Detection and \textit{in vitro} and \textit{in vivo} characterization of porcine circovirus DNA from a porcine-derived commercial pepsin product

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Received 8 July 2004
Accepted 27 July 2004

Non-pathogenic porcine circovirus type 1 (PCV1) and pathogenic PCV2 are widespread in swine herds. In this study, the detection and characterization of PCV1 and PCV2 DNA from porcine-derived commercial pepsin are reported. The complete genomic sequences of the pepsin-derived PCV1 and PCV2 share 76 % nucleotide sequence identity with each other and 95–99 % identity with respective North American PCV1 and PCV2 isolates. However, the PCV-contaminated pepsin lacks infectivity in PK-15 cells. To further assess the infectivity of the contaminating pepsin \textit{in vivo}, 16 5-week-old, specific-pathogen-free pigs were divided randomly into three groups: pigs in group 1 \((n = 5)\) were each inoculated intramuscularly and intranasally with 4 ml PBS buffer as negative controls, those in group 2 \((n = 6)\) were each inoculated with 400 mg contaminated pepsin dissolved in 4 ml PBS and those in group 3 \((n = 5)\) were each inoculated with \(4 \times 10^4\) TCID\textsubscript{50} PCV2 as positive controls. PCV2 viraemia, seroconversion and pathological lesions were detected in group 3 pigs, but not in group 1 or 2 pigs, confirming that the contaminating PCVs were non-infectious. Nevertheless, the detection of PCV DNA in a porcine-derived commercial product raises concern for potential human infection through xenotransplantation.

Porcine circovirus type 1 (PCV1) and type 2 (PCV2) are widespread in commercial swine populations worldwide (Allan & Ellis, 2000; Allan et al., 1998; Fenaux et al., 2000). PCV1 was discovered as a contaminant of the PK-15 cell line (Tischer et al., 1982). Experimental inoculation of pigs with PCV1 does not produce clinical disease and PCV1 is thus generally considered to be non-pathogenic (Allan et al., 1995; Tischer et al., 1986). PCV2 is the primary causative agent of post-weaning multisystemic wasting syndrome (PMWS) in pigs (Allan & Ellis, 2000; Fenaux et al., 2000, 2002; Harding & Clark, 1997; Harms et al., 2001; Laddekrjæer-Mikkelsen et al., 2002; Rovira et al., 2002). The PMWS-associated PCV2 shares about 76 % nucleotide sequence identity with the non-pathogenic PCV1 (Fenaux et al., 2000; Roca et al., 2004). Both PCV1 and PCV2 are non-enveloped, icosahedral viruses with a single-stranded circular DNA genome of about 1·76 kb (Mankertz et al., 2004). Like porcine paroviruses, PCV virions are resistant to inactivation and are difficult to remove from the environment (Royer et al., 2001).

Xenotransplantation with porcine organs, cells and other porcine-derived products has received considerable attention in the last few years and could potentially alleviate the problems associated with the shortage of human organ donors (Meng, 2003; Tucker et al., 2003). However, inadvertent transmission of potentially zoonotic porcine viruses to humans is of major concern. Antibodies to PCV1 have been reportedly detected in many animal species, including humans (Tischer et al., 1986), and PCV2 has been shown to infect BALB/c mice (Kiupel et al., 2001). However, others have reported contradictory results regarding the ability of PCV to cause cross-species infections (Ellis et al., 2000; Quintana et al., 2002). Nevertheless, the use of PCV-contaminated porcine organs, cells and medical products, such as factor VIII, heparin, insulin and pepsin, in humans through xenotransplantation procedures has raised concerns for potential human infections. The objective of this study was to determine whether a porcine-derived commercial pepsin product was contaminated with PCVs.
and, if so, whether or not the contaminating PCVs were still infectious in vitro and in vivo.

Samples of two different porcine-derived pepsin lots (two lyophilized 100 g vials from each lot) from a commercial company were tested for the presence of PCV1 and PCV2 DNA by PCR. For each vial of pepsin, 10 mg lyophilized product was dissolved in 100 µl PBS. DNA was extracted from the dissolved pepsin with a QIAamp DNA mini kit (Qiagen) according to the protocols supplied by the manufacturer. The MCV1 (5’-GCTGAACCTTTTAAGTGAGCCGGG-3’) and MCV2 (5’-TCACACAGTCTCAGTAGTACATCCTCA-3’) primer pair was used to detect PCV1 or PCV2 DNA in the pepsin by PCR, as described previously (Fenaux et al., 2000). To amplify and sequence the complete genomes of PCV1 and PCV2 from the contaminated pepsin product, 11 nested sets of primer pairs were designed, based on the published sequences of PCV1 and PCV2 (Fenaux et al., 2000). PCR products of the expected size were purified and sequenced directly by using the PCR primers.

Of the two lots of pepsin that were tested in this study, one (both vials) was positive for both PCV1 and PCV2 DNA by PCR, and the other one was negative. The complete genomes of the pepsin-derived PCV1 and PCV2 were determined to be 1759 and 1768 bp in length, respectively. To determine the extent of sequence identity between the pepsin-derived PCV1 and PCV2 and other known PCVs, the complete genomic sequences of four PCV1 and 31 PCV2 isolates that were available in GenBank were compared with those of the pepsin-derived PCV1 and PCV2. Pepsin-derived PCV2 displayed 95–99% nucleotide sequence identity with the published PCV2 sequences. Similarly, the pepsin-derived PCV1 shared 98–99% nucleotide sequence identity with the published PCV1 sequences. The pepsin-derived PCV1 and PCV2 shared 76% nucleotide sequence identity with each other. A phylogenetic tree was constructed on the basis of the complete genomic sequences of 37 isolates of PCV1 and PCV2 from different geographical regions with the aid of the PAUP program (David L. Swofford, Smithsonian Institution, Washington, DC, USA) (Fig. 1). Phylogenetic analysis showed that the pepsin-derived PCV2 was related closely to North American PCV2 isolates and formed a minor branch (Fig. 1). Similarly, the pepsin-derived PCV1 clustered with other PCV1 isolates, but formed a distinct minor branch.

Although PCV virions are very resistant to inactivation (Royer et al., 2001), detection of PCV DNA in a commercial pepsin product does not necessarily mean that the contaminating viruses are still infectious. To biologically characterize the infectivity of the contaminating PCV DNA, PK-15 cells that were free of PCV1 contamination (Fenaux et al., 2002) were grown in T-25 flasks and inoculated with the lot of pepsin that was contaminated with PCV1 and PCV2, as well as with the lot of pepsin that was negative for PCV1 and PCV2. The pepsin was dissolved in PBS prior to inoculation. As positive controls, PK-15 cells were inoculated with PCV1 or PCV2 infectious stock (Fenaux et al., 2002). After five blind passages, the inoculated cells were plated on eight-well LabTek chamber slides and subsequently fixed with a solution containing 80% acetone and 20% methanol at 4°C for 20 min. Evidence of PCV1 and PCV2 replication in inoculated cells was detected by immunofluorescence assay (IFA) with a PCV1 mAb (Allan et al., 1995), as described previously (Fenaux et al., 2003, 2004a, b) or with a PCV2-specific polyclonal rabbit antibody, as described previously (Fenaux et al., 2002; Lekcharoensuk et al., 2004). The results showed that the contaminating PCV1 in pepsin was not infectious in vitro, as no PCV1 antigen was detected in inoculated PK-15 cells after five blind passages. Similarly, the contaminating PCV2 also lacked infectivity in vitro. In the positive control, PK-15 cells inoculated with PCV1 and PCV2 infectious virus stocks were both infected, as demonstrated by IFA for the presence of PCV1 or PCV2 antigen in the nucleus. Mock-infected PK-15 cells were negative for PCV1 and PCV2 antigen.

We and others have demonstrated previously that naked PCV2 or PCV1 genomic DNA was infectious when injected intramuscularly into live animals (Fenaux et al., 2002, 2003, 2004a, b; Roca et al., 2004). Therefore, the contaminating PCV DNA in pepsin, although non-infectious in vitro, could still be infectious when injected into animals. To further investigate the infectivity of the contaminating pepsin, 16 SPF pigs of 5 weeks of age were assigned randomly to three groups. Prior to inoculation at −1 day post-inoculation (d.p.i.), serum samples from all pigs in groups 1, 2 and 3 tested negative for PCV1 and PCV2 DNA. To maximize inoculation efficiency, each pig was given 1/4 of the inoculum intramuscularly and 3/4 intranasally. The five pigs in group 1 were each inoculated with 4 ml PBS as negative controls, the six pigs in group 2 were each inoculated with 400 mg PCV-contaminated pepsin dissolved in 4 ml PBS and the five pigs in group 3 were each inoculated with 4 × 10³ TCID₅₀ wild-type PCV2 as positive controls.

All pigs were monitored for clinical signs of disease by a team of two people. Pigs were weighed on a weekly basis. Rectal temperatures and clinical respiratory scores, ranging from 0 to 6 (0, normal; 6, severe) (Halbur et al., 1995), were recorded every other day from 5 to 39 d.p.i. Clinical observations, including evidence of central nervous system disease, liver disease (icterus), musculoskeletal disease and changes in body condition, were recorded daily. There was no difference in weight gain or mean rectal temperatures among any of the groups. From 19 to 42 d.p.i., the mean clinical scores recorded for positive-control group 3 pigs became more severe (P<0.05) than those recorded for animals in groups 1 and 2 (data not shown).

All animals received a complete necropsy at 42 d.p.i. The necropsy team was blinded to the infection status of the pigs at necropsy. An estimated percentage of the lung with grossly visible pneumonia was recorded for each pig, based on a previously described scoring system (Halbur et al.,
Other lesions, such as enlargement of lymph nodes (ranging from 0 for normal to 3 for three times normal size), were scored separately (Fenaux et al., 2004a, b). We examined the pathological lesions that were induced by PCV2 infection only, as PCV1 is non-pathogenic (Allan et al., 1995). The group 1 negative-control pigs had no evidence of gross lymph-node or lung lesions. Two of the six pepsin-inoculated group 2 pigs had moderate enlargement of lymph nodes. The lesions in these two pigs were not attributable to PCV2, as serological and virological evidence of PCV2 infection in these pigs was lacking. The PCV2-inoculated group 3 pigs all had mild to severe enlargement of lymph nodes. The mean gross lesion scores in the lymph nodes of group 1 and group 2 pigs were not significantly different from each other, but were significantly less severe than that of PCV2-inoculated group 3 positive-control pigs. Lungs were free of gross visible pneumonia in all three groups.

Sections for histopathological examination were taken from lungs (five sections), heart, lymph nodes (tracheobronchial), tonsil, thymus, liver, spleen, small intestine and kidney. The tissues were examined in a blinded fashion and given a score for severity of lung, lymph-node, tonsil, spleen and liver lesions (Halbur et al., 1995). Lung scores ranged from 0 (normal) to 3 (severe lymphohistiocytic interstitial pneumonia). Liver scores ranged from 0 (normal) to 3 (severe lymphohistiocytic hepatitis). Lymphoid-tissue

**Fig. 1.** Phylogenetic tree based on the complete genomic sequences of 37 PCV1 and PCV2 isolates available in GenBank. GenBank accession number and geographical origin were used to designate each PCV isolate. The tree was constructed with the aid of the PAUP program. Heuristic-search and midpoint-rooting options were used to produce a consensus tree. Branch-lengths are proportional to the number of character-state changes. Bar, 10 character-state changes.
scores were for an estimated amount of lymphoid depletion (LD) and histiocytic replacement (HR) of follicles, ranging from 0 (normal) to 3 (severe LD and HR of follicles). Mild lymphoplasmacytic and histiocytic broncho-interstitial pneumonia was observed in three of five group 1 pigs and in five of six pepsin-inoculated group 2 pigs (Table 1). All PCV2-inoculated group 3 pigs had mild to moderate lymphoplasmacytic and histiocytic broncho-interstitial pneumonia. In the pepsin-inoculated group 2 pigs, one animal had very mild LD and HR of the lymph-node follicles. All PCV2-inoculated group 3 pigs had mild to moderate LD and HR in lymph nodes and spleen tissues (Table 1).

Sera collected from all pigs at −1, 7, 14, 21, 28, 35 and 42 d.p.i. were tested for PCV2 antibodies by a modified indirect ELISA (Nawagitgul et al., 2002) and by PCR for the presence of PCV1 and PCV2 DNA (Fenaux et al., 2000). Group 1 negative-control pigs and pepsin-inoculated group 2 pigs remained negative for PCV1 and PCV2 viraemia throughout the study (Table 2). In the positive-control group 3 pigs, PCV2 viraemia was first detected at 7 d.p.i. and four of five pigs became viraemic for PCV2 (Table 2). No seroconversion to PCV2 antibodies was detected in any of the group 1 or 2 pigs throughout the study. In the positive-control group, seroconversion to PCV2 antibodies was first detected at 21 d.p.i. in one pig and, by 42 d.p.i., all group 3 pigs inoculated with PCV2 had seroconverted (data not shown).

A PCV2-specific rabbit polyclonal antisera was used for immunohistochemistry to detect PCV2 antigen in lymph-node, spleen, tonsil and thymus tissues that were collected during necropsy at 42 d.p.i., according to the procedures described by Sorden et al. (1999). The amount of PCV2 antigen distributed in the lymphoid tissues was scored in a blinded fashion by assigning a score of 0 (no signal) to 3 (strong positive signal). Lymphoid tissues from the negative-control group 1 pigs, as well as those from the pepsin-inoculated pigs, were negative for PCV2 antigen. In the group 3 positive-control pigs, mild to moderate levels of PCV2 antigen were detected in lymph-node tissues of three of five pigs, and in tonsil and spleen tissues of two of five pigs (data not shown).

Xenozoonosis due to the inadvertent transmission of porcine viruses from pig xenografts to human transplant recipients and the potential subsequent transmission of the virus to others are of major concern in xenotransplantation (Meng, 2003; Tucker et al., 2003). In this study, we found that one of the two lots of porcine-derived commercial pepsin that were tested was positive for both PCV1 and PCV2 DNA. However, we were unable to infect PK-15 cells with the contaminated pepsin, suggesting that the contaminated PCV1 and PCV2 viruses in pepsin were probably inactivated and lacked infectivity in vitro. SPF piglets that were inoculated experimentally with the contaminated pepsin did not become viraemic for PCV1 or PCV2, nor did they seroconvert to PCV2; PCV2 antigen was not detected in the lymphoid tissues of pepsin-inoculated pigs. Compared to the PCV2-inoculated positive-control group, the negative-control pigs and the pepsin-inoculated pigs had no significant gross or microscopic lesions characteristic of PCV2 infection. The lack of PCV infectivity of the contaminated pepsin in vitro and in vivo is probably

### Table 1. Distribution of histopathological lesions in different tissues and organs from inoculated and control pigs

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
<th>Lung</th>
<th>Lymph nodes</th>
<th>Tonsil</th>
<th>Spleen</th>
<th>Liver</th>
<th>Kidney</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LD</td>
<td>HR</td>
<td>LD</td>
<td>HR</td>
<td>LD</td>
<td>HR</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>PBS</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>2</td>
<td>PCV-contaminated pepsin</td>
<td>5/6</td>
<td>0/8</td>
<td>0/6</td>
<td>0/0</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>3</td>
<td>PCV2</td>
<td>5/5</td>
<td>1-4</td>
<td>3/5</td>
<td>0-8</td>
<td>0/5</td>
<td>1/5</td>
<td>2/5</td>
</tr>
</tbody>
</table>

*Values in parentheses are mean histological scores for interstitial pneumonia (0, normal through 6, severe) and for interstitial hepatitis and LD and HR for lymph nodes, tonsils and spleen.

### Table 2. Detection of viraemia by PCR in sera of inoculated and control pigs

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
<th>No. pigs positive/no. pigs tested at (d.p.i.):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−1 7 14 21 28 35 42 Total</td>
</tr>
<tr>
<td>1</td>
<td>PBS</td>
<td>0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td>2</td>
<td>PCV-contaminated pepsin</td>
<td>0/6 0/6 0/6 0/6 0/6 0/6 0/6 0/6</td>
</tr>
<tr>
<td>3</td>
<td>PCV2</td>
<td>0/5 4/5 4/5 4/5 4/5 3/5 3/5 1/5 4/5</td>
</tr>
</tbody>
</table>
due to the pepsin-manufacturing process, which effectively inactivates the PCVs and degrades their genomic DNA. However, other porcine-derived products, such as factor VIII from pig plasma, may contain high titres of infectious PCVs and, thus, the manufacturing process may not completely inactivate the viruses. Porcine parvovirus (Soucie et al., 2000) and porcine endogenous retrovirus (Takefman et al., 2001) were detected in porcine-derived factor VIII. Therefore, it is important to test other porcine-derived medical and research products, such as factor VIII, heparin and insulin, for potential contamination by PCVs.

Acknowledgements

We thank Drs S. M. Boyle, L. A. Eng, R. B. Duncan, J. C. Sible and T. E. Toth for their support and Jillian Fenaux for her editorial assistance. This study was supported by a grant from Genentech Inc., South San Francisco, CA, USA, and in part by a grant from the US Department of Agriculture National Research Initiative Competitive Grant Program (NRI 2004-35204-14213).

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