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

D. J. Lefebvre,1 S. Costers,1 J. Van Doorsselaere,2 G. Misinzo,1 P. L. Delputte1 and H. J. Nauwynck1

Correspondence
H. J. Nauwynck
hans.nauwynck@ugent.be
1Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
2Department of Health Care and Biotechnology, KATHO Catholic University College of South-West Flanders, Wilgenstraat 32, 8800 Roeselare, Belgium

This study examined whether antigenic differences among porcine circovirus type 2 (PCV-2) strains could be detected using monoclonal antibodies (mAbs). A subtractive immunization protocol was used for the genotype 2 post-weaning multisystemic wasting syndrome (PMWS)-associated PCV-2 strain Stoon-1010. Sixteen stable hybridomas that produced mAbs with an immunoperoxidase monolayer assay (IPMA) titre of 1000 or more to Stoon-1010 were obtained. Staining of recombinant PCV-2 virus-like particles demonstrated that all mAbs were directed against the PCV-2 capsid protein. Cross-reactivity of mAbs was tested by IPMA and neutralization assay for genotype 1 strains 48285, 1206, VC2002 and 1147, and 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 of Stoon-1010, 48285, 1206 and 1103, but not VC2002, 1147 and 1121. mAbs 31D5, 48B5, 59C6 and 108E8 did not react with genotype 1 strains or had a reduced affinity compared with genotype 2 strains in the IPMA and neutralization assay. mAb 13H4 reacted in the IPMA with PMWS-associated strains Stoon-1010, 48285, 1206 and VC2002, and the porcine dermatitis and nephropathy syndrome-associated strain 1147, but not with reproductive failure-associated strains 1121 and 1103. mAb 13H4 did not neutralize any of the tested strains. It was concluded that, despite the high amino acid identity of the capsid protein (≥91 %), antigenic differences at the capsid protein level are present among PCV-2 strains with a different genetic and clinical background.

INTRODUCTION

Porcine circovirus type 2 (PCV-2) is widespread in domestic and wild pigs. It belongs to the family Circoviridae, along with porcine circovirus type 1 (PCV-1), which was discovered and characterized as a non-cytopathic contaminant of the continuous porcine kidney cell line PK-15 (ATCC CCL-33) (Tischer et al., 1974, 1982). PCV-1 is not regarded as a pathogen for pigs (Tischer et al., 1986; Allan et al., 1995), whereas PCV-2 is considered as the crucial pathogen in post-weaning 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 & Ellis, 2000). Besides wasting, PCV-2 may also cause reproductive failure (West et al., 1999; Ladekjær-Mikkelsen et al., 2001; Meehan et al., 2001; Sanchez et al., 2001, 2003; Mateusen et al., 2004). PCV-2 has also been isolated from pigs with porcine dermatitis and nephropathy syndrome (PDNS) and a number of other diseases, but neither PDNS nor these other diseases have been reproduced experimentally (Allan et al., 2000; Rosell et al., 2000; Segales et al., 2005, Wellenberg et al., 2004).

The PCV-2 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 kb and 11 putative open reading frames (ORFs) (Hamel et al., 1998). Proteins encoded by three of these ORFs are considered to play a role in the pathogenesis of PCV-2 infections. ORF1 encodes the replication-associated proteins Rep and Rep’ (Mankertz & 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, 2006).

Meerts et al. (2005a) demonstrated biological differences among different PCV-2 strains in vitro. Replication kinetics of PMWS- and PDNS-associated PCV-2 strains were significantly different from reproductive failure-associated PCV-2 strains. Recently, it was demonstrated that the virulence of a PCV-2 isolate originating from a PMWS-affected animal differed significantly from an isolate recovered from a subclinically infected animal. Important differences in serological profile, virus replication and severity of lesions were shown after experimental inoculation of specific-pathogen-free (SPF) pigs (Opriessnig et al., 2006).

Among various strains of PCV-2, 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 the capsid protein (Larochelle et al., 2002). Several studies have suggested that genetic differences in PCV-2 are associated with the geographical region from which the isolates originated (Fenaux et al., 2000; Hamel et al., 2000; Mankertz et al., 2000; Kim & Lyoo, 2002) and a recently proposed classification system (Olvera et al., 2007) divides PCV-2 into two genotypes (1 and 2) and eight clusters (1A–1C and 2A–2E). 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 PCV-2 strain (Fenaux et al., 2000; Meehan et al., 2001; Larochelle et al., 2002; Pogranichnyi et al., 2002; de Boisséson et al., 2004; Grierson et al., 2004). Until now, mouse monoclonal antibodies (mAbs) directed against PCV-2 have not shown major differences in reactivity to different PCV-2 strains (Allan et al., 1999; McNeilly et al., 2001).

In this study, mAbs to PCV-2 were produced, characterized and used to identify antigenic differences among PCV-2 strains with a different genotype and originating from different clinical presentations.

**METHODS**

**Viruses.** Seven different PK-15-adapted PCV-2 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). PCV-1 originated from the persistently infected PK-15 cell line ATCC CCL-33 (Tischer et al., 1974, 1982). All PCV-2 strains used were passaged 11–17 times, except for strain 1121, which was passaged 30 times.

**Recombinant PCV-2 virus-like particles.** PCV-2 virus-like particles (VLPs) were obtained by infecting S9 insect cells with a baculovirus recombinant P054 expressing the ORF2 of PCV-2 strain Stoon-1010. Purification of VLPs was performed in a caesium chloride gradient as described by Nawagitgul et al. (2000).

**Cells.** PCV-negative PK-15 cells and the persistently PCV-1-infected PK-15 cell line ATCC CCL-33 were grown in minimal essential medium (MEM) containing Earle’s salts (Gibco), supplemented with 5 or 10 % fetal bovine serum (FBS), 0.3 mg glutamine ml⁻¹, 100 U penicillin ml⁻¹, 0.1 mg streptomycin ml⁻¹ and 0.1 mg kanamycin ml⁻¹. Cell cultures were maintained at 37 °C in the presence of 5 % CO₂.

**Mouse immunization.** Before immunization, mice were made immunotolerant to PK-15 cells as described by Matthew & Sandrock (1987). Four 6-week-old female BALB/c mice were injected intraperitoneally (i.p.) with 1.5 × 10⁷ PCV-negative PK-15 cells in 300 μl PBS. After 10 min, 24 and 48 h, cyclophosphamide (Sigma) was injected i.p. at a dose of 100 mg (kg body weight)⁻¹ in a total volume of 500 μl PBS. After 3 and 6 weeks, injections with PK-15 cells and cyclophosphamide were repeated. Two weeks after the last treatment, 2.25 × 10⁸ Stoon-1010-inoculated PK-15 cells were injected i.p. in a volume of 300 μl PBS mixed with an equal amount of complete Freund’s adjuvant (Sigma). At this time point and 2 weeks later, serum was collected from mice. Three weeks after the inoculation with PCV-2, one mouse received an i.p. injection with 4.5 × 10⁷ Stoon-1010-inoculated PK-15 cells diluted in 600 μl PBS. Euthanasia was performed 4 days later and the spleen was collected.

**Production and screening of hybridomas.** Hybridoma cells were produced by fusion of spleen cells with SP 2/0 myeloma cells as described by Galfre & Milstein (1981). The resulting hybridoma cells were maintained in RPMI 1640 (Gibco) supplemented with 10 % FBS. PCV-2-specific mAbs in supernatant fluids were demonstrated on

**Table 1. Origins of the PCV-2 strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clinical origin</th>
<th>Genotype</th>
<th>Geographical origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stoon-1010</td>
<td>PMWS-affected piglet</td>
<td>2</td>
<td>Canada</td>
<td>Meehan et al. (1998)</td>
</tr>
<tr>
<td>48285</td>
<td>PMWS-affected piglet</td>
<td>1</td>
<td>France</td>
<td>Meehan et al. (1998)</td>
</tr>
<tr>
<td>1206</td>
<td>PMWS-affected piglet</td>
<td>1</td>
<td>Belgium</td>
<td>Meerts et al. (2005a)</td>
</tr>
<tr>
<td>VC2002</td>
<td>PMWS-affected piglet</td>
<td>1</td>
<td>Belgium</td>
<td>Meerts et al. (2004)</td>
</tr>
<tr>
<td>1147</td>
<td>PDNS-affected piglet</td>
<td>1</td>
<td>UK</td>
<td>Meehan et al. (2001)</td>
</tr>
<tr>
<td>1121</td>
<td>Aborted fetuses</td>
<td>2</td>
<td>Canada</td>
<td>Meehan et al. (2001)</td>
</tr>
<tr>
<td>1103</td>
<td>Aborted fetuses</td>
<td>2</td>
<td>Canada</td>
<td>Meehan et al. (2001)</td>
</tr>
</tbody>
</table>
PCV-negative and Stoon-1010-inoculated PK-15 cells by an immuno-
peroxidase monolayer assay (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
galactosyl antibodies (pAbs) (Dako) in PBS was added for 1 h at
37 °C. After washing twice in PBS, substrate solution was added and
cell cultures were analysed by light microscopy (Olympus Optical Co.). Selected hybridoma cultures were cloned by limiting dilution.

**Determination of mAb class.** The isotype of the produced mAbs
was determined using a peroxidase-based commercial mouse mAb
identification kit (Zymed). This test identifies the IgG1, IgG2a, IgG2b,
IgG3, IgA and IgM isotype classes and the α and β type of the light
chain using monospecific rabbit pAbs. Supernatant fluids of anti-PCV
mAbs 13D12 (IgG1) and 1C11 (IgG2a) (Nauwynck & Pensaert, 1995)
and anti-*Escherichia coli* mAb E7G3 (IgG3) (Tiels et al., 2007) were
used as positive controls.

**Indirect immunofluorescence staining of recombinant PCV-2
VLPs.** 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 incubation with a 1:500 dilution of fluorescein
isothiocyanate-labelled goat anti-mouse pAbs (Molecular Probes)
containing 10% PCV-2-negative goat serum 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) was used for visualization.

**Western blot analysis.** Stoon-1010-inoculated and mock-inocu-
lated PCV-negative PK-15 cells were harvested by scraping. Cells were
pelleted by centrifugation at 15 700 g for 20 min at 4 °C and subsequently
lysed by 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), protease inhibitors (Complete; Roche) and 0.5% SDS.
Cells were centrifuged at 15 700 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). This membrane was then incubated for 1 h
at room temperature in PBS containing 0.1% Tween 20 (PBS-
TWEEN), supplemented with 5% BSA (Sigma). 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 pAbs, originating from a PCV-2-
negative SPF pig inoculated with strain 1121 (Pensaert
et al., 2005a) were used as positive controls. mAbs 13D12 and 1C11 were used as negative controls. A Leica DM/RBE fluorescence microscope (Leica
Microsystems) was used for visualization.

**Reactivity of mAbs to different PCV-2 strains.** PCV-2 strains
Stoon-1010, 48285, 1206, VC2002, 1147, 1121 and 1103 were used to
prepare 96-well IPMA plates as described by Labarque et al. (2000).
PCV-negative PK-15 cells and the persistently PCV-1-infected PK-15
cell line were used for control IPMA plates. The staining procedure
was similar to the IPMA technique described above. Tenfold dilutions of
hybridoma supernatants were prepared in PBS and used as primary
antibodies. 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 three times for each strain.

**Sensitive neutralization assays.** In order to detect the neutralizing
activity of the mAbs, a sensitive neutralization assay was adapted from
the method of Meerts et al. (2005b). Briefly, 10^3.5 TCID₅₀ PCV-2 in a
volume of 200 μl was incubated for 1 h at 37 °C with 200 μl undiluted hybridoma supernatant. After incubation, this mixture was
added to semi-confluent monolayers of PCV-negative PK-15 cells in
four 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 h later. At this time point, the first replication cycle of
PCV-2 was completed (Meerts et al., 2005a). PCV-2-infected PK-15
cells were stained by an IPMA using porcine PCV-2-specific pAbs,
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 reduction in the number of infected cells in comparison
with medium. Assays were performed with all seven strains. Anti-PCV-2
mAb F190 was used as a positive control, mAbs 13D12 and 1C11 were used as negative controls. A Leica DM/RBE fluorescence microscope (Leica
Microsystems) was used for visualization.

**Sequencing of ORF2 from strains 1206 and VC2002.** The Belgian
PCV-2 strains 1206 and VC2002 were purified by ultracentrifugation
at 180 000 g for 5 h through a 30 % sucrose gradient as described by
Delputte et al. (2002). A set of PCR primers was designed based on
alignment of the genome sequences of strains Stoon-1010, 48285,
1147, 1121 and 1103. The primer set PCV-2-FW (5’-AGGCGAC-
CTTCTTCTGTTTTCG-3’) and PCV-2-REV (5’-GAATGGCCGGCG-
CTATCACCTGTAATGTTTATTTATTCG-3’) amplified the
complete ORF2. Two internal oligonucleotides were synthesized:
CV1 (5’-GGGCGTGGCCCTTTGTGKAC-3’) and CV2 (5’-TGTR-
GACCAGTTGGGCTC-3’). These internal oligonucleotides were
as described by Fenaux et al. (2000) with minor modifications, and
were used for sequencing. A 1:200 fraction of proteinase K-treated
ultrapurified PCV-2 was used as template in PCRs using Platinum Pfx
DNA polymerase (Invitrogen) at an annealing temperature of 60 °C
and using the cycling conditions described by the manufacturer. PCR
products (~800 bp) were treated with exonuclease I and antarctic
phosphatase (New England Biolabs) and used directly for cycle
sequencing with a Big Dye Terminator Cycle Sequencing kit v1.1
(Applied Biosystems) and PCV-2 primers. Cycle sequencing reaction
products were purified using ethanol precipitation and separated on an
ABI Genetic Analyzer 310 (Applied Biosystems). Additionally, PCR
products (~800 bp) were gel purified using a QIAquick gel extraction
kit (Qiagen) and cloned in pBluescript II SK (+) cut with EcoRV and
treated with antarctic phosphatase. Clones containing the PCV-2 ORF2
were sequenced using T7 and T3 primers as described above. The
ORF2 sequences (~800 bp) were sequenced using T7 and T3 primers as described above. The
sequences were analysed and compiled using Align, CLUSTAL W
and Sixframe in the Biology Workbench (http://workbench.sdsc.edu)
Phylogenetic relationships among sequences were analysed as described by
Tripathi & Sowdhamini (2006). Briefly, phylogenetic trees were
derived from multiple sequence alignments with PHYLIP v3.67.
Bootstrapping was performed 100 times using SEQBOOT. Pairwise
distances between genomic sequences and protein sequences were
determined with DNADIST and PROTDIST, respectively. Neighbour-
joining (NJ) trees were calculated with neighbour and maximum-
likelihood (ML) trees with DNAML and ProML. Majority rule consensus
trees were obtained with CONSENSE and visualized with
DRAWGRAM.

The ORF2 sequences (702 nt, from ATG to the stop codon) from strains 1206, VC2002-k2 and VC2002-k3 have been deposited in
GenBank under accession numbers EF990644, EF990645 and EF990646, respectively.

**RESULTS**

**Mouse immunization**

Prior to immunization, four BALB/c mice were made immunotolerant 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 in IPMAs. All serum samples taken before immunization were negative for anti-PCV-2 antibodies as determined by IPMA. Two weeks after the first immunization, all mice had anti-Stoon-1010 antibody titres of between 2560 and 40 960. One mouse with an IPMA titre of 10 240 and without reaction to PK-15 cells was selected. It received a boost injection 1 week later and its spleen was used for the production of hybridomas.

**Production and screening of hybridomas**

Forty-four hybridomas that produced mAbs against PCV-2-infected PK-15 cells were frozen. Cloning by limiting dilution resulted in 16 stable hybridomas that produced mAbs with an IPMA titre of 1000 or more to Stoon-1010.

**Determination of mAb class**

A commercial identification kit was used to determine the isotypes of the mAbs. The results are presented in Table 2. Six hybridomas produced IgG1 mAbs and eight hybridomas produced IgG2a mAbs. 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 \( \kappa \) type.

**Indirect immunofluorescence staining of recombinant PCV-2 VLPs**

The reactivity of the mAbs to VLPs was tested by performing indirect immunofluorescence staining of VLPs smeared onto glass slides. All 16 mAbs reacted with the VLPs, indicating that the mAbs were directed against the PCV-2 capsid protein. No staining was observed with irrelevant mAbs.

**Western blot analysis**

The reactivity of the mAbs to Stoon-1010-inoculated PK-15 cells was determined in a Western blot assay. mAbs 31D5, 38C1 and 108E8 gave a strong and specific reaction with a protein of approximately 28 kDa (Fig. 1). For mAb 21C12, a faint but specific band was observed at 28 kDa.

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

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

<table>
<thead>
<tr>
<th>mAb</th>
<th>Isotype</th>
<th>1010 4825</th>
<th>1206 VC2002</th>
<th>1147</th>
<th>1121 Abortion</th>
<th>1103 Abortion</th>
<th>PCV-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PMWS Genotype 2</td>
<td>PMWS Genotype 1</td>
<td>PMWS Genotype 1</td>
<td>PMWS Genotype 1</td>
<td>Genotype 1</td>
<td>Genotype 2</td>
</tr>
<tr>
<td>9C3</td>
<td>IgG1</td>
<td>10 000</td>
<td>10 000</td>
<td>10 000</td>
<td>10 000</td>
<td>10 000</td>
<td>10 000</td>
</tr>
<tr>
<td>13H4</td>
<td>IgG2a</td>
<td>1 000</td>
<td>1 000</td>
<td>1 000</td>
<td>100</td>
<td>100</td>
<td>Negative</td>
</tr>
<tr>
<td>16G12</td>
<td>IgG2a</td>
<td>10 000</td>
<td>10 000</td>
<td>10 000</td>
<td>10 000</td>
<td>10 000</td>
<td>10 000</td>
</tr>
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<td>21C12</td>
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<tr>
<td>31D5</td>
<td>IgG1</td>
<td>10 000</td>
<td>Negative</td>
<td>Negative/1 000*</td>
<td>Negative</td>
<td>Negative</td>
<td>10 000</td>
</tr>
<tr>
<td>38C1</td>
<td>IgG1</td>
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<td>10 000</td>
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<td>No reaction</td>
<td>1 000</td>
<td>Negative</td>
<td>Negative/100*</td>
<td>Negative</td>
<td>Negative</td>
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<tr>
<td>55B1</td>
<td>IgG2a</td>
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<td>59C6</td>
<td>IgG2a</td>
<td>10 000</td>
<td>10</td>
<td>100/10 000*</td>
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<td>100</td>
<td>10 000</td>
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<td>63H3</td>
<td>IgG2a</td>
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<td>108E8</td>
<td>IgG1</td>
<td>1 000</td>
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<td>Negative/1 000*</td>
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<td>1 000</td>
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<tr>
<td>114C8</td>
<td>IgG1</td>
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</tbody>
</table>

* mAbs 31D5, 48B5, 59C6 and 108E8 stained two different populations of infected cells in strain 1206. IPMA titres for the first population (~99% of the infected cells, on the left of the slash) were comparable to those of the genotype 1 strains 48285, VC2002 and 1147. IPMA titres for the second population (~1% of the infected cells, on the right of the slash) were comparable to those of the 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.
Antigenic differences among PCV-2 strains

Reactivity of mAbs to different PCV-2 strains

An IPMA was used to examine the reactivity of hybridoma supernatants to seven different PCV-2 strains (Table 2). Eleven out of 16 hybridomas stained all seven strains with a maximum tenfold variation in titres among 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 genotype 1 strains 48285, VC2002 and 1147, or they had IPMA antibody titres to these strains that were at least 100 times lower than for the genotype 2 strains Stoon-1010, 1121 and 1103. These four mAbs stained two different populations of infected cells in strain 1206. IPMA antibody titres for the first population (~99% of the infected cells) were comparable to those of the other genotype 1 strains. IPMA antibody titres for the second population (~1% of the infected cells) were comparable to those of the 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 four PMWS-associated strains (Stoon-1010, 48285, 1206 and VC2002) and the single PDNS-associated strain (1147), but did not react with the two reproductive failure-associated strains (1121 and 1103). None of the 16 mAbs reacted with PCV-1 or with PK-15 cells.

Table 3. Neutralizing activity of hybridoma supernatants

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

<table>
<thead>
<tr>
<th>mAb</th>
<th>1010 PMWS Genotype 2</th>
<th>48285 PMWS Genotype 1</th>
<th>1206 PMWS Genotype 1</th>
<th>VC2002 PMWS Genotype 1</th>
<th>1147 PDNS Genotype 1</th>
<th>1121 Abortion Genotype 2</th>
<th>1103 Abortion Genotype 2</th>
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<tbody>
<tr>
<td>9C3</td>
<td>94 ± 3</td>
<td>92 ± 5</td>
<td>54 ± 10</td>
<td>1 ± 13</td>
<td>34 ± 20</td>
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<td>−6 ± 14</td>
<td>3 ± 12</td>
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<td>14 ± 4</td>
<td>−10 ± 12</td>
<td>3 ± 12</td>
<td>51 ± 5</td>
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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 seven PCV-2 strains in the IPMA demonstrated neutralization of Stoon-1010 (up to 95%), 48285 (up to 94%), 1206 (up to 57%) and 1103 (up to 61%). The four mAbs (31D5, 48B5, 59C6 and 108E8) that had a higher affinity for genotype 2 strains than for genotype 1 strains in the IPMA demonstrated neutralization of the genotype 2 strains Stoon-1010 (up to 98%) and 1103 (up to 67%). For these four mAbs, neutralization of genotype 1 strains 48285 and 1206 was absent or very low (up to 35%). mAb 13H4 did not neutralize any of the seven tested strains. Only one mAb (21C12) demonstrated some neutralization (32%) of strain VC2002 and only two mAbs (9C3 and 38C1) demonstrated some neutralization (34 and 30%, respectively) of strain 1147. None of the 16 mAbs neutralized strain 1121.

**Sequencing of ORF2 from strains 1206 and VC2002**

The ORF2 of the Belgian PMWS-associated PCV-2 strains 1206 and VC2002 was amplified by PCR and sequenced. Strain 1206 contained an ORF2 of 702 bp (starting from ATG and including the stop codon) encoding a 233 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 and including the stop codon) encoding a protein of 233 aa. Ten other VC2002 clones were almost 100% identical at the nucleotide level with clone k39 with between 1 and 3 nt differences. Clone VC2002-k2 showed 94% identity with k39 at the nucleotide and amino acid levels and 96–99% amino acid identity with strains from China (e.g. NCBI protein database accession nos AAP44186, AAU87508 and AAAT97651), The Netherlands (accession no. AAS65982; Grierson et al., 2004) and a strain isolated from wild boars in Germany (accession no. AAU13781; Knell et al., 2005). Capsid protein similarity among the seven different strains used in this study was determined using pairwise alignments and CLUSTAL W (Fig. 2). The ORF2 amino acid identities of the strains used in this study is shown in Table 4. Fig. 3 shows a phylogenetic tree of the ORF2 protein based on the ML method with the percentage confidence shown on each branch. This tree 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. Genotype 1 strains 48285, 1206, VC2002-k39 and 1147 were assigned to cluster 1A/1B, VC2002-k2 to cluster 1C and genotype 2 strains Stoon-1010, 1121 and 1103 to cluster 2E. The same strain classification was obtained by using the ML method and with ORF2 DNA sequences.
DISCUSSION

This is the first study to demonstrate antigenic diversity among PCV-2 strains. This was established by the production and characterization of mAbs directed to the PCV-2 capsid protein. The cross-reactivity of the mAbs to seven different PCV-2 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 seven PCV-2 strains used in the IPMA. Four other mAbs (31D5, 48B5, 59C6 and 108E8) were able to differentiate the genotype 1 strains 48285, 1206, VC2002 and 1147 from the genotype 2 strains Stoon-1010, 1121 and 1103 by IPMA, as they did not react with genotype 1 strains or had a reduced affinity compared with genotype 2 strains. The IPMA results of the latter four mAbs were also reflected in the neutralization assays. Until now, mAbs have not allowed differentiation of PCV-2 strains (Allan et al., 1999; McNeilly et al., 2001). mAbs 31D5, 48B5, 59C6 and 108E8 also did 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 PCV-2 cell culture adaptation (data not shown).

Using the IPMA, mAbs 31D5, 48B5, 59C6 and 108E8 stained two different populations of infected cells in strain 1206. This suggests that strain 1206 consists of two viral subpopulations, where 99% of the virus behaves as a genotype 1 strain and 1% of the virus behaves as a genotype 2 strain. No signs of the existence of subpopulations were detected by sequencing of strain 1206. This may be explained by the fact that the putative genotype 2 subpopulation was present at a very low level (1%). Sequencing of the VC2002 strain did reveal the existence of two PCV-2 subpopulations in the virus stock. After cloning, two 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 German wild boars (Knell et al., 2005), which documents the putative epidemiological link between PCV-2 infections in pigs in these regions.

### Table 4. ORF2 amino acid identity within PCV-2 strains used in this study

The percentage amino acid identities given are the result of pairwise alignments of the ORF2 proteins. Percentage identities between the genotype 1 strains (except VC2002-k2) are shown in bold; percentage identities between the genotype 2 strains are shown in bold and italics; percentage identities between the VC2002-k2 strain and other strains are underlined.

<table>
<thead>
<tr>
<th>Strain</th>
<th>1206</th>
<th>1147</th>
<th>48285</th>
<th>VC2002-k39</th>
<th>1121</th>
<th>1103</th>
<th>1010</th>
<th>VC2002-k2</th>
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<td>98</td>
<td>98</td>
<td>98</td>
<td>93</td>
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</tr>
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<tr>
<td>VC2002-k2</td>
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<td>98</td>
<td>98</td>
<td>97</td>
<td>97</td>
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</table>

Fig. 3. Unrooted phylogenetic tree constructed using the NJ method. The percentage confidence is indicated on the branches. This tree was based on the ORF2 protein sequences of the PCV-2 strains that were used in the present study (strain names in parentheses), one PCV-1 sequence (outgroup) and 20 PCV-2 sequences that were obtained from Olvera et al. (2007). These sequences are listed in Table 5. *. No NCBI protein accession number was available for the ORF2 protein, so the GenBank nucleotide sequence was used.
lysine was replaced by an isoleucine. Linear antigenic capsid protein. The same applies to position 206, where a proline and isoleucine, this may have major consequences and hydrophobicity between lysine/arginine and threonine, lysine and arginine. Due to the differences in size, charge

These three substitutions all involve the basic amino acids located at positions 63, 88, 89 and 206. At position 63, a threonine was substituted for a lysine or an arginine. At position 88, a lysine was replaced by a proline, and at position 89, an isoleucine was replaced by an arginine. These three substitutions all involve the basic amino acids lysine and arginine. Due to the differences in size, charge and hydrophobicity between lysine/arginine and threonine, proline and isoleucine, this may have major consequences on the secondary and tertiary structure of the PCV-2 capsid protein. The same applies to position 206, where a lysine was replaced by an isoleucine. Linear antigenic determinants of the PCV-2 ORF2 protein, as determined by PEPSCAN, are located at positions 65–87, 113–139, 169–193 and 193–207 (Mahe´, 2000). 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 amino acid substitutions that involve basic amino acids 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 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 the protein. Position 131 is located within, and position 191 is located at the outer border of, an antigenic domain (Mahe´ 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.

Table 5. Name, phylogenetic cluster (according to Olvera et al., 2007) and origin of the ORF2 sequences used in Fig. 3

<table>
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<th>NCBI protein accession no.</th>
<th>GenBank accession no.</th>
<th>Strain</th>
<th>Cluster</th>
<th>Clinical origin</th>
<th>Geographical origin</th>
<th>Reference</th>
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<td>PK-15 cell line</td>
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<td>USA</td>
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</table>

domestic and wild pigs (Cságo et al., 2006). The identification of two different PCV-2 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 PCV-2 infections in the pathogenesis of PCV-2-associated diseases is not clear.

Using protein sequences (NJ and ML methods), 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 methods), the same topology was obtained as by Olvera et al. (2007), with the only difference being that clusters 1A and 1B could not be differentiated in the present study (data not shown). We assume that these differences are a consequence of the reduced number of sequences used.

Putative amino acid substitutions that discriminate the genotype 1 strains 48285, 1206, VC2002-k39 and 1147 from the genotype 2 strains Stoon-1010, 1121 and 1103 are located at positions 63, 88, 89 and 206. At position 63, a threonine was substituted for a lysine or an arginine. At position 88, a lysine was replaced by a proline, and at position 89, an isoleucine was replaced by an arginine. These three substitutions all involve the basic amino acids lysine and arginine. Due to the differences in size, charge and hydrophobicity between lysine/arginine and threonine, proline and isoleucine, this may have major consequences on the secondary and tertiary structure of the PCV-2 capsid protein. The same applies to position 206, where a lysine was replaced by an isoleucine. Linear antigenic
Fenaux et al. (2004) demonstrated that PCV-2 that was passaged 120 times in PK-15 cells (VP120) replicated more efficiently in PK-15 cells than wild-type virus that had been passaged only once (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 amino acid 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 antibodies, whereas their ability to produce non-neutralizing antibodies remains unaffected (Meerts et al., 2005b, 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 seven PCV-2 strains, suggesting that a universal PCV-2 neutralizing epitope does not exist. Neutralization was observed for Stoon-1010, 48285, 1206 and 1103, but not VC2002, 1147 and 1121. No discriminative amino acid motifs that could explain these results were detected. The mAbs that neutralized Stoon-1010, 48285, 1206 and 1103 did not differentiate these strains from VC2002, 1147 and 1121 in the IPMA, indicating that these two different groups of PCV-2 strains have different neutralizing epitopes, and suggesting that these two different groups of PCV-2 strains use different entry pathways in PK-15 cells. Recently, the glycosaminoglycans heparan sulfate and chondroitin sulfate B have both been described as attachment receptors for PCV-2 (Misinzo et al., 2006). Protein binding to these two attachment receptors is restricted to the basic amino acids lysine and arginine (Esko, 1999), suggesting a crucial role of basic amino acid residues in the entry of PCV-2 into the host cell. The positive amino acid charges of lysine and arginine interact three-dimensionally with negatively charged glycosaminoglycan sulfates and carboxylates (Esko, 1999), which indicates that three-dimensional conformation plays a crucial role in interactions between the PCV-2 capsid protein and its receptors.

Until now, it was assumed that no distinct antigenic variation existed among PCV-2 isolates. In this study, we have clearly demonstrated the existence of major antigenic differences among the capsid proteins of PCV-2 strains with a different genotype and isolated from different clinical presentations.

ACKNOWLEDGEMENTS

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