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Simultaneous typing of seven porcine pathogens by multiplex PCR with a GeXP analyser

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A novel high-throughput method was developed for simultaneous detection and differentiation of seven porcine pathogens by multiplex PCR based on a GenomeLab Gene Expression Profiler (GeXP) analyser. The pathogens included in this study were pseudorabies virus (PRV), classical swine fever virus (CSFV), African swine fever virus (ASFV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), porcine circovirus type 2 (PCV-2) and Japanese encephalitis virus (JEV). Seven pairs of chimeric primers, consisting of a pathogen-specific sequence fused to a universal sequence at the 5′ end, were used to initiate the PCR, after which a set of universal primers was used for the subsequent cycles of the PCR. Amplification products were separated by capillary electrophoresis and identified using fluorescence spectrophotometry. The specificity of the GeXP assay was examined with single and mixed pathogen cDNA/DNA templates. The specific DNA product amplification peaks of seven pathogens were observed on the GeXP analyser. Negative controls did not produce DNA products. The sensitivity was evaluated by performing the assay on serial 10-fold dilutions of the plasmids containing the target sequence. Under optimised conditions this assay achieved a sensitivity of 100–1000 copies/μL for a single virus and 1000 copies/μL when all of the seven pre-mixed viral targets were present. Furthermore, the GeXP-PCR assay was 100% specific when 58 clinical samples were tested in comparison with the conventional PCR method. In conclusion, the GeXP assay is a rapid, cost-effective, sensitive, specific and high throughput method for simultaneously detecting seven pathogens that infect swine.

1. Introduction

Pseudorabies virus (PRV), classical swine fever virus (CSFV), African swine fever virus (ASFV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), porcine circovirus type 2 (PCV-2) and Japanese encephalitis virus (JEV) are seven key porcine reproductive pathogens. These viruses cause diseases in pigs with high morbidity and mortality and generate serious economic losses to the swine industry. Furthermore, CSFV, PRRSV, PCV2, PRV, and PPV can also cause immunosuppression creating conditions for secondary illnesses or complications with other pathogens. ASFV is the only known DNA arbovirus and the clinical signs of ASF can be confused with CSF (King et al., 2003). Even though ASF has not been found in China, it is important to develop rapid methods for diagnosing this disease and distinguishing it from other diseases of swine.

Identification and differentiation of porcine reproductive diseases via isolation of the pathogenic agents or by using differential serological tests can be labour intensive and time-consuming procedure. Molecular typing methods have been developed and are currently used for rapid detection and identification of porcine reproductive pathogens (Giridharan et al., 2005; Jiang et al., 2010; Wernike et al., 2013). Although useful, most molecular methods are limited to the detection of a few pathogens in one test tube. Therefore, it may be advantageous to develop a rapid, cost effective and high-throughput method to detect and distinguish reproductive pathogens in one reaction, particularly in swine where simultaneously infected with two or more pathogens under typical

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conditions of intensive swine production (Ogawa et al., 2009). In this study, a multiplex PCR was established and optimised with a GeXP analyser for simultaneous typing of seven porcine reproductive pathogens.

The GenomeLab Gene Expression Profiler (GeXP) analyser (Beckman Coulter, Brea, CA, USA) provides an alternative technology for high-throughput multiplexed quantitative gene expression. The system can detect and quantify up to 35 molecular targets in up to 192 samples within a single reaction (Rai et al., 2009; Ismail et al., 2010). The analytical procedure includes modified reverse transcription and PCR amplification steps, followed by capillary electrophoretic separation. Compared to other multiple detection methods, the GeXP-PCR assay provides high sensitivity and specificity (Yang et al., 2012). GeXP has been successfully used in the study of drug toxicity mechanisms (Hu et al., 2013) and gene expression regulation (Biswas et al., 2013; Rasoli et al., 2014; Zhang et al., 2013), as well as the early diagnosis of cancer research (Huang et al., 2013; Foo et al., 2014) and pathogen detection (Zhou et al., 2013; Xie et al., 2014).

2. Materials and methods

2.1. Pathogens, nucleic acid extraction and reverse transcription

The gene fragments P72 of ASFV were synthesised in Chengdu, China. Porcine reproductive pathogen reference strains, field isolates and other non-reproductive pathogens used in this study are described in Table 1. All vaccines were from commercial sources.

Viral genomic DNA and RNA were extracted from the specimens using the TIANamp Virus RNA/DNA Kit following the manufacturer's protocol (TIANamp, Tiangen Biochemical Technology, Beijing, China). Reverse transcription was performed with the Prime Script™ RT Reagent Kit (Perfect Real Time) at a final volume of 10 μL containing 2 μL of 5× PrimeScript buffer (including dNTP Mixture and Mg^+), 0.5 μL PrimeScript RT Enzyme Mix I (containing RNase Inhibitor), 25 pmol Oligo dT Primer, 200 pmol random 6 mers, 3 μL template, and 2 μL RNase Free dH_2O. After incubation for 15 min at 37 °C, the mixture was heated for 5 s at 85 °C for inactivation of reverse transcriptase. The mixture was then chilled on ice and the DNA/cDNA was stored at −20 °C until tested.

2.2. Clinical samples

Fifty-eight clinical specimens, including lymph nodes, spleen, lung and tonsil tissues, were collected from piglets with reproductive problems from local farms in Sichuan Province, China, during January 2013 to April 2015, and were stored at −20 °C (Table 1). These samples were tested using the optimised GeXP-multiplex PCR assay. All of the clinical samples tested positive for PRV (GB/T 18641-2002), CSFV (GB/T 16551-2008), PRRSV (GB/T 27517-2011), PPV (SN/T 1874-2007), PCV2 (GB/T 21674-2008), or JEV (GB/T 22333-2008).

2.3. GeXP primer design

This assay include one pair of universal primers (TagF: 5′-AGGTGACACTATAGAATA-3′. TagR: 5′-GTACGACTCACTATAGGGA-3′) and seven pairs of chimeric primers (a pathogen-specific sequence fused at the 5′ end to the universal sequence). The forward universal primer was Cy5-labeled at the 5′ end of the sequence. Seven pairs of chimeric primers were designed using GenomeLab GeXP eXpress Profiler software (Beckman Coulter, Brea, CA, USA) on the basis of the multiple-sequence alignments. Gene sequences of seven viruses were obtained through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and were aligned by using Clustalx software. Pathogen-specific primers were designed in a highly conserved region and were evaluated using the NCBI Primer-Blast and Primer Premier 5.0 software. The primer sequences, their target genes and the size of the resulting amplicons are summarised in Table 2. All primers were obtained from Sangon (Shanghai, China).

2.4. Plasmids

Seven specific targets (without the universal sequence) of each porcine virus were amplified using the primers listed in Table 2, the specific amplified DNA (amplicon) for each pathogen was extracted from agarose gels using the E.Z.N.A.™ Gel Extraction kit, following the manufacturer's protocol (OMEGA). Each specific viral target fragment was cloned into the plasmid pMD19-T (TaKaRa). Constructed plasmids were transformed in *Escherichia coli*. The isolation of plasmids was carried out using a plasmid extraction kit (Sangon). The recombinant plasmid DNAs obtained were sequenced. Sequence data were analysed using DNASTAR software and were compared with corresponding sequence data in GenBank to confirm the identity of the cloned fragments.

2.5. Mono GeXP-PCR assay

The mono GeXP assay was developed with DNA/cDNA to evaluate the specificity of each pair of pathogen-specific primers and to ascertain the actual amplicon size of each target region. Nuclease-free sterile distilled water was used as a negative control throughout the assay. The GeXP-PCR assay contained 0.5 μL of DNA/cDNA, 12.5 μL of 2 × Ex Taq, 20 μM each of the forward universal primer and reverse universal primer, and 1 μM each of the forward chimeric primer and reverse chimeric primer. DNAse-free water was added to the PCR reaction to a final volume of 25 μL. The PCR mixture was incubated at 95 °C for 5 min, followed by two steps with different annealing temperatures: step 1, 12 PCR cycles of 30 s at 95 °C for, 30 s at 60 °C, and 25 s at 72 °C; step 2, 20 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 25 s; 5 min at 72 °C, before then being held at 4 °C in a thermal cycler. After amplification, 1 μL of PCR product that was diluted tenfold was added to a 38.75 μL of sample loading solution, along with 0.25 μL of DNA size standard-400 (GenomeLab GeXP Start Kit; Beckman Coulter). Finally, a drop of paraffin oil was added after blending. The GeXP system was then used to analyse PCR products based on size using high-resolution capillary gel electrophoresis. The peak height for each PCR target was reported in the electropherograms and the reaction was considered positive when the dye signal was greater than 2000 arbitrary units (A.U.).

2.6. Establish and optimise multiple GeXP-PCR system

Plasmid constructs containing specific viral target fragments were used as templates for the optimisation of multiple GeXP-PCR. The assay conditions for these experiments were optimised by varying single parameters, while all other assay parameters were unchanged. According to the results of orthogonal experiments to select the optimal proportion of primers, the effects of annealing temperature (chimeric primers from 50 °C to 62 °C) and other reaction conditions were also optimised. After amplification, 1 μL of PCR product, diluted tenfold, was added to 37.75 μL of sample loading solution along with 0.25 μL of DNA size standard-400 (GenomeLab GeXP Start Kit; Beckman Coulter, Fullerton, CA, USA). The GeXP system was used to analyse PCR products.

2.7. Specificity and sensitivity of the multiple GeXP-PCR assay

To determine the specificity of the GeXP-PCR assay, different samples of each virus (except ASFV), uninfected samples and other