Prevalence of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2 and porcine parvovirus from aborted fetuses and pigs with respiratory problems in Korea

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Porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV-2) and porcine parvovirus (PPV) infections were investigated as possible causes of the postweaning multisystemic wasting syndrome (PMWS). Specific primers for RT-PCR and PCR were designed for the differential detection of PRRSV, PCV-2 and PPV. Using PCR, these viruses were detected in homogenized tissue samples from pigs that had respiratory or reproductive problems in the time period between 1998 and 2000; the overall prevalences were: PRRSV 31.4%, PCV-2 46.9%, and PPV 8.1%. PCV-2 was also detected in aborted fetal tissues.

Key words: PMWS, PRRSV, PCV-2, PPV, prevalence, PCR

Introduction

The postweaning multisystemic wasting syndrome (PMWS), which was first identified in Canada, and more recently in the United States, in many European countries and in Asia, can cause great economic losses. This disease which is more frequently being described, occurs in swine herds that usually were in good health. It has a relatively high fatality risk mainly in nursery and early growing pigs [2]. Clinically, PMWS is characterized by a progressive weight loss, dyspnea, anemia, diarrhea and jaundice. The microscopic lesions of PMWS include granulomatous inflammation, interstitial pneumonia, hepatitis and nephritis (E.G. CLARL and HARDING, J.C. 1998. Proceed 15th Int. Pig Vet. Soc., Birmingham, UK, p212).

The porcine reproductive and respiratory syndrome (PRRS) is characterized by late-term abortions and stillbirths, as well as respiratory disease. In weaned pigs, the disease is characterized by fever, pneumonia and lethargy [1, 18]. The PRRS virus is a member of a newly described group of enveloped single-strand RNA viruses that is classified into the family Arteriviridae, genus Arterivirus. The virion contains a 15 kb RNA of positive polarity and its genome consists of eight open reading frames (ORF5s) which encode specific viral proteins [18]. Tests for the detection of PRRS virus include virus isolation (VI), immunohistochemistry (IHC), in situ hybridization and polymerase chain reaction (PCR).

Porcine circovirus (PCV) originally was described as being a contaminant of the continuous pig kidney cell line PK-15(ATCC-CCL 30). PCV is a non-enveloped, small virus containing a single-stranded circular DNA genome of 1.76 kb. It is classified into the virus family Circoviridae [22]. Recently, the existence of two subtypes of PCV has been proposed: a non-pathogenic PCV-1 and a potentially pathogenic PCV-2. PCV-2 is antigenically and genetically distinguishable from the nonpathogenic PCV-1 [6]. The definitive diagnosis of PCV-related diseases is based on the detection of a viral antigen or a nucleic acid that is associated with lesions in diseased pigs. IHC and in situ hybridization assays have been described for the detection of PCV-2 in tissues from field cases of wasting disease and reproductive disorders [17].

Porcine parvovirus (PPV) is classified into the family Paroviridae. The PPV genome is single-strand DNA of 5kb with two large ORFs. PPV is one of the most important infectious agents that cause reproductive failure such as stillbirth, mummification, embryonic death and infertility in swine [19]. For the detection of viral nucleic acid, PCR and nested-PCR assays from infected cell lines

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and clinical samples have been described [3].

The PMWS of pigs often occurs in animals that are co-infected with PRRSV, PCV-2, PPV, or pseudorabies virus [2, 5]. The respiratory disease that is induced by the PRRSV infection and the disease that is associated with PMWS are not distinguishable in their clinical signs and histological lesions. More serious signs have been noted for PMWS when the animals were co-infected by PRRSV and PCV-2 [12]. Therefore it is necessary to have the means to detect both viruses in pigs that have respiratory problems.

The objectives of this study were to establish differential PCR methods for detecting PRRSV, PCV-2 and PPV and to use these tests to examine field samples for the presence of these viruses in Korean pigs.

Materials and Methods

Samples

Samples were obtained from pigs submitted to the Virology Laboratory, College of Veterinary Medicine, Seoul National University during the period from 1998 to 2000. Lungs and lymph nodes from 113 pigs were collected from 6- to 12-week-old pigs from farms where there were respiratory problems; These consisted of 25 farms in 1998, 34 farms in 1999, and 27 farms in 2000. Twenty-one samples were collected from 12 farms where the fetuses had resulted from a mid- or late-stage abortion. Samples were prepared in 50% suspensions with PBS (0.1M, pH 7.2) by chopping and homogenizing. The homogenates were frozen at -20°C until use.

Viruses and cells

The PRRSV, VR-2332 strain was propagated into MARC-145 cells [8] that were grown in minimal essential medium (MEM, Gibco/BRL, Uxbridge, U.S.A.) supplemented with 3% fetal bovine serum (Gibco/BRL), 20mM HEPES, 300mM L-glutamine, 0.2% (w/v) sodium bicarbonate and antibiotics (penicillin G sodium 10,000 unit/ml, streptomycin sulfate 10,000 μg/ml). The PPV, PKV strain, was propagated into PK-15 cell cultures as follows: 50-70% confluent cell monolayers were allowed to adsorb the virus for 30 min, washed with sterile PBS (pH 7.4) and maintained in MEM containing 3% FBS. Persistently PCV-infected PK-15 cells (ATCC-CCL 33) were used for the detection of PCV-1. The cells were maintained in MEM containing 5% FBS.

Extraction of nucleic acid

TRizol (Gibco/BRL), a commercially available mixture of guanidium isothiocyanate and phenol, was used for the RNA extraction. Extraction and purification of total RNA was performed according to the manufacturer's recommendation. Virus infected cell monolayers were lysed directly in a culture flask by adding 60μl of TRizol reagent per 10cm² of cell monolayer area. For the clinical pig samples, 500μl of TRizol reagent was mixed with 200 μl of homogenized sample solution. Following the addition of 100μl of chloroform the sample was centrifuged for 10 min at 12,000×g. The RNA-containing aqueous phase was precipitated with isopropanol that had the same volume, reacted in -20°C for 2 hours and then centrifuged for 10 min at 12,000×g. The RNA pellet was washed with 1,000 μl of 75% ethanol and centrifuged for 10 min at 12,000×g. The supernatant was removed and the pellet was dried. The pellet was resuspended in 50μl of diethylpyrocarbonate (DEPC) - treated deionized water.

For the DNA extraction, 500μl of cell lysis buffer containing 27% sucrose, 1× saline sodium citrate (SSC) (15 mM trisodium citrate and 0.15 M NaCl, pH7.0), 1mM EDTA, 1% sodium dodecyl sulphate (SDS) and 200μg/ml protease K, was mixed with the 200μl of homogenized sample solution, thoroughly vortexed and incubated at 37°C for 2 hours. Following incubation, 500μl of mixture of phenol, chloroform and isomyl alcohol (25:24:1) were added, thoroughly vortexed and centrifuged for 5 min at 12,000×g. The DNA containing aqueous phase was precipitated using the same volume of isopropanol, reacted at -20°C for 2 hours and then centrifuged for 5 min at 12,000×g. The DNA pellet was washed with 1 ml of 70% ethanol, and then centrifuged for 5 min at 12,000×g. The supernatant ethanol was completely removed and the pellet was resuspended in 100μl of Tris-EDTA (TE) buffer.

Design of primers

PRRSV primers were prepared for RT-PCR, based on the nucleotide sequence of the open reading frames 6 and 7 [9]. The site of the sense primer (No. 21) starts at sequence position 14,696 of ORF 6 and that of the antisense primer (No. 26) starts at sequence position 15,362 of ORF 7, respectively. Primers of PCV-2 that were to be targeted to a non-homologous region of the PCV-1 sequence, were designed from ORF 1 of a published PCV sequence (GenBank accession no. U49186, [6]) using a primer computer program (Primer, Scientific and Educational Software); VF-2 starts from the sequence position 61 and VR-2 starts from the sequence position 421. PCR primers for PPV were chosen within the NS-1 gene [20]. Sense (P-1) and antisense (P-6) primers were
Table 1. Nucleotide sequence of primers for porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV-2), and porcine parvovirus (PPV)

<table>
<thead>
<tr>
<th>Target virus</th>
<th>Primer Pairs</th>
<th>Sequence (5' → 3')</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRRSV</td>
<td>No.21</td>
<td>GTA CAT TCT GCC CCC TGC CC</td>
<td>668bp</td>
</tr>
<tr>
<td></td>
<td>No.26</td>
<td>GCC CTA ATT GAA TAG GTG AC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VF-2</td>
<td>GAA GAA TOG AAG AAG CCG</td>
<td>361bp</td>
</tr>
<tr>
<td>PCV-2</td>
<td>VR-2</td>
<td>CTC ACA GCA GTA GAC ACG T</td>
<td></td>
</tr>
<tr>
<td>PPV</td>
<td>P-1</td>
<td>ATA CAA TTC TAT TTC ATG GGC CAG C</td>
<td>330bp</td>
</tr>
<tr>
<td></td>
<td>P-6</td>
<td>TAT GTT CTG GTC TTT OCT GGC ATC</td>
<td></td>
</tr>
</tbody>
</table>

located at sequence positions 1,453 -1,477 and 1,759-1,782, respectively (Table 1), in which a region encodes a non-structural protein that shows a high homology among PPV strains.

**RT-PCR and PCR**

RT-PCR for PRRSV detection was performed as follows: 5 μl of extracted sample RNA was mixed with 10 μl of the reverse primer, denatured at 95°C for 5 min, and then immediately cooled on ice. To make a final volume of 25 μl reaction mixture for the cDNA synthesis, 10 μl of RNase-free deionized water, 5 μl of 5× reaction buffer, 2.5 μl of DTT (10mM), 1 μl of dNTP mixture (200mM), and 1 unit of moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco/BRL) were mixed. The mixture was incubated at 37°C for 1h. For the PCR, 25 μl reaction mixtures contained of 2 μl synthesized cDNA, 2.5 μl of 10x PCR buffer, 2.0 mM MgCl2, 1 μl dNTP mixture (200 mM), 1.0 μM each primer, and 1.0 U Taq DNA polymerase (Promega, Madison, WI, USA). The mixture was subjected to 30 amplification cycles. Each cycle consisted of 30 sec denaturation, 30 sec annealing and a 1.5 min elongation step at temperatures of 94°C, 55°C and 72°C, respectively. A further final elongation step was made for 3 min at 72°C.

For the PCR of PCV-2, 25 μl reaction mixture consisted of 2μl viral template DNA, 2.5μl 10x PCR buffer, 2.0pM MgCl2, 1μl dNTPs mixture (200mM), 1.0 pM each primer and 1.0 U Taq DNA polymerase. The mixture was subjected to 30 amplification cycles. Each cycle consisted of 94°C, 30 sec denaturation, 56°C, 30 sec annealing and a 72°C, 30 sec elongation step. Also a final 3 min at 72°C was the elongation step.

For PPV, reaction mixture consisted of 100μl of 0.5 μM each primer, 2.5 U Taq DNA polymerase, 0.2mM dNTPs, 10 μl of reaction buffer (500Mm KCl, 15mM MgCl2, 10mM Tris-HCl pH 8.0), 10μl of viral extracted DNA and distilled water. The mixture was subjected to 35 amplification cycles. Each cycle consisted of 94°C, 45 sec denaturation, 55°C, 60 sec annealing and a 72°C, 90 sec elongation step. Also a final 10 min at 72°C was the elongation step.

The amplified products of each viral nucleic acid were analyzed using 2.0% agarose gel (Sigma, St. Louis, MO, USA) electrophoresis. After washing the gel for 15 min in 0.5 μg/ml ethidium bromide, the bands were visualized using an UV transilluminator.

**Sequencing of the PCR product**

The specificity of the PCV-2 PCR products was confirmed by using sequencing analysis. PCV-2 PCR products from the field sample were referred to the Medicinal Chemistry Research Center, Korea Institute of Science and Technology for further analysis.

**Results**

**Establishment of the PCR**

The PCR methods established in this study showed that each viral nucleic acid (PRRSV, PCV-2 and PPV) was amplified by specific primers. Positive or negative controls, or either the diagnostic or analytic sensitivity and specificity were been evaluated before the prevalence test of each virus. It was found that the sensitivity and specificity of the established PCRs were similar to those of previous reports [6, 9, 20]. The PRRSV by RT-PCR using the specific primers was identified by extracting RNA from MARC-145 cells infected with the VR-2332 strain. The specific product size was 668bp (Fig. 1). In the case of the PCR for PCV-2, viral DNA was amplified from field samples from pigs that had respiratory problems, but no specific bands were detected using DNA from the PK-15 cells that were persistently infected by the PCV-1 and pseudorabies virus, YS strain (Fig. 2). The specific PCR product size was 361 bp for PCV-2. There was 99% homology in the nucleotide sequence between the field
sample BI-950 and previously reported data [6]. DNA of PPV was detected in PK-15 cells infected with KPV strain of PPV and the specific PCR product size was 330bp (Fig. 3). Therefore the identification of the PCR-amplified products was confirmed.

Fig. 1. Detection of porcine reproductive and respiratory syndrome virus (PRRSV) by RT-PCR from tissues of pigs that have respiratory problems. Lane M: 100bp ladder; lanes 1-7: Field samples BI-386, BI-733, BI-858, IV-329, BI-850, IV-422 and IV-282; lane R: PRRSV, VR2332 strain

Fig. 2. Detection of porcine circovirus 2 by PCR from tissues of pigs that have respiratory problems. Lane M: 7; refer to the footnote of Fig. 1, lane P: PK-15 cells persistently infected porcine circovirus 1, lane A: pseudorabies virus, YS strain

Fig. 3. Detection of porcine parvovirus by PCR from tissues of pigs that have respiratory problems. Lane M: 100bp ladder, lane P: porcine parvovirus, PVK strain, lanes 1-3: Field samples IV-60, BI-850 and BI-961.

Respiratory cases

Of the 113 samples from 86 farms where the pigs had respiratory problems, extracted RNA from 45 tissue samples from 27 farms was positive for PRRSV. Extracted DNAs from 57 tissue samples from 40 farms were positive for PCV-2 and those from 9 field samples from 7 farms were positive for PPV (Table 2). Fig. 1, 2 and 3 are representative of positive and negative PCR results from these samples.

Table 2. Prevalences (% determined by PCR) of porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus 2 (PCV-2) and porcine parvovirus (PPV) for pigs that have respiratory problems in Korea.

<table>
<thead>
<tr>
<th>Virus</th>
<th>1998 (n=25 tested)</th>
<th>1999 (n=34 tested)</th>
<th>2000 (n=27 tested)</th>
<th>Total (n=86 tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRRSV</td>
<td>6 (24.0%)</td>
<td>10 (29.4%)</td>
<td>11 (40.7%)</td>
<td>27 (31.4%)</td>
</tr>
<tr>
<td>PCV-2</td>
<td>10 (40.0%)</td>
<td>14 (41.2%)</td>
<td>16 (59.3%)</td>
<td>40 (46.5%)</td>
</tr>
<tr>
<td>PPV</td>
<td>0 (0%)</td>
<td>5 (14.7%)</td>
<td>2 (7.4%)</td>
<td>7 (8.1%)</td>
</tr>
</tbody>
</table>

As shown in Table 2, PRRSV was detected from 6 to 25 farms (24.0%) in 1998, 10 from 34 farms (29.4%) in 1999 and 11 from 27 farms (40.7%) in 2000. PCV-2 was found 10 from 25 farms (40.0%) in 1998, 14 from 34 farms (41.2%) in 1999 and 16 from 27 farms (59.3%) in 2000. PPV was not detected in 1998, but it was detected in 5 of 34 farms (14.7%) in 1999 and in 2 of 27 farms (7.4%) in 2000.

Co-infection of PRRSV and PCV-2 was detected in 3 cases from 25 farms (12.0%) in 1998, 5 from 34 farms (14.7%) in 1999 and 10 from 27 farms (37.0%) in 2000. In the case of PRRSV and PPV co-infection, it was detected in samples from 1 of 34 farms (2.9%) in 1999 but was not detected in 1998 and 2000. Co-infection with PCV-2 and PPV, was not detected in 1998, but it was detected in 2 of 34 farms (5.9%) in 1999 and in 1 of 27 farms (3.7%) in 2000. Multiple infections of PRRSV, PCV-2 and PPV were not detected in 1998, but they were detected in 1 of 34 farms (2.9%) in 1999 and in 1 of 27 farms (3.7%) in 2000 (Table 3).

Reproductive case

PRRSV and PPV were not detected by PCR in 16 fetal abortus samples from 12 farms where reproductive problems occurred. However, PCV-2 was detected in 4 fetuses from 3 of 12 farms (Fig. 4).
Table 3. Co-infection prevalences (% determined by PCR) of porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV-2), and porcine parvovirus (PPV) for pigs that have respiratory problems in Korea

<table>
<thead>
<tr>
<th>Viruses</th>
<th>1998 (n=25 tested)</th>
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<th>2000 (n=27 tested)</th>
<th>Total (n=86 tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRRSV + PCV-2</td>
<td>3 (12.0%)</td>
<td>3 (14.7%)</td>
<td>10 (37.0%)</td>
<td>16 (18.6%)</td>
</tr>
<tr>
<td>PRRSV + PPV</td>
<td>0 ( 0%)</td>
<td>1 (2.9%)</td>
<td>0 ( 0%)</td>
<td>1 (1.2%)</td>
</tr>
<tr>
<td>PCV-2 + PPV</td>
<td>0 ( 0%)</td>
<td>2 (5.9%)</td>
<td>1 (3.7%)</td>
<td>3 (3.5%)</td>
</tr>
<tr>
<td>PRRSV + PCV-2 + PPV</td>
<td>0 ( 0%)</td>
<td>1 (2.9%)</td>
<td>1 (3.7%)</td>
<td>2 (2.3%)</td>
</tr>
</tbody>
</table>

Fig. 4. Detection of porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV) and porcine circovirus type 2 (PCV-2) by differential PCR from the aborted fetuses Lane M: 100bp ladder, lane R: PRRSV, VR2332 strain, lane P: PPV, PVK strain, lane C: PCV-2 positive control, lanes 1-3: Field samples L-301, BI-721 and IV-459

Discussion

The postweaning multisystemic wasting syndrome (PMWS) is presently being described as being a disease that is being recognized more frequently in swine. Although its causative agent has yet to be definitively identified, PCV-2 has been isolated from PMWS affected animals [15, 16]. Experimental infections with PCV-2 and PPV lead to the typical lesions and signs of PMWS [4]. Available evidence both from naturally occurring disease and experimental data indicates that PCV-2 is the primary infectious viral agent that causes PMWS [14].

An experiment was reported, which suggested that PCV-2 is an essential, primary immuno-suppressive agent, but is not sufficient by itself to cause PMWS in gnotobiotic pigs. Immuno-suppression may be the mechanism that accounts for the synergistic relationship between the PCV-2 and PPV infections and the clinical expression of PMWS [10]. PPV has been recovered from field cases of PMWS and co-infection of PCV-2 and PPV in the piglets appears to result in a wasting disease. Although PPV is not regarded as being a significant cause of mortality in young pigs, its role as a secondary invader in the genesis of severe PMWS in gnotobiotic pigs has been suggested [11].

The use of the PRRSV PCR assay that is described in the present study was found to be directly applicable to field samples taken from a variety of tissues from pigs that showed clinical signs. Virus isolation is difficult to perform because the virus is partially degraded by autolysis [18]. Immunohistochemistry results are influenced by the experimental procedure itself and in situ hybridization finds greater application in research than for use in routine diagnosis. On the other hand, PCR has the potential capability to provide a sensitive, rapid and specific method for detection of viral RNA of PRRSV from the organs of pigs that show various clinical signs and lesions [21, 23]. Therefore, the high sensitivity of the PRRSV PCR assay is ideal for the detection of a very small amount of viral nucleic acid in tissue samples.

We have developed a PCR assay in the laboratory that detects the presence of PCV nucleic acid in tissues that were collected from naturally infected pigs and a multiplex PCR assay was developed to differentiate the PCV-1 that infected the PK-15 cell line from the PCV-2 that was associated with PMWS [13]. A PCR assay that allows for the detection and differentiation between PRRSV and PCV has also been described [7, 12]. Differential PCR tests have likewise been described for the detection of PCV-1 and PCV-2 and the diagnosis by typing of PCV in clinical specimens [7]. PMWS that was associated with infection of PCV-2 could be confirmed by granulomatous lesion and immunohistochemistry (IHC) or in situ hybridization from pig in swine herds that showed growth retardation [4]. In our study, PCV-2 was also detected from pigs that had growth retardation and a granulomatous lesion.

In the present study, a differential PCR for PRRSV, PCV-2 and PPV detection was established for the diagnosis of field samples. PRRSV, PCV-2 and PPV were detected from pig farms where there were reproductive problems in Korea in the past three years. The detection rate of PRRSV has increased gradually year by year and this shows a greater increase. The infection rate of PCV-2 was also highly increased (59.3%). These results were similar to previous results reported in other countries. PCV-2 was readily detected by PCR in 55% of randomly tested pigs which exhibited a wide range of clinical signs and lesions in Canada [7]. In this study, PPV was detected from farms where the pigs had respiratory
problems, but the detection rate of PPV was lower than the results that were previously reported from other countries. The specific viral DNA of PPV was detected in 11 of 34 field samples in Canada [4].

Viral DNA of PCV was also detected in PRRSV positive samples. PCV-2 infection rates in the PRRSV positive farms were 50.0% in 1998, 50.0% in 1999 and 90.9% in 2000. Dual infections by these viruses have gradually increased in recent years, and the infection rate of PCV-2 increased over 20% in the following year of the study as compared with that of the previous year. Also the infection rates of PCV-2 and co-infection with PRRSV showed a high rate in 2000. Based on these results, dual infections by PRRSV and PCV-2 may be widespread in Korea. These two viruses can be present together in swine herds but their possible association or synergism requires further investigation [12]. We found that co-infections by PCV-2 and PPV occurred much less frequently in Korea.

In the case of reproductive problems, PRRSV and PPV are the agents that are mainly detected in fetal abortuses from farms where there is reproductive failure [18, 20]. The fetuses in recent cases of PCV-2 associated abortion, were examined for other viral pathogens which have been associated with reproductive failure in swine. These include PPV, PRRSV and encephalomyocarditis virus (EMCV). The PRRSV, EMCV and PPV antigens were not detected by PCR and immunostaining [24]. In the present study PCV-2 was confirmed in Korean field samples where there were reproductive problems but PRRSV and PPV was not. However, PCV-2 has not been identified to be a primary causative agent in reproductive failure [4]. Further studies are required to determine the role of PCV-2 in swine abortion.

Acknowledgements
This study was supported by the Grants from the Special Research Program (GSRP-MAP) of the Agricultural R&D Promotion Center, Ministry of Agriculture and Forestry, the Research Institute for Veterinary Science of the College of Veterinary Medicine, Seoul National University, and the Brain Korea 21 Project from the Ministry of Education, Korea.

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