Detection of porcine circoviruses in clinical specimens using multiplex PCR in Hubei, central China

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In order to detect and simultaneously discriminate PCV1, PCV2 and PCV3, a multiplex PCR assay was developed and used to detect clinical samples in this study. Each of target genes of PCV1, PCV2 and PCV3 was amplified using the designed primers, while no other porcine viruses genes were detected. The limit of detection of the assay was 10 copies/μL of PCV1, PCV2 and PCV3. The tissue samples from eight pig farms were detected using the multiplex PCR assay. The results showed that PCV1, PCV2 and PCV3 are co-circulating in central China. The PCV1, PCV2 and PCV3 singular infection rate was 52.4% (150/286), 61.2% (175/286) and 45.1% (129/286), respectively, while the PCV1 and PCV2 co-infection rate was 11.2% (32/286), the PCV1 and PCV3 co-infection rate was 5.9% (17/286), the PCV2 and PCV3 co-infection rate was 23.4% (67/286), and the PCV1, PCV2 and PCV3 co-infection rate was 1.7% (5/286), respectively, which were 100% consistent with the sequencing method and Real-time PCR methods. It proved that this multiplex PCR assay could be used as a differential diagnostic tool for monitoring and control of PCVs in the field. The results also indicate that the PCVs infection and their co-infection are severe in Hubei Province, Central China.
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ABSTRACT

In order to detect and simultaneously discriminate PCV1, PCV2 and PCV3, a multiplex PCR assay was developed and used to detect clinical samples in this study. Each of target genes of PCV1, PCV2 and PCV3 was amplified using the designed primers, while no other porcine viruses genes were detected. The limit of detection of the assay was 10 copies/μL of PCV1, PCV2 and PCV3. The tissue samples from eight pig farms were detected using the multiplex PCR assay. The results showed that PCV1, PCV2 and PCV3 are co-circulating in central China. The PCV1, PCV2 and PCV3 singular infection rate was 52.4% (150/286), 61.2% (175/286) and 45.1% (129/286), respectively, while the PCV1 and PCV2 co-infection rate was 11.2% (32/286), the PCV1 and PCV3 co-infection rate was 5.9% (17/286), the PCV2 and PCV3 co-infection rate was
was 23.4% (67/286), and the PCV1, PCV2 and PCV3 co-infection rate was 1.7% (5/286), respectively, which were 100% consistent with the sequencing method and Real-time PCR methods. It proved that this multiplex PCR assay could be used as a differential diagnostic tool for monitoring and control of PCVs in the field. The results also indicate that the PCVs infection and their co-infection are severe in Hubei province, Central China.

**Keywords** Porcine circovirus; PCV1; PCV2; PCV3; viral discrimination; multiplex PCR

**INTRODUCTION**

Porcine circoviruses (PCVs), are non-enveloped and circular DNA viruses, which belong to the genus *Circovirus*, family *Circoviridae* (*Mankertz et al., 2004*). At present, PCVs are smallest animal viruses. Two species of circovirus, PCV1 and PCV2, had been proved as infectious to pigs before 2015 (*Ku et al., 2017*). PCV1 was first isolated and in 1974, which just was a contaminant of the PK-15 cell, and nonpathogenic for pigs (*Tischer et al., 1974; 1986*). PCV2 was first identified from the pigs which was suffering post-weaning multisystemic wasting syndrome (PMWS) in the middle of 1990s (*Allan et al., 1998*). Pigs infected PCV2 have various clinical diseases, which have made the swine industries huge economic losses all over the world (*Opriessnig et al., 2007*). In 2016, a novel circovirus, called PCV type 3 (PCV3), was isolated from diseased pigs in the USA (*Phan et al., 2016; Palinski et al., 2017*). Subsequently, several outbreaks of it were reported from the United Kingdom of Great Britain (*Collins et al., 2017*), Poland (*Stadejek et al 2017*), Italy, Denmark, Spain (*Franzo et al., 2018*), Korean (*Kwon et al., 2017*), Brazil (*Tochetto et al., 2018*), and China (*Fan et al., 2017; Ku et al., 2017; Zhai et al., 2017; Zheng et al., 2017*). PCV1 and PCV3 had been confirmed as potential pathogen associated with many kinds of clinical symptoms, which are similar as PCV2 infection (*Saha et al., 2011; Palinski et al., 2017; Kwon et al., 2017; Ku et al., 2017; Franzo et al., 2018*). And now, PCV3 has been found in about 20 provinces in China (*Fig. 1*).

Both PCV1 and PCV2 infections are common in pig herds all over the world (*Beach et al., 2010*), and PCV3 is the third porcine circovirus type found in swine, which is circulating in the swine population (*Palinski et al., 2017*). The clinical manifestations of PCV3 are similar to those
of PCV2 and to co-infection with PCV1, PCV2 and PCV3 in pig herds in the several countries
described above. The co-infection of PCV3 with PCV2 was reported in clinical samples of
diseased pigs in Hubei province (Ku et al. 2017). And co-infection of PCV2 with PCV1 was
found in Hubei province. However, PCV1, PCV2 and PCV3 or their co-infections were tested
separately using different methods in the previous reports. Considering the similarities between
the clinical manifestations associated with PCV3 and PCV2, and the high impact of PCV2 and
PCV3 on the economy of pig industry, it is necessary to develop a rapid, convenient, sensitive,
and specific diagnostic approach to discriminate PCV1, PCV2 and PCV3 infection.

However, there is no rapid, convenient and specific diagnostic assay capable of
differentiating PCV1, PCV2 and PCV3 infection. Therefore, to understand the current
epidemiology of PCVs in Hubei province, a rapid, simple, specific and sensitive multiplex PCR
assay was developed to detect and discriminate PCV1, PCV2 and PCV3. The accuracy and
applicability of the multiplex PCR were evaluated for detection of PCVs DNA in clinical
samples collected from the eight pig farms in Hubei province (Fig. 1) where co-infection of
PCVs was reported (Ku et al. 2017). The objective of the present study was to investigate the
epidemiological characteristics of PCVs in the Hubei province using the developed multiplex
PCR.

MATERIALS AND METHODS

Cells and viruses

PK15 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco) at 37
°C in an incubator with humidified 5% CO₂. PCV1, PCV2, PCV3 and other viruses, including
classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus
(PRRSV), pseudorabies virus (PRV), porcine parvovirus (PPV), rotavirus (RV), Japanese
encephalitis virus (JEV), porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus
(PDCoV), stored at Key Laboratory of Prevention and Control Agents for Animal Bacteriosis
(Ministry of Agriculture), were used to verify the specificity of the developed multiplex PCR
assay. And the viruses PCV1, PCV2, PCV3 were also used as positive control viruses. Viral
genomic DNA or RNA for the specificity of the proposed multiplex PCR assay were extracted according to our previous study (Yang et al., 2017).

**Primers design**

The primers were designed based on PCV1, PCV2, PCV3 sequences in GenBank database (Table 1). The PCR-amplified PCV1, PCV2 and PCV3 products were 310, 505 and 1021 bp, respectively. The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

**DNA extraction**

Viral DNA samples for proposed multiplex PCR were extracted using the Viral DNA/RNA Miniprep Kit (Axygen Scientific, CA, USA) from the tissue samples. Total DNA was eluted with 30 μL of diethyl pyrocarbonate-treated water, used immediately or stored at -80 °C.

**Optimization of the Multiplex PCR**

In order to obtain the best reaction parameters, the multiplex PCR was optimized by varying single parameters while other parameters were maintained. The optimization was performed in a 50 µL PCR reaction mixture as follows: 10 × PCR buffer 5.0 µL, 10 mM dNTPs 2-4 µL, each 10 µM primer (Table 1) 0.5-1 µL, Taq DNA polymerase (5U/µL) (TaKaRa, Dalian, China) 0.5-1 µL, the DNA template 3.0-5.0 µL, and added distilled water to 50 µL.

The amplifications were performed under the following conditions in a thermal cycler (Bio-Rad, Hercules, CA, USA). After 5 min initial denaturation at 95 °C, 35 cycles were conducted at 94 °C for 40 s, 52-58 °C for 40 s and 72 °C for 50-70 s, followed by a 10-min final extension at 72 °C. The PCR products were detected according to our previous study (Yang et al., 2017). The specific viral targets fragments were cloned into the plasmid pMD18-T (TaKaRa, Dalian, China). Plasmids containing the PCV1, PCV2 or PCV3 gene were purified using a MiniBEST Plasmid Purification Kit (TaKaRa, Dalian, China). Each plasmid sample concentration was determined by measuring the absorbance at 260 nm using a Eppendorf BioSpectrometer (Eppendorf, Hamburg, Germany), and each cloned gene copy numbers was quantified as previously described (Parida et al., 2011). The standard PCV1, PCV2 and PCV3 DNA samples were ten-fold diluted (from 10^7 to 10^2 copies/µL) and stored at −80 °C until use.

**Specificity and sensitivity of the multiplex PCR**

Specificity of the multiplex PCR assay was determined by using the DNA or cDNA of above-
mentioned porcine viruses as templates and ddH$_2$O as a negative control. PCV1, PCV2 and PCV3 were verified by sequencing and the other virus strains (CSFV, PRRSV, PRV, PPV, RV, JEV, PEDV and PDCoV) were identified by serological or PCR methods.

Total DNA from the plasmids was extracted and used to detect the sensitivity of the developed multiplex PCR assay. The ten-fold diluted standard PCV1, PCV2 or PCV3 DNA samples (from $10^7$ to $10^{-2}$ copies/μL) were used as templates for multiplex PCR.

**Clinical Sample Collection**

A total of 286 tissue samples including lung, spleen and lymph node, were collected from diseased pigs from eight farms in Hubei Province (Fig. 1), central China, from July to December, 2017. These pigs were suspected to have clinical signs of PCVs. Specifically, the clinical signs of the diseased pig were as follow: 92 samples of reproductive failure cases, 78 samples of pigs with PMWS, 65 samples of respiratory disorders cases, 36 diarrhea samples and 15 PDNS samples. All samples were collected in accordance with the standards for animal welfare approved by the Animal Ethics Committee of the Hubei province.

**Sample preparation and detection of PCVs**

The tissue samples were 5-fold diluted with phosphate-buffered saline (0.1 M, pH 7.2) and homogenized. All samples were frozen and thawed three times, and centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatants were used for DNA extraction immediately or stored at −80 °C until use.

The DNA of the clinical specimens were detected using the multiplex PCR assay to investigate the epidemiology of PCVs in Hubei province, central China. To confirm the accuracy of the developed protocol, each PCR product of positive samples was cloned into the plasmid pMD18-T and sequenced. And two Real-time PCR methods (*Kim et al., 2017; Li et al., 2013*) were used to detect PCV1, PCV2 and PCV3 for comparison.

**RESULTS**

**Optimization of the Multiplex PCR assay**

The optimum parameters of the proposed multiplex PCR were as follows. A final 50-μL volume
of master mix for the multiplex PCR including 10×Buffer 5.0 μL, Taq polymerase (5 U/μL) 0.5 μL, DNA template 5.0 μL, dNTPs (10 mM) 4 μL, each primer (10 μM) 1.0 μL, and nuclease-free water was added to 50 μL. An optimized experimental protocol consisted of a 5-min denaturation program at 95 °C, and 35 cycles amplification program (denaturation at 94 °C for 40 s, 56 °C for 40 s, and elongation at 72°C for 50 s), followed by an extension at 72°C for 10 min.

DNAs of PCV1, PCV2, PCV3, and ddH2O, the negative control, were detected using the protocol described above and the multiplex PCR amplification results are illustrated in Fig. 2.

**Specificity and sensitivity of the proposed multiplex PCR**

The specificity of the three primer pairs for PCVs was analyzed using the developed multiplex PCR. As illustrated in Fig. 3, the multiplex PCR assay was specific for PCVs because no amplification products occurred with CSFV, PRRSV, PRV, PPV, RV, JEV, PEDV, PDCoV and ddH2O (lanes 4-12), whereas the PCV1, PCV2 and PCV3 target genes were specifically amplified using the three defined primer pairs (lanes 1-3).

The sensitivity of the proposed multiplex PCR assay was defined as the minimum DNA molecules concentration which could be detected. DNA standards, which was 10-fold diluted, with known copy numbers (10⁷ copies/μL to 10⁻² copies/μL) were used for multiplex PCR. As shown in Fig. 4, the detection limits of multiplex PCR were 10 copies/μL plasmid DNA molecules for PCV1, PCV2 and PCV3, which indicated that the sensitivity of the multiplex PCR was 10 copies/μL for PCV1, PCV2 and PCV3.

**Detection of viruses in clinical specimens**

A total 286 clinical samples were detected by the multiplex PCR assay. The results were as follows: The PCV1-positive, PCV2-positive and PCV3-positive rate at the farm level was 62.5% (5/8), 87.5% (7/8) and 62.5% (5/8), respectively. The positive rates of PCV1, PCV2 and PCV3 in these samples were 52.4% (150/286), 61.2% (175/286) and 45.1% (129/286), respectively, which were 100% consistent with the sequencing method and the Real-time PCR methods (Table 2). The results of the multiplex PCR method and subsequent sequencing further demonstrated the accuracy of the developed assay.
Additionally, the PCV1 and PCV2 co-infection rate was 11.2% (32/286), the PCV1 and PCV3 co-infection rate was 5.9% (17/286), the PCV2 and PCV3 co-infection rate was 23.4% (67/286), and the PCV1, PCV2 and PCV3 co-infection rate was 1.7% (5/286), respectively in the samples from eight pig farms (Table 3). The total co-infection rate was 42.3% (121/286) in all of the detected samples.

**DISCUSSION**

PCV3 is associated with nephropathy syndrome, reproductive failure and porcine dermatitis (Palinski et al., 2017), respiratory disease complex, and cardiac and multisystemic inflammation (Phan et al., 2016). PCV2 is clinically characterized by decreased weight gain, wasting, dyspnea, and enlarged lymph nodes. It has also been identified from diseased pigs with various other clinical presentations, such as porcine dermatitis and nephropathy syndrome, porcine respiratory disease complex (PRDC) and reproductive failure (Chen et al., 2016). Therefore, PCV2 is confirmed as an endemic viral disease of swine all over the world. And in modern swine production, it is also recognized as one of the most economically important infectious pathogens (Opriessnig et al., 2008). Although PCV1-seroprevalence at herd level diversifies between 10% and 100% (Puvanendiran et al., 2011), it is generally known that PCV1 has no pathogenicity to pigs (Allan et al., 1998; Tischer et al., 1986). It was found originally as a contaminant of the porcine kidney cell line PK15 (Tischer et al., 1974). However, PCV1 has recently gained focus of attention because it was discovered as contaminant in several live veterinary vaccines and human vaccines (Beach et al., 2010; Pastoret, 2010). Moreover, PCV1 has been identified in cases of congenital tremors in aborted/stillborn piglets and newborn pigs, which indicated that PCV1 can proliferate and may produce pathological change in the lungs of fetal porcine (Saha et al., 2011). There could be a potential damage to the piglets’ immune system caused by PCV1 infection (Cao et al., 2018). Both PCV1 and PCV2 infections are common in pig herds worldwide (Allan & Ellis 2000), and PCV3 is already widely outbreak and distributed on pig farms in many countries (Palinski et al., 2017; Kwon et al., 2017; Ku et al., 2017; Franzo et al., 2018). Single infection of PCV2 or PCV3, or co-infection of PCV1, PCV2 and PCV3 could
cause many kinds diseases in pig herds (Collins et al., 2017; Ku et al., 2017; Kwon et al., 2017).

Veterinary workers should inestable suitable prevention and control policies for PCVs and their novel strains emerging from viral evolution (Liu et al., 2018).

In order to establish prevention and control strategies for PCVs, a convenient and sensitive diagnostic method is necessary to simultaneously detect and discriminate PCVs in clinical samples. PCR assays are sensitive methods to detect a circovirus infection in viremia animals (Denner & Mankertz, 2017). Several different PCR methods, including digital droplet PCR (ddPCR), real-time PCR and quantitative PCR (qPCR) using specific primers have been developed for detection of PCV1 and PCV2 (Brunborg et al., 2004; Chang et al., 2010; Mankertz et al., 2000; Olvera et al., 2004; Quintana et al., 2006; Rovira et al., 2002; Segalés et al., 2005; Wang et al., 2014; Wang et al., 2016; Zhao et al., 2010; Zhao et al., 2015). And recently, two qPCR methods were proposed to detect and quantify PCV3 DNA (Wang et al., 2017; Palinski et al., 2017). However, there is no specific, sensitive and reliable single assay capable of detecting and differentiating infection by PCV1, PCV2 and PCV3. Therefore, a specific, sensitive and rapid multiplex PCR assay to detect and discriminate PCV1, PCV2 and PCV3 in clinical specimens was developed in the present study.

In this study, an efficient and sensitive multiplex PCR assay was developed to detect and discriminate PCV1, PCV2 and PCV3 using three specific primer pairs. The amplified PCR products of PCV1, PCV2 and PCV3 strain from the specific primers are very different, which can be easily differentiated by electrophoresis. The specific primers for multiplex PCR assay successfully amplified the 310-bp PCV1, 505-bp PCV2 or 1021-bp PCV3 gene. Furthermore, the multiplex PCR assay with three sets of PCV1-, PCV2- and PCV3-specific primers simultaneously detected and discriminated PCV1, PCV2 and PCV3 DNA in a single reaction. And the sensitivity of the multiplex PCR assay was 10 copies/μL for PCV1, PCV2 and PCV3, which is similar to several previous studies (Brunborg et al., 2004; Ku et al., 2017; Li et al., 2018; Wang et al., 2017; Palinski et al., 2017; Zhang et al., 2018).

Although multiplex qPCR assays that can discriminate PCV2 and PCV3 have already been
developed, including one study from China (Li et al., 2018), but all the assays must be practiced in an expensive instrument—Fluorescent Quantitative PCR. It is unaffordable for many laboratories especially which in the counties and towns in China, so the multiplex qPCR assays couldn’t be practiced in their laboratories. However, almost all of them are equipped with the conventional PCR. The sensitivity of the multiplex PCR assay in this study was 10 copies/μL for PCV1, PCV2 and PCV3, which is similar to several qPCR assays. Definitely, the multiplex PCR is a more convenient and sensitive diagnostic method to detect and discriminate PCVs in clinical samples in China and any other developing countries.

A total of 286 specimens from eight pig farms in Hubei province, central China, were analyzed using the proposed multiplex PCR. The results were 100% same as those of sequencing method and Real-time PCR methods. The PCV1, PCV2 and PCV3 singular infection rate, the PCV1 and PCV2 or PCV3 co-infection rate, the PCV2 and PCV3 or PCV1, PCV2 and PCV3 co-infection rate were higher than the previous reports (Ku et al., 2017; Zhai et al., 2017; Zhang et al., 2018). The results indicated that the PCVs infection and their co-infection are severe in Hubei Province, Central China. And the epidemiology and genome characterizations of PCVs in Hubei Province would be further studied in the near future.

DNAs of PCV2 and PCV3 were detected using multiplex PCR in aborted fetal tissue samples and respiratory diseased piglet tissue samples. The results suggested that both PCV2 and PCV3 infection are associated with reproductive failure and respiratory disease at the infection pig farms, as previous researches (Palinski et al., 2017; Ku et al., 2017; Wang et al., 2017). These results indicated that co-infections, as well as singular infection of PCV1, PCV2 and PCV3, are common in pig herds, which are similar with previous studies (Ku et al., 2017; Palinski et al., 2017; Wang et al., 2017; Kwon et al., 2017). The PCV singular infection, and PCV1, PCV3 and PCV2 co-infection play an etiological role in porcine circovirus associated disease (PCVAD), which had caused huge economic losses to pig farms all over the world.

The multiplex PCR assay allows to detect PCVs in field samples with a sensitivity level, and also allows to simultaneously discriminate PCV1, PCV2, and PCV3 in a single reaction, which
makes it lower consumption and less time. Considering the prevalence of PCV1, PCV2 and PCV3 co-infection in the field, the multiplex PCR will enable the correct diagnosis of suspected clinical cases and stimulate further epidemiological researches for its control.

**CONCLUSIONS**

In summary, the developed multiplex PCR is a rapid, convenient, sensitive, efficient, and highly specific assay to detect and discriminate PCVs, which will be useful in etiological and epidemiological studies, as well as diagnosis in clinical cases. The accuracy and simplicity of the assay makes it a useful, suitable and powerful tool for PCVs detection, prevention and control in China and any other developing countries.

**ADDITIONAL INFORMATION AND DECLARATIONS**

**Funding**

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**Competing Interests**

The authors declare there are no competing interests.

**Author Contributions**

Keli Yang and Yongxiang Tian conceived and designed the experiments. Danna Zhou, Fangyan Yuan and Zhengying Duan performed the experiments. Rui Guo and Zuwu Jiao performed sampling, and provided the biological material. Keli Yang analyzed the data and wrote the paper. All the authors read and approved the final manuscript.

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Figure 1

FIGURE 1 | Geographical distribution of PCV3 in China (red regions, till June 2018) and the position of pig farms (red stars) in this study
Figure 2

**FIGURE 2| Electrophoresis of multiplex PCR products in optimization conditions.**

Lane M, DL2000 DNA marker; lane 1, PCV1; lane 2, PCV2; lane 3, PCV3; lane 4, ddH$_2$O.

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*
FIGURE 3| Specificity of multiplex PCR for the detection of PCVs. Lane M, DL2000 DNA marker; lane 1, PCV1; lane 2, PCV2; lane 3, PCV3; lane 4, CSFV; lane 5, PRRSV; lane 6, PRV; lane 7, PPV; lane 8, RV; lane 9, JEV; lane 10, PEDV; lane 11, PDCoV; lane 12, ddH₂O.

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*
**Figure 4**

**FIGURE 4| Sensitivity of multiplex PCR for the detection of PCVs.** Lane M, DL2000 DNA marker; lanes 1-10 are: 1, $10^7$; 2, $10^6$; 3, $10^5$; 4, $10^4$; 5, $10^3$; 6, $10^2$; 7, $10^1$; 8, $10^0$; 9, $10^{-1}$; 10, $10^{-2}$ copies/μL.

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*
Table 1 (on next page)

<table>
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<th>Table</th>
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<tr>
<td>Sequences of the primers for multiplex PCR</td>
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Table 1 Sequences of the primers for multiplex PCR

<table>
<thead>
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<th>Primer</th>
<th>Primer sequences (5’-3’)</th>
<th>Origin/target gene</th>
<th>Location</th>
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**Table 2** (on next page)

Table

Detection of clinical specimens by multiplex PCR and sequencing method
Table 2 Detection of clinical specimens by multiplex PCR and sequencing method

<table>
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<tr>
<th>Pig Farm</th>
<th>No. of specimens</th>
<th>multiplex PCR</th>
<th>Sequencing method</th>
<th>Concordance rate (%)</th>
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<td></td>
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<td>PCV1 positive (%)</td>
<td>PCV2 positive (%)</td>
<td>PCV3 positive (%)</td>
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<td>0 (0)</td>
<td>36 (72.0)</td>
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<tr>
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<td>25</td>
<td>22 (88.0)</td>
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<tr>
<td>Total</td>
<td>286</td>
<td>150 (52.4)</td>
<td>175 (61.2)</td>
<td>129 (45.1)</td>
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Table 3 (on next page)

Table

Detection of the co-infection of clinical specimens by multiplex PCR
<table>
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<tr>
<th>Pig Farm</th>
<th>No. of specimens</th>
<th>PCV1 and PCV2 positive (%)</th>
<th>PCV1 and PCV3 positive (%)</th>
<th>PCV2 and PCV3 positive (%)</th>
<th>PCV1, PCV2 and PCV3 positive (%)</th>
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</tr>
<tr>
<td>4</td>
<td>36</td>
<td>8 (22.2)</td>
<td>5 (13.9)</td>
<td>13 (36.1)</td>
<td>3 (8.3)</td>
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<tr>
<td>Total</td>
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<td>17 (5.9)</td>
<td>67 (23.4)</td>
<td>5 (1.7)</td>
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