177.
- Labarque, G.G., Nauwynck, H.J., Mesu, A.P., Pensaert, M.B., 2000. Seroprevalence of porcine circovirus types 1 and 2 in the Belgian pig population. *Vet Q* 22, 234-236.
- Ladekjær-Mikkelsen, A.S., Nielsen, J., Storgaard, T., Bøtner, A., Allan, G., McNeilly, F., 2001. Transplacental infection with PCV-2 associated with reproductive failure in a gilt. *Vet Rec* 148, 759-760.

- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227, 680-685.
- Larochelle, R., Magar, M., D'Allaire, S., 2002. Genetic characterization and phylogenetic analysis of porcine circovirus type 2 (PCV2) strains from cases presenting various clinical conditions. *Virus Res* 90, 101-112.
- Lekcharoensuk, P., Morozov, I., Paul, P.S., Thangthumnyom, N., Wajjawalku, W., Meng, X.J., 2004. Epitope mapping of the major capsid protein of type 2 porcine circovirus (PCV2) by using chimeric PCV1 and PCV2. *J Gen Virol* 78, 8135-8145.
- Liu, J., Chen, I., Kwang, J., 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. *J Virol* 79, 8262-8274.
- Liu, J., Chen, I., Du, Q., Chua, H., Kwang, J., 2006. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. *J Virol* 80, 5065-5073.
- Mahé, D., Blanchard, P., Truong, C., Arnauld, C., Le Cann, P., Cariolet, R., Madec, F., Albina, E., Jestin, A., 2000. Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes. *J Gen Virol* 81, 1815-1824.
- Mankertz, A., Domingo, M., Folch, J.M., Le Cann, P., Jestin, A., Segalés, J., Chmielewicz, B., Plana-Duran, J., Soike, D., 2000. Characterisation of PCV-2 isolates from Spain, Germany and France. *Virus Res* 66, 65-77.
- Mankertz, A., Hillenbrand, B., 2001. Replication of porcine circovirus type 1 requires two proteins encoded by the viral rep gene. *Virology* 279, 429-438.
- Mankertz, A., Mueller, B., Steinfeldt, T., Schmitt, C., Finsterbusch, T., 2003. New reporter gene-based replication assay reveals exchangeability of replication factors of porcine circovirus types 1 and 2. *J Virol* 77, 9885-9893.
- Mateusen, B., Sanchez, R.E., Van Soom, A., Meerts, P., Maes, D.G.D., Nauwynck, H.J., 2004. Susceptibility of pig embryos to porcine circovirus type 2 infection. *Theriogenology* 61, 91-101.
- Matthew, W.D., Sandrock, A.W., 1987. Cyclophosphamide treatment used to manipulate the immune response for the production of monoclonal antibodies. *J Immunol Methods* 100, 73-82.
- McNeilly, F., McNair, I., Mackie, D.P., Meehan, B.M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S., Allan, G.M., 2001. Production, characterization and applications of monoclonal antibodies to porcine circovirus 2. *Arch Virol* 146, 909-922.
- Meehan, B.M., Creelan, J.L., McNulty, M.S., Todd, D., 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. *J Gen Virol* 78, 221-227.
- Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *J Gen Virol* 79, 2171-2179.
- Meehan, B.M., McNeilly, F., McNair, I., Walker, I., Ellis, J.A., Krakowka, S., Allan, G.M., 2001. Isolation and characterization of porcine circovirus 2 from cases of sow abortion and porcine dermatitis and nephropathy syndrome. *Arch Virol* 146, 835-842.
- Meerts, P., Nauwynck, H., Sanchez, R., Mateusen, B., Pensaert, M., 2004. Prevalence of porcine circovirus 2 (PCV2)-related wasting on Belgian farms with or without a history of postweaning multisystemic wasting syndrome. *Vlaams Diergen Tijds* 73, 31-38.
- Meerts, P., Misinzo, G., McNeilly, F., Nauwynck, H.J., 2005a. Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, foetal cardiomyocytes and macrophages. *Arch Virol* 150, 427-441.
- Meerts, P., Van Gucht, S., Cox, E., Vandebosch, A., Nauwynck, H.J., 2005b. Correlation

- between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. *Viral Immunol* 18, 333-341.
- Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Bøtner, A., Kristensen, C.S., Nauwynck, H.J., 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. *BMC Vet Res* 2, 6.
- Misinzo, G., Meerts, P., Bublot, M., Mast, J., Weingartl, H.M., Nauwynck, H.J., 2005. Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. *J Gen Virol* 86, 2057-2068.
- Misinzo, G., Delputte, P.L., Meerts, P., Lefebvre, D.J., Nauwynck, H.J., 2006. Porcine circovirus 2 uses heparan sulfate and chondroitin sulfate B glycosaminoglycans as receptors for its attachment to host cells. *J Virol* 80, 3487-3494.
- Morozov, I., Sirinarumit, T., Sorden, S.D., Halbur, P.G., Morgan, M.K., Yoon, K.J., Paul, P.S., 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. *J Clin Microbiol* 36, 2535-2541.
- Nauwynck, H.J., Pensaert, M.B., 1995. Effect of specific antibodies on the cell-associated spread of pseudorabies virus in monolayers of different cell types. *Arch Virol* 140, 1137-1146.
- Nawagitgul, P., Morozov, I., Bolin, S.R., Harms, P.A., Sorden, S.D., Paul, P.S., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. *J Gen Virol* 81, 2281-2287.
- Nayar, G.P., Hamel, A., Lin, L., 1997. Detection and characterization of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *Can Vet J* 38, 385-386.
- Olvera, A., Cortey, M., Segalés, J., 2007. Molecular evolution of porcine circovirus type 2 genomes: Phylogeny and clonality. *Virology* 357, 175-185.
- Opriessnig, T., McKeown, N.E., Zhou, E.M., Meng, X.J., Halbur, P.G., 2006. Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. *J Gen Virol* 87, 2923-2932.
- Pensaert, M.B., Sanchez, R.E., Ladekjær-Mikkelsen, A.S., Allan, G.M., Nauwynck, H.J., 2004. Viremia and effect of fetal infection with porcine viruses with special reference to porcine circovirus 2 infection. *Vet Microbiol* 98, 175-183.
- Pogranichniy, R.M., Yoon, K.J., Harms, P.A., Sorden, S.D., Daniels, M., 2002. Case-control study on the association of porcine circovirus type 2 and other swine viral pathogens with postweaning multisystemic wasting syndrome. *J Vet Diagn Invest* 14, 449-456.
- Rosell, C., Segalés, J., Ramos-Vara, J.A., Folch, J.M., Rodríguez-Arriola, G.M., Duran, C.O., Balasch, M., Plana-Duran, J., Domingo, M., 2000. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. *Vet Rec* 146, 40-43.
- Sanchez, R.E., Nauwynck, H.J., McNeilly, F., Allan, G., Pensaert, M.B., 2001. Porcine circovirus 2 infection in swine fetuses inoculated at different ages of gestation. *Vet Microbiol* 83, 169-176.
- Sanchez, R.E., Meerts, P., Nauwynck, H.J., Pensaert, M.B., 2003. Change of porcine circovirus 2 target cells in pigs during development from foetal to early postnatal life. *Vet Microbiol* 95, 15-25.
- Segalés, J., Allan, G.M., Domingo, M., 2005. Porcine circovirus diseases. *Anim Health Res Rev* 6, 119-142.
- Segalés, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynck, H., Larsen, L., Dupont, K., McCullough, K., Ellis, J., Krakowka, S., Mankertz, A., Fredholm, M., Fossum, C., Timmusk, S., Stockhofe-Zurwieden, N., Beattie, V., Armstrong, D., Grasland, B., Bækbo, P., Allan, G., 2008. PCV-2 genotype definition and nomenclature. *Vet Rec*

162, 867-868.

- Song, Y., Jin, M., Zhang, S., Xu, X., Xiao, S., Cao, S., Chen, H., 2007. Generation and immunogenicity of a recombinant pseudorabies virus expressing cap protein of porcine circovirus type 2. *Vet Microbiol* 119, 97-104.
- Tiels, P., Verdonck, F., Coddens, A., Ameloot, P., Goddeeris, B., Cox, E., 2007. Monoclonal antibodies reveal a weak interaction between the F18 fimbrial adhesin FedF and the major subunit FedA. *Vet Microbiol* 119, 115-120.
- Tischer, I., Rasch, R., Tochtermann, G., 1974. Characterization of papovavirus- and picornavirus-like particles in permanent pig kidney cell lines. *Zentralbl Bakteriol Parasitenkd Infektionskr Hyg Abt 1 Orig* 26, 153-167.
- Tischer, I., Gelderblom, H., Vetterman, W., Koch, M.A., 1982. A very small porcine virus with circular single-stranded DNA. *Nature* 295, 64-66.
- Tischer, I., Miels, W., Wolff, D., Vagt, M., Griem, W., 1986. Studies on epidemiology and pathogenicity of porcine circovirus. *Arch Virol* 91, 271-276.
- Tripathi, L.P., Sowdhamini, R., 2006. Cross genome comparisons of serine proteases in *Arabidopsis* and rice. *BMC Genomics* 7, 200.
- Wellenberg, G.J., Stockhofe-Zurwieden, N., de Jong, M.F., Boersma, W.J., Elbers, A.R., 2004. Excessive porcine circovirus type 2 antibody titres may trigger the development of porcine dermatitis and nephropathy syndrome: a case-control study. *Vet Microbiol* 99, 203-214.
- West, K.H., Bystrom, J.M., Wojnarowicz, C., Shantz, N., Jacobson, M., Allan, G.M., Haines, D.M., Clark, E.G., Krakowka, S. McNeilly, F., Konoby, C., Martin, K., Ellis, J.A., 1999. Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus 2. *J Vet Diagn Invest* 11, 530-532.

Chapter 3.2. Recombination of two porcine circovirus type 2 strains

Archives of Virology, submitted for publication

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Abstract

Pigs can be concurrently infected with different PCV2 strains. In this study, a cell culture-adapted PCV2 strain, originating from a PMWS-affected pig, was purified by limiting dilution cloning. Three different strains were obtained and one of them was a perfect mosaic of the other two, with recombination breakpoints in ORF2 and ORF1. Incongruence was observed between phylogenetic trees constructed with the whole genome, ORF2 and ORF1. Amplification of ORF2 and ORF1 from original material, followed by cloning and sequencing, resulted in sequences corresponding with the parental strains, but not with the mosaic strain. These results demonstrate that PCV2 can undergo recombination.

1. Introduction

Porcine circovirus type 2 (PCV2) is a member of the genus *Circovirus* of the family *Circoviridae* (Pringle, 1999). PCV2 is a ubiquitous pathogen in pigs. Under certain conditions, PCV2 infection may cause postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease that causes severe growth retardation, weight loss and death in weaned pigs (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998). PCV2 is a small, non-enveloped virus with a circular single-stranded DNA. The ambisense genome contains approximately 1.77 Kb and has 2 major open reading frames (ORFs) (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998). ORF1 encodes 2 non-structural replicase proteins; the 314 amino acid (aa) Rep and the 178 aa spliced and frame-shifted Rep' (Cheung 2003; Mankertz et al., 2003). ORF2 codes for the structural 233-234 aa capsid protein (Nawagitgul et al., 2000; Olvera et al., 2007).

A recently proposed classification system divides PCV2 strains into 3 genotypes - PCV2a, PCV2b and PCV2c - based on their genomic sequences (Olvera et al., 2007; Grau-Roma et al., 2008; Segalés et al., 2008). PCV2b genomes were mainly published in GenBank after 2003, whereas PCV2a genomes were mainly published before 2003, suggesting that PCV2b may be more recent than PCV2a (Olvera et al., 2007). Similar observations have been made worldwide in the field (Allan et al., 2007; Cheung et al., 2007; Carman et al., 2008; Dupont et al., 2008; Timmusk et al., 2008). PCV2c genomes were only found in archived material from the eighties (Dupont et al., 2008).

PCV2 mainly evolves due to mutation and/or recombination (Ma et al., 2007; Olvera et al., 2007; Hughes and Piontkivska, 2008). Putative recombination breakpoints were identified previously by *in silico* analysis and were located within or near the origin of replication, in the beginning of ORF1 and diffused along ORF1, as well as in the beginning and at the ending of ORF2 (Ma et al., 2007; Olvera et al., 2007). Hesse et al. (2008) described a chimeric PCV2 sequence with a PCV2a ORF1 and a PCV2b ORF2. Putative recombination events were also suggested by Cságola et al. (2006). Several authors previously reported the simultaneous presence of different PCV2 genome sequences in a pig *in vivo* (de Boisséson et al., 2004; Cheung et al., 2007; Grau-Roma et al., 2008; Hesse et al., 2008) or in a cell culture *in vitro* (Lefebvre et al., 2008), but none of these authors described parental and recombinant sequences.

This paper describes the presence of parental and a recombinant PCV2 strain in a cell culture *in vitro* and evaluates to what extent this recombinant strain differs from its parental strains.

2. Material and methods

2.1. Viruses and cells

The PK-15 cell-adapted PCV2b strain VC2002 was used in the present study. VC2002 originates from an inguinal lymph node from a Belgian PMWS-affected pig (Meerts et al., 2004). Previously, it was shown that the 11th passage of strain VC2002 on PK-15 cells was a mixture of 2 different PCV2b strains; VC2002-k2 and VC2002-k39 (Lefebvre et al., 2008), respectively classified in phylogenetic clusters 1C and 1A/1B from Olvera et al. (2007). VC2002-k2 and VC2002-k39 have 42 nucleotide (nt) differences in ORF2 and the VC2002-k2 ORF2 is 3 nt longer (Lefebvre et al., 2008).

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. Limiting dilution cloning

In order to obtain pure VC2002-k2 and VC2002-k39 strains, the 13th passage of strain VC2002 on PK-15 cells, containing 10^{4.6} TCID₅₀/ml, was diluted in culture medium by making 2-fold or 10-fold serial dilutions. Serial dilutions were inoculated on semi-confluent monolayers of PCV negative PK-15 cells in 96-well plates. One hour later, fresh culture medium was added. At 72 hours post inoculation, supernatant fluids were collected and PCV2 positive wells were detected by an immunoperoxidase monolayer assay (IPMA) as described before (Labarque et al., 2000). In dilutions with less than 10% PCV2 positive wells and with only one PCV2 positive cell per positive well, it was assumed that these positive wells were the result of one single infectious virus particle. Thereafter,

supernatant fluids from such wells were re-inoculated on PCV negative PK-15 cells and passed 15 times on PK-15 cells.

2.3. Sequencing of ORF2 and ORF1

ORF2 was amplified using primers PCV2-FW and PCV2-REV (Lefebvre et al., 2008) and sequenced using these primers and internal oligonucleotides CV1 and CV2 (Lefebvre et al., 2008). ORF1 was amplified using primers CV1 and CV2 or primers PCV2ORF1FW (5'-CAATATCCGTATAACCATGT-3') and PCV2ORF1REV (5'-ATCTGGCCAAGATGGCTGCG-3'). These primers and internal oligonucleotides FWREP (5'-AGGGTGCTGCTCTGCAACGG-3') and REVREP (5'-CATTGTGGGGCCACCTGGGT-3') were used for sequencing. The position of the primers is shown in Fig. 1. Supernatant fluids from limiting dilution clones and a homogenized tissue suspension from the original inguinal lymph node material were used as templates in PCR reactions using Platinum *Pfx* DNA polymerase (Invitrogen, Merelbeke, Belgium). PCR products from limiting dilution clones were sequenced and sequences were analyzed and compiled as described previously (Lefebvre et al., 2008). PCR products from the original inguinal lymph node material were gel purified using a QiaQuick gel extraction kit (Qiagen Benelux, Venlo, The Netherlands) and cloned in a TOPO-blunt vector (Invitrogen) as described by the manufacturer. Subsequently, clones containing the PCV2 ORF2 or ORF1 were sequenced and sequences were analyzed and compiled as described previously (Lefebvre et al., 2008). Phylogenetic relationships among sequences were analyzed as described by Lefebvre et al. (2008).

The sequences of strains II11A ('strain 1'), 4D4 ('strain 3') and II9F ('strain 2') have been deposited in GenBank under accession numbers EU909686, EU909687 and EU909688, respectively.

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'strain 1' ATGACGTATCCAAGGAGCGTTACCGAAGACGAAGACACCGCCCCCGCAGCCATCTTGGCCAGATCCTCCGCCGCCGCCCTGGCTCGTC 90
'strain 3' ATGACGTATCCAAGGAGCGTTACCGAAGACGAAGACACCGCCCCCGCAGCCATCTTGGCCAGATCCTCCGCCGCCGCCCTGGCTCGTC 90
'strain 2' ATGACGTATCCAAGGAGCGTTACCGGAGAAGAAGACACCGCCCCCGCAGCCATCTTGGCCAGATCCTCCGCCGCCGCCCTGGCTCGTC 90
START ORF2
                                ← FCV2ORF1REV
'strain 1' CACCCCGCCACCCTTACCCTGGGAGGAAAATGGCATCTTCAACACCCCGCTTCCCGGCACATCGGTTATACTGTCAAGCTACC 180
'strain 3' CACCCCGCCACCCTTACCCTGGGAGGAAAATGGCATCTTCAACACCCCGCTTCCCGGCACATCGGTTATACTGTCAAGCTACC 180
'strain 2' CACCCCGCCACCCTTACCCTGGGAGGAAAATGGCATCTTCAACACCCCGCTTCCCGGCACATCGGTTATACTGTCAAGCAGAAC 180
'strain 1' ACAGTCAGAACGCCCTCCTGCAATGTGGACATGATGAGATTAAATATTAATGATTTTCTTCCGCCAGGAGGGGGCTCAAACCCCTCACT 270
'strain 3' ACAGTCAGAACGCCCTCCTGCAATGTGGACATGATGAGATTAAATATTAATGATTTTCTTCCGCCAGGAGGGGGCTCAAACCCCTCACT 270
'strain 2' ACAGTCAGAACGCCCTCCTGGCGGTGGACATGATGAGATTCAATATTAATGATTTTCTTCCGCCAGGAGGGGGCTCAAACCCCGCTCT 270
'strain 1' GTGCCCTTGAATACTACAGAATAAGGAAGGTTAAGTTGAATTCGTGCCCTGTCTCCCAATCACCCAGGGTGACAGGGGAGTGGGCTCC 360
'strain 3' GTGCCCTTGAATACTACAGAATAAGGAAGGTTAAGTTGAATTCGTGCCCTGTCTCCCAATCACCCAGGGTGACAGGGGAGTGGGCTCC 360
'strain 2' GTGCCCTTGAATACTACAGAATAAGGAAGGTTAAGTTGAATTCGTGCCCTGTCTCCCGATCACCCAGGGTGACAGGGGAGTGGGCTCC 360
'strain 1' ACTGCTGTATTCTAGATGATAAATTTGTAACAAAGGCCAATGCGCTAACCTATGACCCCTATGTAATACTCTCTCCGCCATACCATA 450
'strain 3' ACTGCTGTATTCTAGATGATAAATTTGTAACAAAGGCCAATGCGCTAACCTATGACCCCTATGTAATACTCTCTCCGCCATACCATA 450
'strain 2' AGTGTCTATTCTAGATGATAAATTTGTAACAAAGGCCAATGCGCTAACCTATGACCCCTATGTAATACTCTCTCCGCCATACCATA 450
BREAKPOINT ORF2 ← CV1
'strain 1' CCCCAGCCCTTCTCTACCCTCCCGCTATTTCACCCCAACCTGCTCTTATAGGAGAAATCGGTAATCTCAACCAATCAACAAGA 540
'strain 3' CCCCAGCCCTTCTCTACCCTCCCGCTATTTCACCCCAACCTGCTCTTATAGGAGAAATCGGTAATCTCAACCAATCAACAAGA 540
'strain 2' CCCCAGCCCTTCTCTACCCTCCCGCTATTTCACCCCAACCTGCTCTTATAGGAGAAATCGGTAATCTCAACCAATCAACAAGA 540
'strain 1' AATCAACTCTGGCTGAGACTACAACTCTGGAATGTAGACCATGATGAGACTCGGCACATGCGCTCGAATAACAGTAAATACGACAGGAC 630
'strain 3' AATCAACTCTGGCTGAGACTACAACTCTGGAATGTAGACCATGATGAGACTCGGCACATGCGCTCGAATAACAGTAAATACGACAGGAC 630
'strain 2' AATCAACTCTGGCTGAGACTACAACTCTGGAATGTAGACCATGATGAGACTCGGCACATGCGCTCGAATAACAGTAAATACGACAGGAA 630
CV2
'strain 1' TACAATATCCGTATAACCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCACTTAAACCTAAGTGAATAATAAAAAACCAT 720
'strain 3' TACAATATCCGTATAACCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCACTTAAACCTAATGAATAATAAAAAACCAT 720
'strain 2' TACAATATCCGTATAACCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCACTTAAACCTAATGAATAATAAAAAACCAT 720
PCV2ORF1FW → STOP ORF2 ← FCV2-REV
'strain 1' ACGAAGTGATAAAAAAGACTCAGTAATTTATTTTCATATGGAATTCAGGGCATGGGGGGAAAGGGTGACGAACCTGGCCCTTCTCTCCG 810
'strain 3' ACGAAGTGATAAAAAAGACTCAGTAATTTATTTTCATATGGAATTCAGGGCATGGGGGGAAAGGGTGACGAACCTGGCCCTTCTCTCCG 810
'strain 2' ACGAAGTGATAAAAAAGACTCAGTAATTTATTTTCATATGGAATTCAGGGCATGGGGGGAAAGGGTGACGAACCTGGCCCTTCTCTCCG 810
PCV2-REV STOP ORF1
'strain 1' TGGATTGTTCTGTAGCATCTCTCCAAATCCCAAGGAAATCTCTCCGATATGAGAGCTTCTACAGCTGGGACAGCAGTTGAGGAGTACC 900
'strain 3' TGGATTGTTCTGTAGCATCTCTCCAAATCCCAAGGAAATCTCTCCGATATGAGAGCTTCTACAGCTGGGACAGCAGTTGAGGAGTACC 900
'strain 2' TGGATTGTTCTGTAGCATCTCTCCAAATCCCAAGGAAATCTCTCCGATATGAGAGCTTCTACAGCTGGGACAGCAGTTGAGGAGTACC 900
BREAKPOINT ORF1
'strain 1' ATTTCAACGGGGTCTGATTGCTGGTAATCAGAATACTCGGGCCAAAAGAGTACAGTTCCACCTTTAGTCTCAACAGTCAAAGGATATC 990
'strain 3' ATTTCAACGGGGTCTGATTGCTGGTAATCAGAATACTCGGGCCAAAAGAGTACAGTTCCACCTTTAGTCTCAACAGTCAAAGGATATC 990
'strain 2' ATTTCAACGGGGTCTGATTGCTGGTAATCAGAATACTCGGGCCAAAAGAGTACAGTTCCACCTTTAGTCTCAACAGTCAAAGGATATC 990
'strain 1' GATCACAGTCTCAGTAGATCATCCACGGCAGCAGCCATAAAAGTCAATCAACAACCACTTCTTCCACATGGTAACCATCCACC 1080
'strain 3' GATCACAGTCTCAGTAGATCATCCACGGCAGCAGCCATAAAAGTCAATCAACAACCACTTCTTCCACATGGTAACCATCCACC 1080
'strain 2' GATCACAGTCTCAGTAGATCATCCACGGCAGCAGCCATAAAAGTCAATCAACAACCACTTCTTCCACATGGTAACCATCCACC 1080
'strain 1' ACTTGTTCCTAGGTGGTTCCAGTATGTGGTTCCGGGTCTGCAAAATAGCAGCCCATTTGCTTTTGCACACCCAGGTGGCCCAAA 1170
'strain 3' ACTTGTTCCTAGGTGGTTCCAGTATGTGGTTCCGGGTCTGCAAAATAGCAGCCCATTTGCTTTTGCACACCCAGGTGGCCCAAA 1170
'strain 2' ACTTGTTCCTAGGTGGTTCCAGTATGTGGTTCCGGGTCTGCAAAATAGCAGCCCATTTGCTTTTACACACCCAGGTGGCCCAAA 1170
REVREP ←
'strain 1' TGACGTGTACATTCGCTCTTCCAATCAGCTTCTGCAATTTCCCGCTCACTTTCAAAGTTTCAGCAGCCCGGGAAATTTCTGACAACG 1260
'strain 3' TGACGTGTACATTCGCTCTTCCAATCAGCTTCTGCAATTTCCCGCTCACTTTCAAAGTTTCAGCAGCCCGGGAAATTTCTGACAACG 1260
'strain 2' TGACGTGTACATTCGCTCTTCCAATCAGCTTCTGCAATTTCCCGCTCACTTTCAAAGTTTCAGCAGCCCGGGAAATTTCTGACAACG 1260
REVREP
'strain 1' TTACAGGGTGTCTGCTCTGCAACGGTACCAGACTCCCGCTCTCAACAAAGGTAATCACAGCAGTAGAGAGGTCACCTCCGTTGCTCTGAG 1350
'strain 3' TTACAGGGTGTCTGCTCTGCAACGGTACCAGACTCCCGCTCTCAACAAAGGTAATCACAGCAGTAGAGAGGTCACCTCCGTTGCTCTGAG 1350
'strain 2' TTACAGGGTGTCTGCTCTGCAACGGTACCAGACTCCCGCTCTCAACAAAGGTAATCACAGCAGTAGAGAGGTCACCTCCGTTGCTCTGAG 1350
FWREP →
'strain 1' ATCTAGGAGTCCACATTTATCAGTAAGTTGCCTCTTTACTGCAATTTCTTATTCGTGATCTGTTCTCTTCTGCTTTCTCGATGT 1440
'strain 3' ATCTAGGAGTCCACATTTATCAGTAAGTTGCCTCTTTACTGCAATTTCTTATTCGTGATCTGTTCTCTTCTGCTTTCTCGATGT 1440
'strain 2' ATCTAGGAGTCCACATTTATCAGTAAGTTGCCTCTTTACTGCAATTTCTTATTCGTGATCTGTTCTCTTCTGCTTTCTCGATGT 1440
'strain 1' GGCAGCGGGCACCAAAATACCCTTCACTTTATTAAGTCTGCTTTCACAAAATAGCGAACCCCTTAGGTGGGTTCTCGCCTT 1530
'strain 3' GGCAGCGGGCACCAAAATACCCTTCACTTTATTAAGTCTGCTTTCACAAAATAGCGAACCCCTTAGGTGGGTTCTCGCCTT 1530
'strain 2' GGCAGCGGGCACCAAAATACCCTTCACTTTATTAAGTCTGCTTTCACAAAATAGCGAACCCCTTAGGTGGGTTCTCGCCTT 1530
'strain 1' CCTCGTTACTTCTCTCGCAACAATAAAATCAATAGGGAATTGGGAGCTCCCGTATTTCTTGGCTGCTCTCGGAAGGATTAT 1620
'strain 3' CCTCGTTACTTCTCTCGCAACAATAAAATCAATAGGGAATTGGGAGCTCCCGTATTTCTTGGCTGCTCTCGGAAGGATTAT 1620
'strain 2' CCTCGTTACTTCTCTCGCAACAATAAAATCAATAGGGAATTGGGAGCTCCCGTATTTCTTGGCTGCTCTCGGAAGGATTAT 1620
'strain 1' TCAGCGTGAACACCCACTTTTATGTTGGTGGGGTCCGCTTCTTCACTCTTCTGCTGGGCAATGTTGCTGAGTGTGCCAGGTT 1710
'strain 3' TCAGCGTGAACACCCACTTTTATGTTGGTGGGGTCCGCTTCTTCACTCTTCTGCTGGGCAATGTTGCTGAGTGTGCCAGGTT 1710
'strain 2' TCAGAGTGAACACCCACTTTTATGTTGGTGGGGTCCGCTTCTTCACTCTTCTGCTGGGCAATGTTGCTGAGTGTGCCAGGTT 1710
START ORF1
'strain 1' CTGCCGCTGCCAAGTGGCTGTAATACTTACAGCGCACTTCTTTCTGTTTTCAGCT 1767
'strain 3' CTGCCGCTGCCAAGTGGCTGTAATACTTACAGCGCACTTCTTTCTGTTTTCAGCT 1767
'strain 2' CTGCCGCTGCCAAGTGGCTGTAATACTTACAGCGCACTTCTTTCTGTTTTCAGAT 1767
PCV2-FW →

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Fig. 1. DNA alignment of PCV2 'strain 1' (EU909686), 'strain 3' (EU909687) and 'strain 2' (EU909688) characterized in this study. Positions of PCR primers used for amplification and sequencing are shown. Start and stop codons of ORF2 and ORF1 are indicated in italics. Recombination breakpoints in ORF2 and ORF1 of 'strain 3' are located between the vertical lines. Bold letters indicate identity between 'strain 3' and 'strains 1 or 2' at positions where 'strain 1' and 'strain 2' differ. Underlined letter indicates a single nucleotide mutation.

3. Results and discussion

3.1. Limiting dilution cloning of the PK-15 cell-adapted PCV2 strain VC2002

Sequences without ambiguities in the chromatogram were obtained for 14 limiting dilution clones and 3 different strains were obtained. Clone II11A ('strain 1') corresponded with VC2002-k2 and clone II9F ('strain 2') corresponded with VC2002-k39. The full-length sequences (1767 nt) of these 2 strains showed 96% identity with each other (69 nt differences). Clone 4D4 ('strain 3') was a perfect mosaic of 'strains 1 and 2' and had a VC2002-k2 sequence from position 1 (ATG from ORF2) till position 362, a VC2002-k39 sequence from position 401 till position 863 and a VC2002-k2 sequence from position 974 till position 1767 (Fig. 1). Since it seems very unlikely that the Belgian PMWS-affected pig, of which the original inguinal lymph node material was derived, was concurrently infected with 3 different PCV2 strains of which one was a perfect mosaic of the other 2, it can be assumed that 'strain 3' originated from recombination between VC2002-k2 and VC2002-k39.

The full-length sequence (1767 nt) of 'strain 3' showed 98% and 97% identity with 'strains 1 and 2', respectively (25 and 44 nt differences). BlastN with the full-length sequence of 'strain 3' showed that this nt sequence was unique. The Chinese strains JZ (DQ206444) and SD4 (DQ201640) had 13 and 15 nt differences with 'strain 3', respectively. The 'strain 3' capsid protein was 233 aa long and had 6 and 7 aa differences with the 'strain 1' and 'strain 2' capsid proteins, respectively. BlastP with the 'strain 3' capsid protein sequence showed that this sequence was unique. Capsid proteins of strains JZ and SD4 were 1 aa different from the 'strain 3' capsid protein. The 'strain 3' Rep protein was identical to the Rep proteins of 'strain 1' and strains JZ and SD4. The 'strain 3' Rep protein differed from the 'strain 2' Rep protein at aa positions 6, 34, 77 and 105. Twelve out of 14 limiting dilution clones were 'strain 3'.

Clones with other recombination breakpoints were not observed. Although further research is required, these data suggest that 'strain 3' has a better *in vitro* replication capacity than 'strains 1 and 2'.

The breakpoint in the 'strain 3' ORF1 (864-973, with ATG from ORF2 as position 1) was adjacent to breakpoints located at positions 985-1020 and 773, previously identified by *in silico* analysis (Olvera et al., 2007) or by base-by-base comparison (Hesse et al., 2008). However, the breakpoint in the 'strain 3' ORF2 (363-400) did not correspond to any of the previously located breakpoints (9-24 and 569-679) in the PCV2 ORF2 (Olvera et al., 2007). To our knowledge, this breakpoint is the first description of a breakpoint that is located centrally in the PCV2 ORF2. Similar recombination breakpoints in the capsid gene have previously been observed in all 3 genera of the family *Circoviridae*, and more specifically in Beak and feather disease virus (Heath et al., 2004), Chicken anemia virus (He et al., 2007) and TT virus (Manni et al., 2002). Base-by-base comparison of strains JZ (DQ206444) and SD4 (DQ201640), strains with 99% nt identity with 'strain 3', showed that strains JZ and SD4 may originate from recombination between a 'strain 1'-like strain and a 'strain 2'-like strain, with a breakpoint in ORF2 between positions 363-400, just like in 'strain 3', but with a breakpoint in ORF1 between positions 1185-1327 instead of 864-973 (data not shown).

3.2. Phylogenetic analysis

In order to provide further proof for the recombinant nature of 'strain 3' and strains JZ and SD4, phylogenetic analysis was performed by nucleotide distance methods. Figure 2 shows phylogenetic trees of full-length genome, ORF1 and ORF2 based on the Neighbor-Joining (NJ) method with the percentages of confidence along the branches. These trees were constructed with DNA sequences from this study and randomly chosen sequences from the different clusters from Olvera et al. (2007) and Lefebvre et al. (2008). 'Strains 1 and 2' always clustered with 1C and 1A strains, respectively (according to the classification system of Olvera et al., 2007). In the full-length genome NJ tree, 'strain 3' clustered with strains JZ and SD4 on a separate branch in the 1C cluster. A similar tree was obtained with the Maximum-Likelihood (ML) method. In the ORF1 NJ and ML trees, 'strain 3' also clustered with 1C strains. However, in the ORF2 NJ tree, 'strain 3' clustered with strains JZ and SD4 on a

separate branch in the 1A/1B cluster, but in the ML tree, these 3 strains clustered with 1C strains.

It was concluded that the chimeric 'strain 3' clustered with 1C or 1A/1B strains, depending on the algorithm used and the part of the genome taken into account. This implies that caution should be taken when interpreting PCV2 phylogenies based on partial genomic sequences, as indicated previously (Cságola et al., 2006; Ma et al., 2007; Hesse et al., 2008). Furthermore, 'strain 3' and strains JZ and SD4 clustered on separate branches in the respective 1C and 1A/1B clusters in the full-length genome and ORF2 NJ trees, showing that recombination increases genetic diversity within certain clusters. An inconsistent phylogeny (Fig. 2) was also observed for strain HuNan (AY556477), previously classified as 1C by Olvera et al. (2007). Base-by-base comparison of HuNan showed that this strain might be considered as a chimeric 1A/1C/1A sequence (versus 1C/1A/1C for 'strain 3') with a breakpoint in ORF2 between positions 298-479 and a breakpoint in ORF1 between positions 703-862 (data not shown), providing further evidence for the existence of recombination breakpoints located centrally in the PCV2 ORF2.

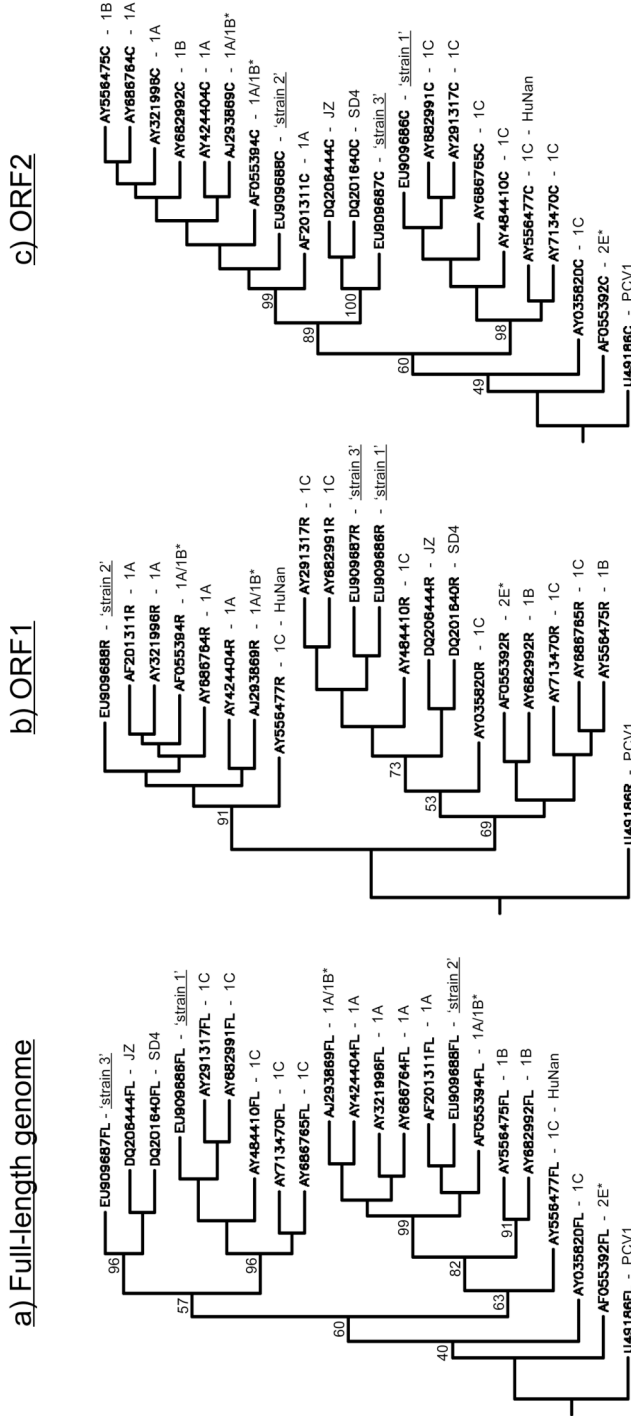


Fig. 2. Unrooted phylogenetic trees constructed by the Neighbor-Joining method. The percentages of confidence are indicated along the branches. Trees are based on DNA sequences of PCV2 strains from the present study ('strain 1', 'strain 2', 'strain 3'), strains JZ and SD4, 13 PCV2b sequences from Olivera et al. (2007), 2 PCV2b and 1 PCV2a (AF055392) sequences from Lefebvre et al. (2008) and 1 PCV1 sequence (outgroup). The genotypes of these strains, according to the classification system of Olivera et al. (2007) (based on full-length genomic sequences), or according to Lefebvre et al. (2008) (based on ORF2 amino acid sequences and indicated with *) are shown. A. Full-length genome. B. ORF1. C. ORF2.

3.3. ORF2 and ORF1 from original inguinal lymph node material

In order to investigate if 'strains 1, 2 and 3' were already present *in vivo*, ORF2 and ORF1 were from the original inguinal lymph node material were amplified by PCR, cloned and sequenced.

Ten ORF2 clones corresponded with 'strain 2' and one ORF2 clone corresponded with 'strain 1'. Chimeric ORF2 clones with a 363-400 breakpoint such as in 'strain 3' were not detected. Twenty-eight ORF1 clones corresponded with 'strain 2', but none of the ORF1 clones corresponded with 'strain 1'. Chimeric ORF1 clones with a 864-973 breakpoint such as in 'strain 3' were not detected. In addition, sequences of ORF2 and ORF1 PCR fragments from the original inguinal lymph node material were pure 'strain 2' sequences, without ambiguities in the chromatogram.

It was concluded that 'strain 2' was most likely more abundantly present in the original inguinal lymph node material than 'strain 1'. Chimeric clones with breakpoints such as in 'strain 3' were not detected. Furthermore, in a PCR experiment using strain-specific primers flanking the ORF2 breakpoint of 'strain 3', it was not possible to amplify a recombinant fragment from the original inguinal lymph node material (data not shown). However, this does not exclude the possibility that 'strain 3' is an *in vivo* recombinant. It is possible that 'strain 3' was not detected in the lymph node material because its number of genomic copies was too low. Other possibilities are that 'strain 3' has a reduced *in vivo* replication capacity compared to 'strains 1 and 2' or that innate and/or specific immunological responses may sort out the more resistant 'strains 1 and 2'. This would require further research.

In conclusion, the present study demonstrates that concurrent replication of different PCV2 strains may lead to recombination. A newly identified recombination breakpoint was located in the PCV2 ORF2. Recombination within PCV2b leads to the emergence of a new sub-cluster.

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References

- Allan, G.M., McNeilly, F., McMenamy, M., McNair, I., Krakowka, S.G., Timmusk, S., Walls, D., Donnelly, M., Minahin, D., Ellis, J., Wallgren, P., Fossum, C., 2007. Temporal distribution of porcine circovirus 2 genogroups recovered from postweaning multisystemic wasting syndrome affected and nonaffected farms in Ireland and Northern Ireland. *J Vet Diagn Invest* 19, 668-673.
- Carman, S., Cai, H.Y., DeLay, J., Youssef, S.A., McEwen, B.J., Gagnon, C.A., Tremblay, D., Hazlett, M., Lulis, P., Fairles, J., Alexander, H.S., van Dreumel, T., 2008. The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease--2004-2006. *Can J Vet Res* 72, 259-268.
- Cheung, A.K., 2003. Transcriptional analysis of porcine circovirus type 2. *Virology* 305, 168-180.
- Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. *Arch Virol* 152, 1035-1044.
- Csárgola, A., Kecskeméti, S., Kardos, G., Kiss, I., Tuboly, T., 2006. Genetic characterization of type 2 porcine circoviruses detected in Hungarian wild boars. *Arch Virol* 151, 495-507.
- de Boissésou, C., Beven, V., Bigarre, L., Thiery, R., Rose, N., Eveno, E., Madec, F., Jestin, A., 2004. Molecular characterization of Porcine circovirus type 2 isolates from postweaning multisystemic wasting syndrome-affected and non-affected pigs. *J Gen Virol* 85, 293-304.
- Dupont, K., Nielsen, E.O., Bækbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. *Vet Microbiol* 128, 56-64.
- Grau-Roma, L., Crisci, E., Sibila, M., López-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A., Segalés, J., 2008. A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. *Vet Microbiol* 128, 23-35.
- Hamel, A.L., Lin, L.L., Nayar, G.P., 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *J Virol* 72, 5262-5267.
- He, C.Q., Ding, N.Z., Fan, W., Wu, Y.H., Li, J.P., Li, Y.L., 2007. Identification of chicken anemia virus putative intergenotype recombinants. *Virology* 366, 1-7.
- Heath, L., Martin, D.P., Warburton, L., Perrin, M., Horsfield, W., Kingsley, C., Rybicki, E.P., Williamson, A.L., 2004. Evidence of unique genotypes of beak and feather disease virus in southern Africa. *J Virol* 78, 9277-9284.

- Hesse, R., Kerrigan, M., Rowland, R.R., 2008. Evidence for recombination between PCV2a and PCV2b in the field. *Virus Res* 132, 201-207.
- Hughes, A.L., Piontkivska, H., 2008. Nucleotide sequence polymorphism in circoviruses. *Infect Genet Evol* 8, 130-138.
- Labarque, G.G., Nauwynck, H.J., Mesu, A.P., Pensaert, M.B., 2000. Seroprevalence of porcine circovirus types 1 and 2 in the Belgian pig population. *Vet Q* 22, 234-236.
- Lefebvre, D.J., Costers, S., Van Doorselaere, J., Misinzo, G., Delputte, P.L., Nauwynck, H.J., 2008. Antigenic differences among porcine circovirus type 2 strains, as demonstrated by the use of monoclonal antibodies. *J Gen Virol* 89, 177-187.
- Ma, C.M., Hon, C.C., Lam, T.Y., Li, V.Y., Wong, C.K., de Oliveira, T., Leung, F.C., 2007. Evidence for recombination in natural populations of porcine circovirus type 2 in Hong Kong and mainland China. *J Gen Virol* 88, 1733-1737.
- Mankertz, A., Mueller, B., Steinfeldt, T., Schmitt, C., Finsterbusch, T., 2003. New reporter gene-based replication assay reveals exchangeability of replication factors of porcine circovirus types 1 and 2. *J Virol* 77, 9885-9893.
- Manni, F., Rotola, A., Caselli, E., Bertorelle, G., Di Luca, D., 2002. Detecting recombination in TT virus: a phylogenetic approach. *J Mol Evol* 55, 563-572.
- Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *J Gen Virol* 79, 2171-2179.
- Meerts, P., Nauwynck, H., Sanchez, R., Mateusen, B., Pensaert, M., 2004. Prevalence of porcine circovirus 2 (PCV2)-related wasting on Belgian farms with or without a history of postweaning multisystemic wasting syndrome. *Vlaams Diergen Tijds* 73, 31-38.
- Morozov, I., Sirinarumitr, T., Sorden, S.D., Halbur, P.G., Morgan, M.K., Yoon, K.J., Paul, P.S., 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. *J Clin Microbiol* 36, 2535-2541.
- Nawagitgul, P., Morozov, I., Bolin, S.R., Harms, P.A., Sorden, S.D., Paul, P.S., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. *J Gen Virol* 81, 2281-2287.
- Olvera, A., Cortey, M., Segalés, J., 2007. Molecular evolution of porcine circovirus type 2 genomes: Phylogeny and clonality. *Virology* 357, 175-185.
- Pringle, C.R., 1999. Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. *Arch Virol* 144, 2065-2070.
- Segalés, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynck, H., Larsen, L., Dupont, K., McCullough, K., Ellis, J., Krakowka, S., Mankertz, A., Fredholm, M., Fossum, C., Timmusk, S., Stockhofe-Zurwieden, N., Beattie, V., Armstrong, D., Grasland, B., Bækbo, P., Allan, G., 2008. PCV-2 genotype definition and nomenclature. *Vet Rec* 162, 867-868.
- Timmusk, S., Wallgren, P., Brunborg, I.M., Wikström, F.H., Allan, G., Meehan, B., McMenemy, M., McNeilly, F., Fuxler, L., Belák, K., Pödersoo, D., Saar, T., Berg, M., Fossum, C., 2008. Phylogenetic analysis of porcine circovirus type 2 (PCV2) pre- and post-epizootic postweaning multisystemic wasting syndrome (PMWS). *Virus Genes* 36, 509-520.

**Chapter 3.3. Clinical and virological outcome of
experimental PCV2a and PCV2b infections in mid-
gestational porcine foetuses**

Theriogenology, submitted for publication

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Abstract

Recently, 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. Seven foetuses were inoculated with PCV2: two with the postweaning multisystemic wasting syndrome (PMWS)-associated PCV2a strain Stoon-1010, two with the reproductive failure-associated PCV2a strain 1121, two with the PMWS-associated PCV2b strain 48285 and one with the porcine dermatitis and nephropathy syndrome-associated PCV2b strain 1147. At 21 days post inoculation, six out of seven PCV2-inoculated foetuses were oedematous and had distended abdomens, whereas the strain 1147-inoculated foetus had a normal external appearance. All PCV2-inoculated foetuses had haemorrhages and congestion in internal organs and an enlarged liver. High PCV2 titres ($> 10^{4.5}$ TCID₅₀ / g tissue) were found in all PCV2-inoculated foetuses, especially in the heart, spleen and liver. High numbers of PCV2-infected cells ($> 1,000$ infected cells / 10 mm² tissue) were observed in the hearts of all PCV2-inoculated foetuses. One strain 1121-inoculated foetus had a low PCV2-specific antibody titre; the other PCV2-inoculated foetuses were negative for PCV2-specific antibodies. Apparent differences in clinical or virological outcome were not observed between PCV2a- and PCV2b-inoculated foetuses. PCV2 did not spread to non-inoculated foetuses. The present study suggests 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; Segalés 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; Ladekjær-Mikkelsen et al., 2001; O'Connor et al., 2001; Farnham et al., 2003; 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 the last third of gestation (Park et al., 2005), by intra-uterine inoculation of SPF sows with PCV2 at insemination (Rose et al., 2007) 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., 2001a; Yoon et al., 2004). The heart is the main target organ for PCV2 infection in foetuses (Sanchez et al., 2001a; Sanchez et al., 2003) and there is a strong correlation between PCV2 replication levels and the severity of the observed histopathological lesions (West et al., 1999; O'Connor et al., 2003; Sanchez et al., 2003; Sanchez et al., 2004; Yoon et al., 2004; Krakowka et al., 2005; 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 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; Segalés et al., 2008). The majority of experimental studies used PCV2a strains to reproduce PCV2-associated reproductive failure in sows (Sanchez et al., 2001a; Johnson et al., 2002; Sanchez et al., 2003; Pensaert et al., 2004; Sanchez 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). To our knowledge, reproductive failure has not been reproduced with PCV2b strains.

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. Material and methods

2.1. Viruses and cells

Four different PK-15 adapted PCV2 strains were used in this study. Their origin, genotype and passage level on PK-15 cells are shown in Table 1. 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 randomly chosen 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.

Table 1. Origin of PCV2 strains used in this study.

Strain	Clinical origin	Geographical origin	Genotype ^a	Passage level in PK-15 cells	Reference
Stoon-1010	PMWS-affected pig ^b	Canada	PCV2a	7	Meehan et al. (1998)
1121	Aborted foetuses	Canada	PCV2a	4	Meehan et al. (2001)
48285	PMWS-affected pig ^b	France	PCV2b	11	Meehan et al. (1998)
1147	PDNS-affected pig ^c	UK	PCV2b	17	Meehan et al. (2001)

^a According to the nomenclature of Segalés et al. (2008).

^b PMWS: postweaning multisystemic wasting syndrome

^c PDNS: porcine dermatitis and nephropathy syndrome

2.2. Experimental design

All Belgian sow herds are PCV2 positive and the seroprevalence of PCV2 is 100 % at the animal level (Labarque et al., 2000; Sanchez et al., 2001b). Due to PCV2 infection-immunity in Belgian sows, experimental PCV2 infections in foetuses have to be performed by direct in utero inoculations of foetuses and not by natural infection routes such as (oro)nasal inoculation of sows during gestation or intra-uterine inoculation of sows at insemination.

Two conventional PCV2 seropositive Landrace sows were submitted to laparotomy at 55 days of gestation. Sows were pre-medicated with azaperonum [Stresnil® 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 anaesthetized 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 and one with PCV2a strain 1121. In the second sow, two foetuses were inoculated with PCV2b strain 48285, one with PCV2b strain 1147 and one with PCV2a strain 1121. Inoculations were performed as described by Sanchez et al. (2001a). Briefly, foetuses were inoculated by trans-uterine injection with 200 µL, containing $10^{4.3}$ TCID₅₀ of PCV2, 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 [Pen-Strep 20/20® 10 mL ip and 10 mL 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 humanly euthanized 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 fetuses 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 authorized and supervised by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University.

2.3. PCV2 replication

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 PCV2-inoculated fetuses, the PCV2 titres in heart, lungs, spleen, liver, kidneys, thymus, tonsils, ileum and cerebrum were determined by virus titration on PK-15 cells as described before (Sanchez et al., 2001a). For non-inoculated fetuses, PCV2 titres were determined in heart, lungs and spleen. Titration experiments were repeated 3 times. For 10 % suspensions, the detection limit of this technique was $10^{2.0}$ TCID₅₀ / g tissue and for 20 % suspensions, the detection limit was $10^{1.7}$ TCID₅₀ / g tissue.

The number of PCV2 antigen positive cells in the hearts of PCV2-inoculated and non-inoculated fetuses was determined by an indirect immunofluorescence staining, adapted from the technique described by Sanchez et al. (2001a). 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 mm² of tissue by using a LEICA DM/RBE fluorescence microscope (Leica Microsystems GmbH, Heidelberg, Germany).

2.4. 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 3 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).

2.5. Statistical analysis

Virus titres were compared between the groups of foetuses inoculated with PCV2a strains and foetuses inoculated with PCV2b strains by applying two-sided Kruskal-Wallis rank sum tests. 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 porcine dermatitis and nephropathy syndrome (PDNS), and between the groups of foetuses from the first sow and foetuses from the second sow. Differences were considered significant when $P < 0.05$. Statistical analyses were performed using S-PLUS (S-PLUS[®] 8.0, Insightful Corporation, Seattle, USA, 2007).

3. Results

3.1. Evaluation of sows

Both sows remained clinically healthy for the entire duration of the study. A rise in their rectal temperatures was not recorded. The first days after laparotomy, 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 pre-serum ranged from 160 to 640 and both pre-sera had a PPV-specific HI Ab titre of 512. Seroconversion against PRRSV or PPV was not observed.

3.2. Gross examinations

All PCV2-inoculated foetuses, except the strain 1147-inoculated foetus, were oedematous and had distended abdomens (Fig. 1.a). Ascites, hydrothorax, hydropericardium, haemorrhages and congestion in internal organs and liver enlargement were observed (Fig. 1.b). Enlarged livers were fragile and easily damaged when manipulated. The strain 1147-inoculated foetus had a normal external appearance, but oedema of the lungs, liver enlargement and generalized lymph node enlargement were observed. Non-inoculated foetuses were normal in appearance.

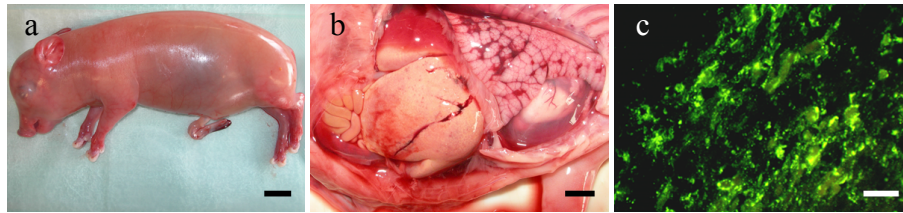


Fig. 1. Effects of PCV2 replication after inoculation of a 55-day-old foetus with PCV2. a) Subcutaneous oedema and abdominal distension. Bar = 2 cm. b) Haemorrhages and congestion in internal organs and liver enlargement. Bar = 1 cm. c) Immunofluorescence staining for the PCV2 capsid protein in the heart. More than 10,000 PCV2 positive cells were present per 10 mm² tissue. Bar = 50 µm.

3.3. PCV2 replication

Table 2 shows the virus titres in different organs of PCV2-inoculated foetuses. The highest PCV2 titres were found in the heart (PCV2a: $10^{6.1\pm 0.9}$ TCID₅₀ / g tissue; PCV2b: $10^{6.2\pm 0.2}$ TCID₅₀ / g tissue), spleen (PCV2a: $10^{5.4\pm 0.3}$ TCID₅₀ / g tissue; PCV2b: $10^{5.3\pm 0.3}$ TCID₅₀ / g tissue), liver (PCV2a: $10^{5.3\pm 0.4}$ TCID₅₀ / g tissue; PCV2b: $10^{5.0\pm 0.7}$ TCID₅₀ / g tissue) and thymus (PCV2a: $10^{4.1\pm 0.7}$ TCID₅₀ / g tissue; PCV2b: $10^{4.3\pm 1.3}$ TCID₅₀ / g tissue). Mean virus titres in other organs ranged from $10^{1.9\pm 0.1}$ TCID₅₀ / g tissue (PCV2a) or $10^{1.6\pm 0.1}$ TCID₅₀ / g tissue (PCV2b) in cerebrum to $10^{3.8\pm 1.1}$ TCID₅₀ / g tissue (PCV2a) or $10^{3.1\pm 1.3}$ TCID₅₀ / g tissue (PCV2b) in the lungs. Non-inoculated foetuses were negative for PCV2. Virus titres were significantly different ($P < 0.05$) in the kidneys of PCV2a-inoculated foetuses ($10^{3.6\pm 0.8}$ TCID₅₀ / g tissue) when compared to PCV2b-inoculated foetuses ($10^{2.3\pm 0.6}$

TCID₅₀ / g tissue), and in the kidneys of foetuses from the first sow ($10^{3.9\pm 0.8}$ TCID₅₀ / g tissue) when compared to foetuses from the second sow ($10^{2.5\pm 0.5}$ TCID₅₀ / g tissue). Statistically significant differences were not observed in any of the other organs. The general virus titre per foetus, defined as the arithmetic mean of virus titres from the organs, was significantly different ($P < 0.05$) in the group of foetuses inoculated with PMWS-derived strains ($10^{5.7\pm 0.3}$ TCID₅₀ / g tissue) when compared to the group of foetuses inoculated with reproductive failure- or PDNS-derived strains ($10^{5.1\pm 0.2}$ TCID₅₀ / g tissue). This was not the case for PCV2a-inoculated foetuses ($10^{5.4\pm 0.6}$ TCID₅₀ / g tissue) vs. PCV2b-inoculated foetuses ($10^{5.4\pm 0.1}$ TCID₅₀ / g tissue) or foetuses from the first sow ($10^{5.6\pm 0.6}$ TCID₅₀ / g tissue) vs. foetuses from the second sow ($10^{5.3\pm 0.3}$ TCID₅₀ / g tissue).

Very high numbers of PCV2 positive cells ($> 10,000$ PCV2 positive cells / 10 mm^2 tissue) were observed in the hearts (Fig. 1.c) of all inoculated foetuses, except for the second strain 48285-inoculated foetus. In this foetus, high numbers of PCV2 positive cells (1,000 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 localized 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 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.

Time of sampling (days of gestation)	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		
76	1010 ^c	PCV2a	1	R4	7.0	4.4	5.3	5.5	3.3	3.2	3.2	3.2	2.3	1.9	
76	1010 ^c	PCV2a	1	R5	6.7	4.9	5.1	5.6	4.8	4.2	4.2	3.0	3.5	1.9	
76	1121	PCV2a	1	L5	5.2	3.3	5.7	4.7	3.5	4.8	2.7	3.0	1.7	1.7	
76	1121	PCV2a	2	R11	5.5	2.4	5.3	5.3	2.9	4.0	<3.0 ^d	<3.0 ^d	1.9	1.9	
76	48285	PCV2b	2	L1	6.0	2.4	5.6	5.6	2.8	5.6	2.2	2.8	1.7	1.7	
76	48285	PCV2b	2	L2	6.4	2.3	5.1	4.2	2.5	4.4	2.1	2.4	1.7	1.7	
76	1147	PCV2b	2	R2	6.2	4.6	5.3	5.3	1.7	3.0	<3.0 ^d	<3.0 ^d	<1.7	<1.7	

^a According to the nomenclature of Segalés 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 Stoon-1010

^d Very limited amounts of tissue material were available. In order to determine the PCV2 titre, the detection limit of the assay was raised from 10^{2.0} TCID₅₀/g tissue to 10^{3.0} TCID₅₀/g tissue.

3.4. Serology

The strain 1121-inoculated foetus of the second sow had a low PCV2-specific IPMA Ab titre of 10. The six other PCV2-inoculated foetuses and all non-inoculated foetuses were negative (< 10) for PCV2-specific IPMA Ab.

4. Discussion

This study confirms previous work from Sanchez et al. (2001a; 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 (Sanchez et al., 2001a; Sanchez et al., 2003). This study also confirms that PCV2 does not rapidly spread from one foetus to another (Sanchez et al., 2001a; Pensaert et al., 2004; Yoon et al., 2004).

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 the pathogenic character of both PCV2 genotypes for foetuses, since previous studies predominantly used PCV2a strains (Sanchez et al., 2001a; Johnson et al., 2002; Sanchez et al., 2003; Pensaert et al., 2004; Sanchez et al., 2004; Yoon et al., 2004).

Recent field observations on the occurrence of PMWS suggest that PCV2b may be more pathogenic than PCV2a, because PCV2b is more frequently isolated from PMWS cases than PCV2a (Carman et al., 2006; Cheung et al., 2007; Gagnon et al., 2007; Carman et al., 2008; Dupont et al., 2008; Grau-Roma et al., 2008; Timmusk et al., 2008; Wiederkehr 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. However, this does not mean that the results of the present study imply that PCV2a and PCV2b are equally pathogenic. First of all, the present study was conducted in a limited number of animals. During mid-gestation, porcine foetuses cannot be easily manipulated without being damaged. Consequently, only a few foetuses per sow can be inoculated using the surgical technique that was used. Thirty-five animals - two

sows, seven PCV2-inoculated foetuses and 26 non-inoculated foetuses - were sacrificed in the present study. Despite the low number of PCV2-inoculated foetuses, sound conclusions can be made regarding foetuses inoculated with PCV2a and foetuses inoculated with PCV2b. The lack of relevant differences (e.g. 1 log₁₀ difference in mean virus titres in the heart) between foetuses inoculated with PCV2a and foetuses inoculated with PCV2b strongly suggests that increasing the number of PCV2-inoculated foetuses in this particular experimental set-up would not lead to other conclusions than those already made at present. It would be unethical to perform a more extensive study. Secondly, PMWS is a disease that develops in weaned pigs that are primary infected with PCV2 and that are not able to mount a protective immune response against PCV2 (Meerts et al., 2006; Fort et al., 2007). Porcine foetuses become immuno-competent at around 80 days of gestation (Salmon, 1984) and more specifically, 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 (Bachmann et al., 1975; Joo et al., 1976b; Sanchez et al., 2001a). 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 one out of seven 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. In this context, the results of the present study might suggest that PCV2a and PCV2b are equally able to replicate in porcine tissues and that the presumed differences in pathogenicity of PCV2 and PCV2b under field conditions might be attributed to different interactions of PCV2a and PCV2b with the pig's immune system. It has been reported that PCV2b may persist more easily in alveolar macrophages than PCV2a (Stevenson et al., 2007). To our knowledge, other reports describing different interactions of PCV2a and PCV2b with the pig's immune system are not available yet. Further research should deal with these issues.

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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References

- Allan, G.M., Ellis, J.A., 2000. Porcine circoviruses: a review. *J Vet Diagn Invest* 12, 3-14.
- Bachmann, P.A., Sheffy, B.E., Vauhan, J.T., 1975. Experimental in utero infection of fetal pigs with a porcine parvovirus. *Infect Immun* 12, 455-460.
- Brunborg, I.M., Jonassen, C.M., Moldal, T., Bratberg, B., Lium, B., Koenen, F., Schonheit, J., 2007. Association of myocarditis with high viral load of porcine circovirus type 2 in several tissues in cases of fetal death and high mortality in piglets. A case study. *J Vet Diagn Invest* 19, 368-375.
- Carman, S., McEwen, B., DeLay, J., van Dreumel, T., Lusi, P., Cai, H., Fairles, J., 2006. Porcine circovirus-2 associated disease in swine in Ontario (2004 to 2005). *Can Vet J* 47, 761-762.
- Carman, S., Cai, H.Y., DeLay, J., Youssef, S.A., McEwen, B.J., Gagnon, C.A., Tremblay, D., Hazlett, M., Lusi, P., Fairles, J., Alexander, H.S., van Dreumel, T., 2008. The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease--2004-2006. *Can J Vet Res* 72, 259-268.
- Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. *Arch Virol* 152, 1035-1044.
- Dupont, K., Nielsen, E.O., Bækbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. *Vet Microbiol* 128, 56-64.
- Farnham, M.W., Choi, Y.K., Goyal, S.M., Joo, H.S., 2003. Isolation and characterization of porcine circovirus type-2 from sera of stillborn fetuses. *Can J Vet Res* 67, 108-113.
- Fort, M., Olvera, A., Sibila, M., Segalés, J., Mateu, E., 2007. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. *Vet Microbiol* 125, 244-255.
- Gagnon, C.A., Tremblay, D., Tijssen, P., Venne, M.H., Houde, A., Elahi, S.M., 2007. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. *Can Vet J* 48, 811-819.
- Grau-Roma, L., Crisci, E., Sibila, M., Lopez-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A., Segalés, J., 2008. A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. *Vet Microbiol* 128, 23-35.

- Johnson, C.S., Joo, H.S., Direksin, K., Yoon, K.J., Choi, Y.K., 2002. Experimental in utero inoculation of late-term swine fetuses with porcine circovirus type 2. *J Vet Diagn Invest* 14, 507-512.
- Joo, H.S., Donaldson-Wood, C.R., Johnson, R.H., 1976a. A standardised haemagglutination inhibition test for porcine parvovirus antibody. *Aust Vet J* 52, 422-424.
- Joo, H.S., Donaldson-Wood, C.R., Johnson, R.H., 1976b. Observations on the pathogenesis of porcine parvovirus infection. *Arch Virol* 51, 123-129.
- Krakowka, S., Ellis, J., McNeilly, F., Waldner, C., Allan, G., 2005. Features of porcine circovirus-2 disease: correlations between lesions, amount and distribution of virus, and clinical outcome. *J Vet Diagn Invest* 17, 213-222.
- Labarque, G.G., Nauwynck, H.J., Mesu, A.P., Pensaert, M.B., 2000. Seroprevalence of porcine circovirus types 1 and 2 in the Belgian pig population. *Vet Q* 22, 234-236.
- Ladekjær-Mikkelsen, A.S., Nielsen, J., Storgaard, T., Bøtner, A., Allan, G., McNeilly, F., 2001. Transplacental infection with PCV-2 associated with reproductive failure in a gilt. *Vet Rec* 148, 759-760.
- Lefebvre, D.J., Costers, S., Van Doorselaere, J., Misinzo, G., Delputte, P.L., Nauwynck, H.J., 2008. Antigenic differences among porcine circovirus type 2 strains, as demonstrated by the use of monoclonal antibodies. *J Gen Virol* 89, 177-187.
- Mateusen, B., Sanchez, R.E., Van Soom, A., Meerts, P., Maes, D.G., Nauwynck, H.J., 2004. Susceptibility of pig embryos to porcine circovirus type 2 infection. *Theriogenology* 61, 91-101.
- Mateusen, B., Maes, D.G., Van Soom, A., Lefebvre, D., Nauwynck, H.J., 2007. Effect of a porcine circovirus type 2 infection on embryos during early pregnancy. *Theriogenology* 68, 896-901.
- McNeilly, F., McNair, I., Mackie, D.P., Meehan, B.M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S., Allan, G.M., 2001. Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. *Arch Virol* 146, 909-922.
- Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *J Gen Virol* 79, 2171-2179.
- Meehan, B.M., McNeilly, F., McNair, I., Walker, I., Ellis, J.A., Krakowka, S., Allan, G.M., 2001. Isolation and characterization of porcine circovirus 2 from cases of sow abortion and porcine dermatitis and nephropathy syndrome. *Arch Virol* 146, 835-842.
- Meerts, P., Misinzo, G., McNeilly, F., Nauwynck, H.J., 2005. Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, fetal cardiomyocytes and macrophages. *Arch Virol* 150, 427-441.
- Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Bøtner, A., Kristensen, C.S., Nauwynck, H.J., 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. *BMC Vet Res* 2, 6.
- O'Connor, B., Gauvreau, H., West, K., Bogdan, J., Ayroud, M., Clark, E.G., Konoby, C., Allan, G., Ellis, J.A., 2001. Multiple porcine circovirus 2-associated abortions and reproductive failure in a multisite swine production unit. *Can Vet J* 42, 551-553.
- Olvera, A., Cortey, M., Segalés, J., 2007. Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. *Virology* 357, 175-185.
- Opriessnig, T., McKeown, N.E., Zhou, E.M., Meng, X.J., Halbur, P.G., 2006. Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. *J Gen Virol* 87, 2923-2932.

- Park, J.S., Kim, J., Ha, Y., Jung, K., Choi, C., Lim, J.K., Kim, S.H., Chae, C., 2005. Birth abnormalities in pregnant sows infected intranasally with porcine circovirus 2. *J Comp Pathol* 132, 139-144.
- Pensaert, M.B., Sanchez, R.E.Jr., Ladekjær-Mikkelsen, A.S., Allan, G.M., Nauwynck, H.J., 2004. Viremia and effect of fetal infection with porcine viruses with special reference to porcine circovirus 2 infection. *Vet Microbiol* 98, 175-183.
- Rose, N., Blanchard, P., Cariolet, R., Grasland, B., Amenna, N., Oger, A., Durand, B., Balasch, M., Jestin, A., Madec, F., 2007. Vaccination of porcine circovirus type 2 (PCV2)-infected sows against porcine Parvovirus (PPV) and Erysipelas: effect on post-weaning multisystemic wasting syndrome (PMWS) and on PCV2 genome load in the offspring. *J Comp Pathol* 136, 133-144.
- Salmon, H., 1984. Immunité chez le foetus et le nouveau-né: modèle porcin. *Reprod Nutr Dev* 24, 197-206.
- Sanchez, R.E., Jr., Nauwynck, H.J., McNeilly, F., Allan, G.M., Pensaert, M.B., 2001a. Porcine circovirus 2 infection in swine foetuses inoculated at different stages of gestation. *Vet Microbiol* 83, 169-176.
- Sanchez, R., Nauwynck, H., Pensaert, M., 2001b. Serological survey of porcine circovirus type 2 antibodies in domestic and feral pig populations in Belgium. In: *Proceedings: ssDNA Viruses of Plants, Birds, Pigs and Primates*, Saint-Malo, France, p. 122.
- Sanchez, R.E., Jr., Meerts, P., Nauwynck, H.J., Pensaert, M.B., 2003. Change of porcine circovirus 2 target cells in pigs during development from fetal to early postnatal life. *Vet Microbiol* 95, 15-25.
- Sanchez, R.E., Jr., Meerts, P., Nauwynck, H.J., Ellis, J.A., Pensaert, M.B., 2004. Characteristics of porcine circovirus-2 replication in lymphoid organs of pigs inoculated in late gestation or postnatally and possible relation to clinical and pathological outcome of infection. *J Vet Diagn Invest* 16, 175-185.
- Segalés, J., Domingo, M., 2002. Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. *Vet Q* 24, 109-124.
- Segalés, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynck, H., Larsen, L., Dupont, K., McCullough, K., Ellis, J., Krakowka, S., Mankertz, A., Fredholm, M., Fossum, C., Timmusk, S., Stockhofe-Zurwieden, N., Beattie, V., Armstrong, D., Grasland, B., Bækbo, P., Allan, G., 2008. PCV-2 genotype definition and nomenclature. *Vet Rec* 162, 867-868.
- Stevenson, L., McNeilly, F., Duffy, C., McNair, I., Adair, B., Allan, G., 2007. Biological comparison of porcine circovirus type 2 (PCV2) isolates. In: *Proceedings of the 5th International Symposium on Emerging and Re-emerging Pig Diseases*, Krakow, Poland, p. 51.
- Timmusk, S., Wallgren, P., Brunborg, I.M., Wikström, F.H., Allan, G., Meehan, B., McMenamy, M., McNeilly, F., Fuxler, L., Belák, K., Pödersoo, D., Saar, T., Berg, M., Fossum, C., 2008. Phylogenetic analysis of porcine circovirus type 2 (PCV2) pre- and post-epizootic postweaning multisystemic wasting syndrome (PMWS). *Virus Genes* 36, 509-520.
- Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J.M., Moonen, P.L.J.M., Zetstra, T., de Boer, E.A., Tibben, H.J., de Jong, M.F., van 't Veld, P., Groenland, G.J.R., van Gennep, J.A., Voets, M.T., Verheijden, J.H.M., Braamskamp, J., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q* 13, 121-130.
- West, K.H., Bystrom, J.M., Wojnarowicz, C., Shantz, N., Jacobson, M., Allan, G.M., Haines, D.M., Clark, E.G., Krakowka, S., McNeilly, F., Konoby, C., Martin, K., Ellis, J.A., 1999. Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus 2. *J Vet Diagn Invest* 11, 530-532.

- Wiederkehr, D.D., Sydler, T., Brugnera, E., Pospischil, A., Buergi, E., Sidler, X., 2008. Pathogenic differences of porcine circovirus type 2 genotypes in Switzerland. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 1, p. 26.
- Yoon, K.J., Jepsen, R.J., Pogranichniy, R.M., Sorden, S., Stammer, R., Evans, L.E., 2004. A novel approach to intrauterine viral inoculation of swine using PCV type 2 as a model. *Theriogenology* 61, 1025-1037.

**Chapter 4. Effect of mitogen stimulation on
porcine circovirus type 2 replication in cells
of the immune system**

**Chapter 4.1. Increased porcine circovirus type 2
replication in porcine leukocytes *in vitro* and *in vivo* by
concanavalin A stimulation**

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Abstract

Previously, it was shown that modulation of the immune system enhances porcine circovirus type 2 (PCV2) replication in pigs. In the present study, the effect of the mitogen concanavalin A (ConA) on PCV2 replication was investigated. Since ConA induces T-lymphocyte activation and initiates the production of interferon-gamma (IFN- γ), a cytokine that enhances PCV2 replication in porcine epithelial and monocytic cell lines *in vitro*, it was examined if the effects observed with ConA were mediated by IFN- γ . In an *in vitro* study, ConA but not IFN- γ enhanced PCV2 replication in peripheral blood mononuclear cells (PBMC). Up to 2.08 % and 0.96 % of PBMC were antigen positive for PCV2 strains 1121 and Stoon-1010 respectively, and a low virus production was observed. PCV2-infected PBMC were identified as CD4⁺ (40 %), CD8⁺ (54 %) and IgM⁺ (11 %). In a subsequent *in vivo* study, caesarean-derived colostrum-deprived piglets were injected with ConA or IFN- γ 12 hours before inoculation and every 3 days for 9 days after inoculation with strain 1121. PCV2 was isolated from inguinal lymph node biopsies from 10 days post inoculation (dpi) in ConA-treated pigs and from 15 dpi in non-treated and IFN- γ -treated pigs. ConA increased PCV2 replication levels, but disease was not observed. Half of the ConA-treated and IFN- γ -treated pigs showed a delayed humoral immune response, but this delay did not result in increased PCV2 replication in these pigs. These experiments demonstrated that ConA enhances PCV2 replication in PBMC *in vitro* and in lymphoid tissues *in vivo*.

1. Introduction

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, single-stranded DNA virus. It is a member of the family of the *Circoviridae* and it is widespread in wild and domestic pigs. In pig dense regions, PCV2 infects all conventional pigs after weaning. The majority of these infections are sub-clinical. Under certain conditions, PCV2 infection causes postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease that causes severe growth retardation, weight loss and death in weaned piglets (Segalés and Domingo, 2002). PMWS is characterized by severe lymphocytic depletion and monocytic infiltration in lymphoid tissues and high amounts of PCV2 are found within these lesions (Segalés and Domingo, 2002).

PCV2 lacks its own polymerases and therefore fully depends on cellular enzymes to complete its infectious cycle. Up till now, only 4 different viral proteins have been described in porcine circovirus-infected cells (Mankertz and Hillenbrand, 2002; Liu et al., 2005), which makes PCV2 incapable of actively modifying the immune response of its host in its own favour, as other more complex viruses do (Tortorella et al., 2000). Therefore, specific conditions in the host intensively influence PCV2 replication. Co-infections with other infectious agents such as porcine parvovirus (PPV) (Allan et al., 1999), porcine reproductive and respiratory syndrome virus (PRRSV) (Allan et al., 2000) and *Mycoplasma hyopneumoniae* (Opriessnig et al., 2004) enhance PCV2 replication and exacerbate the clinical outcome of a PCV2 infection. Injection of immune stimulating drugs such as the soluble protein antigen Keyhole limpet hemocyanin (KLH) in incomplete Freund's adjuvant (Krakowka et al., 2001) and vaccination (Opriessnig et al., 2003) enhance PCV2 replication as well, suggesting that the induction of an immune response might be a key factor in the induction of high levels of PCV2 replication. However, strategies involving immune-stimulating drugs or vaccination have not always been successful in enhancing PCV2 replication and PMWS occurrence (Resendes et al., 2004).

PCV2 replication in lymphoid tissues occurs in B-lymphocytes, T-lymphocytes, monocytes and macrophages (Sanchez et al., 2004). Recently, it was shown that the plant lectin concanavalin A (ConA) is able to induce PCV2 replication in CD3⁺ peripheral blood mononuclear cells (PBMC) that have been inoculated with PCV2 *in vitro* (Yu et al., 2007). Mitogen stimulation also induces PPV replication in porcine peripheral blood lymphocytes (Paul et al., 1979) and TT virus replication in human PBMC (Mariscal et al., 2002). PPV and TT virus are also non-enveloped,

single-stranded DNA viruses. The mitogen ConA causes predominantly T-lymphocyte proliferation and activation (Mosmann et al., 1986), leading to the production of lymphokines. More specifically, ConA induces production of high concentrations of interferon-gamma (IFN- γ) *in vitro* (Verfaillie et al., 2001) and *in vivo* (Miyagi et al., 2004).

Recently, it was shown that interferon-alpha (IFN- α) and especially IFN- γ are able to enhance PCV2 replication in cell lines *in vitro*. When administered after PCV2 inoculation, 1,000 units of IFN- γ / 10^6 cells increased the number of PCV2-infected cells in epithelial PK-15 and monocytic 3D4/31 cells with 691 % and 423 % respectively (Meerts et al., 2005b). However, an *in vivo* study demonstrated that pigs with high IFN- γ mRNA expression levels in PBMC are less susceptible to PCV2 replication (Meerts et al., 2005c). Up till now, the effects of ConA and IFN- γ on PCV2 replication *in vivo* are not known.

It is the aim of the present study to determine the effects of ConA and IFN- γ on PCV2 replication in PBMC *in vitro* and to try to set up a model for the reproduction of PMWS using ConA and IFN- γ *in vivo*.

2. Material and methods

2.1. Cells

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⁻¹ glutamine, 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin and 0.1 mg ml⁻¹ kanamycin.

PBMC were separated from blood by density centrifugation at 750 x g on Ficoll-Paque[®] (Amersham Pharmacia Biotech AB, Uppsala, Sweden). These cells were maintained in RPMI 1640 (Gibco) containing 10 % FBS, 0.3 mg ml⁻¹ glutamine, 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin, 0.1 mg ml⁻¹ kanamycin, 1 % non-essential amino acids (100x; Gibco) and 1 % sodium pyruvate (100 mM; Gibco).

Cells were maintained at 37 °C in the presence of 5 % CO₂.

2.2. Viruses

PCV2 strains 1121 and Stoon-1010 were used in this study. The replication kinetics of these 2 strains have been described in PK-15 cells, foetal cardiomyocytes and alveolar macrophages by Meerts et al. (2005a). For *in vitro* use, 1121 and Stoon-1010 were at a 29th and 19th passage level on PK-15 cells respectively. For *in vivo* inoculation, a 4th passage on PK-15 cells of strain 1121 was used.

2.3. Concanavalin A and recombinant IFN- γ

ConA was purchased from Sigma (Heidelberg, Germany). Recombinant IFN- γ (rIFN- γ) was purchased from R&D Systems (Abingdon, UK).

2.4. Pigs

Six nine-month-old conventional PCV2 seronegative pigs were used as blood donors for the isolation of PBMC.

Seventeen caesarean-derived colostrum-deprived (CD/CD) pigs were obtained from 2 Landrace sows and were raised in sterile conditions in individual isolators.

2.5. In vitro experiments

2.5.1. Inoculation and treatment

After isolation, PBMC were inoculated with a dose of $10^{4.3}$ TCID₅₀ / 10^6 PBMC for 1 h at 37 °C. After inoculation, cells were washed twice, resuspended in fresh medium and seeded in 24-well cell culture plates (Nunc, Roskilde, Denmark) at a concentration of 10^6 PBMC ml⁻¹. Immediately after seeding, ConA or rIFN- γ was added at a dose of 5 μ g / 10^6 PBMC or 1,000 units / 10^6 PBMC respectively. A dose of 5 μ g ConA / 10^6 PBMC is commonly used to induce proliferation in porcine PBMC (Verfaillie et al., 2001). A dose of 1,000 units of rIFN- γ / 10^6 cells was the most efficient dose to increase the number of PCV2-infected cells in PK-15 and 3D4/31 cells (Meerts et al., 2005b). This dose was effective for stimulating PBMC *in vitro*, because 24 hours after treatment, a 5-fold increase in interleukin-12 levels was observed in supernatant fluids of rIFN- γ -treated PBMC when compared to non-

treated PBMC. This was measured with a commercial ELISA for interleukin-12 (R&D Systems).

PBMC inoculated with PK-15 cell culture medium and treated with ConA or rIFN- γ were included as negative controls.

2.5.2. Fixation and stainings

At 0, 12, 24, 36, 48, 72 and 96 hours post inoculation (hpi), PBMC and supernatant fluids were collected. Cells were smeared onto glass slides, fixed in methanol at -20 °C for 10 min and stored at -20 °C until use. Supernatant fluids were centrifuged at 2,900 x g for 10 min and stored at -20 °C until virus titration on PK-15 cells.

Expression kinetics of the non-structural Rep protein and the structural capsid protein were determined with double immunofluorescence stainings using the monoclonal antibody (mAb) F210, directed against the PCV2 Rep protein (McNeilly et al., 2001), and biotinylated purified porcine polyclonal antibodies (pAbs), directed against the PCV2 capsid protein (Meerts et al., 2005a). Briefly, fixed cell smears were air-dried at room temperature for 10 min. Next, they were incubated with an optimal dilution of mAb F210 in phosphate-buffered saline (PBS). Subsequently, a 1 : 500 dilution of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse pAbs (Molecular Probes, Eugene, USA) in PBS was applied. This was followed by an optimal dilution of biotinylated purified porcine pAbs in PBS, followed by incubation with a 1 : 100 dilution of Texas Red-labelled streptavidin (Molecular Probes) in PBS. Each of these incubations were performed for 1 h at 37 °C. Finally, Hoechst 33342 (Molecular Probes) was applied at a concentration of 10 $\mu\text{g ml}^{-1}$ for 10 min at room temperature, in order to visualize the nucleus. Cell smears were washed three times with PBS between the incubations. Stained cell smears were mounted with a glycerol solution containing 1,4-diazabicyclo(2.2.2)octane (DABCO) anti-fading agent (Janssen Chimica, Beerse, Belgium). A LEICA DM/RBE fluorescence microscope (Leica Microsystems GmbH, Heidelberg, Germany) was used for visualization. Expression kinetics of the Rep protein and the capsid protein were assessed at 0, 12, 24, 36, 48, 72 and 96 hpi, by determination of nuclear and cytoplasmic localization of PCV2 antigens in 5,000 PBMC at each time point.

PCV2 positive PBMC (strain 1121) were further characterized at 0, 36 and 72 hpi by using mAbs directed against cell markers CD4, CD8, IgM and SWC3, respectively.

The cell marker CD4 is present on T-helper and memory T-helper lymphocytes, regulatory T-lymphocytes and natural interferon-alpha producing cells (Pescovitz et al., 1984; Charley and Lavenant, 1990). CD8 is present on cytotoxic and gamma-delta T-lymphocytes, memory T-lymphocytes and natural killer cells (Pescovitz et al., 1984; Saamuller et al., 1994). IgM is present on naïve B-lymphocytes, B-lymphoblasts and IgM-producing plasma cells (Van Zaane and Hulst, 1987). SWC3 is present on monocytes, macrophages, dendritic cells and granulocytes (Pescovitz et al., 1984; Paillot et al., 2001). The staining procedure was similar to the technique described above. Cell smears were incubated with optimal dilutions of mAbs against CD4, CD8, IgM or SWC3, respectively, followed by FITC-labelled goat anti-mouse pAbs. Thereafter, cell smears were stained with biotin-conjugated porcine anti-PCV2 pAbs, followed by Texas Red-labelled streptavidin. On each cell smear, all PCV2 positive cells were analyzed by fluorescence microscopy.

2.5.3. Virus titration of PBMC culture supernatant

Ten-fold dilutions of PBMC culture supernatants were inoculated on semi-confluent monolayers of PCV negative PK-15 cells. After 72 hours of incubation, cells were fixed in 4 % paraformaldehyde in PBS. PCV2-infected cells were detected via an immunoperoxidase monolayer assay (IPMA) as described by Labarque et al. (2000).

2.5.4. Statistical analysis

The statistical significance of differences between non-treated PBMC and ConA-treated PBMC and between non-treated PBMC and rIFN- γ -treated PBMC was tested by applying two-sided Kruskal-Wallis tests. Differences were considered significant when $P < 0.05$. Statistical analyses were performed using S-PLUS (S-PLUS 6.1, Insightful Corporation, Seattle, USA, 2002).

2.6. In vivo experiments

2.6.1. Inoculation and treatment

The set up of the experiment is illustrated in Table 1. The 17 CD/CD pigs were randomly divided in 5 groups. At 19 days of age, all pigs from groups A (PCV2/-),

B (PCV2/ConA) and C (PCV2/rIFN- γ) were inoculated intraperitoneally and oronasally with a total dose of $10^{4.3}$ TCID₅₀ of strain 1121. Pigs of groups D (-/ConA) and E (-/rIFN- γ) were mock inoculated with equal volumes of cell culture medium. Treatment regimes with ConA or rIFN- γ were designed based on the results of a preliminary experiment in conventional pigs. In PCV2-inoculated PK-15 cells, addition of plasma of a ConA-treated conventional pig or a rIFN- γ -treated conventional pig increased the number of infected cells with maximum 524 % and 154 % respectively, when compared with plasma samples from the same pigs before ConA or rIFN- γ treatment. As a result from this experiment, pigs from groups B (PCV2/ConA) and D (-/ConA) were injected every 3 days with ConA at a dose of 1.5 mg kg^{-1} body weight. Half of the dose was injected intramuscularly (IM) in the neck and the other half was injected subcutaneously (SC) in the region of the lower abdomen that is drained by the inguinal lymph nodes. The first injection was administered 12 h before PCV2 inoculation of the pigs and repeated at 2, 5 and 8 days post inoculation (dpi). Pigs from groups C (PCV2/rIFN- γ) and E (-/rIFN- γ) were treated at the same time points with rIFN- γ at a dose of 100,000 units (3 μg of recombinant protein) per treatment: 50,000 units were injected IM in the neck and 50,000 units SC in the lower abdomen. Pigs were monitored for a period of 21 days after PCV2 inoculation and humanly euthanized afterwards, by intravenous injection of a barbiturate overdose (Natriumpentobarbital 20 %, Kela, Hoogstraten, Belgium).

Table 1. Distribution of caesarean-derived colostrum-deprived pigs in different groups with specific manipulations per group.

Group	n	PCV2 inoculation	Treatment	Pig numbers
A	4	yes	-	1 to 4
B	4	yes	ConA	5 to 8
C	4	yes	rIFN- γ	9 to 12
D	3	no	ConA	13 to 15
E	2	no	rIFN- γ	16 to 17

2.6.2. Clinical monitoring

Pigs 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).

2.6.3. PCV2 replication

To follow the degree of PCV2 replication in all individual pigs, inguinal lymph node biopsies were collected at different time points as described before (Meerts et al., 2005c). At 10 dpi, a biopsy was taken from the left inguinal lymph node and at 15 dpi a biopsy was taken from the right inguinal lymph node. In order to take lymph node biopsies, the pigs were anaesthetized by IM injection of 2.2 mg tiletamine and 2.2 mg zolazepam (Zoletil[®], Virbac, Wavre, Belgium) dissolved in 0.22 ml 2 % xylazine (XYL-M[®] 2 %, VMD, Arendonk, Belgium) kg⁻¹ body weight. An incision was made in the overlaying skin and half the inguinal lymph node was excised. The wound was closed by mersilene sutures (Mersutures[®], Ethicon, USA). At the time of euthanasia (21 dpi) the remaining parts of both left and right inguinal lymph nodes were collected.

Ten % suspensions were made from these biopsies and from the remaining parts of these lymph nodes, and the level of PCV2 replication in these organs was determined by PCV2 titration on PK-15 cells. The detection limit of this technique was 10^{2.7} TCID₅₀ / g.

Quantification and immunophenotyping of PCV2 positive cells was performed on inguinal lymph node biopsies that were collected at 15 dpi. Serial cryostat sections were fixed in methanol at -20 °C for 10 min. Double immunofluorescence stainings for CD4, CD8, IgM or SWC3, respectively, and for PCV2, were performed as described above. Prior to mounting, autofluorescence was quenched by immersion of the sections for 30 min in a 10 mM dilution of CuSO₄ in a 50 mM NH₄-acetate buffer at pH 5.0. PCV2 positive cells were quantified and immunophenotyped using a fluorescence microscope. The total number of PCV2 positive cells and the total number of double positive cells (cell marker and PCV2) were determined over an area of 10 mm² of tissue.

2.6.4. Humoral immune response against PCV2

At 0, 7, 10, 15 and 21 dpi, blood was taken (1/1 in Alsevers solution) from the jugular vein, in order to monitor total anti-PCV2 Ab titres by an IPMA described previously (Labarque et al., 2000). PCV2 neutralizing Abs were determined by a sensitive neutralization assay described previously (Meerts et al., 2005c).

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

2.6.5. Statistical analysis

The statistical significance of differences between non-treated pigs and ConA-treated pigs and between non-treated pigs and rIFN- γ -treated pigs was tested by applying two-sided Kruskal-Wallis tests. Differences were considered significant when $P < 0.05$. Statistical analyses were performed using S-PLUS (S-PLUS 6.1, Insightful Corporation, Seattle, USA, 2002).

3. Results

3.1. In vitro experiments

3.1.1. PCV2 replication kinetics in PBMC

Figure 1 demonstrates mean percentages of PCV2 positive cells and antigen expression patterns in PCV2-inoculated PBMC of three conventional PCV2 seronegative pigs (pigs 1, 2 and 3). For strain 1121, maximum numbers of antigen positive cells were very low in non-treated PBMC (0.06 % in pig 1 at 48 hpi, 0.14 % in pig 2 at 72 hpi and 0.04 % in pig 3 at 48 hpi). Antigen positive cells were observed starting from 36 hpi (pigs 2 and 3) or 48 hpi (pig 1). In ConA-treated PBMC, maximum numbers of antigen positive cells of 2.08 %, 1.02 % and 2.02 % were observed at 96 hpi, 72 hpi and 96 hpi for pigs 1, 2 and 3 respectively. Nuclear localized viral antigens were detected for the first time at 12 hpi for pig 2 and at 36 hpi for pigs 1 and 3. Cytoplasmic localized viral antigens were detected starting from 36 hpi. At this time point, 20 % to 50 % of cells with viral antigens in the nucleus showed viral antigens in the cytoplasm and all cells with viral antigens in the cytoplasm showed viral antigens in the nucleus. From 36 hpi to 96 hpi, the numbers of total PCV2 antigen positive cells, positive cells with antigens in the nucleus and positive cells with antigens in the cytoplasm were significantly different in ConA-treated PBMC when compared to non-treated PBMC. In rIFN- γ -treated PBMC, maximum numbers of antigen positive cells were very low (0.06 % in pig 1

at 72 hpi, 0.18 % in pig 2 at 72 hpi and 0.06 % in pig 3 at 36 and 48 hpi). Antigen positive cells were observed starting from 24 hpi (pig 2), 36 hpi (pig 3) or 72 hpi (pig 1).

For Stoon-1010, maximum numbers of antigen positive cells were very low in non-treated PBMC (0.02 % in pig 1 at 96 hpi, 0.22 % in pig 2 at 96 hpi and 0.00 % in pig 3). Antigen positive cells were observed starting from 12 hpi (pig 2) or 96 hpi (pig 1). In ConA-treated PBMC, maximum numbers of antigen positive cells of 0.96 %, 0.84 % and 0.34 % were observed at 48 hpi for pig 1 and at 72 hpi for pigs 2 and 3 respectively. Nuclear localized viral antigens were detected for the first time at 24 hpi for pig 1 and at 12 hpi for pigs 2 and 3. Cytoplasmic localized viral antigens were detected for the first time at 24 hpi for pigs 1 and 2 and at 72 hpi for pig 3. At these time points, 12 % to 33 % of cells with viral antigens in the nucleus showed viral antigens in the cytoplasm and all cells with viral antigens in the cytoplasm showed viral antigens in the nucleus. From 24 hpi to 72 hpi, the numbers of total PCV2 antigen positive cells and positive cells with antigens in the nucleus were significantly different in ConA-treated PBMC when compared to non-treated PBMC. At 96 hpi, the numbers of positive cells with antigens in the cytoplasm were significantly different in ConA-treated PBMC when compared to non-treated PBMC. In rIFN- γ -treated PBMC, maximum numbers of antigen positive cells were very low (0.02% in pig 1 at 36 hpi, 0.16 % in pig 2 at 72 hpi and 0.02 % in pig 3 at 72 hpi). Antigen positive cells were observed starting from 12 hpi (pig 2), 36 hpi (pig 1) or 72 hpi (pig 3).

In ConA-treated PBMC, all cells with nuclear localized capsid protein also contained Rep protein in their nucleus. In 89 % (1121) and 81 % (Stoon-1010) of the cells with nuclear localized Rep protein, nuclear localized capsid protein was found. Cells with cytoplasmic staining for capsid protein, without nuclear staining for Rep or capsid proteins, were observed starting from 48 hpi (pig 2 and 3) or 72 hpi (pig 1) for strain 1121 and starting from 48 hpi (pig 1), 72 hpi (pig 2) or 96 hpi (pig 3) for Stoon-1010. This staining pattern was accompanied with morphological changes typical for cellular degeneration. Rep protein was never detected in the cytoplasm of PCV2 positive cells, except in a small fraction of cells with typical morphological signs of degeneration.

Positive reactions were not observed when staining non-inoculated PBMC for PCV2 antigens or when staining PCV2-inoculated PBMC with an irrelevant isotype-matched mAb and with porcine PCV2-negative control pAbs.

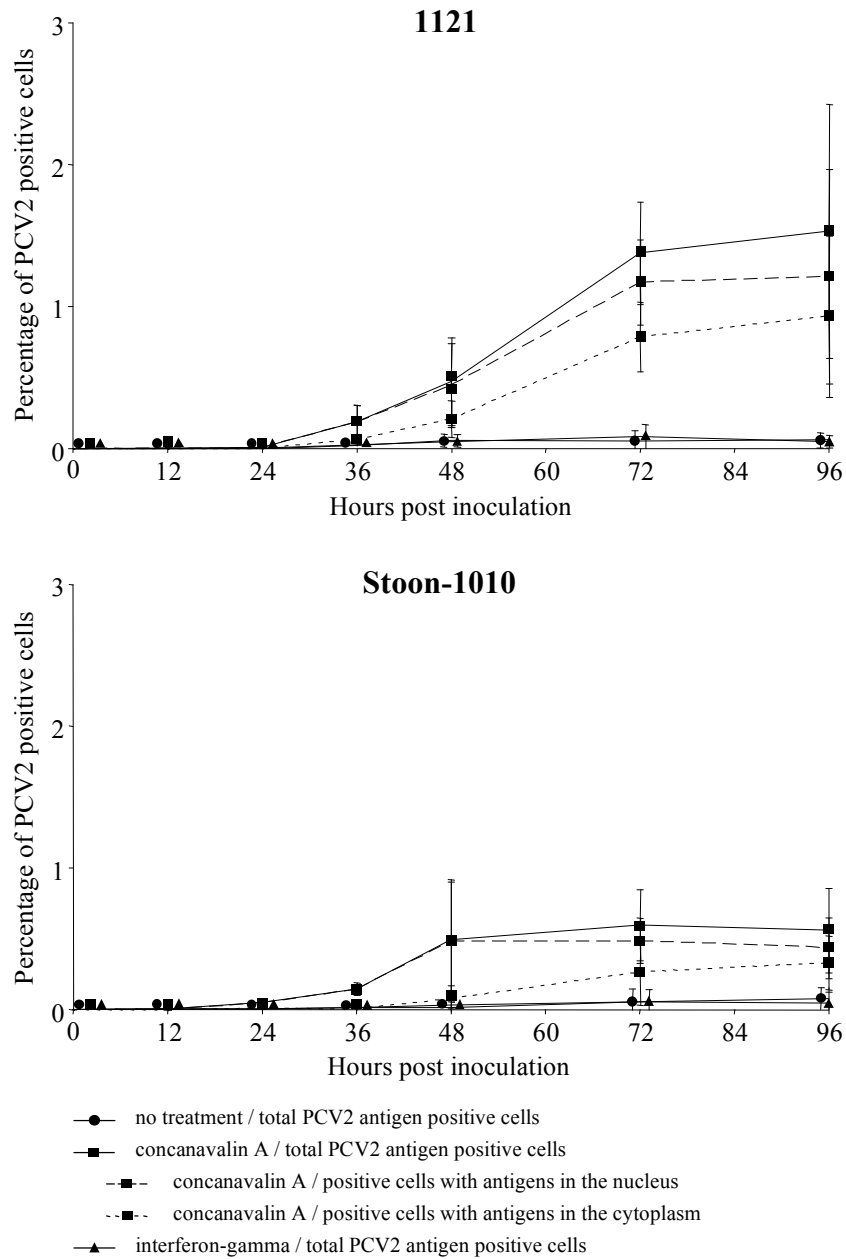


Fig. 1. Mean percentage (\pm SD) of PCV2 antigen positive cells and antigen expression patterns in non-treated, concanavalin A-treated or recombinant interferon-gamma-treated, PCV2-inoculated PBMC of three conventional PCV2 seronegative pigs at different time points after *in vitro* inoculation of PBMC with strains 1121 and Stoon-1010 (pigs 1, 2 and 3).

3.1.2. Identification of PCV2 positive PBMC

PBMC were obtained from three conventional PCV2 seronegative pigs (pigs 4, 5 and 6). The proportions of ConA-treated PCV2 positive PBMC per cell smear, identified as CD4⁺, CD8⁺, IgM⁺ or SWC3⁺ after inoculation with strain 1121, were determined at 0, 36 and 72 hpi. At 0 hpi, PCV2 positive cells were not observed in ConA-treated PBMC. At 36 hpi, PCV2 positive cells were identified as CD4⁺ (48 % ± 5 %), CD8⁺ (56 % ± 4 %) or IgM⁺ (12 % ± 3 %). At 72 hpi, 40 % ± 5 % of PCV2 positive cells were CD4⁺, 54 % ± 11 % were CD8⁺ and 11 % ± 4 % were IgM⁺. Viral antigen positive SWC3⁺ PBMC were not found. The numbers of non-treated and rIFN- γ -treated PCV2 positive PBMC per cell smear were too low for a correct interpretation of results.

3.1.3. Virus titration of PBMC culture supernatant

Figure 2 demonstrates the evolution of mean virus titres in culture supernatants of PCV2-inoculated PBMC of three conventional PCV2 seronegative pigs (pigs 1, 2 and 3). Time-dependent rises in titres were seen for supernatants of ConA-treated PBMC, starting from 72 hpi for strain 1121 and from 48 hpi for Stoon-1010. Maximum virus titres of 10^{3.2} TCID₅₀ / ml supernatant and 10^{4.1} TCID₅₀ / ml supernatant were observed for strain 1121 and Stoon-1010 at 96 hpi. Significant differences between virus titres in supernatants of non-treated PBMC and ConA-treated PBMC were observed at 72 and 96 hpi for strain 1121 and at 36, 48 and 96 hpi for Stoon-1010. A small but statistically significant difference between virus titres in supernatants of non-treated PBMC and rIFN- γ -treated PBMC was observed for strain 1121 at 96 hpi.

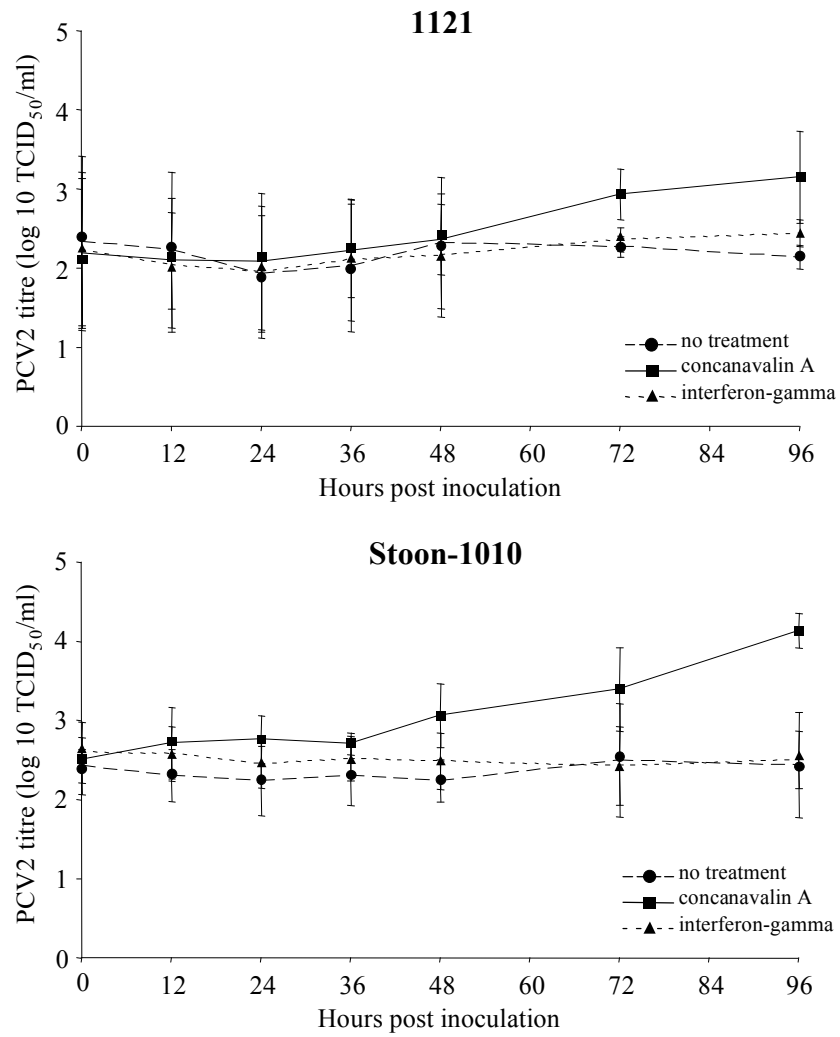


Fig. 2. Evolution of mean (\pm SD) PCV2 titres in culture supernatants of non-treated, concanavalin A-treated or recombinant interferon-gamma-treated, PCV2-inoculated PBMC of three conventional PCV2 seronegative pigs (pigs 1, 2 and 3).

3.2. In vivo experiments

3.2.1. Clinical monitoring

Clinical signs suggestive of PMWS were not observed. Differences in general conditions were not observed between any of the pigs. Inguinal lymph nodes of all pigs of groups B (PCV2/ConA), C (PCV2/rIFN- γ), D (-/ConA) and E (-/rIFN- γ) were noticeably enlarged and pale. This was observed from the first time a biopsy was taken, up to the end of the study. Enlarged, pale inguinal lymph nodes were not observed in any of the pigs of group A (PCV2-inoculated, non-treated pigs). Pig 6 (group B, PCV2/ConA) died during blood sampling at 15 dpi.

3.2.2. PCV2 replication

The evolution in PCV2 titres in the inguinal lymph nodes of individual pigs is shown in Fig. 3. From all pigs of groups A, B and C (PCV2-inoculated pigs), PCV2 was isolated at at least one time point. PCV2 could be detected for the first time at 15 dpi in pigs of groups A (PCV2/-) and C (PCV2/rIFN- γ). In pigs of group B (PCV2/ConA), PCV2 was already isolated from the lymph node biopsies taken at 10 dpi. Titres at this time point ranged between $10^{3.0}$ and $10^{4.3}$ TCID₅₀ / g. At 15 and 21 dpi, PCV2 titres in pigs of group B (PCV2/ConA) were generally higher than in pigs of groups A (PCV2/-) and C (PCV2/rIFN- γ), but due to the high variation in PCV2 titres between pigs in the same groups and due to the limited number of pigs per group, a significant difference between groups A (PCV2/-) and B (PCV2/ConA) could only be observed at 10 dpi and not at 15 and 21 dpi.

The general evolution in PCV2 titres in time was similar in all pigs from groups A, B and C (PCV2-inoculated pigs), except in one pig (pig 7) from group B (PCV2/ConA). Eleven out of twelve pigs of groups A, B and C (PCV2-inoculated pigs) showed a peak of PCV2 replication at 15 dpi. Afterwards, the PCV2 titres decreased in all these pigs. Pig 7 (group B, PCV2/ConA) showed a gradual increase in PCV2 titre until the end of the study.

In pigs of groups D and E (non-inoculated pigs), PCV2 was never isolated during the study.

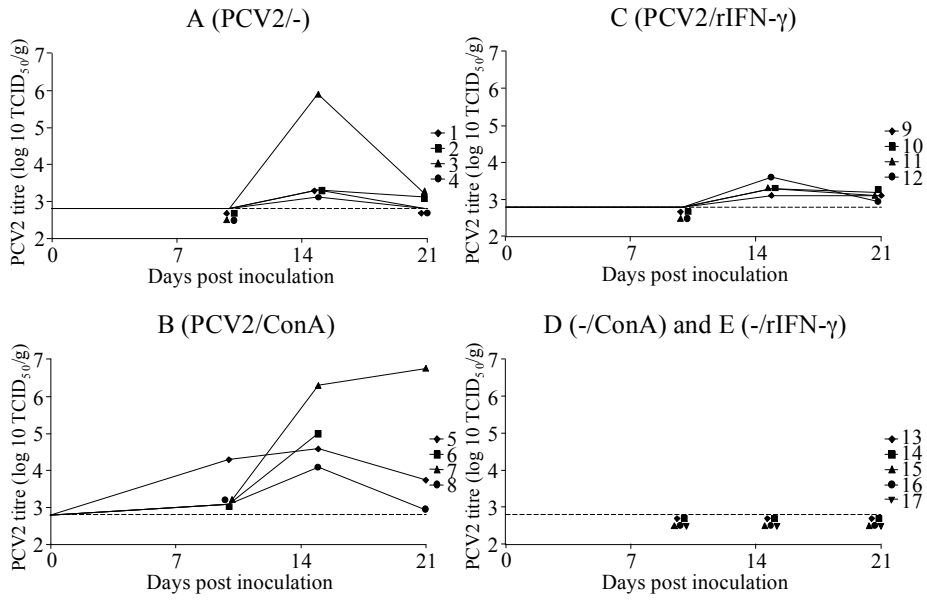


Fig. 3. Evolution of PCV2 titres in inguinal lymph nodes of PCV2-inoculated caesarean-derived colostrum-deprived pigs of groups A (non-treated), B (treated with concanavalin A, ConA) and C (treated with recombinant interferon-gamma, rIFN- γ), and of non-inoculated pigs of groups D (treated with concanavalin A, ConA) and E (treated with recombinant interferon-gamma, rIFN- γ). The dashed line represents the detection limit of the assay ($10^{2.7}$ TCID₅₀ / g).

Table 2 shows the results of the quantification and immunophenotyping of PCV2 positive cells in inguinal lymph node biopsies at 15 dpi. In biopsies from pigs of groups A (PCV2/-) and C (PCV2/rIFN- γ), low numbers of PCV2 positive cells were observed (less than 20 PCV2 positive cells per 10 mm²). In these pigs, PCV2 was mainly localized in SWC3⁺ cells, but in some pigs small proportions of PCV2 positive cells were CD4⁺, CD8⁺ or IgM⁺. PCV2 positive cells were mainly observed as single cells in follicular and parafollicular regions.

The number of PCV2 positive cells was significantly higher in biopsies from group B (PCV2/ConA) pigs 5, 6 and 7 (389 - 664 PCV2 positive cells per 10 mm²), when compared to pigs from group A (PCV2/-). In pigs 5, 6 and 7, PCV2 was found in CD4⁺, CD8⁺, IgM⁺ and SWC3⁺ cells. In pigs 6 and 7, SWC3⁺ cells were the predominantly PCV2 positive cell type (75 % and 66 % respectively). In pig 5, the percentages of CD4⁺, CD8⁺, IgM⁺ and SWC3⁺ PCV2 positive cells ranged from 14 % to 33 %. In pigs 5, 6 and 7, the vast majority of PCV2 positive cells were located in the peripheral areas of the follicular regions and in the parafollicular regions. In these pigs, PCV2 antigens were occasionally (< 5 %) observed in the nucleus of CD4⁺, CD8⁺, IgM⁺ and SWC3⁺ cells. For pig 8 (group B, PCV2/ConA), similar observations were made as for pigs from groups A (PCV2/-) and C (PCV2/rIFN- γ). PCV2 positive cells were not observed in any of the pigs of groups D and E (non-inoculated pigs).

Table 2. Quantification and immunophenotyping of PCV2 positive cells at 15 days post inoculation in inguinal lymph node biopsies of PCV2-inoculated caesarean-derived colostrum-deprived pigs of groups A (non-treated), B (treated with concanavalin A, ConA) and C (treated with recombinant interferon-gamma, rIFN- γ). Pigs from groups D and E (non-inoculated pigs) did not have PCV2 positive cells.

Group	Pig no.	Treatment	Virus titre (log ₁₀ TCID ₅₀ /g)	Identification of PCV2 positive cells ^a				
				CD4 ⁺	CD8 ⁺	IgM ⁺	SWC3 ⁺	
A	1	-	3.3	2 / 9 (22%)	1 / 5 (20%)	1 / 5 (20%)	5 / 7 (71%)	
	2	-	3.3	0 / 4 (0%)	0 / 10 (0%)	0 / 2 (0%)	1 / 1 (100%)	
	3	-	5.8	4 / 18 (22%)	0 / 13 (0%)	3 / 10 (30%)	7 / 11 (64%)	
	4	-	3.0	0 / 2 (0%)	0 / 2 (0%)	0 / 4 (0%)	1 / 1 (100%)	
B	5	ConA	4.5	140 / 434 (32%)	60 / 432 (14%)	89 / 389 (23%)	136 / 418 (33%)	
	6	ConA	5.0	77 / 635 (12%)	69 / 664 (10%)	70 / 555 (13%)	339 / 454 (75%)	
	7	ConA	6.3	72 / 570 (13%)	84 / 555 (15%)	44 / 537 (8%)	394 / 601 (66%)	
	8	ConA	4.0	0 / 4 (0%)	0 / 5 (0%)	0 / 1 (0%)	4 / 4 (100%)	
C	9	rIFN-gamma	3.0	1 / 8 (13%)	0 / 4 (0%)	0 / 7 (0%)	12 / 16 (75%)	
	10	rIFN-gamma	3.3	0 / 2 (0%)	0 / 1 (0%)	0 / 2 (0%)	1 / 2 (50%)	
	11	rIFN-gamma	3.3	0 / 1 (0%)	0 / 2 (0%)	0 / 1 (0%)	2 / 2 (100%)	
	12	rIFN-gamma	3.5	1 / 10 (10%)	1 / 2 (50%)	0 / 6 (0%)	10 / 15 (67%)	

^a Number of both PCV2 and cell marker positive cells / total number of PCV2 positive cells per 10 mm² tissue.

3.2.3. Humoral immune response against PCV2

The evolution in PCV2-specific Abs, as demonstrated by IPMA, is shown in Fig. 4. Anti-PCV2 Abs were demonstrated in all pigs from groups A, B and C (PCV2-inoculated pigs), except in the pig that died at 15 dpi (pig 6, group B, PCV2/ConA). Differences were observed in the time points at which anti-PCV2 Abs were detected for the first time. Pigs from group A (PCV2/-) seroconverted at 10 or 15 dpi. In groups B (PCV2/ConA) and C (PCV2/rIFN- γ), only 2 out of 4 pigs seroconverted at 15 dpi. In the other pigs of groups B (PCV2/ConA) and C (PCV2/rIFN- γ), seroconversion against the virus was delayed until 21 dpi. Statistically significant differences were not detected at any of the time points. PCV2 neutralizing Abs evolved in a similar way as PCV2-specific Abs detected by IPMA (data not shown). None of the pigs from groups D or E (non-inoculated pigs) seroconverted against PCV2.

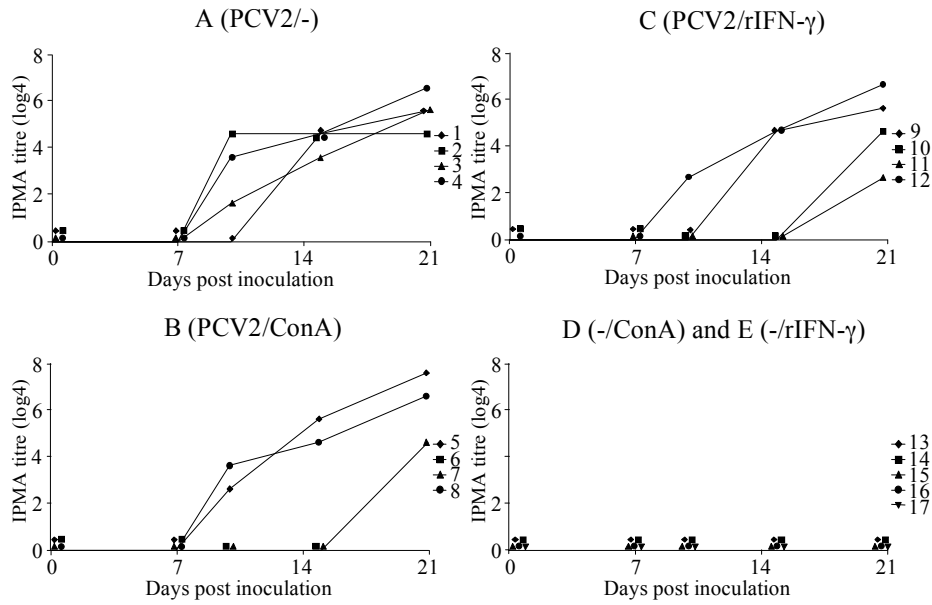


Fig. 4. Evolution of anti-PCV2 antibody titres (IPMA) in PCV2-inoculated caesarean-derived colostrum-deprived pigs of groups A (non-treated), B (treated with concanavalin A, ConA) and C (treated with recombinant interferon-gamma, rIFN- γ), and in non-inoculated pigs of groups D (treated with concanavalin A, ConA) and E (treated with recombinant interferon-gamma, rIFN- γ).

4. Discussion

In the present study, it was shown that the mitogen ConA increases PCV2 replication in porcine leukocytes both *in vitro* and *in vivo*. An effect of rIFN- γ on PCV2 replication in leukocytes was not observed under the conditions of the present study. Expression of PCV2 antigens in ConA-stimulated PBMC was observed starting from 12 hpi to 36 hpi, depending on the PCV2 strain and the blood donor that were used. This is in agreement with another study, in which PCV2 replication was observed in ConA-stimulated PBMC from 18 hpi onwards (Yu et al., 2007). In the present study, the first detectable viral antigens were localized in the nucleus. These antigens were identified as Rep proteins and capsid proteins. This suggests that in the early stages of infection, newly synthesized Rep and capsid proteins are efficiently transported to the nucleus where genome replication and encapsidation take place. As infection progressed, the number of cells with capsid proteins in the cytoplasm increased, suggesting the translocation of assembled virions from the nucleus to the cytoplasm, as suggested previously by Finsterbusch et al. (2005). At the late stages of infection, cells with intense cytoplasmic staining but without nuclear staining were observed. This was accompanied with morphological changes typical for cellular degeneration, suggesting that PCV2 infection may lead to cell death. Only in these degenerated cells, the Rep protein was occasionally found in the cytoplasm, indicating a very strong affinity of the Rep protein for the nucleus in viable cells. The results further suggest that the rise in PCV2 titres in culture supernatants coincided with the appearance of these degenerated cells. The fact that PCV2 antigens were most frequently found in the nucleus of PBMC early in replication contradicts with earlier findings in PK-15 cells. The majority of PCV2-infected PK-15 cells have capsid antigens in the cytoplasm without having Rep or capsid antigens in the nucleus during the early stages of the replication cycle (Meerts et al., 2005a). Since it is known that cellular transcription factors of lymphoid and epithelial cell types can have different specificities for promoters, enhancers and co-activators (Foster et al., 1985; Forsberg and Westin, 1991), it can be speculated that in PK-15 cells the RNA polymerase II transcription-initiation complex might have a higher affinity for the promoter region of the capsid gene than for the promoter region of the Rep gene, leading to expression of the capsid protein in a large number of cells without expression of the Rep protein. In ConA-treated PBMC, the affinities

of this complex for both promoters may be equal, leading to a simultaneous expression of the Rep protein and the capsid protein.

In the present study, it was demonstrated that PCV2 replicates in CD4⁺, CD8⁺ and IgM⁺ PBMC, suggesting that not only circulating T-lymphocytes, which was demonstrated previously by Yu et al. (2007), but also circulating B-lymphocytes may be susceptible to PCV2 replication *in vitro*. Since CD4 is also present on e.g. natural IFN- α producing cells (Charley and Lavenant, 1990) and since CD8 is also present on e.g. natural killer cells (Saalmuller et al., 1994), we cannot exclude the possibility that *in vitro* stimulation with ConA makes these cell types susceptible to PCV2 replication as well. Further research should clarify this issue. As in other *in vitro* studies (Yu et al., 2007), we failed to demonstrate PCV2 replication in PBMC-derived monocytes.

Meerts et al. (2005b) have previously shown that IFN- γ enhances PCV2 replication *in vitro* in porcine epithelial and monocytic cell lines. This enhancing effect by IFN- γ was caused by an increased internalization of bound virions into the cell. The influence of IFN- γ on the internalization of PCV2 in PBMC was not investigated in the present study. In cell lines, active cellular polymerases allow PCV2 to complete its infectious cycle. Therefore, we assume that the enhancing effect of ConA on PCV2 replication in PBMC *in vitro* was merely due to its high mitotic activity. We assume that the lack of high mitotic activity of rIFN- γ , under the conditions of the present study, was the main reason why rIFN- γ did not have an effect on PCV2 replication in PBMC *in vitro*.

In preliminary experiments, PCV2 replication was also observed in PBMC stimulated with phytohaemagglutinin and a combination of ionomycin and phorbol dibutyrate but not with LPS (data not shown). The best results were obtained with ConA and therefore this mitogen was selected for the present study. The technique that has been described is inexpensive, easy to perform and therefore it is potentially suitable for detailed *in vitro* studies of the PCV2 replication cycle in lymphocytes, studies that are nowadays merely performed in cell lines.

In the present study, it was shown that treatment of CD/CD pigs with ConA renders them more susceptible to an increased PCV2 replication. Injecting the pigs with rIFN- γ , following the protocol that was used in the present study, could not induce this increased PCV2 replication. The different outcomes of the ConA and rIFN- γ treatments could be due to various mechanisms. ConA is known to cause proliferation and activation of mainly T-lymphocytes (Mosmann et al., 1986), but it

also affects other immune cell types such as B-lymphocytes (Andersson et al., 1972) and monocytes/macrophages (Smith and Goldman, 1972). ConA also induces various cytokines and IFN- γ is just one of them. ConA thus has a much wider range of biological activities than IFN- γ . ConA also induces much higher concentrations and a more prolonged production of IFN- γ in animals compared to the IFN- γ concentration that can be reached by injecting rIFN- γ . Injected rIFN- γ is very quickly removed from the body (Lortat-Jacob et al., 1996), while ConA induces a continuous production of IFN- γ by the T-lymphocytes of the animal. Unfortunately, we were not able to demonstrate the presence of IFN- γ in plasma samples of the CD/CD pigs of the present study, nor with a bio-assay on PK-15 cells, nor with a commercial ELISA for IFN- γ (Biosource Europe, Nivelles, Belgium), indicating that IFN- γ blood levels were very low at the times of blood sampling (data not shown). Nevertheless, the treatments of pigs with ConA or rIFN- γ were effective since inguinal lymph nodes of treated pigs were clearly enlarged, whether these pigs were inoculated with PCV2 or not. Therefore, results from the present study are indicative that the enhancing effect of ConA treatment on PCV2 replication was due to the overall effect of ConA on the pig's immune system and not to the effect of IFN- γ alone. Obviously, more research needs to be performed to address this issue.

A remarkable difference between ConA-treated pigs and non-treated and rIFN- γ -treated pigs was observed in inguinal lymph node biopsies collected at 15 dpi. High numbers of PCV2-infected cells were observed in 3 out of 4 ConA-treated pigs. PCV2-infected cells were identified as CD4⁺, CD8⁺, IgM⁺ or SWC3⁺, as demonstrated previously by Sanchez et al. (2004). Although PCV2 replicated to high levels in cells of the immune system, only 2 of these 3 pigs showed a delayed onset of the humoral immune response against PCV2. High levels of PCV2 replication in cells of the immune system were not observed in non-treated and rIFN- γ -treated pigs. Still, a delayed onset of the humoral immune response was observed in 2 out of 4 rIFN- γ -injected pigs. This suggests that the delayed humoral immune response observed in 2 out of 4 ConA-treated and in 2 out of 4 rIFN- γ -treated pigs was not caused by high levels of PCV2 replication in cells of the immune system, but might have been caused by a common effect of ConA and rIFN- γ treatments in these specific pigs. Possible responsible mechanisms include the inhibitory effects of ConA-activated suppressor T-cells on B-cell proliferation and antibody production (Redelman et al., 1976), and the inhibitory effect of IFN- γ on the early stages of B-cell activation (Abed et al., 1994). The present results

further suggest that an impaired humoral immune response against PCV2 is not sufficient to cause high levels of PCV2 replication. Further research should deal with these issues.

In the present study, it was again demonstrated that it can be difficult to reproduce PCV2-associated disease in CD/CD pigs. Even in the ConA-treated pigs that experienced an increased PCV2 replication, clinical signs were not observed. Still, the ConA treatment might provide a valuable tool to investigate the link between high PCV2 replication and PMWS. Several treatments of CD/CD pigs that influence the pig's immune system have been described until now. It has been demonstrated that immune stimulation (Krakowka et al., 2001), immune suppression by Cyclosporin A (Krakowka et al., 2002; Meerts et al., 2005c) and mitogen treatment (the present study) are able to increase PCV2 replication in pigs. This observation may indicate that there is an unstable equilibrium between a specific immune response that is able to neutralize PCV2 and to eliminate PCV2-infected cells, and an immune response that facilitates the replication of the virus in the pig. This fragile balance might be the underlying mechanism responsible for the high variation that is observed in PCV2 replication and clinical outcome between individual pigs and the even higher variation between different studies.

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References

- Abed, N.S., Chace, J.H., Cowdery, J.S., 1994. T cell-independent and T cell-dependent B cell activation increases IFN-gamma R expression and renders B cells sensitive to IFN-gamma-mediated inhibition. *J Immunol* 153, 3369-3377.
- Allan, G.M., Kennedy, S., McNeilly, F., Foster, J.C., Ellis, J.A., Krakowka, S.J., Meehan, B.M., Adair, B.M., 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. *J Comp Pathol* 121, 1-11.
- Allan, G.M., McNeilly, F., Ellis, J., Krakowka, S., Meehan, B., McNair, I., Walker, I., Kennedy, S., 2000. Experimental infection of colostrum deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. *Arch Virol* 145, 2421-2429.
- Andersson, J., Edelman, G.M., Moller, G., Sjoberg, O., 1972. Activation of B lymphocytes by locally concentrated concanavalin A. *Eur J Immunol* 2, 233-235.
- Charley, B., Lavenant, L., 1990. Characterization of blood mononuclear cells producing IFN alpha following induction by coronavirus-infected cells (porcine transmissible gastroenteritis virus). *Res Immunol* 141, 141-151.
- Finsterebusch, T., Steinfeldt, T., Caliskan, R., Mankertz, A., 2005. Analysis of the subcellular localization of the proteins Rep, Rep' and Cap of porcine circovirus type 1. *Virology* 343, 36-46. Erratum in: *Virology* (2006) 349, 492.
- Forsberg, M., Westin, G., 1991. Enhancer activation by a single type of transcription factor shows cell type dependence. *EMBO J* 10, 2543-2551.
- Foster, J., Stafford, J., Queen, C., 1985. An immunoglobulin promoter displays cell-type specificity independently of the enhancer. *Nature* 315, 423-425.
- Krakowka, S., Ellis, J.A., McNeilly, F., Ringler, S., Rings, D.M., Allan, G., 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). *Vet Pathol* 38, 31-42.
- Krakowka, S., Ellis, J.A., McNeilly, F., Gilpin, D., Meehan, B., McCullough, K., Allan, G., 2002. Immunologic features of porcine circovirus type 2 infection. *Viral Immunol* 15, 567-582.
- Labarque, G.G., Nauwynck, H.J., Mesu, A.P., Pensaert, M.B., 2000. Seroprevalence of porcine circovirus types 1 and 2 in the Belgian pig population. *Vet Q* 22, 234-236.
- Liu, J., Chen, I., Kwang, J., 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. *J Virol* 79, 8262-8274.
- Lortat-Jacob, H., Baltzer, F., Grimaud, J.A., 1996. Heparin decreases the blood clearance of interferon-gamma and increases its activity by limiting the processing of its carboxyl-terminal sequence. *J Biol Chem* 271, 16139-16143.
- Mankertz, A., Hillenbrand, B., 2002. Analysis of transcription of Porcine circovirus type 1. *J Gen Virol* 83, 2743-2751.
- Mariscal, L.F., Lopez-Alcorocho, J.M., Rodriguez-Inigo, E., Ortiz-Movilla, N., de Lucas, S., Bartolomé, J., Carreno, V., 2002. TT virus replicates in stimulated but not in nonstimulated peripheral blood mononuclear cells. *Virology* 30, 121-129.
- McNeilly, F., McNair, I., Mackie, D.P., Meehan, B.M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S., Allan, G.M., 2001. Production, characterization and applications of monoclonal antibodies to porcine circovirus 2. *Arch Virol* 146, 909-922.
- Meerts, P., Misinzo, G., McNeilly, F., Nauwynck, H.J., 2005a. Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, foetal cardiomyocytes and macrophages. *Arch Virol* 150, 427-441.

- Meerts, P., Misinzo, G., Nauwynck, H.J., 2005b. Enhancement of porcine circovirus 2 replication in porcine cell lines by IFN-gamma before and after treatment and by IFN-alpha after treatment. *J Interf Cytok Res* 25, 684-693.
- Meerts, P., Van Gucht, S., Cox, E., Vandebosch, A., Nauwynck, H.J., 2005c. Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. *Viral Immunol* 18, 333-341.
- Miyagi, T., Takehara, T., Tatsumi, T., Suzuki, T., Jinushi, M., Kanazawa, Y., Hiramoto, N., Kanto, T., Tsuji, S., Hori, M., Hayashi, N., 2004. Concanavalin A injection activates intrahepatic innate immune cells to provoke an antitumor effect in murine liver. *Hepatology* 40, 1190-1196.
- Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L., 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136, 2348-2357.
- Opriessnig, T., Yu, S., Gallup, J.M., Evans, R.B., Fenaux, M., Pallares, F., Thacker, E.L., Brockus, C.W., Ackermann, M.R., Thomas, P., Meng, X.J., Halbur, P.G., 2003. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. *Vet Pathol* 40, 521-529.
- Opriessnig, T., Thacker, E.L., Yu, S., Fenaux, M., Meng, X.J., Halbur, P.G., 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. *Vet Pathol* 41, 624-640.
- Paillet, R., Laval, F., Audonnet, J.C., Andreoni, C., Juillard, V., 2001. Functional and phenotypic characterization of distinct porcine dendritic cells derived from peripheral blood monocytes. *Immunology* 102, 396-404.
- Paul, P.S., Mengeling, W.L., Brown, T.T. Jr., 1979. Replication of porcine parvovirus in peripheral blood lymphocytes, monocytes, and peritoneal macrophages. *Infect Immun* 25, 1003-1007.
- Pescovitz, M.D., Lunney, J.K., Sachs, D.H., 1984. Preparation and characterization of monoclonal antibodies reactive with porcine PBL. *J Immunol* 133, 368-375.
- Redelman, D., Scott, C.B., Sheppard, H.W. Jr., Sell, S., 1976. In vitro studies of the rabbit immune system. V. Suppressor T cells activated by concanavalin A block the proliferation, not the induction of antierythrocyte plaque-forming cells. *J Exp Med* 143, 919-936.
- Resendes, A., Segalés, J., Balasch, M., Calsamiglia, M., Sibila, M., Ellerbrok, H., Mateu, E., Plana-Duran, J., Mankertz, A., Domingo M., 2004. Lack of an effect of a commercial vaccine adjuvant on the development of postweaning multisystemic wasting syndrome (PMWS) in porcine circovirus type 2 (PCV2) experimentally infected conventional pigs. *Vet Res* 35, 83-90.
- Saalmuller, A., Hirt, W., Maurer, S., Weiland, E., 1994. Discrimination between two subsets of porcine CD8⁺ cytolytic T lymphocytes by the expression of CD5 antigen. *Immunology* 81, 578-583.
- Sanchez, R.E.Jr., Meerts, P., Nauwynck, H.J., Ellis, J.A., Pensaert M.B., 2004. Characteristics of porcine circovirus-2 replication in lymphoid organs of pigs inoculated in late gestation or postnatally and possible relation to clinical and pathological outcome of infection. *J Vet Diagn Invest* 16, 175-185.
- Segalés, J., Domingo, M., 2002. Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. *Vet Q* 24, 109-124.
- Smith, C.W., Goldman, A.S., 1972. Effects of concanavalin A and pokeweed mitogen in vivo on mouse peritoneal macrophages. *Exp Cell Res* 73, 394-398.
- Tortorella, D., Gewurz, B.E., Furman, M.H., Schust, D.J., Ploegh, H.L., 2000. Viral subversion of the immune system. *Annu Rev Immunol* 18, 861-926.

- Van Zaane, D., Hulst, M.M., 1987. Monoclonal antibodies against porcine immunoglobulin isotypes. *Vet Immunol Immunopathol* 16, 23-26.
- Verfaillie, T., Cox, E., To, L.T., Vanrompay, D., Bouchaut, H., Buys, N., Goddeeris, B.M., 2001. Comparative analysis of porcine cytokine production by mRNA and protein detection. *Vet Immunol Immunopathol* 81, 97-112.
- Yu, S., Vincent, A., Opriessnig, T., Carpenter, S., Kitikoon, P., Halbur, P.G., Thacker, E., 2007. Quantification of PCV2 capsid transcript in peripheral blood mononuclear cells (PBMCs) in vitro. *Vet Microbiol* 123, 34-42.

Chapter 5. General discussion

Since the 90's, postweaning multisystemic wasting syndrome (PMWS) affects pig production all over the world. PMWS is caused by PCV2, a small ubiquitous virus that mostly causes sub-clinical infections. PCV2 has an uncomplicated structure. The PCV2 genome codes for one structural protein (Nawagitgul et al., 2000), 2 non-structural replicase proteins (Cheung, 2003a; Cheung, 2003b) and 1 non-structural protein that may be associated with apoptosis (Liu et al., 2005). PCV2 fully depends on cellular polymerases to complete its infectious cycle and the PCV2 genome does not code for viral proteins that help to evade the host's immune system as other more complex viruses do (Tortorella et al., 2000; Vossen et al., 2002). Intriguingly, the pathogenesis of PMWS is rather complicated and interactions of PCV2 with the host's immune system seem to play a crucial role in the development of PMWS (Krakowka et al., 2001; Krakowka et al., 2002; Darwich et al., 2004; Segalés et al., 2004; Segalés and Mateu; 2006). Still, a lot of uncertainties exist on how virus-specific and host-specific factors influence these interactions. The studies included in this thesis were conducted in order to define virus-specific and host-specific factors that influence the pathogenesis of PCV2 infections and the development of PMWS.

In the first part of this thesis, it was examined to what extent PCV2 strains may be different from antigenic, genetic and pathogenetic point of view. Already in 1998, it was observed that genetic differences were present between PCV2 strains isolated from different geographic regions (Meehan et al., 1998). During the next years, other researchers made similar observations (Fenaux et al., 2000; Hamel et al., 2000; Mankertz et al., 2000; Kim and Lyoo, 2002), but at that time it was assumed that these genetic differences did not code for antigenic differences and that only one PCV2 serotype existed. This way of thinking was enforced by the observation that monoclonal antibodies (mAbs) against PCV2 reacted similarly with PCV2 strains originating from different geographic regions (Allan et al., 1999; McNeilly et al., 2001). In this thesis, a panel of 16 mAbs was developed against the capsid protein of Stoon-1010, a Canadian PCV2a (genotype 2) strain originating from a PMWS-affected pig. In an immunoperoxidase monolayer assay (IPMA), 11 mAbs reacted similarly with Stoon-1010 and 6 PCV2 strains with a different genetic and/or clinical background. Four mAbs did not react with PCV2b (genotype 1) strains or had a reduced reactivity compared to PCV2a (genotype 2) strains. One mAb reacted with strains originating from cases of PMWS or Porcine Dermatitis and Nephropathy Syndrome (PDNS) but not with strains originating from cases of

reproductive failure. This was the first and at present the only proof that antigenic differences exist between the capsid proteins of PCV2 strains with a different genotype and isolated from different clinical presentations. At present, other laboratories are trying to make similar panels of discriminating mAbs (G. Allan, personal communication). In previous studies (Allan et al., 1999; McNeilly et al., 2001), mAbs against PCV2 were produced by injecting mice 3 to 4 times with PCV2 and in 2 or 3 of these injections PCV2 was mixed with an adjuvant. Since the immune system has the tendency to mount a response towards abundant or immunodominant epitopes (Matthew and Sandrock, 1987; Sleister and Rao, 2002), a classical immunization protocol presumably favours the development of antibodies against a common immunodominant PCV2 epitope and presumably handicaps the development of antibodies against less abundant or poorly immunogenic PCV2 epitopes. In this thesis, a different immunization protocol was applied. Firstly, mice were tolerized to PK-15 cells with the immunosuppressive drug cyclophosphamide that suppresses humoral immune responses by affecting proliferating B-lymphocytes and T-lymphocytes (Stockman et al., 1973; Willers and Sluis, 1975; el-Sady et al., 1986). Secondly, mice were immunized with only one injection of PCV2 with an adjuvant followed by a second injection of PCV2 without adjuvant. By doing so, 3 different types of mAbs were obtained; i) 11 mAbs reacted similarly with all 7 tested PCV2 strains in the IPMA and it may be speculated that these mAbs were directed against a common immunodominant PCV2 epitope that may correspond with the epitope recognised by previously produced mAbs (Allan et al., 1999; McNeilly et al., 2001), ii) 4 mAbs had no or a reduced reactivity with PCV2b (genotype 1) strains in the IPMA and it may be speculated that these mAbs were directed against a typical PCV2a (genotype 2) epitope, iii) 1 mAb did not react with strains originating from cases of reproductive failure in the IPMA and it may be speculated that this mAb was directed against an epitope typical for strains originating from cases of PMWS or PDNS. Further research should reveal if these assumptions are true or not.

Interestingly, none of the 11 mAbs that reacted similarly with all 7 tested PCV2 strains in the IPMA were able to neutralize 3 out of 7 tested PCV2 strains in the neutralization assay. Similar observations were made with the 4 mAbs that had no or a reduced reactivity with PCV2b (genotype 1) strains in the IPMA. The results of the neutralization assay were independent of the genotype and the clinical background of the 3 strains that could not be neutralized. This indicates that these 3

strains have a different neutralizing epitope and suggests that these 3 strains use a different entry pathway in PK-15 cells. Further research should deal with this issue. Recently, it has been observed that the PCV2 genome is rapidly evolving. PCV2b (genotype 1) genomes were mainly published in GenBank after 2003, whereas PCV2a (genotype 2) genomes were mainly published before 2003, suggesting that PCV2b may be more recent than PCV2a (Olvera et al., 2007). Similar observations have been made in the field in Canada (Carman et al., 2006; Gagnon et al., 2007; Carman et al., 2008), USA (Cheung et al., 2007), Ireland and Northern Ireland (Allan et al., 2007), Denmark (Dupont et al., 2008), Sweden (Timmusk et al., 2008) and Switzerland (Wiederkehr et al., 2008). PCV2c (genotype 3) genomes were only found in archived material from the 80's (Dupont et al., 2008). For torque teno virus (TTV) and TTV-like mini virus (TLMV), both members of the genus *Anellovirus* of the family *Circoviridae*, it is very well known that the extremely high genetic variability that exists among these viruses is caused by mutations and viral co-infections with subsequent recombination events, leading to evasion of the host's immune system and the establishment of persistent infections (Ball et al., 1999; Nishizawa et al., 1999; Okamoto et al., 1999; Prescott et al., 1999; Worobey, 2000; Biagini et al., 2001; Biagini, 2004; Jelcic et al., 2004). Among the single-stranded DNA plant viruses of the family *Geminiviridae*, inter-species and inter-genus recombination with other geminiviruses and inter-familial recombination with nanovirus-like pathogens play a crucial role in the emergence of new geminivirus diseases and in the pathogenesis and aggravation of geminivirus disease complexes (Hou and Gilbertson, 1996; Zhou et al., 1997; Padidam et al., 1999; Saunders and Stanley, 1999; Pita et al., 2001; Stanley, 2004). The PCV2 genome is not extremely variable and PCV2 does not recombine with other viral species, but there are strong indications that recombination accounts for some of the genetic variability observed in the PCV2 genome (Cságola et al., 2006; Ma et al., 2007; Olvera et al., 2007; Hesse et al., 2008). In this thesis, a cell culture-adapted PCV2 isolate, containing 2 genetically different PCV2 strains, was purified by limiting dilution cloning *in vitro*. Remarkably, not only the 2 targeted strains were cloned, but also a third strain was obtained, and this third strain was a perfect mosaic of the 2 targeted strains, suggesting that this mosaic strain originated from recombination between the 2 targeted strains. To our knowledge, this was the first study that successfully cloned a mixed viral strain into individual viruses by limiting dilution cloning. The major advantage of this technique is that cloned, real viruses are obtained, which is not the

case when viral genomes are amplified by PCR and subsequently cloned into a plasmid. In this way, limiting dilution cloning may also be a very good tool for the purification of other viruses in the future. To our knowledge, this was also the first study that obtained both parental PCV2 strains as well as the resultant recombinant PCV2 strain. Moreover, a previously unreported recombination breakpoint, located between nucleotide positions 363 and 400 (with ATG from the capsid gene as position 1), was identified in the capsid gene and PCV2 genomes with this specific breakpoint in the capsid gene formed a separate cluster in different phylogenetic trees, confirming that recombination increases genetic variability among PCV2 strains.

PCV2 entry pathways differ between different types of host cells and at present, a universal PCV2 internalization receptor does not seem to exist (Vincent et al., 2003; Misinzo et al., 2005; Misinzo et al., 2008a; Misinzo et al., 2008b). Furthermore, it seems that the PCV2 genome is rapidly evolving due to recombination and mutation (Hughes and Piontkivska, 2008). Based on these observations, it can be speculated that PCV2 is a virus that accidentally arose in the pig population and that is constantly adapting to its host and getting fitter. In this way, it may be just a matter of time before PCV2d arises. Porcine circoviruses (PCVs) do not infect humans, cattle, sheep, horses or rabbits (Allan et al., 1994a; Tischer et al., 1995; Allan et al., 2000; Ellis et al., 2000; Ellis et al., 2001; Quintana et al., 2002; Garkavenko et al., 2004), but PCV2 may replicate in mice after experimental inoculation and cause microscopic lesions in lymphoid organs (Kiupel et al., 2001; Quintana et al., 2002; Kiupel et al., 2005; Liu et al., 2006; Zhao et al., 2007; Cságola et al., 2008). Although PCVs do not infect humans, PCV gene expression and genome replication take place in some human cell lines, but the infection is non-productive (Hattermann et al., 2004b). At present, a human circovirus that may recombine with PCVs has not been found and PCVs are not considered as a major risk for xenotransplantation (Garkavenko et al., 2004; Hattermann et al., 2004a). However, it cannot be excluded that sooner or later PCV2 and the closely related human TT virus evolve in such a way that these viruses may recombine with each other and create a new zoonotic virus that is pathogenic for pigs and humans. Taking into account that pigs can be infected with PCV2 by oral consumption of unprocessed PCV2 positive pig meat (Opriessnig et al., 2008) and that PCV2 is resistant to temperatures below 80° C (Allan et al., 1994b; Welch et al., 2006; O'Dea et al., 2008), it becomes clear that during the next decades the genetic evolution of the *Circoviridae* should be followed

with Argus' eyes, in order to avoid that people would become infected with a new zoonotic circovirus due to the consumption of pig meat.

Besides the observation that the PCV2 genome is changing over time, it has recently been observed in the field that PCV2b (genotype 1) strains are more frequently isolated from PMWS cases than PCV2a (genotype 2) strains, suggesting that PCV2b may be more pathogenic than PCV2a (Carman et al., 2006; Cheung et al., 2007; Gagnon et al., 2007; Dupont et al., 2008; Carman et al., 2008; Grau-Roma et al., 2008; Timmusk et al., 2008; Wiederkehr et al., 2008). Since it was shown in this thesis that PCV2a (genotype 2) and PCV2b (genotype 1) have different capsid epitopes, it was investigated if these different capsid epitopes could be responsible for differences in pathogenicity between PCV2a (genotype 2) and PCV2b (genotype 1) in pigs under experimental conditions. To minimize the chance that the obtained results would be a reflection of strain-specific interactions between a certain PCV2 strain and the host's immune system, rather than a reflection of the intrinsic properties of that PCV2 strain, the infection experiments were performed in 55-day-old immuno-incompetent foetuses. At 21 dpi, all 7 PCV2-inoculated foetuses, whether they were inoculated with a PCV2a (genotype 2) strain or a PCV2b (genotype 1) strain, showed liver enlargement, lymph node enlargement and haemorrhages and congestion in internal organs, and high numbers of PCV2-infected cells ($> 1,000$ infected cells / 10 mm^2 tissue) were observed in their hearts. High PCV2 titres ($> 10^{4.5}$ TCID₅₀ / g tissue) were found in all 7 PCV2-inoculated foetuses, especially in the heart, spleen and liver. The complete lack of relevant clinical and virological differences between foetuses inoculated with PCV2a (genotype 2) and foetuses inoculated with PCV2b (genotype 1) might suggest that both PCV2 genotypes are equally able to replicate in tissues from immuno-incompetent foetuses. This also suggests that the observed differences in pathogenicity of PCV2 and PCV2b under field conditions might be attributed to different interactions of PCV2a and PCV2b with the pig's immune system. Further studies have to elucidate putative differential interactions of PCV2a and PCV2b with the pig's immune system.

Unfortunately, PCV2 research still faces several obstacles. At present, a universally reproducible experimental model for the induction of high levels of PCV2 replication and subsequent PMWS does not exist, and primary immune cells, the most important target cell population *in vivo*, do not sustain productive PCV2 infection *in vitro*. In the second part of this thesis, an attempt was made to render

primary immune cells susceptible to productive PCV2 infection *in vitro* and to develop an experimental PMWS model, based on the knowledge that stimulation of the immune system is one of the factors that enhance PCV2 replication and the subsequent development of PMWS. This attempt was made by using the plant lectin concanavalin A (ConA). ConA is a potent mitogen that mainly causes proliferation (Quade and Roth, 1999; Dorn et al., 2002) and activation (Mosmann et al., 1986) of T-lymphocytes, but it also stimulates other cell types such as B-lymphocytes (Andersson et al., 1972), monocytes/macrophages (Smith and Goldman, 1972; Schmidt et al., 1973), natural killer cells (Miyagi et al., 2004), neutrophils (Asahi et al., 1993) and epithelial cells (Jans et al., 1992). ConA is a potent inducer of various cytokines *in vivo* (Tiegs, 1997; Andersson et al., 2007) and *in vitro* (Dozois et al., 1997; Verfaillie et al., 2001; Yancy et al., 2001). In this thesis, it was found that ConA induced a maximum 26-fold increase (from 0.06 % to 1.54 % for strain 1121 at 96 hours post inoculation) in the number of PCV2 positive peripheral blood mononuclear cells (PBMC) *in vitro* and induced a maximum 50-fold increase (from $10^{2.4}$ TCID₅₀ / ml to $10^{4.1}$ TCID₅₀ / ml for strain Stoon-1010 at 96 hours post inoculation) in virus titres in supernatant fluids of PCV2-inoculated PBMC. Primary immune cell types sustaining PCV2 replication *in vitro* were demonstrated to be CD4⁺, CD8⁺ or IgM⁺, suggestive for T-lymphocytes and B-lymphocytes. However, further research needs to be performed to elucidate if e.g. natural IFN- α producing cells, that are CD4⁺, (Charley and Lavenant, 1990) or natural killer cells, that are CD8⁺, (Saalmuller et al., 1994) are susceptible to PCV2 replication *in vitro* or not. Monocytes/macrophages were not susceptible to PCV2 replication after ConA stimulation. ConA is inexpensive and easy to use. The technique that has been described in this thesis may be used for detailed *in vitro* studies of different aspects of the PCV2 replication cycle in primary immune cells and research results obtained in primary immune cells may even be more truthful than those obtained in cell lines because cell lines can be biologically different from primary cells (Pan et al., 2008). PCV2 was already isolated from inguinal lymph node biopsies from 10 days post inoculation (dpi) in ConA-treated, caesarean-derived colostrum-deprived (CD/CD) piglets versus 15 dpi in non-treated piglets. ConA treatment induced a 13-fold increase (from $10^{3.9}$ TCID₅₀ / g to $10^{5.0}$ TCID₅₀ / g) in mean virus titres and a 56-fold increase (from 7 cells / 10 mm² to 391 cells / 10 mm²) in the mean number of PCV2 positive cells in inguinal lymph nodes of CD/CD piglets at 15 dpi. ConA treatment delayed seroconversion from 10 or 15 dpi to 21 dpi in half of the ConA-treated

piglets. Still, clinical signs suggestive of PMWS or histopathological lesions associated with PMWS (data not shown) were not observed. The technique that has been described in this thesis may be a good tool to increase PCV2 replication levels, but unfortunately it does not seem to be the right tool to reproduce PMWS. At present, it seems that the highest chance of experimentally reproducing PMWS is obtained when piglets are co-infected with another porcine pathogen such as porcine parvovirus or porcine reproductive and respiratory syndrome virus (Tomás et al., 2008).

This thesis aimed to identify virus-specific and host-specific factors that influence the pathogenesis of PCV2 infections and the development of PMWS. It was shown that the PCV2 genome may rapidly evolve due to recombination and that distinct antigenic differences are present between the 2 major PCV2 genotypes and between PCV2 strains originating from different clinical presentations. However, these antigenic differences did not seem to cause differences in clinical or virological outcome of PCV2 infections in immuno-incompetent foetuses. It requires further investigation if these distinct antigenic differences among PCV2 strains are responsible for pathogenic differences that have recently been observed in the field, or if on the other hand, other virus-specific, host-specific or environmental factors are involved. Further, it was shown that mitogen stimulation of lymphocytes increased PCV2 replication in pigs, but PMWS could not be reproduced.

References

- Allan, G.M., McNeilly, F., Foster, J.C., Adair, B.M., 1994a. Infection of leucocyte cell cultures derived from different species with pig circovirus. *Vet Microbiol* 41, 267-279.
- Allan, G.M., Phenix, K.V., Todd, D., McNulty, M.S., 1994b. Some biological and physico-chemical properties of porcine circovirus. *Zentralbl Veterinarmed B* 41, 17-26.
- Allan, G.M., McNeilly, F., Meehan, B.M., Kennedy, S., Mackie, D.P., Ellis, J.A., Clark, E.G., Espuna, E., Saubi, N., Riera, P., Bötner, A., Charreyre, C.E., 1999. Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. *Vet Microbiol* 66, 115-123.
- Allan, G.M., McNeilly, F., McNair, I., Curran, M.D., Walker, I., Ellis, J., Konoby, C., Kennedy, S., Meehan, B., 2000. Absence of evidence for porcine circovirus type 2 in cattle and humans, and lack of seroconversion or lesions in experimentally infected sheep. *Arch Virol* 145, 853-857.
- Allan, G.M., McNeilly, F., McMenamy, M., McNair, I., Krakowka, S.G., Timmusk, S., Walls, D., Donnelly, M., Minahin, D., Ellis, J., Wallgren, P., Fossum, C., 2007. Temporal distribution of porcine circovirus 2 genogroups recovered from

- postweaning multisystemic wasting syndrome affected and nonaffected farms in Ireland and Northern Ireland. *J Vet Diagn Invest* 19, 668-673.
- Andersson, J., Edelman, G.M., Möller, G., Sjöberg, O., 1972. Activation of B lymphocytes by locally concentrated concanavalin A. *Eur J Immunol* 2, 233-235.
- Andersson, M., Berg, M., Fossum, C., Jensen-Waern, M., 2007. Development of a microarray for studying porcine cytokine production in blood mononuclear cells and intestinal biopsies. *J Vet Med A Physiol Pathol Clin Med* 54, 161-168.
- Asahi, M., Taniguchi, T., Hashimoto, E., Inazu, T., Maeda, H., Yamamura, H., 1993. Activation of protein-tyrosine kinase p72syk with concanavalin A in polymorphonuclear neutrophils. *J Biol Chem* 268, 23334-23338.
- Ball, J.K., Curran, R., Berridge, S., Grabowska, A.M., Jameson, C.L., Thomson, B.J., Irving, W.L., Sharp, P.M., 1999. TT virus sequence heterogeneity in vivo: evidence for co-infection with multiple genetic types. *J Gen Virol* 80, 1759-1768.
- Biagini, P., Gallian, P., Attoui, H., Touinssi, M., Cantaloube, J., de Micco, P., de Lamballerie, X., 2001. Genetic analysis of full-length genomes and subgenomic sequences of TT virus-like mini virus human isolates. *J Gen Virol* 82, 379-383.
- Biagini, P., 2004. Human circoviruses. *Vet Microbiol* 98, 95-101.
- Carman, S., McEwen, B., DeLay, J., van Dreumel, T., Lulis, P., Cai, H., Fairles, J., 2006. Porcine circovirus-2 associated disease in swine in Ontario (2004 to 2005). *Can Vet J* 47, 761-762.
- Carman, S., Cai, H.Y., DeLay, J., Youssef, S.A., McEwen, B.J., Gagnon, C.A., Tremblay, D., Hazlett, M., Lulis, P., Fairles, J., Alexander, H.S., van Dreumel, T., 2008. The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease--2004-2006. *Can J Vet Res* 72, 259-268.
- Charley, B., Lavenant, L., 1990. Characterization of blood mononuclear cells producing IFN alpha following induction by coronavirus-infected cells (porcine transmissible gastroenteritis virus). *Res Immunol* 141, 141-151.
- Cheung, A.K., 2003a. Transcriptional analysis of porcine circovirus type 2. *Virology* 305, 168-180.
- Cheung, A.K., 2003b. The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. *Virology* 313, 452-459.
- Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. *Arch Virol* 152, 1035-1044.
- Csárgola, A., Kecskeméti, S., Kardos, G., Kiss, I., Tuboly, T., 2006. Genetic characterization of type 2 porcine circoviruses detected in Hungarian wild boars. *Arch Virol* 151, 495-507.
- Csárgola, A., Cadar, D., Tuboly, T., 2008. Replication and transmission of porcine circovirus type 2 in mice. *Acta Vet Hung* 56, 421-427.
- Darwich, L., Segalés, J., Mateu, E., 2004. Pathogenesis of postweaning multisystemic wasting syndrome caused by Porcine circovirus 2: An immune riddle. *Arch Virol* 149, 857-874.
- Dorn, A.D., Waters, W.R., Byers, V.M., Pesch, B.A., Wannemuehler, M.J., 2002. Characterization of mitogen-stimulated porcine lymphocytes using a stable fluorescent dye (PKH2) and multicolor flow cytometry. *Vet Immunol Immunopathol* 87, 1-10.
- Dozois, C.M., Oswald, E., Gautier, N., Serthelon, J.P., Fairbrother, J.M., Oswald, I.P., 1997. A reverse transcription-polymerase chain reaction method to analyze porcine cytokine gene expression. *Vet Immunol Immunopathol* 58, 287-300.
- Dupont, K., Nielsen, E.O., Bækbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in

- genotypes with time. *Vet Microbiol* 128, 56-64.
- Ellis, J.A., Wiseman, B.M., Allan, G., Konoby, C., Krakowka, S., Meehan, B.M., McNeilly, F., 2000. Analysis of seroconversion to porcine circovirus 2 among veterinarians from the United States and Canada. *J Am Vet Med Assoc* 217, 1645-1646.
- Ellis, J.A., Konoby, C., West, K.H., Allan, G.M., Krakowka, S., McNeilly, F., Meehan, B., Walker, I., 2001. Lack of antibodies to porcine circovirus type 2 virus in beef and dairy cattle and horses in western Canada. *Can Vet J* 42, 461-464.
- el-Sady, E., Parker, D., Turk, J.L., 1986. The effect of cyclophosphamide in vivo on the expression of lymphocyte markers, detected by monoclonal antibodies, in the rat. *Int J Immunopharmacol* 8, 961-966.
- Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.J., 2000. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. *J Clin Microbiol* 38, 2494-2503.
- Gagnon, C.A., Tremblay, D., Tijssen, P., Venne, M.H., Houde, A., Elahi, S.M., 2007. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. *Can Vet J* 48, 811-819.
- Garkavenko, O., Croxson, M.C., Irgang, M., Karlas, A., Denner, J., Elliott, R.B., 2004. Monitoring for presence of potentially xenotic viruses in recipients of pig islet xenotransplantation. *J Clin Microbiol* 42, 5353-5356.
- Grau-Roma, L., Crisci, E., Sibila, M., López-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A., Segalés, J., 2008. A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. *Vet Microbiol* 128, 23-35.
- Hamel, A.L., Lin, L.L., Sachvie, C., Grudeski, E., Nayar, G.P., 2000. PCR detection and characterization of type-2 porcine circovirus. *Can J Vet Res* 64, 44-52.
- Hattermann, K., Maerz, A., Slanina, H., Schmitt, C., Mankertz, A., 2004a. Assessing the risk potential of porcine circoviruses for xenotransplantation: consensus primer-PCR-based search for a human circovirus. *Xenotransplantation* 11, 547-550.
- Hattermann, K., Roedner, C., Schmitt, C., Finsterbusch, T., Steinfeldt, T., Mankertz, A., 2004b. Infection studies on human cell lines with porcine circovirus type 1 and porcine circovirus type 2. *Xenotransplantation* 11, 284-294.
- Hesse, R., Kerrigan, M., Rowland, R.R., 2008. Evidence for recombination between PCV2a and PCV2b in the field. *Virus Res* 132, 201-207.
- Hou, Y.M., Gilbertson, R.L., 1996. Increased pathogenicity in a pseudorecombinant bipartite geminivirus correlates with intermolecular recombination. *J Virol* 70, 5430-5436.
- Hughes, A.L., Piontkivska, H., 2008. Nucleotide sequence polymorphism in circoviruses. *Infect Genet Evol* 8, 130-138.
- Jans, D.A., Jans, P., Luzius, H., Fahrenholz, F., 1992. N-glycosylation plays a role in biosynthesis and internalization of the adenylate cyclase stimulating vasopressin V2-receptor of LLC-PK1 renal epithelial cells: an effect of concanavalin A on binding and expression. *Arch Biochem Biophys* 294, 64-69.
- Jelcic, I., Hotz-Wagenblatt, A., Hunziker, A., Zur Hausen, H., de Villiers, E.M., 2004. Isolation of multiple TT virus genotypes from spleen biopsy tissue from a Hodgkin's disease patient: genome reorganization and diversity in the hypervariable region. *J Virol* 78, 7498-7507.
- Kim, J.H., Lyoo, Y.S., 2002. Genetic characterization of porcine-circovirus-2 field isolates from PMWS-pigs. *J Vet Sci* 3, 31-39.
- Kiupel, M., Stevenson, G.W., Choi, J., Latimer, K.S., Kanitz, C.L., Mittal, S.K., 2001. Viral replication and lesions in BALB/c mice experimentally inoculated with porcine

- circovirus isolated from a pig with postweaning multisystemic wasting disease. *Vet Pathol* 38, 74-82.
- Kiupel, M., Stevenson, G.W., Galbreath, E.J., North, A., HogenEsch, H., Mittal, S.K., 2005. Porcine circovirus type 2 (PCV2) causes apoptosis in experimentally inoculated BALB/c mice. *BMC Vet Res* 1, 7.
- Krakowka, S., Ellis, J.A., McNeilly, F., Ringler, S., Rings, D.M., Allan, G., 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). *Vet Pathol* 38, 31-42.
- Krakowka, S., Ellis, J.A., McNeilly, F., Gilpin, D., Meehan, B., McCullough, K., Allan, G., 2002. Immunologic features of porcine circovirus type 2 infection. *Viral Immunol* 15, 567-582.
- Liu, J., Chen, I., Kwang, J., 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. *J Virol* 79, 8262-8274.
- Liu, J., Chen, I., Du, Q., Chua, H., Kwang, J., 2006. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. *J Virol* 80, 5065-5073.
- Ma, C.M., Hon, C.C., Lam, T.Y., Li, V.Y., Wong, C.K., de Oliveira, T., Leung, F.C., 2007. Evidence for recombination in natural populations of porcine circovirus type 2 in Hong Kong and mainland China. *J Gen Virol* 88, 1733-1737.
- Mankertz, A., Domingo, M., Folch, J.M., Le Cann, P., Jestin, A., Segales, J., Chmielewicz, B., Plana-Duran, J., Soike, D., 2000. Characterisation of PCV-2 isolates from Spain, Germany and France. *Virus Res* 66, 65-77.
- Matthew, W.D., Sandrock, A.W., 1987. Cyclophosphamide treatment used to manipulate the immune response for the production of monoclonal antibodies. *J Immunol Methods* 100, 73-82.
- McNeilly, F., McNair, I., Mackie, D.P., Meehan, B.M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S., Allan, G.M., 2001. Production, characterization and applications of monoclonal antibodies to porcine circovirus 2. *Arch Virol* 146, 909-922.
- Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *J Gen Virol* 79, 2171-2179.
- Misinzo, G., Meerts, P., Bublot, M., Mast, J., Weingartl, H.M., Nauwynck, H.J., 2005. Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. *J Gen Virol* 86, 2057-2068.
- Misinzo, G., Delputte, P.L., Lefebvre, D.J., Nauwynck, H.J., 2008a. Porcine circovirus 2 infection of epithelial cells is clathrin-, caveolae- and dynamin-independent, actin and Rho-GTPase-mediated, and enhanced by cholesterol depletion. *Virus Res*, in press.
- Misinzo, G., Delputte, P.L., Nauwynck, H.J., 2008b. Inhibition of endosome-lysosome system acidification enhances porcine circovirus 2 infection of porcine epithelial cells. *J Virol* 82, 1128-1135.
- Miyagi, T., Takehara, T., Tatsumi, T., Suzuki, T., Jinushi, M., Kanazawa, Y., Hiramatsu, N., Kanto, T., Tsuji, S., Hori, M., Hayashi, N., 2004. Concanavalin A injection activates intrahepatic innate immune cells to provoke an antitumor effect in murine liver. *Hepatology* 40, 1190-1196.
- Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L., 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136, 2348-2357.
- Nawagitgul, P., Morozov, I., Bolin, S.R., Harms, P.A., Sorden, S.D., Paul, P.S., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. *J Gen Virol* 81, 2281-2287.

- Nishizawa, T., Okamoto, H., Tsuda, F., Aikawa, T., Sugai, Y., Konishi, K., Akahane, Y., Ukita, M., Tanaka, T., Miyakawa, Y., Mayumi, M., 1999. Quasispecies of TT virus (TTV) with sequence divergence in hypervariable regions of the capsid protein in chronic TTV infection. *J Virol* 73, 9604-9608.
- O'Dea, M.A., Hughes, A.P., Davies, L.J., Muhling, J., Buddle, R., Wilcox, G.E., 2008. Thermal stability of porcine circovirus type 2 in cell culture. *J Virol Methods* 147, 61-66.
- Okamoto, H., Takahashi, M., Nishizawa, T., Ukita, M., Fukuda, M., Tsuda, F., Miyakawa, Y., Mayumi, M., 1999. Marked genomic heterogeneity and frequent mixed infection of TT virus demonstrated by PCR with primers from coding and noncoding regions. *Virology* 259, 428-436.
- Olvera, A., Cortey, M., Segalés, J., 2007. Molecular evolution of porcine circovirus type 2 genomes: Phylogeny and clonality. *Virology* 357, 175-185.
- Opriessnig, T., Patterson, A.R., Meng, X.J., Halbur, P.G., 2008. Porcine circovirus type 2 in muscle and bone marrow is infectious and transmissible to naïve pigs by oral consumption. *Vet Microbiol* doi:10.1016/j.vetmic.2008.06.018
- Padidam, M., Sawyer, S., Fauquet, C.M., 1999. Possible emergence of new geminiviruses by frequent recombination. *Virology* 265, 218-225.
- Pan, C., Kumar, C., Bohl, S., Klingmueller, U., Mann, M., 2008. Comparative proteomic phenotyping of cell lines and primary cells to assess preservation of cell type specific functions. *Mol Cell Proteomics*, in press.
- Pita, J.S., Fondong, V.N., Sangaré, A., Otim-Nape, G.W., Ogwal, S., Fauquet, C.M., 2001. Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. *J Gen Virol* 82, 655-665.
- Prescott, L.E., MacDonald, D.M., Davidson, F., Mokili, J., Pritchard, D.I., Arnot, D.E., Riley, E.M., Greenwood, B.M., Hamid, S., Saeed, A.A., McClure, M.O., Smith, D.B., Simmonds, P., 1999. Sequence diversity of TT virus in geographically dispersed human populations. *J Gen Virol* 80, 1751-1758.
- Quade, M.J., Roth, J.A., 1999. Dual-color flow cytometric analysis of phenotype, activation marker expression, and proliferation of mitogen-stimulated bovine lymphocyte subsets. *Vet Immunol Immunopathol* 67, 33-45.
- Quintana, J., Balasch, M., Segalés, J., Calsamiglia, M., Rodríguez-Arriola, G.M., Planadurán, J., Domingo, M., 2002. Experimental inoculation of porcine circoviruses type 1 (PCV1) and type 2 (PCV2) in rabbits and mice. *Vet Res* 33, 229-237.
- Saalmuller, A., Hirt, W., Maurer, S., Weiland, E., 1994. Discrimination between two subsets of porcine CD8+ cytolytic T lymphocytes by the expression of CD5 antigen. *Immunology* 81, 578-583.
- Saunders, K., Stanley, J., 1999. A nanovirus-like DNA component associated with yellow vein disease of *Ageratum conyzoides*: evidence for interfamilial recombination between plant DNA viruses. *Virology* 264, 142-152.
- Schmidt, M.E., Douglas, S.D., Rubin, A.D., 1973. Human monocyte activation by supernatants from concanavalin A (con A) stimulated lymphocytes. *Cell Immunol* 9, 45-59.
- Segalés, J., Domingo, M., Chianini, F., Majó, N., Domínguez, J., Darwich, L., Mateu, E., 2004. Immunosuppression in postweaning multisystemic wasting syndrome affected pigs. *Vet Microbiol* 98, 151-158.
- Segalés, J., Mateu, E., 2006. Immunosuppression as a feature of postweaning multisystemic wasting syndrome. *Vet J* 171, 396-397.
- Sleister, H.M., Rao, A.G., 2002. Subtractive immunization: a tool for the generation of discriminatory antibodies to proteins of similar sequence. *J Immunol Methods* 261, 213-220.

- Smith, C.W., Goldman, A.S., 1972. Effects of concanavalin A and pokeweed mitogen in vivo on mouse peritoneal macrophages. *Exp Cell Res* 73, 394-398.
- Stanley, J., 2004. Subviral DNAs associated with geminivirus disease complexes. *Vet Microbiol* 98, 121-129.
- Stockman, G.D., Heim, L.R., South, M.A., Trentin, J.J., 1973. Differential effects of cyclophosphamide on the B and T cell compartments of adult mice. *J Immunol* 110, 277-282.
- Tiegs, G., 1997. Experimental hepatitis and role of cytokines. *Acta Gastro-Ent Belg* 60, 176-179.
- Timmusk, S., Wallgren, P., Brunborg, I.M., Wikström, F.H., Allan, G., Meehan, B., McMenamy, M., McNeilly, F., Fuxler, L., Belák, K., Pödersoo, D., Saar, T., Berg, M., Fossum, C., 2008. Phylogenetic analysis of porcine circovirus type 2 (PCV2) pre- and post-epizootic postweaning multisystemic wasting syndrome (PMWS). *Virus Genes* 36, 509-520.
- Tischer, I., Bode, L., Apodaca, J., Timm, H., Peters, D., Rasch, R., Pociuli, S., Gerike, E., 1995. Presence of antibodies reacting with porcine circovirus in sera of humans, mice, and cattle. *Arch Virol* 140, 1427-1439.
- Tomás, A., Fernandes, L.T., Valero, O., Segalés, J., 2008. A meta-analysis on experimental infections with porcine circovirus type 2 (PCV2). *Vet Microbiol* doi:10.1016/j.vetmic.2008.05.023.
- Tortorella, D., Gewurz, B.E., Furman, M.H., Schust, D.J., Ploegh, H.L., 2000. Viral subversion of the immune system. *Annu Rev Immunol* 18, 861-926.
- Verfaillie, T., Cox, E., To, L.T., Vanrompay, D., Bouchaut, H., Buys, N., Goddeeris, B.M., 2001. Comparative analysis of porcine cytokine production by mRNA and protein detection. *Vet Immunol Immunopathol* 81, 97-112.
- Vincent, I.E., Carrasco, C.P., Herrmann, B., Meehan, B.M., Allan, G.M., Summerfield, A., McCullough, K.C., 2003. Dendritic cells harbor infectious porcine circovirus type 2 in the absence of apparent cell modulation or replication of the virus. *J Virol* 77, 13288-13300.
- Vossen, M.T., Westerhout, E.M., Söderberg-Nauclér, C., Wiertz, E.J., 2002. Viral immune evasion: a masterpiece of evolution. *Immunogenetics* 54, 527-542.
- Welch, J., Bienek, C., Gomperts, E., Simmonds, P., 2006. Resistance of porcine circovirus and chicken anemia virus to virus inactivation procedures used for blood products. *Transfusion* 46, 1951-1958.
- Wiederkehr, D.D., Sydler, T., Brugnera, E., Pospischil, A., Buergi, E., Sidler, X., 2008. Pathogenic differences of porcine circovirus type 2 genotypes in Switzerland. In: *Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 1*, pp. 26.
- Willers, J.M., Sluis, E., 1975. The influence of cyclophosphamide on antibody formation in the mouse. *Ann Immunol (Paris)* 126, 267-279.
- Worobey, M., 2000. Extensive homologous recombination among widely divergent TT viruses. *J Virol* 74, 7666-7670.
- Yancy, H., Ayers, S.L., Farrell, D.E., Day, A., Myers, M.J. 2001. Differential cytokine mRNA expression in swine whole blood and peripheral blood mononuclear cell cultures. *Vet Immunol Immunopathol* 79, 41-52.
- Zhao, L., Song, C., Yang, Z., 2007. Experimental inoculation of porcine circovirus type 2 (PCV2) in nude mice and BALB/c mice. In: *Proceedings of the 5th International Symposium on Emerging and Re-emerging Pig Diseases, Krakow, Poland*, pp. 54.
- Zhou, X., Liu, Y., Calvert, L., Munoz, C., Otim-Nape, G.W., Robinson, D.J., Harrison, B.D., 1997. Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. *J Gen Virol* 78, 2101-2111.

Chapter 6. Summary - Samenvatting

Porcine circovirus type 2 (PCV2) is a ubiquitous virus that infects young pigs after weaning. The vast majority of these infections are sub-clinical. However, under certain conditions, PCV2 infection causes postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease that causes severe growth retardation, weight loss and death in weaned piglets. The past decade, substantial knowledge has been acquired on the pathogenesis of PCV2 infections and PMWS, but a lot of factors that influence the clinical outcome of a PCV2 infection are still unknown.

In [chapter 1](#), an introduction is given on PCV2 taxonomy, morphological and physical characteristics, genomic organization, replication, phylogeny and virus-host cell interactions. The current knowledge on the pathogenesis of PCV2 infections and PCV2-associated diseases is reviewed.

In [chapter 2](#), the aims of the thesis are outlined. A major aim was to identify virus-specific factors that influence the pathogenesis of PCV2 infections. Secondly, it was examined if it was possible to establish a reproducible experimental PMWS model.

In [chapter 3](#), it was examined to what extent PCV2 strains originating from cases of PMWS, reproductive failure or porcine dermatitis and nephropathy syndrome (PDNS), may be different from antigenic, genetic and pathogenetic point of view.

In [chapter 3.1](#), the antigenic structure of PCV2 was examined. More specifically, it was examined if antigenic differences among PCV2 strains could be detected by using monoclonal antibodies (mAbs). Prior to immunization, Balb/c mice were made immunotolerant to PCV negative PK-15 cells by repeated injection with cyclophosphamide and PCV negative PK-15 cells. Afterwards, PK-15 cells infected with the Canadian PMWS-associated PCV2a (genotype 2) strain Stoon-1010 were used for immunization. Sixteen stable hybridomas that produced mAbs with an immunoperoxidase monolayer assay (IPMA) titre of 1,000 or more to Stoon-1010 were obtained. Staining of recombinant PCV2 virus-like particles demonstrated that all 16 mAbs were directed against the PCV2 capsid protein. Further, mAbs 21C12, 31D5, 38C1 and 108E8 reacted with the 28 kDa PCV2 capsid protein in a Western blot assay. Cross-reactivity of mAbs was tested by IPMA and neutralization assay for 6 other cell-culture adapted PCV2 strains. These strains were PCV2b (genotype 1) strains 48285 (PMWS, France), 1206 (PMWS, Belgium), VC2002 (PMWS, Belgium) and 1147 (PDNS, UK), and PCV2a (genotype 2) strains 1121 (reproductive failure, Canada) and 1103 (reproductive failure, Canada). Eleven mAbs (9C3, 16G12, 21C12, 38C1, 43E10, 55B1, 63H3, 70A7, 94H8, 103H7 and 114C8) recognized all 7 strains in the IPMA and demonstrated neutralization to

strains Stoon-1010, 48285, 1206 and 1103, but not to strains VC2002, 1147 and 1121. Monoclonal Abs 31D5, 48B5, 59C6 and 108E8 did not react with PCV2b (genotype 1) strains or had a reduced reactivity compared to PCV2a (genotype 2) strains in the IPMA and neutralization assay. Monoclonal Ab 13H4 reacted in the IPMA with PMWS-associated strains Stoon-1010, 48285, 1206 and VC2002, and the PDNS-associated strain 1147, but not with reproductive failure-associated strains 1121 and 1103. Monoclonal Ab 13H4 did not neutralize any of the 7 tested strains. It was concluded that despite the high amino acid identity of the capsid protein ($\geq 91\%$), antigenic differences at the capsid protein level are present between PCV2 strains with a different genotype and isolated from different clinical presentations. In this study, it was also observed that the Belgian PMWS-associated strains VC2002 and 1206 were mixtures of different PCV2 viruses. It was shown that strain VC2002 was a mixture of 2 PCV2b (genotype 1) strains (VC2002-k2 and VC2002-k39, respectively) and that strain 1206 was a mixture of a PCV2a (genotype 2) strain and a PCV2b (genotype 1) strain.

In [chapter 3.2](#), it was examined if recombination between 2 different PCV2 viruses may give rise to a new, genetically different, recombinant virus in PMWS-affected pigs. In this study, the cell culture-adapted PCV2 strain VC2002, a mixture of PCV2 strains VC2002-k2 and VC2002-k39, was purified by limited dilution cloning in PK-15 cells. Following limited dilution, several clones were sequenced and 3 different strains were obtained. One strain ('strain 1') corresponded with VC2002-k2 and another strain ('strain 2') corresponded with VC2002-k39. The third strain ('strain 3') was a chimeric VC2002-k2/VC2002-k39/VC2002-k2 strain, with a recombination breakpoint in the ORF2 (capsid) gene located between positions 363-400 (with ATG from ORF2 as position 1) and a recombination breakpoint in the ORF1 (Rep) gene located between positions 864-973. The breakpoint in the ORF1 gene was similar to breakpoints previously described in literature. The breakpoint in the ORF2 gene had not been described in literature before, although similar breakpoints were already described in the 3 different genera of the family *Circoviridae*, and more specifically in Beak and feather disease virus, Chicken anemia virus and TT virus. However, analysis with BlastN of PCV2 genomic sequences available in databases revealed that also other PCV2 strains have a recombination breakpoint in the ORF2 gene located between positions 363-400 (with ATG from ORF2 as position 1). Subsequent phylogenetic analysis provided further evidence for the recombinant origin of 'strain 3'; 'strain 3' clustered with

similar recombinant strains on separate branches in the respective 1C or 1A/1B phylogenetic clusters (according to the classification system of Olvera et al., 2007^a), depending on the algorithm used and the part of the genome taken into account. In order to determine if the recombinant 'strain 3' originated from recombination in a PMWS-affected pig *in vivo*, ORF2 and ORF1 genes from the original lymph node material were amplified by PCR, and subsequently cloned into a vector and sequenced. Sequences corresponded with 'strains 1 and 2' but not with the recombinant 'strain 3'. It was concluded that PCV2 may undergo recombination and that recombination may increase genetic diversity within PCV2.

In [chapter 3.3](#), it was examined if the genetic and antigenic differences that were observed among PCV2 strains with different genotypes and originating from different clinical presentations, could induce differences in viral replication in porcine foetuses. The clinical and virological outcome of surgical inoculation of 55-day-old immuno-incompetent porcine foetuses were determined. Seven foetuses were inoculated with PCV2; 2 with the PMWS-associated PCV2a (genotype 2) strain Stoon-1010, 2 with the reproductive failure-associated PCV2a (genotype 2) strain 1121, 2 with the PMWS-associated PCV2b (genotype 1) strain 48285 and one with the PDNS-associated PCV2b (genotype 1) strain 1147. At 21 days post inoculation, 6 out of 7 PCV2-inoculated foetuses were oedematous and had distended abdomens, whereas the strain 1147-inoculated foetus had a normal external appearance. All 7 PCV2-inoculated foetuses had haemorrhages and congestion in internal organs and an enlarged liver. High PCV2 titres ($> 10^{4.5}$ TCID₅₀ / g tissue) were found in all 7 PCV2-inoculated foetuses, especially in the heart, spleen and liver. High numbers of PCV2-infected cells ($> 1,000$ infected cells / 10 mm² tissue) were observed in the hearts of all 7 PCV2-inoculated foetuses. One of the 2 foetuses inoculated with strain 1121 had a low PCV2-specific antibody titre; the other PCV2-inoculated foetuses were negative for PCV2-specific antibodies. PCV2 did not spread to non-inoculated foetuses. Apparent differences in clinical or virological outcome were not observed between any of the PCV2-inoculated foetuses, suggesting that PCV2 strains with different genotypes and originating from different clinical presentations induce similar gross pathological lesions and replicate to similar high titres in organs of 55-day-old immuno-incompetent porcine foetuses.

^a Olvera, A., Cortey, M., Segalés, J., 2007. *Virology* 357, 175-185.

In [chapter 4](#), it was examined if it was possible to establish an experimental PMWS model based on the knowledge that stimulation of the immune system enhances PCV2 replication in pigs. More specifically, it was examined in [chapter 4.1](#) if stimulation of the immune system with the mitogen concanavalin A (ConA) could increase PCV2 replication in immune cells and subsequently cause PMWS. Since ConA mainly induces T-lymphocyte activation and also initiates the production of interferon-gamma (IFN- γ), a cytokine that enhances PCV2 replication in porcine epithelial and monocytic cell lines *in vitro*, it was also examined if IFN- γ had similar effects on PCV2 replication as ConA. In an *in vitro* study, ConA but not IFN- γ enhanced PCV2 replication in peripheral blood mononuclear cells (PBMC). Up to 2.08 % and 0.96 % of PBMC were antigen positive for PCV2 strains 1121 and Stoon-1010 at 96 and 48 hours post inoculation (hpi), respectively. Infectious virus was demonstrated in culture supernatants of PCV2-inoculated PBMC, with maximum titres of $10^{3.2}$ TCID₅₀ / ml supernatant and $10^{4.1}$ TCID₅₀ / ml supernatant for strains 1121 and Stoon-1010 at 96 hpi, respectively. At 72 hpi, PCV2 positive PBMC were identified as CD4⁺ (40 % \pm 5 %), CD8⁺ (54 % \pm 11 %) or IgM⁺ (11 % \pm 4 %). In a subsequent *in vivo* study, caesarean-derived colostrum-deprived (CD/CD) piglets were injected with ConA or IFN- γ 12 hours before inoculation and every 3 days for 9 days after inoculation with strain 1121. PCV2 was isolated from inguinal lymph node biopsies from 10 days post inoculation (dpi) in ConA-treated piglets and from 15 dpi in non-treated and IFN- γ -treated piglets. ConA but not IFN- γ increased mean PCV2 replication levels (from $10^{3.9}$ TCID₅₀ / g tissue to $10^{5.0}$ TCID₅₀ / g tissue) and the mean number of PCV2 positive cells (from 7 cells / 10 mm² tissue to 391 cells / 10 mm² tissue) in inguinal lymph node biopsies of CD/CD piglets at 15 dpi. PCV2 positive cells were identified as CD4⁺ (14 % \pm 11 %), CD8⁺ (10 % \pm 3 %), IgM⁺ (11 % \pm 8 %) or SWC3⁺ (69 \pm 22 %). Two out of 4 ConA-treated and 2 out of 4 IFN- γ -treated piglets showed a delayed humoral immune response from 10 or 15 dpi to 21 dpi, but this delay did not result in increased PCV2 replication. Clinical signs suggestive of PMWS were not observed in any of the pigs. It was concluded that ConA enhances PCV2 replication in PBMC *in vitro* and in lymphoid tissues *in vivo*.

As a general conclusion, it can be stated that PCV2 strains are genetically and antigenically more diverse than they were presumed to be. Despite this diversity, it does not seem that different pathotypes or serotypes of PCV2 exist at present. Despite scientific progress during the last decade, the pathogenesis of PMWS remains partially unexplained.

Porcien circovirus type 2 (PCV2) is een alomtegenwoordig virus dat jonge varkens infecteert na het spenen. Het merendeel van deze infecties verloopt subklinisch. Onder bepaalde omstandigheden kunnen PCV2 infecties echter aanleiding geven tot het wegwijnsyndroom. Dit is een multifactoriële ziekte die ernstige groeivertraging, gewichtsverlies en sterfte veroorzaakt bij gespeende biggen. Het afgelopen decennium werd er belangrijke wetenschappelijke kennis vergaard over de pathogenese van PCV2 infecties en het wegwijnsyndroom. Desalniettemin zijn er nog steeds meerdere ongekende factoren die een effect hebben op de klinische uitkomst van PCV2 infecties.

In hoofdstuk 1 wordt een overzicht gegeven over de taxonomie, morfologische en fysische eigenschappen, genomorganisatie, replicatie, fylogenie en virus-gastheercel interacties van PCV2. Daarnaast wordt er een overzicht gegeven van de bestaande kennis van de pathogenese van PCV2 infecties en PCV2-geassocieerde ziektesyndromen.

In hoofdstuk 2 worden de doelstellingen van deze thesis uiteengezet. De voornaamste doelstelling was het identificeren van virusspecifieke factoren die de pathogenese van PCV2 infecties beïnvloeden. Er werd eveneens onderzocht of het mogelijk is om een reproduceerbaar experimenteel model voor het wegwijnsyndroom te ontwikkelen.

In hoofdstuk 3 werd nagegaan in welke mate PCV2 stammen die geïsoleerd werden uit gevallen van wegwijnsyndroom, reproductiestoornissen of porcien dermatitis en nephropathy syndroom (PDNS), zouden kunnen verschillen op antigenisch, genetisch en pathogenetisch vlak.

In hoofdstuk 3.1 werd de antigenische structuur van PCV2 onderzocht. Er werd nagegaan of het mogelijk is om antigenische verschillen te detecteren tussen PCV2 stammen door gebruik te maken van monoklonale antistoffen. Alvorens te worden geïmmuniseerd, werden Balb/c muizen immunotolerant gemaakt tegenover PCV-negatieve PK-15 cellen door middel van injecties met cyclophosphamide en PCV-negatieve PK-15 cellen. Vervolgens werden de muizen geïmmuniseerd met PK-15 cellen die geïnfecteerd waren met de Canadese wegwijnsyndroom-geassocieerde PCV2a (genotype 2) stam Stoon-1010. Na fusie werden er 16 stabiele hybridoma's verkregen die monoklonale antistoffen produceerden met een minimale immunoperoxidase monolaag assay (IPMA) titer van 1000 of meer tegenover Stoon-1010. Alle 16 monoklonale antistoffen reageerden met recombinante PCV2-achtige partikels, waardoor werd aangetoond dat ze gericht waren tegen het PCV2 capsid

eiwit. Bovendien reageerden monoklonale antistoffen 21C12, 31D5, 38C1 en 108E8 met het 28 kDa PCV2 capsid eiwit in een Western blot assay. De kruisreactiviteit van de monoklonale antistoffen tegenover 6 andere celcultuurgeadapteerde PCV2 stammen werd onderzocht door middel van IPMA en virusneutralisatietesten. Deze 6 stammen waren de PCV2b (genotype 1) stammen 48285 (wegkwijnsyndroom, Frankrijk), 1206 (wegkwijnsyndroom, België), VC2002 (wegkwijnsyndroom, België) en 1147 (PDNS, Verenigd Koninkrijk), en de PCV2a (genotype 2) stammen 1121 (reproductiestoornissen, Canada) en 1103 (reproductiestoornissen, Canada). Elf monoklonale antistoffen (9C3, 16G12, 21C12, 38C1, 43E10, 55B1, 63H3, 70A7, 94H8, 103H7 and 114C8) reageerden met alle 7 stammen in de IPMA en neutraliseerden stammen Stoon-1010, 48285, 1206 and 1103, maar niet stammen VC2002, 1147 and 1121. Monoklonale antistoffen 31D5, 48B5, 59C6 and 108E8 reageerden niet met PCV2b (genotype 1) stammen of hadden een gereduceerde reactiviteit met deze stammen in vergelijking met PCV2a (genotype 2) stammen in de IPMA en de virusneutralisatietesten. Monoklonale antistof 13H4 reageerde in de IPMA met de wegkwijnsyndroom-geassocieerde stammen Stoon-1010, 48285, 1206 en VC2002, en de PDNS-geassocieerde stam 1147, maar niet met de reproductiestoornis-geassocieerde stammen 1121 and 1103. Monoklonale antistof 13H4 neutraliseerde geen enkele van de 7 geteste stammen. Er werd geconcludeerd dat ondanks de hoge aminozuur identiteit op het niveau van het capsid eiwit ($\geq 91\%$), er wel degelijk antigenische verschillen bestaan tussen PCV2 stammen met een verschillend genotype en tussen PCV2 stammen die geïsoleerd werden uit verschillende klinische presentaties. In deze studie werd er eveneens waargenomen dat de Belgische wegkwijnsyndroom-geassocieerde stammen VC2002 en 1206 een mengsel waren van verschillende PCV2 virussen: VC2002 was een mengsel van 2 PCV2b (genotype 1) stammen (VC2002-k2 en VC2002-k39) en stam 1206 was een mengsel van een PCV2a (genotype 2) stam en een PCV2b (genotype 1) stam.

In [hoofdstuk 3.2](#) werd er onderzocht of recombinatie tussen 2 verschillende PCV2 virussen die gelijktijdig aanwezig waren in een varken met wegkwijnsyndroom zou kunnen aanleiding geven tot het ontstaan van een nieuw, genetisch verschillend, recombinant virus. In deze studie werd de celcultuurgeadapteerde PCV2 stam VC2002, een mengsel van PCV2 stammen VC2002-k2 en VC2002-k39, opgezuiverd door middel van een limiterende verdunningsklonering in PK-15 cellen. Na deze klonering werden verschillende klonen gesequeneerd en er werden 3 verschillende stammen gedetecteerd. Eén stam ('stam 1') kwam overeen met

VC2002-k2 en een andere stam ('stam 2') kwam overeen met VC2002-k39. De derde stam ('stam 3') was een chimere VC2002-k2/VC2002-k39/VC2002-k2 stam met een recombinatie breekpunt in het ORF2 (capsid) gen gelokaliseerd tussen posities 363-400 (met ATG van ORF2 als positie 1) en een recombinatie breekpunt in het ORF1 (Rep) gen gelokaliseerd tussen posities 864-973. Het breekpunt in het ORF1 gen was gelijkaardig aan breekpunten die reeds eerder in de literatuur beschreven werden, doch het breekpunt in het ORF2 gen werd niet eerder beschreven. Gelijkaardige breekpunten werden wel reeds eerder beschreven in de 3 verschillende genera van de familie *Circoviridae*, en meer specifiek in Beak and feather disease virus, Chicken anemia virus en TT virus. Analyse met BlastN van PCV2 genoomsequenties beschikbaar in databanken maakte echter duidelijk dat er wel degelijk ook andere PCV2 stammen zijn met een recombinatie breekpunt in het ORF2 (capsid) gen gelokaliseerd tussen posities 363-400 (met ATG van ORF2 als positie 1). De daaropvolgende fylogenetische analyse verschaftte extra bewijs voor de recombinante oorsprong van 'stam 3'. 'Stam 3' groepeerde zich steeds met gelijkaardige recombinante stammen op afzonderlijke takken binnen de 1C of 1A/1B fylogenetische clusters (volgens het classificatiesysteem van Olvera et al., 2007^a), afhankelijk van het gebruikte algoritme en het gedeelte van het genoom dat werd onderzocht. Om uit te maken of de recombinante 'stam 3' ontstaan is *in vivo* door recombinatie in een varken met wegwijnsyndroom, werden de ORF2 en ORF1 genen van het originele lymfeknoopmateriaal geamplificeerd door middel van PCR en vervolgens gekloneerd in een vector en gesequeneerd. Er werden enkel sequenties teruggevonden die overeenstemden met 'stam 1' en 'stam 2', doch niet met de recombinante 'stam 3'. Er werd geconcludeerd dat PCV2 recombinatie kan ondergaan en dat recombinatie de genetische diversiteit van PCV2 kan verhogen.

In hoofdstuk 3.3 werd er onderzocht of de genetische en antigenische verschillen die werden waargenomen tussen PCV2 stammen met een verschillend genotype en met een verschillende klinische achtergrond, aanleiding zouden kunnen geven tot verschillen in virale replicatie in weefsels van varkens. In dit licht werden de klinische en virologische uitkomst van experimentele inoculaties van 55-dagen-oude immuno-incompetente porciene foetussen bepaald. Zeven foetussen werden geïnoculeerd met PCV2: 2 met de wegwijnsyndroom-geassocieerde PCV2a (genotype 2) stam Stoon-1010, 2 met de reproductiestoornis-geassocieerde PCV2a (genotype 2) stam 1121, 2 met de wegwijnsyndroom-geassocieerde PCV2b (genotype 1) stam 48285 en 1 met de PDNS-geassocieerde PCV2b (genotype 1)

^a Olvera, A., Cortey, M., Segalés, J., 2007. *Virology* 357, 175-185.

stam 1147. Eenentwintig dagen na de inoculatie waren 6 van de 7 geïnoculeerde foetussen oedemateus en ze hadden een uitgezet abdomen, doch de stam 1147-geïnoculeerde foetus had een normaal uitwendig voorkomen. Alle 7 geïnoculeerde foetussen hadden een vergrootte lever en vertoonden bloedingen en stuwings in de inwendige organen. Hoge PCV2 titers ($> 10^{4.5}$ TCID₅₀ / g weefsel) werden teruggevonden bij alle 7 geïnoculeerde foetussen, en dan vooral in het hart, de milt en de lever. Hoge aantallen PCV2-geïnfecteerde cellen (> 1000 geïnfecteerde cellen / 10 mm^2 weefsel) werden waargenomen in het hart van alle 7 geïnoculeerde foetussen. Eén van de 2 foetussen geïnoculeerd met stam 1121 had een lage PCV2-specifieke antistoftiter, de andere PCV2-geïnoculeerde foetussen hadden geen PCV2-specifieke antistoffen. PCV2 verspreidde zich niet naar niet geïnoculeerde foetussen. Er werden geen duidelijke verschillen in klinische of virologische uitkomst geobserveerd tussen de PCV2-geïnoculeerde foetussen, wat er enigszins of wijst dat PCV2 stammen met een verschillend genotype of met een verschillende klinische achtergrond gelijkaardige macroscopische letsels induceren en tot vergelijkbare titers repliceren in de organen van 55-dagen-oude immuno-incompetente porciene foetussen.

In hoofdstuk 4 werd er onderzocht of het mogelijk was om een experimenteel model voor het wegkwijnsyndroom te ontwikkelen gebaseerd op de kennis dat stimulatie van het immuunsysteem de PCV2 replicatie verhoogt in varkens. Meer specifiek werd er in hoofdstuk 4.1 nagegaan of stimulatie van het immuunsysteem met concanavalin A (ConA) de PCV2 replicatie in immuuncellen kan verhogen en of dit *in vivo* PMWS kan induceren. Aangezien ConA voornamelijk T-lymfocyten activeert, alsook de productie van interferon-gamma (IFN- γ) initieert - een cytokine dat de PCV2 replicatie verhoogt in porciene epitheliale en monocytische cellijnen *in vitro* - werd er eveneens nagegaan of stimulatie van het immuunsysteem met IFN- γ hetzelfde effect had op de PCV2 replicatie als ConA. In een *in vitro* studie werd er vastgesteld dat ConA, doch niet IFN- γ , de PCV2 replicatie verhoogde in perifere bloed mononucleaire cellen (PBMC). Tot 2,08 % en 0,96 % van de PBMC waren antigeenpositief voor PCV2 stammen 1121 en Stoon-1010 op respectievelijk 96 en 48 uur na inoculatie (uni). Infectieus virus werd aangetoond in supernatans vloeistof van PCV2-geïnoculeerd PMBC, met maximale titers van $10^{3.2}$ TCID₅₀ / ml supernatans and $10^{4.1}$ TCID₅₀ / ml supernatans voor stammen 1121 en Stoon-1010 op 96 uni. Op 72 uni, werden de PCV2 positieve PBMC geïdentificeerd als CD4⁺ (40 % \pm 5 %), CD8⁺ (54 % \pm 11 %) of IgM⁺ (11 % \pm 4 %). In een daaropvolgende *in*

vivo studie werden gnotobiotische biggen met ConA of IFN- γ geïnjecteerd 12 uur voor de inoculatie en om de 3 dagen gedurende 9 dagen na de inoculatie met PCV2 stam 1121. PCV2 kon geïsoleerd worden uit lymfeknoopbiopsieën van ConA-behandelde biggen vanaf 10 dagen na inoculatie (dni), en uit lymfeknoopbiopsieën van niet behandelde en van IFN- γ -behandelde biggen vanaf 15 dni. ConA doch niet IFN- γ verhoogde de gemiddelde PCV2 titer (van $10^{3.9}$ TCID₅₀ / g weefsel tot $10^{5.0}$ TCID₅₀ / g weefsel) en het gemiddeld aantal PCV2 positieve cellen (van 7 cellen / 10 mm^2 weefsel tot 391 cellen / 10 mm^2 weefsel) in lymfeknoopbiopsieën van gnotobiotische biggen op 15 dni. PCV2 positieve cellen werden geïdentificeerd als CD4⁺ (14 % \pm 11 %), CD8⁺ (10 % \pm 3 %), IgM⁺ (11 % \pm 8 %) of SWC3⁺ (69 \pm 22 %). Twee van de 4 ConA-behandelde biggen en 2 van de 4 IFN- γ -behandelde biggen hadden een vertraagde humorale immuunrespons van 10 of 15 dni tot 21 dni, doch deze vertraging resulteerde niet in een verhoogde PCV2 replicatie. Ziekte tekens geassocieerd met het wegwijnsyndroom werden bij geen enkele van de biggen waargenomen. Er werd geconcludeerd dat ConA de PCV2 replicatie verhoogt in PBMC *in vitro* en in lymfoïede weefsels *in vivo*.

Als algemene conclusie kan er gesteld worden dat PCV2 stammen genetisch en antigenisch meer verschillen vertonen dan aanvankelijk werd gedacht. Ondanks deze verschillen lijkt het er op dit ogenblik niet op dat er verschillende PCV2 pathotypes of serotypes bestaan. Ondanks de wetenschappelijke vooruitgang gedurende het afgelopen decennium blijft de pathogenese van het wegwijnsyndroom gedeeltelijk onopgehelderd.

Curriculum vitae

Personal particulars

David Lefebvre was born in Gent, Belgium, on March 17th, 1979. In 1997, he completed secondary education at the Sint-Lodewijkscollege in Brugge. In 2003, he graduated with the degree of Doctor of Veterinary Medicine from the Faculty of Veterinary Medicine, Ghent University. From 2003 to 2004, he worked as a veterinary surgeon in the rural veterinary practice of Cosne d'Allier, located in the Auvergne region in France. From December 1st, 2004 till November 30th, 2008, he received a Ph.D. scholarship from Ghent University, funded by the European Union (Sixth Framework Programme, Project No. 513928, "Control of Porcine Circovirus Diseases (PCVDs): Towards Improved Food Quality and Safety"). During this period, he studied the pathogenesis of porcine circovirus type 2 infections in pigs in the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University.

Currently, David Lefebvre is a part-time practical assistant in the Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, and a part-time contractual researcher in the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University.

Publications

Publications in international scientific peer-reviewed journals

- Meerts, P., Misinzo, G., **Lefebvre, D.**, Nielsen, J., Bøtner, A., Kristensen, C.S., Nauwynck, H.J., 2006. Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. *BMC Veterinary Research* 2, 6.
- Misinzo, G., Delputte, P.L., Meerts, P., **Lefebvre, D.J.**, Nauwynck, H.J., 2006. Porcine circovirus 2 uses heparan sulfate and chondroitin sulfate B glycosaminoglycans as receptors for its attachment to host cells. *Journal of Virology* 80, 3487-3494.
- Mateusen, B., Maes, D.G., Van Soom, A., **Lefebvre, D.**, Nauwynck, H.J., 2007. Effect of a porcine circovirus type 2 infection on embryos during early pregnancy. *Theriogenology* 68, 896-901.
- Misinzo, G., Delputte, P.L., **Lefebvre, D.J.**, Nauwynck, H.J., 2008. Increased yield of porcine circovirus-2 by a combined treatment of PK-15 cells with interferon-gamma and inhibitors of endosomal-lysosomal system acidification. *Archives of Virology* 153, 337-342.
- Lefebvre, D.J.**, Costers, S., Van Doorselaere, J., Misinzo, G., Delputte, P.L., Nauwynck, H.J., 2008. Antigenic differences among porcine circovirus type 2 strains, as demonstrated by the use of monoclonal antibodies. *Journal of General Virology* 89, 177-187.
- Lefebvre, D.J.**, Meerts, P., Costers, S., Misinzo, G., Barbé, F., Van Reeth, K., Nauwynck, H.J., 2008. Increased porcine circovirus type 2 replication in porcine leukocytes in vitro and in vivo by concanavalin A stimulation. *Veterinary Microbiology* 132, 74-86.
- Costers, S., **Lefebvre, D.J.**, Delputte, P.L., Nauwynck, H.J., 2008. Porcine reproductive and respiratory syndrome virus modulates apoptosis during replication in alveolar macrophages. *Archives of Virology* 153, 1453-1465.
- Atanasova, K., Van Gucht, S., Barbé, F., **Lefebvre, D.J.**, Chiers, K., Van Reeth, K., 2008. Lung cell tropism and inflammatory cytokine-profile of porcine respiratory coronavirus infection. *The Open Veterinary Science Journal* 2, 117-126.

- Misinzo, G., Delputte, P.L., **Lefebvre, D.J.**, Nauwynck, H.J., 2008. Porcine circovirus 2 infection of epithelial cells is clathrin-, caveolae- and dynamin-independent, actin and Rho-GTPase-mediated, and enhanced by cholesterol depletion. *Virus Research*, doi:10.1016/j.virusres.2008.09.005.
- Costers, S., **Lefebvre, D.J.**, Goddeeris, B., Delputte, P.L., Nauwynck, H.J., 2008. Absence of PRRSV-specific cytotoxic T-lymphocyte activity in freshly isolated and PRRSV restimulated peripheral blood mononuclear cells of PRRSV-infected pigs. *Veterinary Research*, conditionally accepted for publication.
- Lefebvre, D.J.**, Barbé, F., Atanasova, K., Nauwynck, H.J., 2008. Clinical and virological outcome of experimental PCV2a and PCV2b infections in mid-gestational porcine fetuses. *Theriogenology*, submitted for publication.
- Lefebvre, D.J.**, Van Doorsselaere, J., Delputte, P.L., Nauwynck, H.J., 2008. Recombination of two porcine circovirus type 2 strains. *Archives of Virology*, submitted for publication.
- Costers, S., **Lefebvre, D.J.**, Van Doorsselaere, J., Vanhee, M., Delputte, P.L., Nauwynck, H.J., 2008. GP4 of PRRSV contains a neutralizing domain that is susceptible to immuno-selection in vitro. *Archives of Virology*, submitted for publication.
- Lefebvre, D.J.**, Van Reeth, K., Vangroenweghe, F., Maes, D., Van Driessche, E., Laitat, M., Nauwynck, H.J., 2008. Serosurvey for viruses associated with reproductive failure in newly introduced gilts and in multiparous sows in Belgian sow herds. *The Veterinary Journal*, submitted for publication.

Publications in popular journals

- Lefebvre, D.**, 2008. Serologische vensters voor een gericht vaccinatiebeleid ter preventie van virale reproductiestoornissen bij gelten en zeugen. *Varkensbedrijf* 6, 26-29.
- Lefebvre, D.**, Laitat, M., 2008. Bilans sérologiques pour une stratégie de vaccination contre les troubles reproducteurs d'origine virale des cochettes et des truies. *L'essentiel du Porc* 4, 15-17.

Abstracts in proceedings of international congresses

- Meerts, P., **Lefebvre, D.**, Cox, E., Nauwynck, H.J., 2005. Correlation between differences in the immune response against porcine circovirus 2 and evolution in replication in pigs. In: Proceedings of the International conference on animal circoviruses and related diseases, Belfast, Northern Ireland, p. 60.
- Misinzo, G., Delputte, P.L., Meerts, P., **Lefebvre, D.J.**, Nauwynck, H.J., 2005. Heparan and dermatan sulphate glycosaminoglycans mediate porcine circovirus 2 attachment to the porcine monocytic line 3D4/31. In: Proceedings of the International conference on animal circoviruses and related diseases, Belfast, Northern Ireland, p. 63.
- Lefebvre, D.J.**, Meerts, P., Misinzo, G., Nielsen, J., Bøtner, A., Kristensen, C.S., Nauwynck, H.J., 2006. Absence of PCV2-neutralizing antibodies in PMWS-affected pigs. In: Proceedings of the 19th International Pig Veterinary Society Congress, Copenhagen, Denmark, Volume 1, p. 175.
- Nauwynck, H., **Lefebvre, D.**, Misinzo, G., Meerts, P., Mateusen, B., Sanchez, R., Delputte, P., 2007. Pathogenesis of porcine circovirus 2 infections. In: Proceedings of the 38th Annual Meeting of the American Association of Swine Veterinarians, Orlando, USA, pp. 489-495.
- Lefebvre, D.J.**, Costers, S., Delputte, P.L., Nauwynck, H.J., 2007. Monoclonal antibodies demonstrate antigenic differences between porcine circovirus type 2 strains. In: Proceedings of the 5th International Symposium on Emerging and Re-emerging Pig Diseases, Krakow, Poland, p. 47.
- Lefebvre, D.J.**, Barbé, F., Atanasova, K., Nauwynck, H.J., 2008. Inoculation of porcine fetuses with different genotypes and doses of PCV2. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 1, p. 38.
- Lefebvre, D.J.**, Van Reeth, K., Vangroenweghe, F., Castryck, F., Maes, D., Laitat, M., Nauwynck, H.J., 2008. Serosurvey for viruses associated with reproductive failure in newly introduced gilts and in multiparous sows in Belgian sow herds. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 1, p. 93.
- Lefebvre, D.J.**, Meerts, P., Costers, S., Misinzo, G., Nauwynck, H.J., 2008. Increased PCV2 replication by concanavalin A in porcine mononuclear cells in vitro and in gnotobiotic pigs in vivo. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 2, p. 50.

- Misinzo, G., Delputte, P.L., **Lefebvre, D.J.**, Nauwynck, H.J., 2008. Entry of porcine circovirus 2 in its host cells. In: Proceedings of the 20th International Pig Veterinary Society Congress, Durban, South Africa, Volume 2, p. 64.
- Costers, S., **Lefebvre, D.J.**, Delputte, P.L., Nauwynck, H.J., 2008. Porcine reproductive and respiratory syndrome virus modulates apoptosis during replication in alveolar macrophages. In: Proceedings of the XIth International Nidovirus Symposium, Oxford, UK, p. 38.
- Costers, S., **Lefebvre, D.J.**, Delputte, P.L., Nauwynck, H.J., 2008. PRRSV-infected macrophages are protected against MHC-I-restricted cytotoxicity. In: Proceedings of the XIth International Nidovirus Symposium, Oxford, UK, p.75.

Oral presentations on international congresses

- Lefebvre, D.**, Meerts, P., Cox, E., Nauwynck, H.J., 2005. Correlation between differences in the immune response against porcine circovirus 2 and evolution in virus replication in pigs. International conference on animal circoviruses and related diseases, Belfast, Northern Ireland, September 13th.
- Lefebvre, D.J.**, Meerts, P., Misinzo, G., Nielsen, J., Bøtner, A., Kristensen, C.S., Nauwynck, H.J., 2006. Absence of PCV2-neutralizing antibodies in PMWS-affected pigs. 19th International Pig Veterinary Society congress, Copenhagen, Denmark, July 18th.
- Lefebvre, D.J.**, Costers, S., Delputte, P.L., Nauwynck, H.J., 2007. Monoclonal antibodies demonstrate antigenic differences between porcine circovirus type 2 strains. 5th International Symposium on Emerging and Re-emerging Pig Diseases, Krakow, Poland, June 25th.
- Lefebvre, D.J.**, Barbé, F., Atanasova, K., Nauwynck, H.J., 2008. Inoculation of porcine fetuses with different genotypes and doses of PCV2. 20th International Pig Veterinary Society congress, Durban, South Africa, June 25th.
- Lefebvre, D.J.**, Van Reeth, K., Vangroenweghe, F., Castryck, F., Maes, D., Laitat, M., Nauwynck, H.J., 2008. Serosurvey for viruses associated with reproductive failure in newly introduced gilts and in multiparous sows in Belgian sow herds. 20th International Pig Veterinary Society congress, Durban, South Africa, June 25th.

Patent

Nauwynck, H.J., **Lefebvre, D.J.**, Delputte, P.L., Arnouts, S., 2008. Methods and compositions in the treatment of porcine circoviral infection. UK Intellectual Property Office. Patent application file number GB0712160.1.

