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Simultaneous detection of porcine pseudorabies virus, porcine parvovirus and porcine circovirus type 2 by multiplex real-time PCR and amplicon melting curve analysis using SYBR Green I

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ABSTRACT: Porcine parvovirus, porcine pseudorabies virus and porcine circovirus type 2 can cause reproductive failure in pigs, and swine are often simultaneously infected by combinations of the three viruses. We here report the development of a SYBR Green I-based multiplex real time PCR assay for simultaneous detection of porcine parvovirus, porcine pseudorabies virus and porcine circovirus type 2. Three pairs of specific primers were designed for the porcine parvovirus-VP2, porcine pseudorabies virus-gH and porcine circovirus type 2-ORF2 genes. Viral genomes were identified based on their distinctive melting temperatures in singleplex PCR reactions. The melting temperature was 74.5 °C for the 313 bp amplicon of porcine parvovirus-VP2 gene, 87.5 °C for the 355 bp amplicon of porcine pseudorabies virus-gH gene and 80.5 °C for the 171 bp amplicon of the porcine circovirus type 2-ORF2 gene, respectively. The detection limit of the method ranged from 0.01–0.03 $TCID_{50}$/ml for the three viruses. In addition, porcine parvovirus, porcine pseudorabies virus and porcine circovirus type 2 viral loads were measured in 100 field samples, and the result showed that the concordance between real-time PCR and conventional PCR was 60.42%. The sensitivity and specificity of real-time PCR were 100% and 100%, while those of conventional PCR were 40.83% and 72.22%, respectively.

Keywords: pig; swine; specific primer

In intensive swine production, it is very common for swine to be infected with two or more viral pathogens simultaneously (Xu et al. 2012; Zeng et al. 2014; Lung et al. 2016). Clinical signs of disease caused by different pathogens can be very similar. Definitive diagnosis of multiple infections is often difficult and thus fails to provide a clear indication based on clinical symptoms. As a result, a number of costly, individual, virus-specific tests are performed initially to expedite the testing procedure. The conventional diagnostic method is virus isolation, which is time-consuming and not suitable for all viruses. Gene sequencing and microarray analysis are accurate diagnostic methods (Chiu 2013; Takeichi et al. 2013), but both of them are costly with long turnaround times. SYBR Green I-based

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multiplex real-time PCR assays have widely been used to simultaneously identify and distinguish a variety of viral pathogens in a single sample on the basis of amplicon size and distinctive melting temperatures (Perez et al. 2011; Perez et al. 2012; Singh and Mustapha 2014; Elkins et al. 2016).

Porcine circovirus (PCV) is a small, non-enveloped, single-stranded circular DNA virus with a genome of about 1.76 kb belonging to the *Circoviridae* family (Allan et al. 1998). Two antigenically and genetically distinct species, PCV1 and PCV2, have been identified. PCV1 is considered non-pathogenic while PCV2 may be subclinical or elicit a range of multifactorial disease syndromes collectively described as porcine circovirus-associated disease (PCVD), which are characterised by progressive weight loss, respiratory signs and jaundice (Opriessnig et al. 2007; Opriessnig and Langohr 2013; Ellis 2014). Porcine parvovirus (PPV) is a small, non-enveloped, single-stranded DNA virus classified in the *Parvoviridae* family, and is a pathogenic factor that primarily induces severe reproductive problems in pregnant swine, such as stillbirth, mummification, embryonic death and infertility, which result in extensive losses to the swine industry worldwide (Ellis et al. 1999; Streck et al. 2015). Porcine pseudorabies virus (PRV) is a member of the genus Varicellovirus of the family *Herpesviridae* that causes respiratory problems, nervous symptoms and abortion and inflicts serious economic losses in the swine industry worldwide (Nauwynck et al. 2007). In this study, the conserved gH gene of PRV, VP2 gene of PPV and ORF2 gene of PCV2 were the targets as has been described previously (Xu et al. 2012; Fu et al. 2016).

The aim of this study was to develop and validate an effective multiplex SYBR Green I real-time PCR assay to detect PPV, PRV and PCV2 simultaneously in one sample and to investigate the effects of diagnostic specimen choice on assay performance. This method could be used to test different kinds of samples such as semen, tissues (lymph nodes, lungs, spleens and livers) and nasal swabs from naturally infected conventional pigs.

MATERIAL AND METHODS

Viruses and clinical samples. The PPV standard strain 7909 was purchased from the China Institute of Veterinary Drug Control, Beijing, China. The PRV HN strain and the PCV2 HN strain were both isolated from clinical specimens of swine and were then identified in the Key Laboratory for Animal-Derived Food Safety of Henan Province, China. These three viruses were propagated in PK15 cells which were also purchased from the China Institute of Veterinary Drug Control, Beijing, China. Porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CFSV), Japanese encephalitis virus (JEV) and porcine circovirus type 1 (PCV1) were provided by the Key Laboratory for Animal-Derived Food Safety of Henan Province, China.

A total of 100 clinical samples were collected on different pig farms in several provinces in China from pigs that were suspected to harbour PPV, PRV and PCV2 infections. Sampled tissues and organs included lymph nodes, colon, duodenum, jejunum, hearts, kidneys, liver, tonsils, lungs, thymus, gonads as well as spleens collected from 4 to 8-week-old sick piglets and different abortion cases during the period of June 2012 to July 2016. Another two healthy pig tissue samples were collected from a pig farm as the negative controls. Tissue samples were stored separately at –70 °C.

Primer design and synthesis. Target genes of PPV, PRV and PCV2 were selected based on the number of sequences available for highly conserved regions in GenBank. The primers were designed based on the most conserved region of the target genes in order to cover as many sequences as possible. The primers were designed according to the PPV-VP2 nucleotide sequences, PRV-gH nucleotide sequences and PCV2-ORF2 nucleotide sequences retrieved from GenBank. Forward primer (F): 5'-TGGGAGGGCTTGGTTAGA-3' and reverse primer (R): 5'-TGGTGGTGAGGTTGCGAT-3') were used to amplify a fragment with a length of 313 bp for the PPV-VP2 gene (Gen-Bank Accession number NC001718). F: 5'-CGTGGA ACGAGCCCTTCAG-3' and R: 5'-AGAGCG GGTTGGCGATGT-3' were used to amplify a fragment with a length of 355 bp for the PRV-gH gene (GenBank Accession number U02513.1). F: 5'-TAACTACTCCTCCCGCCATAC-3' and R: 5'-GCCTACGTGGTCTACATTTCC-3' were used to amplify a fragment with a length of 171 bp for the PCV2-ORF2 gene (GenBank Accession number AF027217). These primers were designed and synthesised by Takara, China.

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DNA extractions. Viral genomic DNA of PPV, PCV2 and PRV was extracted using a DNA extraction kit (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was isolated either from 20 mg of frozen clinical specimens or from 500 µl lysates of PK15 cells infected with the three viruses.

Preparation of standard plasmid DNA. The VP2 gene of PPV, gH gene of PRV and ORF2 gene of PCV2 were amplified using the Ex Taq PCR kit (Takara, Mountain View, USA) for the standard plasmid DNA construction. The PCR products were sub-cloned into the pGEM-T Easy vector (Promega, USA) and purified with a QIAGEN plasmid Maxi Kit and then quantified by measuring OD_{260} using a NanoDrop spectrophotometer (Ultrospec® 20000, Pharmacia, UK). All selected colonies were confirmed by sequencing. The exact number of DNA molecules was calculated using a previously described formula (Huang et al. 2009). For multiplex reactions, standard plasmids were mixed in equal ratios and adjusted to a concentration of 10^{10} viral copies/µl for PPV, PRV and PCV2, and the mixed plasmids were used to make 10-fold serial dilutions to construct triplex standard curves for analytical validation.

Optimization of the PPV/PRV/PCV2 SYBR Green I real-time PCR assay. PCR cycling parameters, primers and reagent concentrations and SYBR PreMix concentration were optimized in order to yield the best results in the PPV/PRV/PCV2 multiplex SYBR Green I real-time PCR protocol. Different annealing/elongation temperatures and cycling conditions were also evaluated.

Singleplex and multiplex SYBR Green I real-time PCR. The optimised PCR assays were run on a LightCycler 2.0® instrument (Roche Applied Science, Mannheim, Germany). To avoid the possibility of preferential amplification of one target sequence over another, a touchdown thermal profile for both singleplex and multiplex real-time PCR assays was used. After the PCR cycles, a melting curve was generated (0 s at 95 °C, 15 s at 65 °C, 0 s at 95 °C) to discriminate between the specific amplicons and non-specific amplification products. Fluorescence was measured after extension at each cycle. All real-time reactions (unknown samples, standard and negative controls) were performed in duplicate in neighbouring wells on the sample plate. Reported results are the average of the duplicates.

After each run, a melting curve analysis was performed, and the melting peaks were analysed to distinguish PPV-, PRV- and/or PCV2-specific amplicons. The Tm value was defined as the peak of the curve. Fluorescence normalisation and data analysis were performed using the thermal cycler's software (MxPro-Mx3005P v 3.00; Stratagene, La Jolla, USA).

Sensitivity, specificity and reproducibility of the real-time PCR. Ten-fold serial dilutions of PPV, PRV and PCV2 standard plasmids were made to generate concentrations from 10^{10} to 10^{0} copies/µl in PBS. DNA in each dilution was extracted and subjected to singleplex and multiplex real-time PCR using the optimised conditions. The lowest detectable concentration was defined as the detection limit of the real-time PCR. The analytical specificity of the real-time PCR was assessed by analysis of the DNA of four other porcine viruses (PRRSV, CSFV, PCV1 and JEV). Each virus was tested in triplicate. The reproducibility of the assay was determined using three different DNA concentrations (high/intermediate/low) of the standard PPV, PRV and PCV2 plasmid preparations. The selected DNA concentrations were 1×10^{-2} to 1×10^{-5} $TCID_{50}$/ml (PPV), 1×10^{-2} to 1×10^{-5} $TCID_{50}$/ml (PCV-2) and 3×10^{-2} to 3×10^{-5} $TCID_{50}$/ml (PRV). To assess the intra-assay variability, the coefficients of variation for CT values obtained for each dilution were analysed in triplicate. To determine inter-assay variability, the standard plasmid was diluted from 10^{7} to 10^{0} copies/µl and tested in six different runs performed by two different operators on different days. The variation among the three testers at each time was analysed.

Data analyses. After the SYBR Green I real-time PCR run, data acquisition and analyses were done using one-way analysis of variance (ANOVA) in SAS version 7.0 software. The fluorescence of SYBR Green I compared to that of the internal passive reference dye, ROX (ΔRn) is measured at the end of each cycle, and the threshold value of ΔRn was 0.2. A P-value of < 0.05 was considered statistically significant.

Conventional PCR. The conventional PCR assay was conducted as described previously (Wu et al. 2008). The PCR reaction in which a 171 bp product was amplified from the PCV2-ORF2 gene was performed in a thermal cycler (Eppendorf, Mastercycler) under the following conditions: one

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cycle of 3 min at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 1 min at 55 °C and elongation for 1 min at 72 °C. This was followed by a final extension step of 5 min at 72 °C. The PRV-gH gene and PPV-VP2 gene PCRs were performed similarly, but with an annealing temperature of 54 °C and 57 °C, respectively. The amplicons were visualised in 1% agarose gels and with a DNA ladder. Negative and positive controls were used in each PCR reaction.

Detection of field samples. A total of 100 field samples diagnosed clinically as PPV, PRV and PCV2 infections were collected. DNA was extracted and then analysed in parallel by conventional PCR and the multiplex real-time PCR. Five positive samples and five negative samples were randomly subjected to virus isolation to validate the reliability of the multiplex real-time PCR.

RESULTS

Real-time PCR strategy optimisation and melting curve analysis

The real-time PCR procedure was optimised with respect to the duration of cycles, SYBR Green I master mixture and primer concentrations, and denaturing/extension temperatures. The optimised parameters were used in the multiplex real-time PCR. Each reaction contained a total volume of 25 µl, including 2 µl DNA (10.1 $\log_{10}$ copies/µl), 12.5 µl 2 × SYBR Green Premix (5 U/µl) and 0.5 µl of each primer (25 pmol/µl). The reaction conditions were the following: pre-denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s and extension at 72 °C for 20 s.

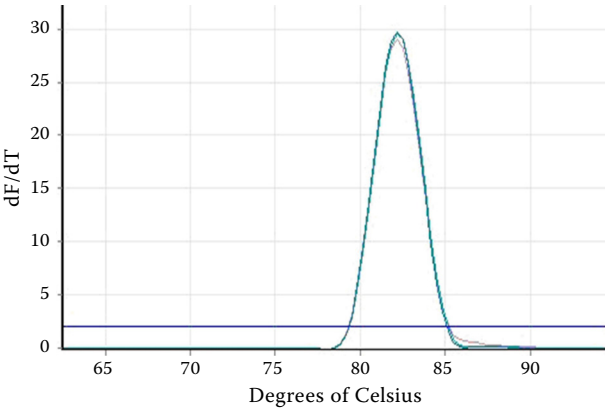

Figure 2. Melting curve analysis for the singleplex SYBR Green I real time PCR of porcine pseudorabies virus

The specificity of the primer pairs for each virus was first analysed in singleplex PCR. As shown in Figures 1–3, specific melting peaks for PCV2 (Tm was about 81.85 °C), PRV (Tm was about 87.45 °C) and PPV (Tm was about 78.55 °C) were obtained. These amplification products were also confirmed by electrophoresis in 1% agarose gel and by sequencing (data not shown).

Multiplex real-time PCR fragments were detected by melting curve analysis. The SYBR Green I multiplex real-time PCR assay that used all the primer sets in a single reaction mix was able to discriminate between each specific amplification product. Hence, the different lengths and compositions of three amplicons for PPV, PRV and PCV2 allowed easy discrimination on the basis of specific Tm values. Melting curve analysis revealed that the PPV melting curve showed a Tm of around 74.5 °C,

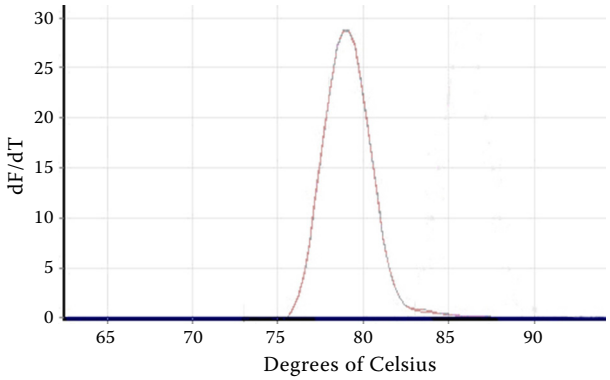

Figure 1. Melting curve analysis for the singleplex SYBR Green I real time PCR of porcine parvovirus

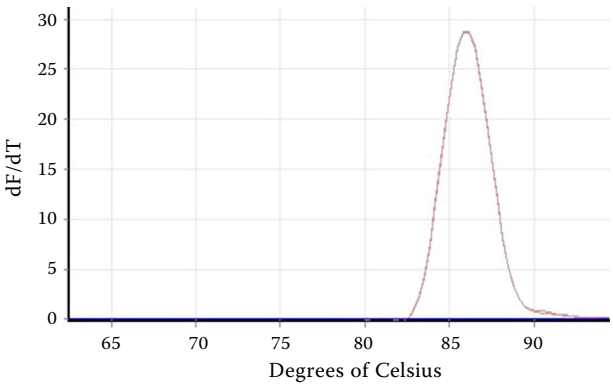

Figure 3. Melting curve analysis for the singleplex SYBR Green I real time PCR of porcine circovirus type 2

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that of PRV was around 87.5 °C and that of PCV2 was around 80.5 °C (Figure 4).

Analytical sensitivity of the singleplex and multiplex real-time PCR assays

The analytical sensitivity of SYBR Green I PCR was determined by gene copy numbers. Ten-fold serial dilution of PPV, PRV and PCV2 plasmid DNA was used as a template for amplification to measure the detection limit. Matrix effect on the analytical sensitivity, Ct values and Tm values for the multiplex real-time PCR system was assessed. The linearity of the reactions between the input plasmid DNA and the CT values with regression coefficients (r^2) greater than 0.99 were obtained for the three viruses. The Tm values of the specific melting peaks decreased when samples were used as matrices ($P < 0.05$). Likewise, the Ct values for multiplex real-time system were higher when samples were used as matrices ($P < 0.05$). Although the use of samples affected the Ct values (increasing) and Tm values (decreasing), this matrix did not affect the analytical sensitivity of the assay. However, the analytical sensitivity of the multiplex real-time PCR system was altered when tissue samples were used as matrices. The detection limits of the singleplex real-time PCR was determined to be 16 copies/µl for PPV, 16 copies/µl for PRV and 12 copies/µl for PCV2, whereas the detection limits of the multiplex real-time PCR were determined to be 14 copies/µl for PPV, 14 copies/µl for PRV and 10 copies/µl for PCV2, equivalent to 0.01 $TCID_{50}$/ml – 0.03 $TCID_{50}$/ml, respectively, and about ten times more sensitive than those of the conventional PCRs (0.1 $TCID_{50}$/ml for PPV, PRV and PCV2, respectively). Therefore, similar but slightly lower sensitivities were observed for the multiplex real-time PCR compared to the real-time PCR assays.

Assay specificity

The specificity of the multiplex real-time PCR was also evaluated. No other porcine viruses such as JEV, PRRSV, PCV1 and CFSV were specifically amplified. In Figure 5, it can be seen that the Tm values of PPV, PRV and PCV2 were around 74.5 °C, 87.5 °C and 80.5 °C, respectively, while the other four detected viruses showed no specific melting peaks. After multiplex real-time PCR amplification, the specific bands of 313 bp for PPV, 355 bp for PRV and 171 bp for PCV2 were also confirmed by agarose gel electrophoresis. No bands of the expected sizes were observed from the other four viruses (data not shown).

Reproducibility of the real-time PCR

The standard plasmids of PPV, PRV and PCV2 were serially diluted and then amplified in the real-time PCR assay by three people on six separate occasions, and the obtained results were similar. The inter-assay variation coefficient among the three testers on the six separate occasions were 1.05, 1.13 and 1.15, respectively. The coefficients of variation (CVs) of CT values between different intra-assay and inter-assay runs for each input DNA copy number were less than 2% for PPV, PRV and PCV2 detection channels. The multiplex real-time PCR system demonstrated a high repeatability with coefficients of variation within runs (intra-assay

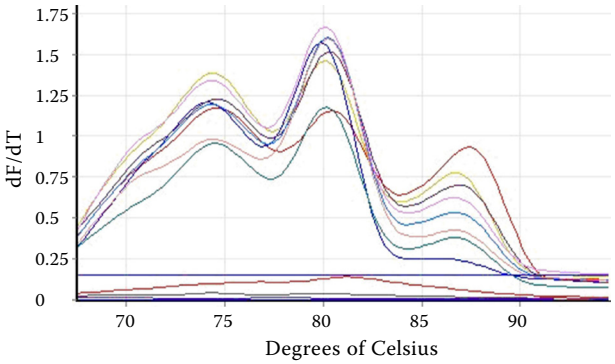

Figure 4. Melting curve analysis for the multiplex SYBR Green I-based real-time PCR

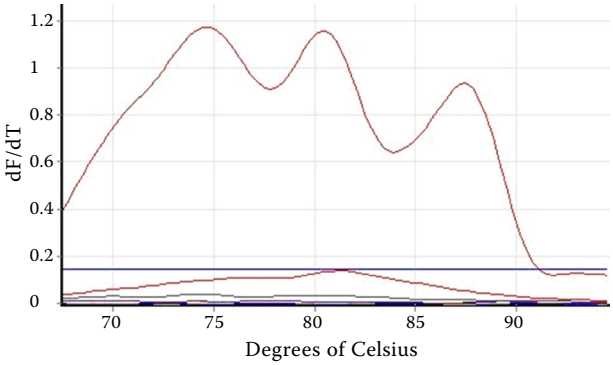

Figure 5. Melting curve analysis of the specificity of the multiplex SYBR Green I-based real-time PCR

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variability) and between runs (inter-assay variability) that ranged from 0.02% to 0.06% and from 0.04% to 0.08%, respectively.

Testing on clinical samples

One hundred field samples were tested in the multiplex real-time PCR and conventional PCR, respectively. The multiplex real-time PCR results showed that 28 samples were positive for PPV, 26 samples were positive for PRV and 35 samples were positive for PCV2. There were 19 samples that were positive for co-infection with PPV and PRV, 22 samples were positive for co-infection with PPV and PCV2, 19 samples were positive for co-infection with PRV and PCV2 and nine samples were infected with all three viruses. The multiplex real time PCR system tests were 60.42% consistent with the conventional PCR testing of samples (Table 1).

DISCUSSION

Multiple viral infections are common in pigs under intensive production conditions, and simultaneous infections with various viruses are known to result in more severe clinical signs and lesions (Kim et al. 2001; Ellis et al. 2008; Blomstrom et al. 2010). Since PPV, PRV and PCV2 are very common pathogens in swine production systems and can cause similar clinical symptoms, and because some strains do not cause visible clinical signs but may affect pig growth, especially that of piglets, it is difficult to diagnose these viral diseases based on clinical symptoms.

Table 1. Performance of multiplex real time PCR and conventional PCR

	Samples from diseased pigs						
	A^+	B^+	C^+	A^+B^+	A^+C^+	B^+C^+	$A^+B^+C^+$
Real-time PCR	28	26	35	19	22	19	9
Conventional PCR	24	20	35	15	20	17	6
Concordance (%)	53.13	59.38	68.75				
Average agreements	60.42						

A = porcine parvovirus, B = porcine pseudorabies virus, C = porcine circovirus type 2

$^+$Positive sample

The rapid and reliable detection and differentiation of porcine viruses are essential for disease surveillance and control. Conventional tests, such as antigen-capture ELISA, the immunoperoxidase monolayer assay (IPMA) and the immunofluorescent assay (IFA), can be used to detect and differentiate the three viruses but with low sensitivity and specificity. Rapid and accurate methods for detection and identification of PPV, PRV and PCV2 are essential. In China, standard detection assays for the three viruses employ PCR. However, it would be more efficient if more than one virus could be detected in one sample. Multiplex real-time PCR can be used for simultaneous amplification of two or more DNA targets in a single reaction vessel and may be carried out using uniquely labelled probes for each target. This reduces the number of samples required, which is especially important when the sample material is scarce. Additional benefits are time-saving in reaction setup and lower costs compared to simple, single reactions (Elnifro et al. 2000; Markoulatos et al. 2002; Persson et al. 2005; Belak 2007).

In this study, a SYBR Green I-based multiplex real-time PCR with melting curve analysis for the simultaneous detection of PPV, PRV and PCV2 genomes in a single reaction vessel was developed and validated by detection and differentiation of nucleic acid targets (Tam et al. 2009). This assay offers a rapid, high-throughput, and reliable screening system for the three tested porcine viruses. Furthermore, it is more sensitive compared to virus isolation and nested PCR (nPCR) assays which consume much time and are labour-intensive (Larochelle et al. 2001; Varga and James 2005; Wilhelm et al. 2005; Kerr 2006; Zheng et al. 2013). The results of specificity analysis indicated that no cross-amplification or non-specific amplification was observed. The sensitivity analysis showed that the limit of detection was in the range of 0.01–0.03 $TCID_{50}$/ml for PPV, PRV and PCV2, values which were at least ten times more sensitive than the conventional PCR. We also compared the sensitivity of mPCR and singleplex real-time PCR assays on the basis of Ct values, and the results indicated that there was no noticeable interference or reduced sensitivity observed by multiplexing the three targets into one sample.

In addition, the SYBR Green I-based multiplex real-time PCR assay is able to discriminate between

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different viral strains based on genomic sequence variability (Niu et al.2016). Variations in the Tm values of the amplicons generally allow differentiation between viral species or strains through melting curve analysis (Howard et al. 2015). Therefore, it has been used to detect the genotypes of different viral agents. We proposed that this assay represents a suitable alternative method to other tests, particularly for large-scale PCR screening, because the SYBR Green I PCR method is not influenced by different genetic backgrounds. Amplicons can be distinguished by melting curve analysis, in which the melting temperature (Tm) of PCR products is determined by the reduction in relative fluorescence as all dsDNA is denatured to its single-stranded form. The shape and peak location of the melting curves are functions of the GC/AT ratio, length and sequence of the fragment (Wernike et al. 2013; Ikten et al. 2016).

An inherent limitation of multiplex real-time PCR assays is potential interaction among oligonucleotides in the same reaction that can cause reduced amplification sensitivity (Wernike et al. 2013; Forghani et al. 2015). In this study, the primer pairs were very carefully designed. The best output scores for each primer pair were chosen to avoid the effect of SYBR Green I translocation. The reaction conditions were carefully optimised to obtain maximum sensitivity. As a result, despite the presence of three primer sets in the PCR reaction mixture, the multiplex PCR was able to detect all viruses at a high level of sensitivity.

The results from clinical sample testing indicated that PPV, PRV and PCV2 co-infections were broadly prevalent in the swine herds. Out of 100 clinical specimens (all from clinically diseased pigs) collected from several provinces in China, 28 samples contained PPV, 35 samples contained PCV2 and 26 samples contained PRV. Co-infection rate of PPV and PRV was 19% (19/100), 22% (22/100) for PPV and PCV2, and 19% (19/100) for PRV and PCV2. The rate of simultaneous infection with PPV, PCV2 and PRV was 9% (9/100). Simultaneous detection and differentiation of the three viruses should be incorporated into the disease control of swine herds in China.

In conclusion, the SYBR Green I-based multiplex detection real-time PCR system described in this study allows the simultaneous detection and differentiation of PPV, PRV and PCV2 in swine viral infections. Further, the assay is easy to carry out, is rapid and is characterised by high sensitivity and specificity. It could be potentially used in routine swine disease surveillance and diagnostics of PPV, PRV and PCV2 infections.

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