Scientific Research

Development of a drtqPCR Assay for Differentiation of PCV-2a and 2b in Shanghai Area

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Abstract

A dual real-time quantitative polymerase chain reaction assay (drtqPCR) was established to detect and differentiate between Porcine circovirus-2a (PCV-2a) and Porcine circovirus-2b (PCV-2b). Genotype-specific primer sets and probes were designed by using sequence data published for different PCV-2 strains. Specificity and sensitivity of the drtqPCR were examined by using PCV-2 isolates with known genotype. Among 367 tissue samples, 44.69% (164/367) were PCV-2 positive. From 164 PCV-2 positive samples, 10.98% (18/164), 92.56% (137/164), and 3.31% (9/164) were positive for PCV-2a, PCV-2b, and both genotypes, respectively. These results suggest that the differential drtqPCR can be used to detect PCV-2 and to differentiate the 2 genotypes from field samples. The PCV-2 infection is quite common in swine of Shanghai area. Furthermore, the PCV-2b infective ratio is far higher than PCV-2a, and PCV-2a/2b mixed infections are also observed but at a lower prevalence in Shanghai area.

Keywords

Genotype Differentiation, drtqPCR, PCV-2

1. Introduction

Porcine circovirus-2 (PCV-2) is a very small, circular, single stranded DNA virus about 1.76 kb in length [1]. Viral DNA possesses at least 3 functional open reading frames (ORFs): ORF1 encodes the Rep proteins involved in virus replication; ORF2 encodes the nucleocapsid (Cap) protein; and ORF3 encodes a protein that induces apoptosis and is involved in viral pathogenesis *in vivo* [2]. The virus has been associated with several dis-

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ease syndromes in pigs, including postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome, reproductive failure, and porcine respiratory disease complex. These syndromes together are considered porcine circovirus-associated disease (PCVAD) [1] [3].

In general, PCV-2 strains from different geographical areas were also shown to be differentiated into genetic groups 1 and 2. PCV-2 groups 1 and 2 were found to contain 1767 and 1768 nucleotides, respectively [4] [5]. In phylogenetic trees, group 1 viruses were classified in the same cluster of PCV-2b, whereas group 2 viruses were classified in the cluster of PCV-2a. PCV-2 genotypes have been classified by several methods, including viral genomic sequencing, restriction fragment length polymorphism (RFLP) analysis, situ hybridization assay, nested polymerase chain reaction assay, and genotype-specific PCR [6]-[8]. Therefore, a dual real-time quantitative polymerase chain reaction (drtqPCR) assay was developed to detect with high sensitivity PCV-2 strains present in tissue samples of pigs, as well as this PCR assay was able to identify PCV-2 genotypes by targeting the ORF2 gene. Each of these methods has advantages and disadvantages in obtaining the results. It is generally accepted that drtqPCR is less time consuming and labor consuming than RFLP, nPCR, and sequencing methods. In this study, we will use the drtqPCR assay to show the PCV-2a and PCV-2b infection in pigs of Shanghai area.

2. Materials and Methods

2.1. Reference Strains

Download reference strains sequences from GenBank (Table 1). The 12 gene sequences of PCV-2 strains in GenBank were from different countries in North America, Europe, and Asia.

2.2. Primer Sets and Probes Design for drtqPCR

Primer sets that could detect the 2 genotypes of PCV-2 were designed in the ORF2 region. By phylogenetic tree, PCV-2a and PCV-2b are obviously different in 1454-1488 regions; therefore the two probes combining PCV-2a and PCV-2b respectively were designed in the variant regions, and prime sets were designed in the common regions. For example PCV-2a, the forward primer sequences were C-F (1418) 5'-CCA, GAA, TTC, AAC, YTT, AAC, CTT, YCT, TAT-3', and the reverse primer sequences were C-R (1535) 5'-GRC, RGT, GGA, CAT, GMT, GAG, A-3'. The probes were designed in their variant region 1454-1488, so the probe of PCV-2a was C-2a-Probe (1459) FAM-AGG, GTA, TAG, AGA, TTT, TGT, TGG, TCC, CCC, CTC-BHQ, and the probe of PCV-2b was C-2b-Probe (1478) HEX-CAA, ACC, CCC, kCw, CTG, TGC, CCT, TTG, A-BHQ (Y=C or T; M=A or C; R=A or G; K=G or T; W=A or T).

2.3. Porcine Circovirus-2 Recombinant Plasmids

Extraction of KF850469 and KF850463 isolates of DNA was performed using the Qiagen QIAamp DNA Mini Kit according to the manufacturer's instructions. The PCR products encompassing the partial viral genome of both strains were produced using the primers: C-F (5'-CCA, GAA, TTC, AAC, YTT, AAC, CTT, YCT,

Table 1. PCV-2 strains for sequence comparison.

Entry number	Entry time	Genotype	Origin
AB072302	2001	PCV-2a	Japan
AF085695	1998	PCV-2a	Canada
AF264038	2000	PCV-2a	USA
AY180397	2002	PCV-2a	Chinese Taipei
AY322004	2003	PCV-2a	France
DQ104423	2005	PCV-2a	China
EF452365	2007	PCV-2a	USA
AY321984	2003	PCV-2b	France
AY484413	2003	PCV-2b	USA
AY847748	2004	PCV-2b	Singapore
DQ141322	2005	PCV-2b	China
DQ151643	2005	PCV-2b	China

TAT-3'), C-R (5'-GRC, RGT, GGA, CAT, GMT, GAG, A-3'). The PCR products were subsequently cloned in the plasmid vector pMD18-T. Finally, the recombinant plasmids (pMD18-T/PCV-2a and pMD18-T/PCV-2b) were sequenced to verify the accuracy of the nucleotide sequences and were used as DNA templates and positive samples to validate the new drtqPCR diagnostic assay.

2.4. drtqPCR Assays

In the dual reaction, 2 µl of sample containing the DNA template was added to a 28-µl reaction mixture (Final concentration: dATP, dTTP, dGTP and dCTP 0.24 mM respectively; Mg^{2+} 2.4 mM; C-F and C-R 0.32 µM; C-2a-Prob and C-2b-Probe 5 µM; Taq1 U/µL; Taq protection 0.2 U/µL). The PCR reaction started with an initial denaturation and polymerase-activating step of 94°C for 3 min, followed by 40 amplification cycles of a 3-step PCR (94°C for 15 sec; 60°C for 40 sec and detecting fluorescent light). Fluorescence channels are FAM and HEX.

2.5. Standard Curves

The standard curve were assayed for assessing the stability of the drtqPCR. The recombinant plasmids pMD18-T/PCV-2a and pMD18-T/PCV-2b were 10-fold serial diluted with the original concentrations 5.4 ng/µl and 7.2 ng/µl by Thermo NANODROP 2000 respectively.

2.6. Sensitivity and Specificity

To assess the sensitivity and specificity of the drtqPCR assay and to determine its ability to differentiate the 2 PCV-2 genotypes, 10-fold serial dilutions of pMD18-T/PCV-2a and pMD18-T/PCV-2b were assayed. The plasmids were then quantified by Thermo NANODROP 2000. Furthermore, all 12 Shanghai PCV-2 isolates (PCV-2a: KF850458, KF850466, and KF850468; PCV-2b: KF850459, KF850460, KF850461, KF850462, KF850463, KF850464, KF850465, KF850467, and KF850469) were tested by drtqPCR. The specificities of the primer-probe sets were determined by testing several specimens from different animals, bacterial DNA, and nucleic acids of swine viruses. The samples consisted of PCV-2-negative porcine tissues, semen, and sera, as well as bovine tissues and various pathogens isolated by the SCADC diagnostic laboratory, such as *Escherichia coli*, *Haemophilus parasuis*, *Lawsonia intracellularis*, *Pasteurella multocida*, *Salmonella* spp., *Staphylococcus hyicus*, *Streptococcus suis*, porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), swine influenza virus (SIV) H_9N_2, porcine pseudorabies virus (PRV), PCV-1, porcine parvovirus (PPV).

2.7. Application of drtqPCR Assay on Tissue Samples

A total of 367 tissue samples from pigs were randomly selected from Shanghai swine farms. 173 samples were from dead pigs tissue for disease, and 194 samples were healthy pigs tissue (Table 2).

3. Results

3.1. Standard Curves

The standard curves of drtqPCR were performed, and R^2 were 0.998 for PCV-2a and 0.984 for PCV-2b. The slope values were −3.578 and −3.477, and calculated E value were 90.3% and 93.9% respectively (Figure 1).

3.2. Sensitivity of drtqPCR

The analytical sensitivity of the drtqPCR was examined by 10-fold serially diluted plasmid of pMD18-T/PCV-2a and pMD18-T/PCV-2b. The PCV-2a with an original concentrations 5.4 ng/µl was detected up to 10^{-6} dilution, and the PCV-2a sensitivity was 5.4 fg/µl. The PCV-2b with an original concentrations 7.2 ng/µl was up to 10^{-5} dilution, and the PCV-2b sensitivity was 0.72 fg/µl (Figure 2).

Table 2. Origin of samples.

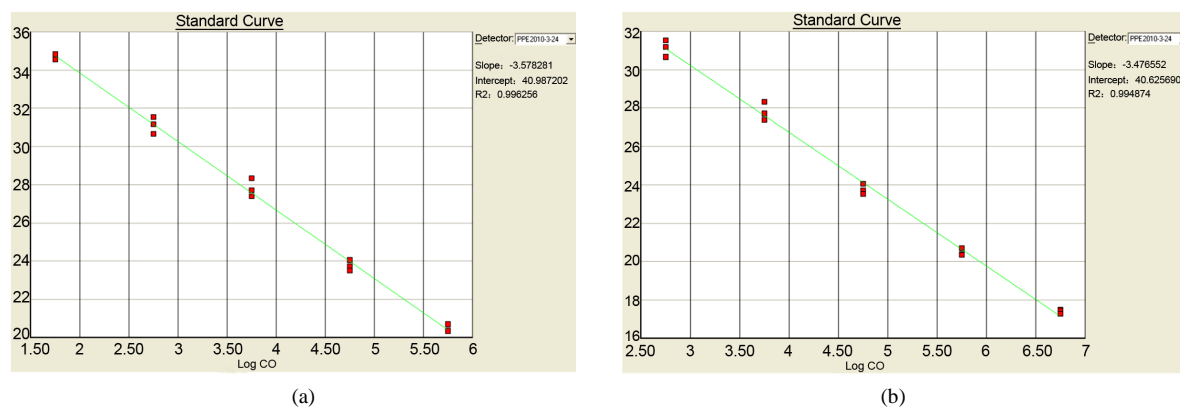| Years	Dead for diseases	Healthy	Total
2009	24	10	34
2010	111	53	164
2011	38	131	169
Total	173	194	367

Figure 1. The standard curves of drtqPCR. (a) PCV-2a: R^2 0.998, slope value −3.578, E value 90.3%; (b) PCV-2b: R^2 0.984, slope value −3.477, E value 93.9%. (a) The standard curves of drtqPCR in PCV-2a; (b) The standard curves of drtqPCR in PCV-2b.
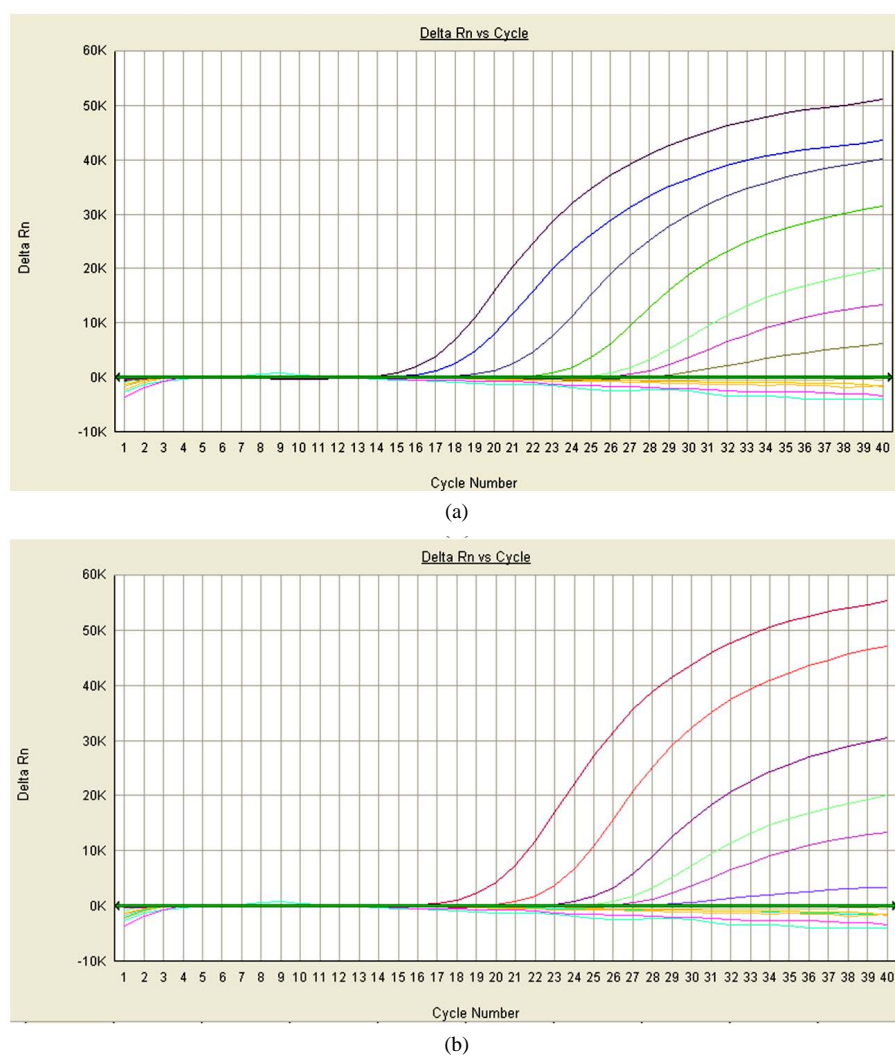

(a)

(b)

Figure 2. Detection of pMD18-T/PCV-2a and pMD18-T/PCV-2b with the original concentrations 5.4 ng/μl and 7.2 ng/μl respectively. (a) PCV-2a; form left to right: 10^{-1} - 10^{-12} dilution of pMD18-T/ PCV-2a; (b) PCV-2b; form left to right: 10^{-1} - 10^{-12} dilution of pMD18-T/PCV-2b. (a) The sensitivity of drtqPCR in PCV-2a; (b) The sensitivity of drtqPCR in PCV-2b.

3.3. Specificity of drtqPCR

The drtqPCR had not appeared positive curves in detecting samples except for PCV-2a/2b positive isolates (PCV-2a (KF850468), PCV-2b (KF850463)) (**Figure 3**). The samples consisted of PCV-2a/2b positive isolates, PCV-2-negative porcine tissues, semen, sera, and various pathogens isolated by the SCADC diagnostic laboratory, such as *Escherichia coli*, *Haemophilus parasuis*, *Salmonella* spp., *Staphylococcus hyicus*, *Streptococcus suis*, porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), porcine pseudorabies virus (PRV), PCV-1, porcine parvovirus (PPV), and uninfected porcine kidney (PK)-15 cells. The positive curves only occurred in PCV-2a/2b positive isolates, and no nonspecific curve appeared in other samples.

3.4. PCV-2 Results of Samples

164 samples were PCV-2 positive in 367 tissue samples. From 2009 to 2011, the positive ratio in PCVAD samples was 54.17%, 54.05%, 65.79% respectively, and the positive ratio in healthy samples was 20.00%, 22.64%, 39.69% (**Table 3**).
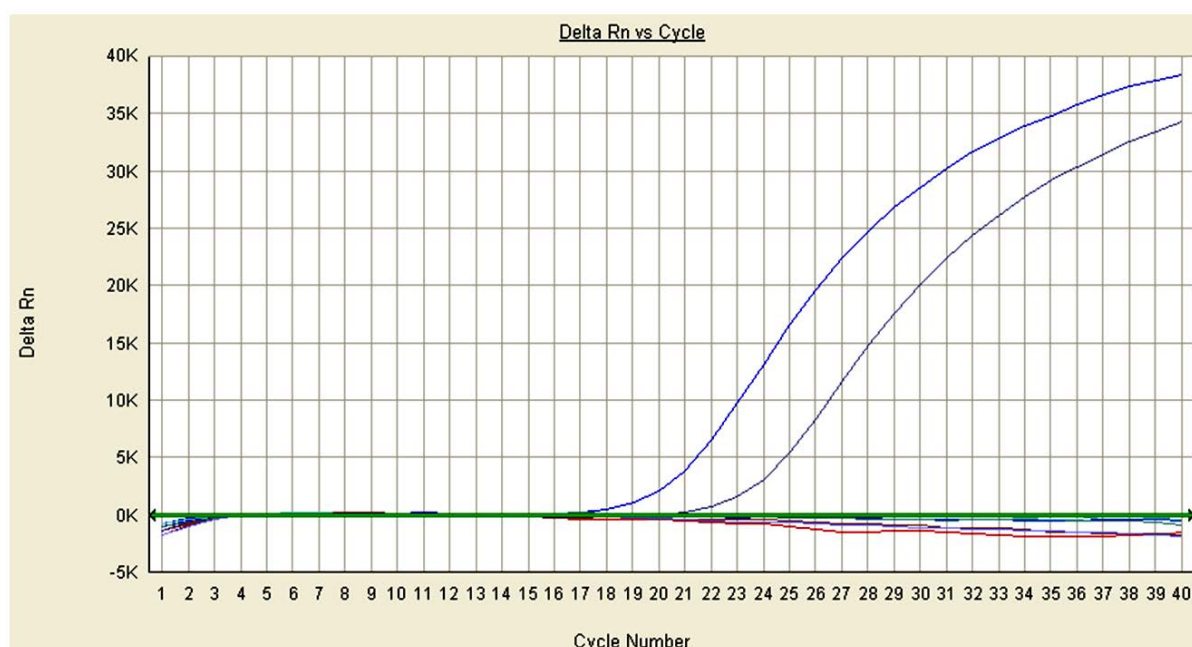

Figure 3. The specificity of drtqPCR. Detecting samples including:PCV-2a (KF850468), PCV-2b (KF850463), PCV-2-negative porcine tissues, semen, sera, *Escherichia coli*, *Haemophilus parasuis*, *Salmonella* spp., *Staphylococcus hyicus*, *Streptococcus suis*, PRRSV, CSFV, PRV, PCV-1, PPV, uninfected porcine kidney (PK)-15 cell. Positive curves were only PCV-2a (right) and PCV-2b (left).

Table 3. PCV-2 results of samples.

Years	Dead for diseases			Healthy			Total		
	Total samples	Positive amount	Positive ratio	Total samples	Positive amount	Positive ratio	Total samples	Positive amount	Positive ratio
2009	24	13	54.17%	10	2	20.00%	34	15	44.12%
2010	111	60	54.05%	53	12	22.64%	164	72	43.90%
2011	38	25	65.79%	131	52	39.69%	169	77	45.56%
Total	173	98	54.34%	194	66	34.02%	367	164	44.69%

3.5. PCV-2a/2b in PCV-2 Positive Samples

From 2009 to 2011, PCV-2a single positive ratio was 13.33%, 4.17% and 16.88% respectively, PCV-2b single positive ratio was 86.67%, 91.67% and 75.32%, and PCV-2a/2b co-infection ratio was 0%, 4.17% and 7.79% (**Table 4**).

3.6. PCV-2a/2b in PCV-2 Positive Samples from Dead for Disease

In PCV-2 positive samples from dead for disease, the PCV-2a single positive ratio was 7.69%, 3.57% and 0 in 2009, 2010 and 2011 respectively, the PCV-2b single positive ratio was 92.31%, 91.07% and 88.00%, and the PCV-2a/2b co-infected ratio was 0, 5.36% and 12.00% (**Table 5**).

3.7. PCV-2a/2b in PCV-2 Positive Samples from Healthy Swine Farms

In healthy swine farms samples, the PCV-2a single positive ratio was 50.00%, 12.50% and 23.08% in 2009, 2010 and 2011 respectively, the PCV-2b single positive ratio was 50.00%, 87.50% and 71.15%, and the PCV-2a/2b co-infected ratio was 0, 0 and 5.77% (**Table 6**).

4. Discussion

Porcine circovirus-2 is now considered a global disease and the cause of significant economic losses in the swine industry [9]. Nonetheless, it strongly suggests that PCV-2b virus could be more virulent than PCV-2a virus. Moreover, others have reported that genetic variations may have a great impact on the virulence of PCV-2 [10]. One of the main objectives of the swine industry and health scientists is to establish if there is virulence variation between PCV-2a and PCV-2b viruses. Consequently, it has become critical for swine producers and veterinarians to establish which PCV-2 genotypes are present in their herds [11]. In the present study, the established drtqPCR was believed that it combines specificity and sensitivity, and reduces the labor and time

Table 4. PCV-2a/2b differential results in PCV-2 positive samples.

Years	PCV-2a single positive ratio	PCV-2b single positive ratio	PCV-2a/2b co-infected ratio	Total
2009	13.33% (2/15)	86.67% (13/15)	0 (0/15)	15
2010	4.17% (3/72)	91.67% (66/72)	4.17% (3/72)	72
2011	16.88% (13/77)	75.32% (58/77)	7.79% (6/77)	77
Total	10.98% (18/164)	83.54% (137/164)	5.49% (9/164)	164

Table 5. PCV-2a/2b in PCV-2 positive samples from dead for disease.

Years	PCV-2a single positive ratio	PCV-2b single positive ratio	PCV-2a/2b co-infected ratio	Total
2009	7.69% (1/13)	92.31% (12/13)	0 (0/13)	13
2010	3.57% (2/56)	91.07% (51/56)	5.36% (3/56)	56
2011	0 (0/25)	88.00% (22/25)	12.00% (3/25)	25
Total	3.19% (3/94)	90.43% (85/94)	6.38% (6/94)	94

Table 6. PCV-2a/2b in PCV-2 positive samples from healthy swine farms.

Years	PCV-2a single positive ratio	PCV-2b single positive ratio	PCV-2a/2b co-infected ratio	Total
2009	50.00% (1/2)	50.00% (1/2)	0 (0/2)	2
2010	12.50% (2/16)	87.50% (14/16)	0 (0/16)	16
2011	23.08% (12/52)	71.15% (37/52)	5.77% (3/52)	52
Total	21.43% (15/70)	74.29% (52/70)	4.29% (3/70)	70

intensiveness of diagnosing PCV-2a and PCV-2b. The drtqPCR was verified by various samples in this study.

In the present study, the ratio of PCV-2 in dead pigs was 54.34%, as 34.02% in healthy. The ratio in dead pigs was higher than in healthy, and both of them raised in recent years of Shanghai area. This suggests that dead pigs for infectious diseases was related of PCV-2, but not all pigs infected with PCV-2 virus may lead to death, and pigs infected PCV-2a/2b could be no any clinics. 9 PCV-2a/2b co-infections were also observed but at a lower prevalence in Shanghai area (2.45%). It was remarkable that the co-infection ratio was rising in recent years. The ratio of PCV-2b was much higher than PCV-2a. The ratio of PCV-2a/2b co-infected rose from 2009 to 2011, whereas PCV-2a was low. This suggests PCV-2b was the most viruses in Shanghai area, and PCV-2a/2b co-infected would impact the farms of Shanghai in the future.

5. Conclusion

In conclusion, the new drtqPCR diagnostic assay is suitable for the sensitive identification and differentiation of PCV-2 and is a more convenient approach than RFLP [12], partial or entire viral genome sequencing, and conventional PCR techniques [13]. Furthermore, it could efficiently quantify PCV-2 in submitted samples and provide a risk assessment in regard to the odds of developing death.

Acknowledgements

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