In vitro and in vivo characterization of an infectious clone of a European strain of porcine circovirus type 2

M. Roca, M. Balasch, J. Segalés, M. Calsamiglia, E. Viaplana, A. Urniza, K. Hattermann, A. Mankertz, J. Plana-Durán and M. Domingo

The aim of this study was to describe the generation of a PCV2 (porcine circovirus type 2) infectious clone (pIC-PCV2) and its infectivity under in vitro and in vivo conditions. The constructed pIC-PCV2 contained the whole PCV2 genome from a German isolate together with a partial duplication of 467 bp. PK-15 cells were transfected with pIC-PCV2 and an indirect immune fluorescence assay (IFA) was performed 7 days post-transfection. The PCV2 Cap gene was expressed in approximately 20% of the cultured cells, and only the recombination product, and not pIC-PCV2, was subsequently detected by PCR and Southern blot. This result indicated that infection by pIC-PCV2 delivered genomic PCV2 DNA specifically into susceptible cells and led to the expression of a functional virus genome.

Eighteen 30- to 40-day-old conventional pigs were distributed into three groups. Group 1 pigs (n = 6) were inoculated intranasally (i.n.) with a Spanish isolate of PCV2 propagated in cell culture; pigs from group 2 (n = 6) were inoculated with pIC-PCV2 intramuscularly (i.m.), and the last group of pigs (n = 6) was inoculated with pIC-PCV2 intraperitoneally (i.p.). All pigs remained clinically healthy during the whole experimental period (35 days). Pigs that received pIC-PCV2 i.p. and i.m., as well as those PCV2 i.n. inoculated, became infected based on an in situ hybridization (ISH), PCR, TaqMan PCR and serological results. The results of this study confirm that cloned PCV2 genomic DNA is infectious both in vitro and in vivo, and is able to cause PMWS-like lesions in i.p. and i.m. experimentally inoculated pigs.

INTRODUCTION

Porcine circoviruses (PCVs) are small (17 nm in diameter), non-enveloped, spherical viruses that contain a single-stranded DNA genome, and are classified in the Circoviridae family, genus Circovirus (Todd et al., 2000). Two types of PCVs have been recognized to date. PCV type 1 (PCV1) was originally identified as a contaminant of porcine kidney cell cultures (PK-15 ATCC CCL-33) (Tischer et al., 1982) and is considered non-pathogenic for swine (Allan et al., 1995; Tischer et al., 1986). On the other hand, PCV type 2 (PCV2) is now accepted as the major infectious agent involved in postweaning multisystemic wasting syndrome (PMWS) (Allan et al., 1999; Bolin et al., 2001; Kennedy et al., 2000).

PMWS usually affects 2- to 3.5-month-old pigs, although the disease has been described in a wider age range, between 3 days and 6 months (Hirai et al., 2001; Segales & Domingo, 2002). The disease is characterized clinically by growth retardation, paleness of the skin, dyspnoea and enlargement of inguinal lymph nodes; occasionally, jaundice and diarrhoea are observed (Harding & Clark, 1997; Rosell et al., 1999). At necropsy, the most frequent lesions are enlargement of lymph nodes and non-collapsed, tan-mottled lungs (Rosell et al., 1999). The main histological lesions of PMWS consist of a variable degree of lymphocyte depletion with loss of follicles together with histiocytic and multinucleate giant cell infiltration in the lymphoid tissues, and lymphohistiocytic inflammatory infiltrations in a wide range of tissues (Clark, 1997; Rosell et al., 1999). Although PCV2 has been systematically associated with PMWS microscopic lesions, the full clinicopathological spectrum of the disease has been traditionally difficult to reproduce using experimental models with PCV2 only (Allan et al.,...
1999; Balasch et al., 1999; Kennedy et al., 2000; Magar et al., 2000). These results, and the fact that concomitant viral infections (Allan et al., 1999; Harms et al., 2001; Krakowka et al., 2000; Rovira et al., 2002) or non-infectious immunostimulation (Krakowka et al., 2001) seem to trigger the clinical disease, suggest that PMWS is a multifactorial disease where PCV2 is strictly necessary but not a sufficient factor to develop the clinical outcome observed under field conditions (Allan et al., 2000a, b; Ellis et al., 1999; Krakowka et al., 2000; Rovira et al., 2002). However, it must be remarked that some experimental studies have reproduced the disease in a significant number of animals using, apparently, only PCV2 as the inoculum (Allan et al., 2002; Bolin et al., 2001; Harms et al., 2001).

The virus inocula used in several trials were obtained from tissue homogenates corresponding to PMWS-affected pigs or PCV2 isolated and propagated in cultured cells. These sources of virus may contain other common swine agents, as has been observed in at least one experiment (Ellis et al., 1999). Therefore, it is possible that the virus stocks used in these experiments were not pure and the reproduced disease and/or pathological lesions may not be attributable to PCV2 infection only (Fenaux et al., 2002). This problem should be resolved with the use of infectious DNA clones of a pure source of PCV2. The availability of infectious clones offers an opportunity for analysis and modification of viral genomes at the molecular level and has greatly aided research on virus replication, pathogenesis and vaccine development (Boyer & Haenni, 1994). In fact, a previous study used a molecular DNA clone of a USA strain of PCV2 injected directly into the liver and lymph nodes; this work was carried out to demonstrate that a biologically pure and homogeneous infectious virus stock could be used for PCV2 pathogenesis studies. In this experiment, mild histopathological PMWS characteristic lesions were reproduced (Fenaux et al., 2002). Furthermore, chimeric infectious DNA clones of PCV2 have been developed, demonstrating that the one containing ORF2 capsid gene of pathogenic PCV2 cloned into the non-pathogenic PCV1 genomic backbone induces specific antibody response to the pathogenic PCV2 capsid antigen, but is attenuated in pigs (Fenaux et al., 2003).

These latter studies (Fenaux et al., 2002, 2003) used a USA PCV2 strain for the construction of the chimeric and non-chimeric DNA infectious clones. Although differences in pathogenicity among PCV2 strains recovered from PMWS cases have not been clearly established, certain differences in genotype by geographical regions have been indicated (Larochelle et al., 2002; Mankertz et al., 2000). The purpose of this study is to describe the generation of an infectious clone of a European strain of PCV2 and its testing under in vitro and in vivo conditions. For the latter purpose, the infectious PCV2 DNA clone was inoculated in conventional pigs by intramuscular and intraperitoneal routes.

**METHODS**

**PCV2 DNA clone construction.** DNA from a PCV2 isolate from Germany (GER3, GenBank reference AF201307) was extracted using the QiaGene DNA minikit (Qiagen) according to the manufacturer’s instructions. Primers were designed to perform two PCRs that allowed amplification of the whole PCV2 genome: F129 (5'–AGGTTGGGGGATTGTATG-3') and B130 (5'–AGCGTAGACAGGTCACCTCAAGTCCAGTCAC-3'). The reaction generated a 911 bp DNA fragment (nt 287–1186). Primer F51 (5'–ACAACGGAGTGACCTGTCATCTGC-3') and B52 (5'–CAGTTAACATGCCTCAAGGCTACCAGC-3') amplified another subgenomic DNA fragment of 1134 bp (nt 1163–581). PCR products were cloned into plasmid pcR2.1 using the TOPO TA cloning kit (Invitrogen), resulting in plasmids pS594-129 (carrying the F129/B130 amplicon) and pS594-51 (with the F51/B52 amplicon) (Fig. 1). The two inserts (generated by F129/ B130 and F51/B52) were subsequently joined via an internal BamHI site at position 384 into pUC18 (NEB) (Fig. 1). The resultant plasmid, pIC-PCV2, carried an overlength fragment of the PCV2 genome positions 1163–1768; 1–1186, with a 24 bp duplication (positions 1163–1186). To increase the size of the duplicated fragment, a 482 bp fragment excised from plasmid pSLV-rep(PCV2), carrying the rep gene of PCV2, was ligated (Mankertz et al., 2003). The final infectious clone (pIC-PCV2) carried a PCV2 fragment from positions 1163–1768; 1–1629, thereby displaying a duplication of 467 bp (1163–1629). pIC-PCV2 plasmid DNA was prepared using the Qiagen plasmid minikit (Qiagen) according to the manufacturer's instructions. Primers were designed to perform two PCRs that allowed amplification of the whole PCV2 genome: F129 (5'–AGGTTGGGGGATTGTATG-3') and B130 (5'–AGCGTAGACAGGTCACCTCAAGTCCAGTCAC-3'). The reaction generated a 911 bp DNA fragment (nt 287–1186). Primer F51 (5'–ACAACGGAGTGACCTGTCATCTGC-3') and B52 (5'–CAGTTAACATGCCTCAAGGCTACCAGC-3') amplified another subgenomic DNA fragment of 1134 bp (nt 1163–581). PCR products were cloned into plasmid pcR2.1 using the TOPO TA cloning kit (Invitrogen), resulting in plasmids pS594-129 (carrying the F129/B130 amplicon) and pS594-51 (with the F51/B52 amplicon) (Fig. 1). The two inserts (generated by F129/ B130 and F51/B52) were subsequently joined via an internal BamHI site at position 384 into pUC18 (NEB) (Fig. 1). The resultant plasmid, pIC-PCV2, carried an overlength fragment of the PCV2 genome positions 1163–1768; 1–1186, with a 24 bp duplication (positions 1163–1186). To increase the size of the duplicated fragment, a 482 bp fragment excised from plasmid pSLV-rep(PCV2), carrying the rep gene of PCV2, was ligated (Mankertz et al., 2003). The final infectious clone (pIC-PCV2) carried a PCV2 fragment from positions 1163–1768; 1–1629, thereby displaying a duplication of 467 bp (1163–1629). pIC-PCV2 plasmid DNA was prepared using the Qiagen plasmid minikit (Qiagen) and was used to transform Escherichia coli XL1 Blue. The bacteria were selected in Luria-Bertani (LB) medium containing 100 µg ampicillin ml⁻¹. The resultant infectious clone contained an overlength genome of a German PCV2 isolate with a partial duplication that enabled the virus to recircularize by homologous recombination and jump out of the plasmid backbone to initiate a natural infection.

---

**Fig. 1.** Scheme of the construction of plasmid pIC-PCV2. Details are given in the Methods section.
Transfection of PK-15 cells with pIC-PCV2. To test the infectivity of the molecular DNA clone in vitro, PK-15 cells were seeded in 24-well plates (2 × 10⁴ cells per well). When the PK-15 cells were approx. 60% confluent, the cells were transfected with 200 ng of pIC-PCV2 DNA using the Qiagen Effectene protocol (Qiagen). pUC18-transfected cells were included as a negative control. Cells transfected with 200 ng of PCV2 DNA were used as a positive control. Seven days after transfection, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature. After washing a further three times, cells were blocked with 3% fetal calf serum and 0.1% Tween in PBS for 1 h at 37°C in a humid atmosphere. Once blocked, cells were washed three times with PBS and incubated with polyclonal sera specific for PCV1 (diluted 1:400 in blocking solution) and PCV2 (diluted 1:200 in blocking solution), respectively. Subsequently, cells were washed three times with PBS and incubated with species-specific FITC-labelled anti-IgG antibodies (Dianova 1:200 in blocking solution) for 1 h. Cells were washed again three times in PBS and a fourth time in double-distilled water. Afterwards the cells were covered with Immu-mount (Shandon). The samples on microscopic slides were analysed for expression of viral antigen using confocal laserscan microscopy (Zeiss LSM510).

Monitoring of the recombination event by PCR. Since expression of viral antigen could originate from plasmid pIC-PCV2 as well as from the recombinant virus genome, the presence of pIC-PCV2 and PCV2 as a product of homologous recombination was monitored by PCR. For this purpose, DNA was isolated from cells 7 days after transfection and amplified with primers F199 (5'-CCACATCGAGAAAGCGAAAGGAAC-3') and B413 (5'-TCGGTGAGCTGTTCTGTAGC-3') to distinguish between the input DNA and the recombinant product.

Southern hybridization. Low molecular mass DNA was isolated from cells, separated on a 1% agarose gel, transferred onto a nylon membrane and visualized by Southern hybridization as described earlier (Mankertz et al., 1997). A PCV2-specific DIG-labelled probe of 366 bp (nt 37–402, cap gene) was used, which was generated by PCR. For this purpose, a DIG-labelling PCR reaction (Roche MolecularBiochemicals) was set up with plasmid pIC-PCV2 DNA as the template and using primers F243 (5'-TTAGGGTTTTAATGTTGAGGGTGTC-3') and B244 (5'-CAGGGTGACAGGGGAGTG-3') (Mankertz & Hillenbrand, 2001).

Animals. Eighteen pigs from three different litters, selected for a low serological PCV2 titre in sows, were used. The piglets were weaned at 2 weeks of age, bled, ear tagged and kept in isolated experimental facilities. All pigs were found to have low titres of antibodies to PCV2 (1:20 to 1:320) by an immunoperoxidase monolayer assay (IPMA) (Rodriguez-Arrioja et al., 2000) when between the age of 30 and 40 days (day 0 of the experiment). The pigs were seronegative to porcine reproductive and respiratory virus (PRRSV), pseudorabies virus (PRV), porcine parvovirus (PPV), swine influenza virus (SIV), transmissible gastroenteritis coronavirus (TGEV), porcine respiratory coronavirus (PRCV), and Actinobacillus pleuropneumoniae, Pasteurella multocida, Bordetella bronchiseptica and swine erysipelas. Also at day 0 of experiment, serum, and nasal and rectal swabs were taken to be analysed by a PCV2 PCR described previously (Quintana et al., 2002). All the pigs were found to be negative for PCV2.

PCV2 inoculum. Swine kidney (SK) cells free of PCV1 were inoculated with the Spanish PCV2 isolate SPA3 (GenBank reference AF201310), which came from a lymphoid tissue homogenate of a PMWS-affected pig. This homogenate had been previously tested by PCR and/or IPMA to exclude the presence of PRRSV, PPV, PRV, SIV, TGEV and PCV1. After three serial passages of SK cells, the supernatant was recovered, clarified by centrifugation at 650 g, and further concentrated by ultrafiltration. The viral pellet was resuspended in Dulbecco’s modified Eagle’s medium, titrated with non-infected SK cells (10⁻⁵.⁴ TCID₅₀ ml⁻¹) aliquoted, and frozen at −80°C until used.

In vivo experimental design. Pigs were distributed randomly into three groups and kept in different isolation rooms. On day 0 of the experiment, the first group of six pigs was inoculated intranasally (i.n.) with 5 ml of PCV2 inoculum (10⁶ TCID₅₀ per pig). The second group of six animals was inoculated intramuscularly (i.m.) with 2 ml of PCV2 infectious clone (300 µg of pcIC-PCV2 per pig). Finally, the last group of six pigs was inoculated intraperitoneally (i.p.) with 2 ml of the infectious clone (300 µg of pcIC-PCV2 per pig). Pigs were clinically monitored daily, weighed weekly, rectal temperature was measured three times a week and blood samples were collected weekly. Pigs were euthanized on day 35 post-inoculation (p.i.) by an overdose of intravenous sodium thiobarbital, in accordance with the European Guidelines for Animal Welfare. A complete necropsy was performed of all pigs, and lung, intestinal superficial lymph nodes, tonsil, thymus, spleen, liver, kidney, ileum and bone marrow samples were collected and fixed by immersion in 10% buffered formalin.

Pathological studies. Pig tissues were subsequently dehydrated through graded alcohols and embedded in paraffin wax, sectioned at 4 µm thick and stained with haematoxylin and eosin (HE). Moreover, serial sections were mounted on silane-treated slides and an in situ hybridization (ISH) technique to detect PCV2 nucleic acid using a specific 40 nt probe was performed as described previously (Rosell et al., 1999, 2000).

PCV2 antibody detection. IPMA described previously (Rodriguez-Arrioja et al., 2000) was performed to detect antibodies to PCV2 in serum samples obtained weekly (0, 7, 14, 21, 28 and 35 days p.i.).

Polymerase chain reaction (PCR) to detect PCV2 genome. DNA was extracted from 200 µl of serum sample using Nucleo Spin Blood (Macherey-Nagel) according to the manufacturer’s instructions. The PCR protocol used has been described previously (Quintana et al., 2002). The amplified products were run using a 2% agarose gel and visualized by staining with 0.5 µg ethidium bromide ml⁻¹. The length of the amplicon for this primer pair was 656 bp.

Sequence analysis, sequence alignments and phylogenetic analysis. Positive PCV2 PCR serum samples corresponding to one pig of each inoculated group PCV2 i.n., PCV-PCV2 i.m. and pIC-PCV2 i.p. were selected to complete the sequence and confirm that it corresponded to the original sequences of the viruses (SPA3 and GER3). The extracted products were amplified with a variety of sequencing primers (data not shown). To extract and purify DNA from the agarose gel, the MinElute gel extraction kit (Qiagen) was used according to the manufacturer’s protocol. Finally, computer analysis and alignment were done using the CLUSTALW multiple alignment function of the BIOEDIT program (version 5.0.9, North Carolina State University). Phylogenetic trees were constructed using the Neighbour-Joining program in the MEGA2 package (version 2.1) taking into account 1000 bootstraps.

TaqMan PCR for PCV2. To quantify PCV2 DNA in serum samples at 35 days p.i., a real-time fluorescent-probe PCR was used (Olvera et al., 2004). The assay was performed in triplicate.

Statistical analyses. Statistical analyses were performed using the SAS system (SAS/STAT Users’ Guide: Statistics, version 8. SAS Institute). A mixed linear model (Verbeke & Molenberghs, 2000) was designed using the mixed procedure for the statistical analysis of the weight and rectal temperatures between different groups. The
results of the ISH and the PCR were analysed by Fisher’s Exact Test. Finally, the results of the IPMA were analysed by a negative binomial test using the Genmod procedure. A generalized linear model was used for the statistical analysis of the TaqMan PCR results.

RESULTS

In vitro evaluation of pIC-PCV2 infectivity in transfected PK-15 cells

Expression of a PCV2-specific protein was observed in approximately 20% of the transfected cells on day 7 post-transfection (Fig. 2). The antiserum used for IFA had been raised against purified virus particles and recognizes the major structural protein of the virus, the Cap protein. The Cap gene can be expressed from the input DNA pIC-PCV2 as well as from PCV2, the product of homologous recombination. Therefore, which DNA is responsible for expression of the Cap protein cannot be distinguished by IFA. To discern between the input DNA and the recombination product, a PCR was performed on cells at 7 days post-transfection. In all cases, primers F199/B413 amplified a 591 bp fragment, which was indicative of the presence of the virus genome. An amplification product of 2450 bp (it includes the vector and only a short part of PCV2 genome), which would be generated from the input DNA, was not observed. Only viral DNA, but no DNA corresponding in size to the infectious clone was observed. This result was further confirmed with Southern hybridization (data not shown).

In vivo evaluation of PCV2/pIC-PCV2 infectivity in conventional pigs

Pigs that received pIC-PCV2 by i.p. and i.m. routes became infected based on ISH, PCR in serum, TaqMan PCR in serum and serological results. Low amounts of virus were detected by ISH in the inguinal superficial lymph node, tonsil, thymus (Fig. 3), ileum, spleen and/or lung of a proportion of pigs on day 35 p.i. (two pigs from the i.n. PCV2 inoculated group, and four pigs from each of the i.m. and i.p. pIC-PCV2 inoculated groups). The PCV2 nucleic acid was mainly located in follicles of the lymph nodes, Peyer’s patches, tonsil and thymus. In the spleen, PCV2 nucleic acid was found in the periarteriolar lymphoid sheets. In all cases, virus was detected in the cytoplasm of dendritic-like cells and macrophages. The PCV2 genome was detected by PCR at 7 days p.i. in the PCV2 i.n. inoculated group, whereas the viral genome was detected for the first time on day 14 in animals inoculated with pIC-PCV2 infectious clone i.m. and i.p. (Table 1). However, no significant differences were observed in the dynamics of PCV2 viraemia among all groups. At the end of the experiment, PCV2 DNA was present in all pigs studied. Three random positive PCR serum samples corresponding to one pig i.n. inoculated with PCV2 (28 days p.i.), another pig i.m. inoculated with pIC-PCV2 (28 days p.i.) and one pig i.p. inoculated with pIC-PCV2 (35 days p.i.) were sequenced. The three sequences were compared with a total of 44 PCV2 sequences from GenBank. The corresponding phylogenetic tree showed that the sequence found

Fig. 2. Transfection of PK-15 cells with pIC-PCV2. Cells were transfected and subsequently subjected to an indirect immune fluorescence assay using a PCV2 antiserum. (A) PK-15 cells, mock-transfected. (B) PK-15 cells transfected with pIC-PCV2.

Fig. 3. Detection of PCV2 DNA in thymus (dark colour) of a pIC-PCV2 i.p. inoculated animal. In situ hybridization to detect PCV2, fast green counterstain. Bar, 150 μm.
in the PCV2 i.n. inoculated pig had 99.6% similarity to the Spanish PCV2 isolate, SPA3; the pIC-PCV2 i.m. and i.p. inoculated pigs had 99.4 and 99.7% similarity to the German isolate, GER3, respectively.

The mean PCV2 load on day 35 p.i. in the PCV2 i.n. pigs was 6.2 × 10^6 copies ml⁻¹ (range 1.0 × 10^6–1.2 × 10^7 copies ml⁻¹). Mean viral load in the pIC-PCV2 i.m. and i.p. inoculated pigs was 4.14 × 10^6 (range 3.5 × 10^5–1.5 × 10^7) and 2.6 × 10^6 (range 2.3 × 10^5–1.4 × 10^6) copies ml⁻¹, respectively, on day 35 p.i. No significant differences in serum PCV2 loads were observed between the different groups.

Finally, seroconversion was observed in all groups (Table 1). PCV2 i.n. inoculated pigs seroconverted as early as 14 days p.i., while the other groups started seroconverting on days 21 (pIC-PCV2 i.p. inoculated pigs) and 28 p.i. (pIC-PCV2 i.m. inoculated pigs). These differences were statistically significant among all groups (P < 0.001). At the end of the experimental period (35 days p.i.), all pigs inoculated with PCV2 i.n. and four out of six pIC-PCV2 i.p. inoculated pigs had high PCV2 antibody titres (ranging from 1 : 5120 to 1 : 20,480). On the other hand, the remaining two pIC-PCV2 i.m. inoculated pigs. These differences were statistically significant among all groups (P < 0.001).

Serol., no. of seropositive pigs to PCV2 (titre >1/320), PCR, no. of pigs with a positive PCR in serum.

<table>
<thead>
<tr>
<th>Day(s) p.i.</th>
<th>PCV2 IN</th>
<th>pIC-PCV2 IM</th>
<th>pIC-PCV2 IP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serol.</td>
<td>PCR</td>
<td>Serol.</td>
<td>PCR</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>35</td>
<td>6</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Lung*</th>
<th>LN‡</th>
<th>Liver†</th>
<th>Kidney‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV2 IN (6)</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>pIC-PCV2 IM (6)</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>pIC-PCV2 IP(6)</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*No. of pigs with mild interstitial pneumonia.
†No. of pigs with mild lymphocyte depletion with mild granulomatous infiltration.
‡No. of pigs with mild mononuclear hepatitis/nephritis.

Clinical and pathological evaluation of PCV2/pIC-PCV2 inoculated pigs

All pigs remained clinically healthy during the whole experimental period. No significant differences were observed in rectal temperatures between the three studied groups. Numerically, the weight increase over the experimental period was higher in the pIC-PCV2 i.m. inoculated group followed by the pIC-PCV2 i.m. inoculated group, and finally by the PCV2 i.n. inoculated group. However, significant differences (P < 0.01) in body weight were observed only on day 35 p.i. between the PCV2 i.n. and pIC-PCV2 i.p. inoculated pigs. No significant differences in body weight were observed among the groups during the rest of the experimental period.

Macroscopically, three out of six PCV2 i.n. inoculated pigs had a slight increase in the size of the mesenteric and mediastinal lymph nodes. Only one out of six animals that received the pIC-PCV2 i.m. had a mild increase in the size of the mesenteric lymph node. Finally, two out of six pIC-PCV2 i.p. inoculated pigs had a mild increase in the size of the mesenteric lymph node. No other significant gross lesions were observed in the inoculated pigs.

Microscopic lesions are summarized in Table 2. Lesions observed in lymphoid tissues consisted of a mild lymphocyte depletion with moderate histiocytic infiltration; multinucleate giant cells were very occasionally seen in the lymphoid organs. Lung lesions consisted of a mild interstitial pneumonia. Also, one pig that received pIC-PCV2 i.m. had a mild multifocal lymphoplasmacytic hepatitis. Three animals from the PCV2 i.n. and one pig from the pIC-PCV2 i.p. inoculated groups had mild interstitial nephritis.

**DISCUSSION**

This work represents the first description of an infectious clone generated with a PCV2 European strain and its in vitro and in vivo testing. This cloned PCV2 genomic DNA was shown to be infectious for PK-15 cells and conventional pigs when injected i.p. and i.m., although it was not able to produce PMWS in the inoculated animals. These results are very similar to those obtained by Fenaux et al. (2002), using a USA strain of PCV2 DNA infectious clone.

The generated infectious clone contained an overlength genome of a German PCV2 isolate with a partial duplication that enabled the virus to recircularize by homologous recombination and jump out of the plasmid backbone to initiate a natural infection. When this plasmid was applied in vitro, 20% of the cells transfected with pIC-PCV2 showed expression of viral antigen, as determined by IFA. It is known that if two identical or similar sequences are present within one DNA molecule, this molecule may undergo homologous recombination (Valerie & Povirk, 2003; West, 2003). Therefore, to discriminate between...
expression from the original input DNA and the product of recombination, a monitoring PCR was used. This approach led to the identification of recombinant virus, while no input DNA was detected. Taken together, these results show that infection by pIC-PCV2 delivers genomic PCV2 DNA specifically into susceptible cells and leads to the expression of a functional virus genome.

In the experimental trial, all pigs that received the PCV2 infectious clone became infected. Pathological, serological and virological (detection and quantification of the virus) results of these animals were very similar to those obtained for the IN infected pigs with a PCV2 strain propagated in cell culture. However, none of the pigs from this study developed clinical PMWS, a condition where PCV2 is considered the major infectious agent involved (Allan et al., 1999; Bolin et al., 2001; Krakowka et al., 2001), although it is not necessarily sufficient to trigger the clinical disease (Segales & Domingo, 2002). Only mild pathological lesions characteristic of PMWS such as lymphoid depletion and granulomatous infiltration (Rosell et al., 1999) were observed in lymphoid tissues. In fact, the present results are also very similar to published work which tested an infectious PCV2 clone based on an American strain of the virus (Fenaux et al., 2002). In this latter report, the PCV2 genomic DNA clone was injected directly into the liver and superficial iliac lymph node of SPF pigs. These authors used those inoculation routes based on the fact that PCV2 replicates in the lymph nodes and liver during the natural (Choi & Chae, 2000; Kiupel et al., 1999; Rosell et al., 1999, 2000) and experimental (Allan et al., 1999; Bolin et al., 2001; Harms et al., 2001; Krakowka et al., 2000) infection. In the present study, the i.m. and i.p. routes of inoculation were chosen. The former one is a traditional and well described route for inoculation of DNA vaccines and plasmids (Ulmer et al., 1997; van Drunen Littel-vanden Hurk et al., 2000; Wolff et al., 1990), and it has been demonstrated to be an efficient route to generate both humoral and cellular immunity against several pathogens in different species, including swine (Cox et al., 1993; Gerdts et al., 1997; Haagmans et al., 1999; van Rooij et al., 1998).

The intraperitoneal route, however, is not a usual route to inoculate DNA plasmids in pigs. This route was thought to be useful since the virus would be transported, either after phagocytosis by intraperitoneal macrophages or in suspension in the lymph, towards the cranial sternal lymph nodes of the ventral thoracic lymphocentrum, as has been demonstrated in other species (Marco et al., 1992). Therefore, the intraperitoneal route would represent a very direct way to reach the lymph nodes of the pig and easier than direct inoculation into lymph nodes. In the present study, the intraperitoneal route was as effective or more than the intramuscular route, at least in terms of infection based on the serological, PCR and TaqMan PCR results. Moreover, the PCV2 genomic DNA clone did not show any difference in viral load at 35 days p.i. when compared to the i.n. route using a PCV2 isolate propagated in cell culture.

Certain differences were observed among groups regarding dynamics of seroconversion and viral detection in serum. Although almost all animals seroconverted by day 35 p.i., pigs that received the PCV2 strain propagated in cell culture seroconverted before groups that received the infectious clone. Furthermore, the pigs that received the DNA clone i.p. seroconverted before (and had higher serological titres at different days p.i.) the ones that received it i.m. These results may suggest that to generate humoral immunity the intraperitoneal route is more efficient than the intramuscular route; however, these results need to be confirmed in an experiment with a higher number of inoculated animals.

The low level of maternal antibodies in the inoculated pigs at the start of the experiment did not seem to have any confounding influence on the onset of viraemia or seroconversion. Similarly, in a previous experiment performed by our group, we also observed that pigs with a low level of maternal antibodies developed PCV2 infection and even PMWS (Rovira et al., 2002). However, we were not able to make a definitive assessment as to whether low levels of maternal antibodies could prevent infection or modify its outcome.

The results of the present study further confirm that cloned PCV2 genomic DNA is infectious in vitro and in vivo, and that it is able to cause PMWS-like lesions in i.p. and i.m. experimentally infected pigs, administration routes that have not been tested previously. The use of infectious DNA clones removes the need for infectious virus stocks in cell cultures, decreases the risk of cell culture contamination with other virus and allows the generation of a biologically pure infectious PCV2. Although it was not the purpose of the present study, the results obtained open the possibility to explore the potential use of this PCV2 infectious clone as a DNA vaccine to control PMWS and other PCV2-associated diseases. In fact, recent published information on the pathogenic and immunogenic characteristics of a chimeric infectious DNA clone (Fenaux et al., 2003) have shown the potential use of these constructs as genetically engineered live-attenuated vaccines.

ACKNOWLEDGEMENTS

We thank R. Dolz, E. Huerta, S. López, M. Mora, A. Olvera, M. Pérez, B. Pérez and J. Plana-Maslopart for technical assistance and Llorenç Badiella from the Servei de Estadística de la Universitat Autònoma de Barcelona for data statistical assistance. This work was supported by project QLRT-PL-199900307 from Fifth Framework Programme 1998–2002 of the European Commission.

REFERENCES


Rosell, C., Segalés, J., Plana-Duran, J. & 7 other authors (1999). Pathological, immunohistochemical, and in-situ hybridization studies...


