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Development of an EvaGreen-based multiplex real-time PCR assay with melting curve analysis for simultaneous detection and differentiation of six viral pathogens of porcine reproductive and respiratory disorder

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A B S T R A C T

Concurrent infection of pigs with two or more pathogens is common in pigs under intensive rearing conditions. Porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), Japanese encephalitis virus (JEV) and pseudorabies virus (PRV) are all associated with reproductive or respiratory disorders or both and can cause significant economic losses in pig production worldwide. An EvaGreen-based multiplex real-time PCR (EG-mPCR) with melting curve analysis was developed in this study for simultaneous detection and differentiation of these six viruses in pigs. This method is able to detect and distinguish PCV2, PPV, PRRSV, CSFV, JEV and PRV with the limits of detection ranging from 100 to 500 copies/μL, high reproducibility, and intra-assay and inter-assay variation ranging from 0.11 to 3.20%. After validation, a total of 118 field samples were tested by the newly developed EG-mPCR. PCV2 was identified in 23%, PPV in 15%, PRRSV in 17% and PRV in 5% of the samples. Concurrent PCV2 and PRRSV infection was detected in 6.7%, PCV2 and PPV in 5% and PPV2 and PRRSV infection was detected in 5% of the cases. The agreement of the EG-mPCR and conventional PCR tests was 99.2%. This EG-mPCR will be a useful, rapid, reliable and cost–effective alternative for routine surveillance testing of viral infections in pigs.

1. Introduction

It is common for pigs to be infected concurrently with two or more viral pathogens with the development of scale production units and intensive pig rearing (Ogawa et al., 2009). Porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), Japanese encephalitis virus (JEV) and pseudorabies virus (PRV) are considered the primary agents associated with reproductive or respiratory disorders or both and frequently occur concurrently. Occurrence of these pathogens is commonly closely linked to economic losses to swine production in many parts of the world (Wu et al., 2013). Clinical signs are variable or non-specific making a primary diagnosis often difficult (Caliendo, 2011). It is therefore important to develop an effective and reliable approach to demonstrate multiple pathogens with one single assay for epidemiological surveillance and disease management.

Conventional gel-based multiplex PCR technology for detecting simultaneously several viruses in a single sample is only possible by working with different fragment sizes and separation by electrophoresis (Xu et al., 2012). Conventional gel-based PCR that is not only labor intensive and time consuming, but also has the risk of potential PCR product carry-over contamination (Barletta et al., 2013). At present these limitations can be overcome by using a multiplex real-time PCR, due to absence of post-amplification procedures, allowing rapid analysis with a greater sample throughput (Garrido et al., 2012). The multiplex real-time PCR namely integrates fluorescence(s) with a multiplex PCR, which are generally classified into fluorescent-probe-based assays and dsDNA-binding fluorescent-dye-based assays.

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Table 1
Primers designed for PCV2, PPV, PRRSV, CSFV, JEV and PRV standard construct and primers for detection of six swine viruses by an EvaGreen-based multiplex real-time PCR.

Primer	Sequence (5′–3′)	Final concn. (μM)	Amplicon position	Amplicon size (bp)	Reference strain
Primers for standard construct and conventional PCR					
PCV2-F	CGAGAAAGCGAAAGGAACMGA	0.20	299–669	371	GQ996404
PCV2-R	GGTAACCATCCCACCACTT				
PPV-F	AAAGTTAGAATAGGATGCGAGGAA	0.20	1759–2469	711	EU790642
PPV-R	GCGGCGTCTGATGGATTA				
PRRSV-F	GAGTTTCAGCGGAACAATGG	0.20	14,269–14,719	451	JX317648
PRRSV-R	GCCGTTGACCGTAGTGGAG				
CSFV-F	GCTCCCTGGGTGGTCTAAG	0.20	148–390	243	AY805221
CSFV-R	AGTGATTCAACTCCATGTGCC				
JEV-F	AAGCAAGCACAATCGGAGG	0.20	3230–3691	462	JN604986
JEV-R	TCAGTGTAAGTGATACCCC				
PRV-F	CGCACCTGCTGTACTTTATCG	0.20	317–667	351	EF645837
PRV-R	GCGTCAGGAATCGCATCA				
Primers for multiplex real-time PCR detection					
PCV2-F	CGAGAAAGCGAAAGGAA	0.26	299–369	71	
PCV2-R	ACTCGATCAGTAAGTTGCC				
PPV-F	AGTTAGAATAGGATGCGAGGAA	0.12	1761–1863	103	
PPV-R	AAGTCCAAAATCACCTGGC				
PRRSV-F	ATGATAGCACAGCTCCACAGAA	0.14	14313–14400	88	
PRRSV-R	CCGCGACTTACCTTTAGAGC				
CSFV-F	GCTCCCTGGGTGGTCTAAG	0.13	148–237	90	
CSFV-R	CTCGTCCACGTAGCATCTCG				
JEV-F	CAGGGACCTTGGGATGAGA	0.06	3270–3472	203	
JEV-R	CCGTACCAGCAGCCATTTT				
PRV-F	CGACGGCGTGAACATCCT	0.13	510–606	97	
PRV-R	GAACTTGTACGTGCGGTGCT				

Compared to dye-based assays, probe-based assays are expensive, and this assay type cannot be used for multiplexing five reactions due to lack of availability of five compatible dyes for use with the current available real-time PCR instruments (Agindotan et al., 2007). However, a dye-based multiplex real-time PCR can satisfy the need to detect more than five targets simultaneously. The most widely used dye that has been utilized successfully in multiplex system is SYBR Green I (SG) (Zheng et al., 2012; Chai et al., 2013), but problems that include inhibition of the PCR reacting, preferential binding to GC-rich sequences, and an effect on melting curve analysis have been reported previously (Gudnason et al., 2007) and could limit insurance of similar or equal amplification efficiency between different templates. EvaGreen (EG), a new saturated dye that intercalated every single nucleotide of the double-stranded DNA, has been shown to be more suitable than SG for multiplex real-time PCR because it is highly sensitive and extremely stable (Khan et al., 2011). The EG-based PCR assay has been proven to be a flexible and reliable approach that ensured reaction specificity and melt peaks of different amplicons which could be obviously identified using melting curve analysis (Cheng et al., 2013).

The objective of the present study was to develop a novel EG-based multiplex real-time PCR (EG-mPCR) that incorporated multiple primers from several viruses followed by melting curve analysis of the amplicons to simultaneously detect and differentiate PCV2, PPV, PRRSV, CSFV, JEV and PRV in a single-tube run. Furthermore, the potential application of the assay in diagnosis was assessed on clinical samples.

2. Materials and methods

2.1. Viruses

PPV vaccine (no. 0040401) was purchased from Beijing Zhonghai Animal Health Science and Technology Co., Ltd. and inactivated PRV and JEV vaccines were supplied by Wuhan Keqian Animal Biological Products (no. 090206 and no. 081138, Wuhan, China). PRRSV (DQ269472), CSFV (Hangzhou strain) and PCV2 (GQ996404) were maintained in the authors' laboratory. The negative controls consisted of PCV1 (HZ2006, EF533941), transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV) vaccine (Harbin Weike Biotechnology Development Company, Harbin, China, no. 030718) and porcine bocavirus (PBoV) (maintained in the authors' laboratory).

2.2. Clinical samples

Sixty tissue samples (lymph nodes, lungs, livers and kidneys) were collected from five pork markets in Zhejiang Province, China, and 58 serum samples were collected from clinically healthy pigs on different pig farms in several provinces of China during 2009–2012. The animal experiments were performed in accordance with international standards for animal welfare.

2.3. Nucleic acid extraction

Viral genomic DNA and RNA were simultaneously extracted from vaccines, virus strains or from clinical specimens using RNA/DNA Extraction Kit Ver. 3.0 (TaKaRa, Dalian, China) according to the manufacturer's protocol.

2.4. Primer design

All of the genomic sequences of the viruses utilized in this study were derived from GenBank nucleotide sequence database. The highly conserved regions within each virus genome were aligned with Clustal-W (DNAStar Inc., Madison, WI, USA). Primers corresponding to conserved regions the viral genomes were designed using Primer Premier 5.0 (Primer Biosoft International, Palo Alto, CA, USA). Six pairs of primers were designed to amplify PCV2, PPV, PRRSV, CSFV, JEV or PRV and are outlined in Table 1. The amplicons were used to construct standard plasmid templates and conventional PCR assay. Another six pairs of primers were selected within the range of the amplicons that were capable to amplify all six selected swine viruses simultaneously in an EG-mPCR reaction (Table 1). Successful detection of a virus was determined mainly based on the amplicon T_m value, which was considered the outcome of the assay. For optimal reaction conditions, it was important to adjust the annealing temperature of