Colostral transmission of porcine circovirus 2 (PCV-2): reproduction of post-weaning multisystemic wasting syndrome in pigs fed milk from PCV-2-infected sows with post-natal porcine parvovirus infection or immunostimulation

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Post-weaning multisystemic wasting syndrome (PMWS) was reproduced in pigs fed colostrum and milk from porcine circovirus 2 (PCV-2)-infected sows and infected post-natally with porcine parvovirus (PPV) or immunostimulated. Pregnant sows were inoculated intranasally with either PCV-2 (n=5) or PCV-2-free PK-15 cell lysates (control, n=10) 3 weeks before the expected farrowing date. Newborn piglets from five of the control sows were introduced to PCV-2-infected sows (n=6 for each sow) and allowed to feed on the colostrum for 12 h and then given 15 ml milk five times a day for 7 days. Newborn piglets from the other five control sows were fed colostrum and milk from their own sows. After 7 days, two piglets from each group were randomly selected to confirm PCV-2 infection. Twenty-one pigs fed by PCV-2-infected sows were randomly divided into three groups and subjected to post-natal PPV infection (group 1), immunostimulation (group 2) or no post-natal treatment (group 3). Twenty-one pigs fed by uninfected sows were also randomly divided and subjected to post-natal PCV-2 and PPV infection (group 4), post-natal PCV-2 infection (group 5) or no treatment (group 6, negative control). Body weight was significantly greater in group 6 than in groups 1, 2 and 4 at 49, 52, 56, 59 and 63 days of age. The typical granulomatous inflammatory reaction and lymphoid depletion of PMWS was observed in the lymph nodes of groups 1, 2 and 4 at 63 days of age. Group 3 had significantly fewer PCV-2-positive cells than groups 1, 2 and 4. In conclusion, PCV-2 shed from colostrum and milk is infectious and reproduces PMWS with post-natal PPV infection or immune stimulation.

INTRODUCTION

Porcine circovirus 2 (PCV-2) is a small, non-enveloped, circular, single-stranded DNA virus and is the primary aetiological agent of post-weaning multisystemic wasting syndrome (PMWS) (Allan & Ellis, 2000; Chae, 2004). The characteristic clinical symptoms of PMWS include a marked increase in mortality, progressive weight loss, dyspnoea and lymph node enlargement (Chae, 2004). The hallmark histopathological lesions are lymphoid depletion, granulomatous inflammation or both in the lymphoid tissues (Chae, 2004).

Experimental reproduction of PMWS appears to be dependent on immune stimulation and viral co-infection and has been achieved in piglets via inoculation with PCV-2 alone or in PCV-2-infected pigs co-infected with porcine parvovirus (PPV) or porcine reproductive and respiratory syndrome virus (PRRSV). PMWS has also been reproduced by immunostimulating PCV-2-infected piglets with injections of an immunogen emulsified in an oil-based macrophage-targeted adjuvant (Allan et al., 1999, 2000; Choi & Chae, 2000; Ellis et al., 1999; Kim et al., 2003; Krakowka et al. 2001; Kyriakis et al., 2002).

PCV-2 infection may result from several routes of exposure, including oro-nasal, intranasal and subcutaneous (Allan et al., 1999; Bolin et al., 2001; Pogranichnyy et al., 2000). Infection results in the shedding of virus in body fluids. PCV-2 has been found to be shed in oropharyngeal swabs, nasal swabs, faeces, whole blood and serum in experimentally infected pigs (Allan et al., 1999; Bolin et al., 2001; Pogranichnyy et al., 2000; Shibata et al., 2006), and shedding in colostrum and milk from naturally and experimentally infected sows has been reported (Ha

†These authors contributed equally to this work.
et al., 2009; Shibata et al., 2006). PCV-2 has been detected by immunohistochemistry and in situ hybridization in macrophages in the lumen of mammary glands from sows experimentally infected with PCV-2 (Park et al., 2009). These observations suggest oral transmission through milk secretions as a potentially important mode of post-natal PCV-2 infection. However, whether PCV-2 found in milk is infectious and whether piglets infected by milk will develop PMWS during the post-natal period has not yet been determined. The aim of the present study was to reproduce PMWS in piglets fed milk from sows experimentally infected with PCV-2 followed by post-natal PPV infection or immunostimulation.

RESULTS

Clinical signs in sows

The five infected sows remained clinically normal, did not farrow prematurely and delivered 54 live-born piglets and three stillborn animals. No clinical signs were observed in the ten control sows.

Detection of PCV-2

PCV-2 was detected in the colostrum of all five sows experimentally infected with PCV-2 and in the serum samples from all pigs in groups 1–3. The mean real-time quantitative PCR (qPCR) cycle threshold (Ct) values obtained for the PCV-2-infected colostrum were 42 [corresponding to approximately 1 × 10^−8 50% tissue culture infective dose (TCID50) ml⁻¹], 42, 45 (1 × 10^−9 TCID50 ml⁻¹), 38 (1 × 10^−7 TCID50 ml⁻¹) and 45. The range of mean qPCR Ct values for 7-day-old pigs in groups 1–3 was 31–37 (1 × 10^−5–1 × 10^−6 TCID50 ml⁻¹) (Table 1). PCV-2 was not detected in the colostrum of the ten uninfected sows or in serum samples from piglets born to uninfected sows and collected at 1 day old.

Virus isolation

PCV-2 was isolated from milk whey and pellets collected from the five PCV-2-infected sows. No virus was isolated from the milk whey and pellets collected from any of the control sows.

Maternal PCV-2 antibody titres in piglets

At 7 days old, all pigs that fed on milk from PCV-2-infected sows were seropositive for PCV-2. The PCV-2 antibody titres varied from 160 to 640 by immunoperoxidase monolayer assay (IPMA; Table 1).

Confirmation of PCV-2 infection by oral transmission

Using in situ hybridization, infection with PCV-2 by feeding on colostrum and milk was confirmed by the detection of PCV-2 in the lymphoid tissues and small intestines of ten piglets (two piglets per sow) that were born to uninfected sows and then fed colostrum and milk from PCV-2-infected sows. No histopathological lesions were seen in any of the ten piglets. However, PCV-2 DNA was detected in the lymph nodes (Fig. 1), spleen, tonsils and Peyer’s patches of the small intestine. Occasionally, PCV-2 DNA was also detected in resident macrophages of the lamina propria of the ileum (Fig. 2). No PCV-2 DNA was detected in lymphoid tissues from ten piglets that were born to uninfected sows and then fed colostrum and milk from their own sows.

Clinical signs in piglets

Clinical signs were observed in all 21 piglets from groups 1, 2 and 4, whereas the seven control piglets (group 6) that received uninfected cell lysates remained clinically normal. The 21 piglets from groups 1, 2 and 4 were observed to be thin and failed to gain weight between 49 (28 days post-inoculation) and 56 (35 days post-inoculation) days of age. Four piglets from group 2 and three piglets from group 4

Table 1. Results of quantification of PCV-2 by qPCR and maternal antibody titre of PCV-2 by IPMA in pigs at 7 days of age fed colostrum and milk from PCV-2-infected sows

<table>
<thead>
<tr>
<th>Group</th>
<th>Assay</th>
<th>Pig no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
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<tr>
<td></td>
<td>IPMA†</td>
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<td>320</td>
<td>160</td>
<td>320</td>
<td>160</td>
<td>640</td>
<td>320</td>
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<tr>
<td>2</td>
<td>qPCR*</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>34</td>
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<td>160</td>
<td>640</td>
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<td>320</td>
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</tr>
<tr>
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<td>34</td>
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<td>36</td>
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<td>160</td>
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<td></td>
</tr>
</tbody>
</table>

*Ct number.
†Antibody titre.
became anorectic at 49 days of age (28 days post-inoculation). Fourteen piglets from groups 3 and 5 were observed to be slightly thin at 63 days of age (42 days post-inoculation).

**Gross lesions**

Tracheobronchial, mesenteric and mediastinal lymph nodes were moderately enlarged and pale in piglets from groups 1, 2 and 4 at 63 days of age. No gross lesions were observed in the piglets from groups 3, 5 and 6 at 63 days of age.

**Body weight**

No significant differences in body weight were detected among the six groups at 28 days of age. The body weight of control pigs from uninfected sows (group 6) was significantly greater than that of PCV-2-positive pigs infected post-natally with PPV (group 1) at 52 ($P=0.035$), 56 ($P=0.013$), 59 ($P=0.006$) and 63 ($P=0.001$) days of age; immunostimulated PCV-2-infected pigs (group 2) at 56 ($P=0.032$), 59 ($P=0.013$) and 63 ($P=0.002$) days of age; pigs infected post-natally with PCV-2 and PPV (group 4) at 49 ($P=0.037$), 52 ($P=0.014$), 56 ($P=0.008$), 59 ($P=0.006$) and 63 ($P=0.001$) days of age (Fig. 3).

**Histopathology**

The typical granulomatous inflammatory reaction and lymphoid depletion associated with PCV-2 infection in pigs with PMWS was observed in the lymph nodes of piglets in groups 1, 2 and 4 (Table 2). Granulomatous inflammation was also seen in Peyer’s patches of the small intestine from one pig in group 1 and two pigs in group 4, in the spleen from one pig in group 1 and two pigs in groups 2 and 3.

The lymph nodes were depleted of mature lymphocytes and contained pyknotic basophilic nuclei and adjacent karyorrhectic debris; germinal centres were reduced or absent. Multifocal granulomatous inflammation was characterized by infiltrates of epithelioid macrophages and multinucleated giant cells. Reactive lymphoid hyperplasia and germinal centre formation were present. Occasionally, lymph nodes contained pyknotic basophilic nuclei and...
adjacent karyorrhectic debris. No histopathological changes were seen in piglets from groups 3, 5 and 6.

The mean lesion scores for the inguinal lymph nodes from pigs of groups 1–5 were significantly different from those of the group 6 control pigs. The mean lesion scores for the inguinal lymph nodes from pigs in groups 1, 2 and 4 were significantly higher than those of group 3 ($P<0.026$, $P<0.017$ and $P<0.001$, respectively). The mean lesion scores for the inguinal lymph nodes from pigs in group 4 were significantly higher than those of group 5 ($P<0.001$).

**PCV-2 distribution by in situ hybridization**

PCV-2 DNA was detected in the lymph nodes, spleen, tonsils and Peyer’s patches of the small intestine from all pigs in groups 1–5. Positive cells typically exhibited a dark brown colour, mainly in the cytoplasm but occasionally in the nucleus, without background staining. A strong PCV-2 hybridization signal was detected in the cytoplasm of epithelioid macrophages and multinucleated giant cells from the inguinal lymph nodes (Fig. 4) and Peyer’s patches of the small intestine from piglets in groups 1, 2 and 4. Scattered macrophages from the inguinal lymph nodes of piglets in groups 3 and 5 exhibited a less intense and dispersed hybridization signal in their cytoplasm. Hybridization signals were also observed in the resident macrophages of the lamina propria of the small intestines from pigs in groups 1–3, which were fed colostrum and milk from PCV-2-infected sows. No consistent hybridization signal was seen in tissue sections pre-treated with DNase A. Sections from the seven negative-control piglets (group 6) showed no PCV-2 hybridization signal.

Statistical analysis of the mean number of PCV-2-positive cells per unit area of inguinal lymph node showed significant differences among the groups (Fig. 5). Pigs in groups 1 and 2 had significantly more PCV-2-positive cells than those in groups 3 and 5 ($P<0.001$). Pigs in group 4 had significantly more PCV-2-positive cells than those in groups 3 and 5 ($P<0.001$). There was no difference in PCV-2-positive cells among piglets from groups 1, 2 and 5.

![Fig. 4. PCV-2 DNA was detected by in situ hybridization in clusters of histiocytes and multinucleated giant cells in lymph nodes of a pig fed with milk from PCV-2-infected sows with postnatal stimulation with KLH/ICFA (group 2) and euthanized at 63 days of age. The DNA probe was detected using substrate nitro blue tetrazolium/5-bromocresyl-3-indolylphosphate, and the section counterstained with methyl green.](image)

![Table 2. Results of granulomatous inflammation in seven pigs from each group](image)

<table>
<thead>
<tr>
<th>Histology in lymph node</th>
<th>Group</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
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<td>Granulomatous lesion</td>
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<td>0/7</td>
<td>7/7</td>
<td>0/7</td>
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<td>0/7</td>
<td>2/7</td>
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<td>0/7</td>
</tr>
</tbody>
</table>

**Fig. 5.** Number of PCV-2-positive cells in lymph nodes in pigs fed milk from PCV-2-infected sows with post-natal PPV infection (group 1), immune stimulation with KLH/ICFA (group 2) or no additional viral infection and stimulation (group 3); in pigs fed milk from uninfected control sows with post-natal PCV-2 and PPV co-infection (group 4) or post-natal PCV-2 infection alone (group 5); or in uninfected control pigs (group 6). *, Pigs in groups 1, 2 and 4 had significantly more PCV-2-positive cells than those from groups 3 and 5 ($P<0.001$).
PPV distribution by in situ hybridization

PPV DNA was detected in the lymph nodes, spleen, tonsils and Peyer’s patches of the small intestine from all pigs in groups 1 and 4. Scattered macrophages from the lymphoid tissues exhibited positive hybridization signals in the cytoplasm. No PPV DNA was detected in any tissues from other groups (groups 2, 3, 5 and 6) because the pigs in these groups were not inoculated with PPV.

DISCUSSION

The results of the present study demonstrated that PCV-2 can be transmitted orally from sow to neonatal piglets through the colostrum and milk. PCV-2 was detected in both the milk whey and cell fraction. The transmission was possible whether free PCV-2 or cell-associated PCV-2 was presented to suckling neonatal piglets. Although no evidence of an absorptive mechanism has yet been elucidated, free colostral PCV-2 can cross intestinal epithelial cells and infect resident macrophages in the lamina propria. The observation of PCV-2 antigen in resident macrophages of the lamina propria supports PCV-2 crossing the intestinal epithelial cells and infecting resident macrophages. Although the precise mechanism of how PCV-2 crosses the intestinal epithelial barrier is not well known, it is interesting that PCV-2 co-existed with maternal anti-PCV-2 antibodies in the colostrum. Therefore, maternal anti-PCV-2 antibody may play an important role in assisting or facilitating free PCV-2 in crossing intestinal epithelial barriers, similar to human immunodeficiency virus (Lehner et al., 1991). Maternal anti-PCV-2 antibodies in the colostrum could bind PCV-2, forming a macromolecule that is easily absorbed by epithelial cells and facilitates viral entry, although this mechanism has not yet been described for PCV-2 transportation. Further studies are needed to elucidate the precise mechanism of PCV-2 transport into the intestinal epithelial barrier.

Another possible mechanism is cell-associated transmission of PCV-2 via colostrum. Maternal colostral macrophages are numerous in the mammary gland secretions of sows (Le Jan, 1996) and PCV-2 is detected mainly in alveoli macrophages in the mammary glands of PCV-2-infected sows (Park et al., 2009). Maternal colostral leukocytes are absorbed from the siblings’ digestive tract and migrate into blood (Williams, 1993), indicating that maternal-origin PCV-2-infected macrophages may cross the intestinal epithelium and migrate into blood in the neonates. In the PMWS model, cell-associated viraemia has been reported and contributes to viral distribution through the lymphoid tissues (Kim et al., 2003). These observations suggest that maternal colostral macrophages play an important role as vehicles for oral transmission of PCV-2. In addition, it is possible that PCV-2 within the maternal colostral macrophages may easily be protected from maternal anti-PCV-2 antibodies. Further study is needed to confirm maternal-origin PCV-2-infected macrophages crossing the intestinal epithelia in the neonates.

Once neonatal piglets are infected with PCV-2 by oral transmission through the colostrum and milk, the infection may induce clinical PMWS following post-natal PPV infection and immunostimulation. The infection decreased piglet body weight compared with uninfected piglets and induced the typical PMWS-associated microscopic lesions in the lymph nodes. Therefore, the piglets in group 1 met the three criteria for the diagnosis of PMWS: (i) the presence of compatible clinical signs; (ii) the presence of characteristic microscopic lesions; and (iii) the presence of PCV-2 within these lesions (Allan & Ellis, 2000; Chae, 2004).

Although little is known about the mechanism for optimal or maximal replication of PCV-2 in vivo, oral PCV-2 transmission to piglets with post-natal PPV infection (group 1) or immunostimulation (group 2) resulted in significantly more PCV-2-positive cells in the lymph nodes compared with pigs infected with PCV-2 alone. Post-natal PPV infection or immunostimulation may potentiate the maximal or optimal replication of PCV-2 to induce PMWS in piglets infected with PCV-2 via oral transmission through colostrum and milk.

In conclusion, PCV-2 transmitted to neonatal piglets via colostrum and milk is infectious. Oral transmission of PCV-2 may trigger the disease and lesions associated with PMWS during the post-natal period. Also, PPV infection and immune stimulation potentiate the progression of PMWS during the post-natal period.

METHODS

Building. To avoid environmental contamination, the building in which the pigs were kept was completely emptied, cleaned three times with hot (>95 °C) water and disinfected for 3 days with 2% potassium peroxymonosulfate and a sodium chloride-based product (Virkon S; Antec International). The building was left empty for 21 days before the sows were introduced, and each sow was housed separately. The buildings were cleaned with hot (>95 °C) water and disinfected with 2% potassium peroxymonosulfate and the sodium chloride-based product every other day after the sows were introduced. Personnel were assigned to individual sows to avoid PCV-2 contamination.

Sow inoculation. PCV-2 strain SNUVR000470 was isolated from the lymph nodes of sows that were submitted to the diagnostic laboratory for respiratory disease (Jung et al., 2006). Fifteen pregnant sows with known breeding dates were purchased from a commercial herd deemed free of PMWS based on herd history, clinical signs and diagnostic tests; all were first-parity sows. The sows were identified as being free of PCV-, PPV- and PRRSV-specific antibodies.

The sows were allocated randomly to an infected (n=5) or control (n=10) group. The viral inoculum contained the isolated PCV-2 strain at its third passage in PCV-free PK-15 cells, and the sows were inoculated intranasally with 6 ml tissue culture fluid containing 1.2 × 10⁵ median TCID₅₀ ml⁻¹ of the PCV-2 strain SNUVR000470 at 93 days of gestation. The ten pregnant control sows were similarly exposed to uninfected cell culture supernatant. Each inoculum was...
instilled into both nostrils over a period of 4–5 min. The sows were housed in isolation facilities and allowed to farrow naturally, but the farrowings were attended. All sows remained clinically normal, did not farrow prematurely and delivered live-born piglets at 114 (infected sows) or 115 (control sows) days of gestation.

**Feeding of colostrum and milk.** Prior to removal and introduction, blood was collected from all piglets born to the control sows. During colostrum feeding, each attending technician kept the newborn piglets from contacting faeces and oro-nasal secretions of the PCV-2-infected sows. After the 12 h colostrum feeding, all newborn piglets were removed and housed separately in isolators and examined at regular intervals. The newborn piglets from uninfected sows and those fed colostrum from PCV-2-infected sows were fed 15 ml milk from the PCV-2-infected sows five times a day for 7 days.

**Piglet allocation.** The five infected sows delivered 54 live-born piglets and three stillborn animals. All piglets born to the infected sows were immediately removed from their sows after farrowing and not used further in this experiment. The ten uninfected sows delivered 105 live-born piglets and two stillborn animals. Sixty-two piglets from the ten uninfected sows were used and 43 piglets were not used further.

Thirty-one newborn piglets from five of the control sows were introduced to the PCV-2-infected sows (n=6 or 7 piglets per sow) and allowed to feed on the colostrum for 12 h. Thirty-one newborn piglets from the other five control sows were fed colostrum and milk from their own uninfected sows. After 7 days of lactation nutrition, ten piglets born to control sows and fed by the five PCV-2-infected sows (two piglets per sow) and ten piglets from the five control sows (two piglets per sow) were randomly selected and euthanized to confirm PCV-2 infection from feeding. The superficial inguinal lymph nodes, lungs, liver, kidneys, spleen, tonsils, thymus, myocardium, stomach, pancreas, jejunum and ileum were collected at necropsy and fixed in 10 % (v/v) phosphate-buffered formalin for 1–2 days before processing for histopathological examination and in situ hybridization. For the experimental study, 21 piglets born to control sows and fed by the five PCV-2-infected sows were used and randomly divided into three groups designated groups 1, 2 and 3 (n=7 each). Twenty-one piglets born to control sows and fed by their own sows were used and randomly divided into three groups designated groups 4, 5 and 6 (n=7 each).

**Experimental design.** The 21 piglets born to control sows and fed PCV-2-infected colostrum and milk were randomly divided into three groups (n=7 each). In group 1, piglets were inoculated intranasally with 2 ml of a 1:20 dilution of PPV (1.3×10⁴ TCID₅₀ ml⁻¹) at 28 days of age. In group 2, piglets were inoculated at two sites, the left axilla and hip, with 2.0 ml (1.0 ml each site) of 1.0 mg keyhole limpet haemocyanin emulsified in incomplete Freund’s adjuvant (KLH/ICFA) at 7 days of age. The left-side injections were repeated at 21 days of age. In group 3, the piglets were not infected with any virus and were used as the milk-transmitted PCV-2 control.

The 21 piglets born to control sows and fed colostrum and milk from their own sows were also randomly divided into three groups (n=7 each). In group 4, piglets were inoculated intranasally with 2 ml of a mixture containing equal volumes of 1:20 dilutions of the PCV-2 (1.2×10⁴ TCID₅₀ ml⁻¹) and PPV (1.3×10⁴ TCID₅₀ ml⁻¹) pools at 28 days of age. In group 5, piglets were inoculated intranasally with 1 ml of a 1:20 dilution of the PCV-2 pools at 28 days of age. In group 6, piglets were inoculated intranasally with PCV- and PPV-free PK-15 cell lysates (Table 3). All groups were housed separately in isolators (one pig per isolator) and examined at regular intervals. Body weight was measured at 21, 24, 28, 31, 35, 38, 42, 45, 49, 52, 56, 59 and 63 days of age.

**Collection of tissues.** All pigs were euthanized for necropsy at 63 days of age. The superficial inguinal lymph nodes, lungs, liver, kidneys, spleen, tonsils, thymus, myocardium, stomach, pancreas, jejunum and ileum were collected at necropsy and fixed in 10 % (v/v) phosphate-buffered formalin for 1–2 days before processing for histopathological examination and in situ hybridization. Superficial inguinal lymph nodes were selected for in situ hybridization because they were previously shown to exhibit consistent and intense labelling for PCV-2 and typical granulomatous lesions (Kim et al., 2003). The methods were approved by the Seoul National University Institutional Animal Care and Use Committee.

**Collection of colostrum and milk.** Colostrum and milk were collected from PCV-2-infected and uninfected sows at 1 and 7 days of lactation. For sampling procedures, the udders were cleaned using warm water and disinfected with alcohol. The first few spurts of milk were discarded before a sample was taken.

**Real-time qPCR.** Real-time qPCR was performed to detect PCV-2 in the colostrum of PCV-2-infected sows and in serum samples taken at 7 days of age from piglets (groups 1, 2 and 3) fed by PCV-2-infected sows. The upper fat layer was removed and the middle aqueous layer (milk whey) was collected. DNA was extracted from the milk whey and serum using a commercial kit (Qiagen) according to the manufacturer’s instructions. The qPCR was performed as described previously (Gagnon et al., 2008). For the detection of PCV-2, a conserved region of the PCV-2

**Table 3.** Experimental design for the treatment of pigs with PCV-2, PPV and KLH/ICFA used in this study

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
</tr>
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<tbody>
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<td>1</td>
</tr>
<tr>
<td>Post-natal PPV infection</td>
<td>2</td>
</tr>
<tr>
<td>Post-natal PCV-2 infection</td>
<td>3</td>
</tr>
<tr>
<td>Post-natal KLH/ICFA injection</td>
<td>4</td>
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<tr>
<td></td>
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genome was targeted using the following primers and probe: Circo-Gen-F (5'-GGCCΑCCTGГTГТГГТΑΑΑ-3'), Circo-Gen-R (5'-CCCCCCΑCCTGTГΤГГТГ-3') and Circo-Gen-Probe (5'-6-FAM-TTTGCAГGCCGАΑΑΑΑΑСАААААААААТГГГ-3').

**Virus isolation from colostrum and milk.** The milk whey and pellet were used to isolate PCV-2 using PCV-free PK-15 cell lines (Ha et al., 2009).

**Detection of maternal PCV-2 antibody in serum.** Serum samples were collected from all pigs in the six groups to determine the maternal PCV-2 antibody titre. PCV-2 antibodies were measured by an IPMA as described previously (Ballasch et al., 1999).

**In situ hybridization.** PCR was performed as described previously (Kim et al., 2001) and the product purified with a 30 kDa cut-off membrane filter. The nucleotide sequences of the purified PCR products were determined using BigDye chemistry with an ABI Prism Sequencer (Applied Biosystems). Sequencing was performed before the PCR products were labelled by random priming with digoxigenin-dUTP (Boehringer Mannheim) according to the manufacturer's instructions. In situ hybridization for PCV-2 and PPV was carried out as described previously (Kim et al., 2003).

**Morphometric analysis.** Single sections were taken from each formalin-fixed lymph node from the virus-infected pigs for morphometric analysis as described previously (Kim et al., 2003). To obtain quantitative data, morphometric analysis of the in situ hybridization slides was performed using the ImageJ program (National Institutes of Health, Bethesda, MA, USA). In each case, three fields were randomly selected, the number of positive cells per unit area (0.25 mm2) was calculated and the mean values were calculated.

**Lesion score.** Lymph nodes were given lesion scores (Kim & Chae, 2004) with respect to the estimated amount of lymphoid depletion of the follicles. The scores ranged from 0 (normal, no lymphoid depletion or granulomatous replacement) to 5 (severe lymphoid depletion and granulomatous replacement).

**Statistical analysis.** Statistical analyses were performed using the spss 12.0 statistical package (SPSS Inc.). A one-way analysis of variance/Tukey's test was used for group comparisons of body weight. A non-parametric Kruskal–Wallis test and Mann–Whitney test were carried out for comparison of the mean lesion score and number of PCV-2-positive cells per unit area among groups. Statistical significance was taken as P<0.05.

**ACKNOWLEDGEMENTS**

This research was supported by the Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries, Republic of Korea. The research was also supported by the Research Institute for Veterinary Science (RIVS) of the College of Veterinary Medicine and the Brain Korea 21 Program for Veterinary Science in the Republic of Korea. This work was also supported by a National Research Foundation of Korea grant funded by the Korean Government (KRF-2006-005-J02902).

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