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Real-time RPA assay for rapid detection and differentiation of wild-type pseudorabies and gE-deleted vaccine viruses

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ABSTRACT

The objective of this study was to develop a dual real-time recombinase polymerase amplification (RPA) assay using exo probes for the detection and differentiation of pseudorabies virus (PRV). Specific RPA primers and probes were designed for gB and gE genes of PRV within the conserved region of viral genome. The reaction process can be completed in 20 min at 39 °C. The dual real-time RPA assay performed in the single tube was capable of specific detecting and differentiating of the wild-type PRV and gE-deleted vaccine strains, without cross-reactions with other non-targeted pig viruses. The analytical sensitivity of the assay was 10^2 copies for gB and gE genes. The dual real-time RPA demonstrated a 100% diagnostic agreement with the real-time PCR on 4 PRV strains and 37 clinical samples. Through the linear regression analysis, the R^2 value of the real-time RPA and the real-time PCR for gB and gE was 0.983 and 0.992, respectively. The dual real-time RPA assay provides an alternative useful tool for rapid, simple, and reliable detection and differentiation of PRV, especially in remote and rural areas.

Introduction

Pseudorabies (PR), also known as Aujeszky's disease, is an economically important viral disease of pigs and other animals in many countries. The disease is caused by Pseudorabies virus (RPV), an enveloped double strand DNA virus of the genus Varicellovirus, subfamily Alphahepersvirinae, and family Herpesviridae [1]. Pigs are the natural host and reservoir of PRV and the only species that can survive infection with the virus [2]. PRV infects pigs at various production phases and causes high mortality rates and nervous system disorders in newborn piglets, respiratory disorders in older pigs, and reproductive failure in pregnant sows [3]. In addition to infecting pigs, PRV can infect numerous other species of mammals, including ruminants, carnivores and rodents, and the infection is normally fatal [1].

Bartha-K61 is the most popular attenuated strain to produce PR vaccines because of the lack of the gE gene [4]. Although PRV vaccine containing attenuated virus prevents the expression of clinical signs, such vaccination does not eliminate wild-type PRV in previously infected pigs nor does it prevent subsequent infection by wild-type strains [5]. PRV can establish a lifelong infection in infected pigs, and once activated, these latent PRV infections could cause the spread of the wild-type virus [6]. Since late 2011, PRV variants with high pathogenicity have emerged in a large number of Bartha-K61-vaccinated swine herds in many regions of China, and the infections have caused significant economic impact to the swine production [3]. Therefore, it is very important to develop a simple and rapid diagnostic tool to identify pigs infected with wild-type PRV or immunized with the PRV gE-deleted vaccine.

Different methods based on DNA amplification have been developed for the detection and differentiation of PRV, including real-time polymerase chain reaction (PCR) [7], loop-mediated isothermal amplification (LAMP) [8] and nanoparticle-assisted PCR (nanoPCR) [5]. However, the developed real-time PCR assay depends on specialized and expensive equipment, and the developed LAMP and nanoPCR assay depend on agarose gel electrophoresis. As a result, it is difficult for these assays to be used for the detection and differentiation of PRV in the field. Recombinase polymerase amplification (RPA), an isothermal gene amplification method [9], has been developed for rapid detection of many pathogens and considered to be the most applicable approach for the field and point-of-care diagnosis of infectious diseases [10]. Yang et al. had developed the real-time RPA assay and RPA lateral flow dipstick (LFD) assay to detect PRV [11], but the assays were based on the gD gene and could not differentiate the wild-type PRV and vaccine strain.

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In this study, we developed a dual real-time RPA assay for rapid detection and differentiation of wild-type PRV and gE-deleted vaccine strain. Real-time detection of the RPA amplification depends on the exo probes and the fluorescence signal was measured in real-time via a scanner Genie III (OptiGene Limited, West Sussex, UK) which has a two-color fluorescence excitation and detection system. Based on two different fluorescence signals derived from two differently labeled exo probes, the method can differentiate PRV strains in one tube simultaneously. The Genie III weighs only 1.75 kg and incorporates a rechargeable battery that can support operation for a complete day, making it suitable for point of care testing.

Materials and methods

Virus strains and clinical samples

Pseudorabies virus (PRV, strains Fa, SH151218 and NQY160308), porcine circovirus-2 (PCV-2, strain HB-MC1) [12], respiratory and reproductive syndrome virus (PRRSV, strain HB-Xl) [13] and encephalomyocarditis virus (EMCV, strain BD) [14] were kept in our laboratory. PRV strains SH151218 and NQY160308 were emergent PRV variants isolated from the Bartha-K61–vaccinated piglets in Hebei Province, China (unpublished data). PRV (strain Bartha-K61), classical swine fever virus (CSFV, strain AV1412) and porcine parvovirus (PPV, strain BJ-2) were all from the commercial attenuated live vaccine (Ringpu Bio-Pharmacy, Baoding, China).

Seventeen clinical samples (cerebrum, lymph node, lung, kidney and whole blood) were collected from the dead piglets and the dead raccoon dogs in Hebei Province in 2016. The piglets were 3–5 days old, and the raccoon dogs exhibited the typical clinical manifestations of pseudorabies and died after consumption of the dead piglets. The other 20 clinical samples (cerebrum, lymph node) were collected from the clinically healthy pigs from the slaughterhouse. For the tissue samples, 10 mg of each sample was homogenized in 1 ml sterile phosphate-buffered saline (PBS). After centrifugation at 3,000 g for 10 min, the supernatant was collected for viral DNA extraction. For the whole blood sample, 1 ml of blood was used for viral DNA extraction.

DNA/RNA extraction and RNA reverse transcription

Viral DNA and RNA were extracted using the TIANamp Virus genomic DNA/RNA kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. 100 ng of viral RNA was reverse transcripted to cDNA using the Primescript II 1st strand cDNA Synthesis kit (Takara, Dalian, China) according to the manufacturer's instructions. Viral DNA and cDNA were quantified using a ND-2000c spectrophotometer (Wilmington, USA). All DNA and cDNA templates were stored at −80 °C until needed.

RPA primers and exo probe

Because gE gene has been deleted from all commercial PRV vaccines and gB gene was highly conserved in the PRV genome, two sets of primers and exo probes specific to certain regions of gB and gE genes were designed following RPA manufacturer guidelines (TwistDx Inc. Cambridge, UK) for general detection (gB) and differentiation (gE) of field and vaccine strains of PRV based on sequence information available in GenBank. The sequence of all the primers and exo probes were summarized in Table 1, and all the primers and probes were synthesized by Sangon (Sangon Biotech, Shanghai, China).

Generation of standard DNA

The complete coding sequences of the gB and gE genes were amplified from the PRV strain Fa genomic DNA using pRVgB-F/pRVgB-R and pRVgE-F/pRVgE-R as primers, respectively (Table 1), and then inserted into the vector pMD19-T (TaKaRa, Dalian, China) for standards. The resulting pRV-gB and pRV-gE were amplified in E. coli DH5α, purified with the SanPrep Plasmid MiniPrep Kit (Sangon Biotech, Shanghai, China) and quantified using a ND-2000c spectrophotometer (NanoDrop, Wilmington, USA). The copy number was calculated by the following formula [15]: Amount (copies/μL = [DNA concentration (g/μL)/(plasmid length × 660)] × 6.02 × 10^{23}. Ten-fold dilutions of the pRV-gB and pRV-gE, ranging from 10^6 to 10^0copies/μL, were prepared in sterile water and aliquots of each dilution were stored at −80 °C.

Table 1
Sequence of primers and probes for PRV real-time PCR and real-time RPA.

Name	Sequence 5′-3′	Amplicon (bp)
PCR		
pRVgB-F	ATGCCCGCTGGTGGCGGTCTTTGG	2745
pRVgB-R	CTACAGGGCGTCGGGGTCC	
pRVgE-F	ATGCGGCCCTTTCTGCTGC	1740
pRVgE-R	TTAAGCGGGGCGGGACATCAA	
Real-time PCR		
gB718F	ACAAGTTCAAGGCCCACATCTAC	94
gB812R	GTCYGTGAAGCGGTTCGTGAT	
gB785P	FAM-ACGTCATCGTCACGACC-BHQ1	
gE694F	CTTCCACTCGCAGCTCTTCTC	72
gE765R	GTRAAGTTCTCGCGCGAGT	
gE708P	HEX-TTCGACCTGATGCCGC-BHQ1	
Real-time RPA		
RPA-gB-F	GCTCTTCAAGGAGAACATCGCCCCGCACAA	333
RPA-gB-R	GCGCCGATCTTGGTGTAGGTGTCGTTGGTGG	
RPA-gB-P	TCTACTACAAGAACGTCATCGTCACGACCG(FAM-dT)(THF)(BHQ1-dT)-GGTCCGGGAGCACGTA-C3	
RPA-gE-F	ACCCCGAGGACGAGTTCAGCAGCGACGAGGAC	156
RPA-gE-R	TCAACAGGCGGTTGGCGGTCACGCCATAGTT	
RPA-gE-P	CCGAGGAGGCGCCCCGCTCCGGCTTCGACG(ROX-dT)(THF)(BHQ2-dT)GGTTCCGCGATCCGGA-C3	

Real-time RPA

The dual real-time RPA reactions were performed in a 50 μL volume using a TwistAmp™ exo kit (TwistDX, Cambridge, UK) according to the manufacturer's instructions. Experiments were performed to optimize the primers and exo probes concentration in the PRV dual real-time RPA reaction system. The RPA-gB-F/RPA-gB-R and RPA-gE-F/RPA-gE-R concentrations ranged from 200 nM to 600 nM, and the RPA-gB-P and RPA-gE-P concentrations ranged from 60 nM to 200 nM. The reaction system also included 29.5 μL rehydration buffer, 2.5 μL magnesium acetate (280 mM), 1 μL of viral or sample DNA, and 11.9 μL ddH_2O. All reagents except for the viral template and magnesium acetate were prepared in a master mix, which was distributed into each 0.2 ml freeze-dried reaction tube containing a dried enzyme pellet. One μL of viral DNA was added to the tubes. Subsequently, magnesium acetate was pipetted into the tube lids, then the lids were closed carefully, the magnesium acetate was centrifuged into the rehydrated material using a minispin centrifuge. The sample was vortexed briefly and spun down once again, and the tubes were immediately placed in the Genie III scanner device to start the reaction at 39 °C for 20 min. The fluorescence signal for gB was collected in the Channel-1 (Blue, excitation 470 nm and emission 510-560 nm), meanwhile the fluorescence signal for gE was collected in the Channel-2 (Yellow, excitation 590 nm and emission > 620 nm). Samples produced an exponential amplification curve above the threshold of the negative control within 20 min were considered positive. The threshold time (TT) was calculated basing on the “fluorescence increase above threshold” by the Genie Explorer software when the RPA reaction was completed.