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Development of PCR for the Identification of Porcine Circovirus Type 2 (PCV-2) Genotype PCV-2a and PCV-2b

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Abstract: PCR was developed to evaluate for its ability to simultaneously detect viral infections of swine. Specific primers were designed for each sub-type of porcine circovirus type 2 (PCV-2); porcine circovirus type 2a (PCV-2a) and porcine circovirus type 2b (PCV-2b). Each target produced specific amplicon with a size of 229 bp (PCV-2a) and 785 bp (PCV-2b). The assay was sensitive and specific in detecting the target agent in clinical specimens. In conclusion, the PCR has the potential to be useful for routine molecular diagnosis and epidemiology.

Key words: Porcine circovirus type 2 (PCV-2), porcine circovirus type 2a (PCV-2a), porcine circovirus type 2b (PCV-2b), agent, target, China

INTRODUCTION

PCV-2 is member of the virus family circoviridae. The virion is icosahedral, non-enveloped and 17 nm in diameter. A singe capsid protein with approximately 30 kDa has been identified for PCV-2 (Nawagitgul *et al*., 2000).

The genome of PCV-2 is circular, covalently closed, single-stranded DNA (ssDNA) with 1765~1766 nt in length (Chae, 2004), dominated by two open reading frames. ORF1 is 945 bases and codes for two replicase proteins, Rep and Rep. The 702 nt ORF2 codes for the capsid protein which forms the icosahedral capsid (Nawagitgul *et al*., 2000).

The capsid protein interacts with a heparin sulfate receptor on cells and is considered to be the principal target for the induction of protective antibody (Horlen *et al*., 2008; Misinzo *et al*., 2006). A 3rd gene, *ORF3* is in a different reading frame embedded within ORF1. The ORF3 protein is not essential for replication in PK-15 cells but may be important for pathogenesis by inducing apoptosis through activation of the caspase-8 pathway (Liu *et al*., 2005).

Porcine circovirus type 2 (PCV-2) has been implicated as the etiological agent for Porcine Multisystemic Wasting Syndrome (PMWS) (Allan *et al*., 1999; Kennedy *et al*., 2000) and could be divided into two groups (1 and 2) and eight clusters (1a-b and 2a-e). The 3rd genotype (PCV-2c) was detected in Denmark with only three sequences to date (Dupont *et al*., 2008). In this study, a differientive PCR was development to detect PCV-2 variants PCV-2a from PCV-2b in South China pig farms.

MATERIALS AND METHODS

Viral isolation: Porcine circovirus types 1 and porcine circovirus types 2, porcine respiratory coronavirus, porcine parvovirus, haemophilus parasuis and porcine reproductive and respiratory syndrome virus were used in this study.

Field samples: Form the archived necropsy material of the college of veterinary, lung, spleen and liver tissues from 60 positive pigs of the years 2008 through 2009 were selected and investigated by PCR and ELISA.

DNA extraction and RNA extraction: Lymph node, lung and spleen tissues were kept frozen at -80°C previously to DNA extraction. DNA was isolated from the tissue samples using Genomic DNA mini kit (Geneaid) according to the manufacturer's instructions.

Viral genomic RNA were simultaneously extracted from the homogenized tissues and from lysates of infected cell cultures with the TRLzolR® reagent (TaKaRa) according to the manufacturer's protocol.

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Primer design and PCR reaction condition: The primers used for this study were designed based on 18 PCV-2 genomes (Accession No: AY556475, AY556473, 556476, AY686763, EF565350, EF565355, EF565357, EU545543, EU545544, FJ644559, FJ905460, FJ905461, FJ716704, FJ716703, FJ667587, FJ667593) published by the National Center for Biotechnology Information and sequence alignment was performed using DNASTAR Version 5.

Two pairs (PCV-2aF, PCV-2aR, PCV-2bF and PCV-2bR) were designed based on the PCV-2 sequences (Table 1), respectively. CR was carried out using EX Taq DNA polymerase Roche mastermix in a 25 uL final reaction volume.

The mastermix contains per deionized water, 10×EX buffer, 2.5 mM dNTP, 0.5 uL of each primer, 2U EX Taq polymerase and 2 uL template. Cycling parameters were 94°C for 10 min; 30 (10 sec, 94°C; 10 sec; optimal annealing temperature (Table 1), 72°C for 40 sec); 72°C for 10 min. Amplified PCR products were analyzed by agarose-gel electrophoresis and sequenced (invitrogen).

Standard plasmid of PCR: The amplified PCR products of PCV-2a and PCV-2b were cloned using the pMD18-T vector system (TaKaRa) and were propagated in *E. coil* according to the manufacturer's instructions. Plasmids were purified by the H.Q and Q Plasmid mini kit (OMEGA) and were quantified by measuring OD260 with ND-1000 Spectrophotometer, NanoDrop.

The viral copy of the extracted plasmids was calculated using the formula. Each plasmid was optimized to 1×10^{10} viral copy uL^{-1} and used to evaluate the diagnostic sensitivity of the PCR.

Specificity of PCR assays: In the specificity studies of PCR assays, PCV-1, PRV, PPV, HPs and PRRSV were tested with primers for PCV-2a and PCV-2b.

Sensitivity of PCR assays: The 10-fold serial dilutions of the standard plasmid from 10^{10} to 1 viral copy uL^{-1} of each genotype were tested to determine the detection limits of PCR.

Table 1: Primer pairs used for amplification and sequencing of PCV-2a and PCV-2b

Numbers	Sequence	Fragment length (bp)	Optimal annealing temp. (°C)
PCV-2aF	TGTTGGCGAGGAGGGTAATGAGGAA	229	70
PCV-2aR	AGACAGGTCACTCCGTTGTCCCTGA		
PCV-2bF	AGAATGTGGAGCTCCTAGATCTCAA	785	62
PCV-2bR	GTAGGCCTCGGCACTGCGTTCGAAA		

Virus isolation and sequencing: To verify the results of PCR assays, all the positive samples were sent to Porcine circovirus team for isolation and all the isolates were sequenced and analyzed.

RESULTS

Analytic sensitivity of PCR: The detection limit of PCR was determined to be 10^7 viral copy uL^{-1} for PCV-2a (Fig. 1), 10^6 For PCV-2b of the standard plasmid (Fig. 2).

Specificity of PCR: The specificity of primer pairs for each genotype was analyzed with PCR. As shown in Fig. 3 and 4, both PCR were specific for the target viral agent because no amplification occurred with PRV, PPV, PCV-1, HPs and ddH_2O (lanes 1-7) whereas each viral target gene was specifically amplified using its defined primer pair (lanes 8-12).

Detection of virus in known-positive specimens: DNA was extracted from tissue samples infected with PCV-2. The PCR correctly detected the virus (Fig. 5 and 6). The PCR products were 229 and 785.

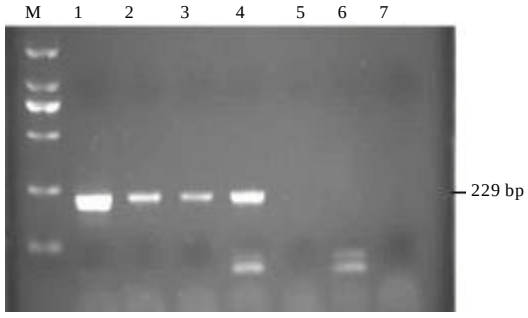

Fig. 1: Sensitivity of PCR for PCV-2a; M: DL2000 DNA Marker; 1: 10^{-3} dilute; 2: 10^{-4} diluted; 3: 10^{-5} diluted; 4: 10^{-6} diluted; 5: 10^{-7} diluted; 6: 10^{-8} diluted; 7: Negative control

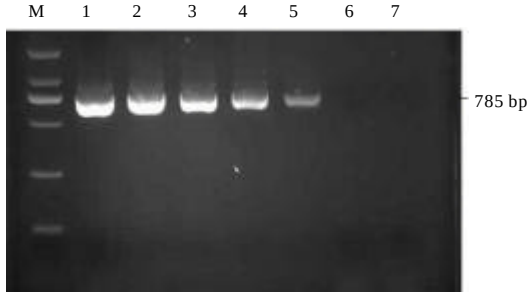

Fig. 2: Sensitivity of PCR for PCV-2b; M: DL2000 DNA Marker; 1: 10^{-3} dilute; 2: 10^{-4} diluted; 3: 10^{-5} diluted; 4: 10^{-6} diluted; 5: 10^{-7} diluted; 6: 10^{-8} diluted; 7: Negative control

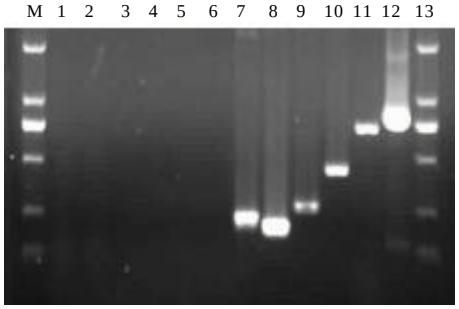

Fig. 3: Specificity of PCR for PCV-2a; M: DL2000DNA Marker; 1: PRV; 2: PPV; 3: PCV-1; 4: PRRSV; 5: HPs; 6: Negative control; 7: PCV-2a PCR; 8: PRV PCR; 9: PPV PCR; 10: PCV-1 PCR; 11: PRRSV PCR; 12: HPs PCR, 13: DL2000DNA marker

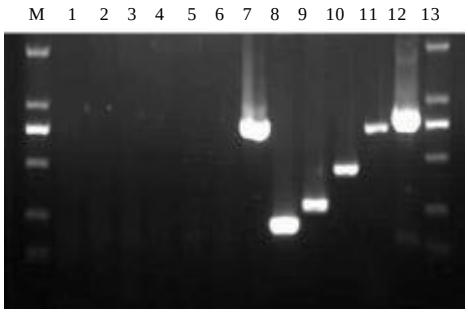

Fig. 4: Specificity of PCR for PCV-2b; M: DL2000 DNA Marker; 1: PRV; 2: PPV; 3: PCV-1; 4: PRRSV; 5: HPs; 6: Negative control; 7: PCV-2b PCR; 8: PRV PCR; 9: PPV PCR; 10: PCV-1 PCR; 11: PRRSV PCR; 12:HPs PCR, 13: DL2000DNA marker

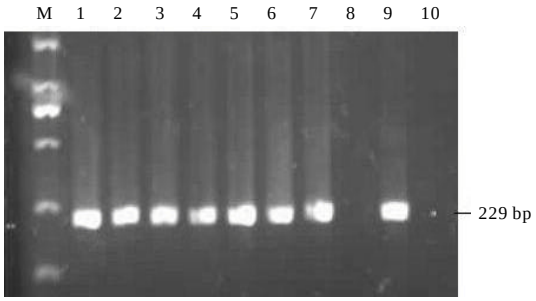

Fig. 5: PCR reaction in positive sample for PCV-2a; M: DL2000 DNA Marker: 1 = No. 1; 2 = No. 2; 3 = No. 3; 4 = No. 4; 5 = No. 5; 6 = No. 6; 7 = No. 7: 8 = No. 8; 9 = No. 9 and 10: Negative control

Detection of viruses in clinical positives specimens by PCR: A total of 60 clinical specimens were subjected to PCR. The results of PCR was identical in all the cases (Table 2).

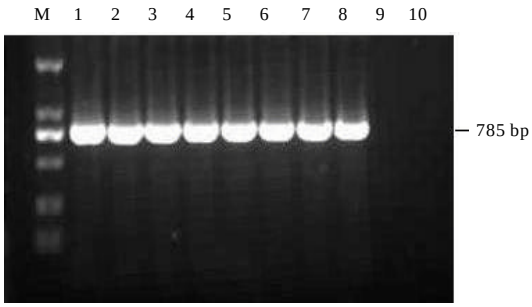

Fig. 6: PCR reaction in positive sample for PCV-2b; M: DL2000 DNA Marker: 1 = No. 1; 2 = No. 2; 3 = No. 3; 4 = No. 4; 5 = No. 5; 6 = No. 6; 7 = No. 7; 8 = No. 8; 9 = No. 9and 10: Negative control

Table 2: Clinical specimens screened with PCR for PCV-2a and PCV-2b virus DNA

No. of samples	No. of PCV-2a and PCV-2b positives	No. of PCV-2b positives	No. of PCV-2a positives
60	32.0	19.0	9.0
100%	53.3%	31.7%	15.0%

DISCUSSION

The global shift from PCV-2a to PCV-2b over time confirms the Danish observations and indicates that PCV-2a could be more adapted or even more pathogenic form of PCV-2. The results suggest that PCV-2a infections were more than PCV-2b in clinical specimens. Epidemiological investigations could be carried out by this PCR assay.

In present study, the sensitivity of PCR assays established was 10^7 viral uL^{-1} (PCV-2a) and 10^6 viral uL^{-1} (PCV-2b), respectively which was high enough for clinical detection. Specific primers were also evaluated for their specificity using PRV, PRRSV, PCV-1 and HPS which can also infect pigs and no specific PCR bands were obtained which demonstrate that the primers we designed were so specific to distinguish the clinical pathogens for pigs as mentioned before.

The positive results of PCR assays for samples sourced from swine farms in south China during the year of 2008-2009 were confirmed again by the PCR assays developed in the present study and positive rates was 53% of 60 samples. Of these positive samples, the infection rates of PCV-2a was 53.3% and PCV-2b was 31.7% indicating that the genotype of PCV-2b was accounted for PMWS in Guangdong province. The PCR assay developed in the study can be used for the molecular epidemiological investigation of PCV-2 infected in swines.

CONCLUSION

In this study, the PCR has the potential to be useful for routine molecular diagnosis and epidemiology.

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