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Short communication

Development of an isothermal recombinase polymerase amplification assay for rapid detection of pseudorabies virus

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ABSTRACT

Recombinase polymerase amplification assays using real-time fluorescent detection (real-time RPA assay) and lateral flow dipstick (RPA LFD assay) were developed targeting the gD gene of pseudorabies virus (PRV). Both assays were performed at 39 °C within 20 min. The sensitivity of the real-time RPA assay and the RPA LFD assay was 100 copies per reaction and 160 copies per reaction, respectively. Both assays did not detect DNAs from other virus or PRV negative samples. Therefore, the developed RPA assays provide a rapid, simple, sensitive and specific alternative tool for detection of PRV.

1. Introduction

Pseudorabies (PR), also known as Aujeszky's disease, is caused by suid alphaherpesvirus 1 or named pseudorabies virus (PRV), which is a member of the family *alphaherpesvirinae* [1]. PRV primarily infects pigs but also shows to be able to infect cattle, dogs, sheep and rodents. In infected pigs with PRV, the clinical symptoms vary from subclinical signs to deaths. The infected young piglets show neurological signs and deaths, while older piglets appear respiratory disorders and pregnant pigs show abortion [2]. Due to effective disease control efforts and implementation of national eradication programs, pseudorabies has been eradicated from domesticated pigs in North America and a number of European countries. However, PR remains an economically important viral disease of pigs in many countries worldwide.

Accurate and rapid detection of PRV plays a critical role in the PR control and eradication programs. Various methods, such as virus isolation, serological tests for detection of PRV antibody and polymerase chain reaction (PCR) assays were developed successfully in detection of PRV [3–7]. However, these tests have certain limitations. Virus isolation is the gold standard for the detection of the virus, but this assay is time-consuming (at least 2–3 days) and has a low sensitivity. Serological tests such as virus neutralization test (VNT) and ELISA assay are the most common assays used for PRV diagnosis and are efficient and sensitive once an animal has seroconverted. However, this testing showed to be lowly sensitive during the acute stage of the infection, resulting in false negative. Real-time PCR is a fast, sensitive, and accurate test system which has potential of detecting PRV during the acute phase of the infection or before seroconversion. But the real-time PCR assay still depends on specialized and expensive equipment and usually takes over more than an hour to complete, as a result it is difficult to be used in the field or in poorly equipped laboratories. Loop-mediated isothermal amplification (LAMP), an isothermal method to amplify viral DNA, was developed for detection of PRV [8,9] with good sensitivity and specificity. LAMP assay could be performed using a water bath and the results could be directly read by the naked eye. However, the assay requires six primers and still needs more than 30 min to complete.

Recombinase polymerase amplification (RPA) assay is a sensitive, inexpensive and powerful tool for rapid detection of pathogens, which can generate testing results at from 37 °C to 42 °C in 20 min or less with simple instrumentation [10]. RPA employs a mixture of bacterial enzymes (strand displacing DNA polymerase, recombinase, and single strand binding protein). Recombinase is

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used to anneal oligonucleotide primers to template DNA for extension and a polymerase for amplification at an isothermal temperature. Single strand binding protein binds to displaced strands of DNA and prevents the primers from being displaced. Real-time detection of RPA amplicons can be performed by appending exo probe (readout specific for fluorescence) and exonuclease III (cutting exo probe at the internal tetrahydrofuran). As an alternative to real-time detection, RPA amplicons could be visualized on lateral flow dipstick by adding nfo probe (readout specific for color in the LFD) and endonuclease IV (cutting nfo probe at the internal tetrahydrofuran). In this study, a PRV real-time RPA assay and a PRV RPA LFD assay were developed for specific detection of PRV and its performance was evaluated using clinical samples.

2. Results and discussion

10% (w/vol) tissue suspensions were prepared by homogenizing twenty-two PRV-free tissue samples (heart, spleen, tonsil, lung and lymph nodes) in PBS. Following a brief centrifugation, the homogenized tissue samples were spiked with 10^5 $TCID_{50}$/mL of PRV Fa strain and stored at −80 °C until used. Seventy-six clinical samples (tonsil, heart, spleen, lymph nodes, lung and serum) were collected from pig farms in Shandong Province (China), and twenty-six clinical samples (lymph nodes, tonsil and serum) were collected from healthy pigs. The PRV gD gene segments (287 bp, corresponding to 370 bp to 656 bp of AY217094), named pPRV/RPA were synthesized by Genewiz (Suzhou, China) and used as the standard viral DNA for the determination of the assay sensitivity.

All primers and probes were designed based on recommendation by Twist (Cambridge, UK) and synthesized by Sangon Biotech (Shanghai, China). Different combinations of primer pairs (PRV-F1 to PRV-F3, PRV-R1 to PRV-R3) and probe (PRV-P) (Table S1) were chosen to perform PRV real-time RPA assay and PRV RPA LFD assay. Based on signal strength (Fig. S1), the primer pair of PRV-F1 and PRV-R3 with the probe PRV-P was chosen selected for subsequent evaluation. The PRV real-time RPA assay was performed using TwistAmp® exo lyophilized kit (Twist, Cambridge, UK) on Agilent Mx3005P thermocycler machine for 60 cycles at 39 °C for 20 s. PRV RPA LFD assay was conducted using TwistAmp® nfo lyophilized kit (Twist, Cambridge, UK) in a water bath at 39 °C for 20 min, and then the lateral flow dipstick (LFD) strips (Milenia Biotec GmbH, Germany) were used to detect the amplicons.

To test the sensitivity (detection limit) of PRV real-time RPA assay, a dilution range from 10^6 to 10^1 copies per reaction of pPRV/RPA DNA was tested for eight replicates. As shown in Fig. 1A and B, standard DNA ranging from 10^6 to 10^2 copies per reaction produced positive results for all eight replicates within 20 min. The detection limit of PRV real-time RPA at 95% probability was 10^2 copies per reaction (probit analysis, $p \leq 0.05$) (Fig. 1C). To further evaluate its detection limit, a dilution range from 2×10^3 $TCID_{50}$/50 μL to 2×10^{-1} $TCID_{50}$/50 μL copies per reaction of PRV Fa strain was tested. The results showed that the PRV real-time RPA assay was able to detect 2 $TCID_{50}$/50 μL of PRV (Fig. S2). The assay was then further evaluated with PRV-spiked samples (n = 22) and non-PRV-spiked samples (n = 22). The results showed that all 22 PRV-spiked samples were positive for PRV DNA, which were consistent with PRV real-time qPCR assay (CT value ranging from 16 to 27) [9,10]. All non-PRV-spiked samples were negative for PRV. There is a correlation between threshold time (real-time RPA) and the CT values (real-time qPCR) (Fig. S3). In evaluating the specificity of PRV real-time RPA assay, no cross reactions were observed with foot-and-mouth disease virus (FMDV), porcine circovirus virus type 2 (PCV2), porcine parvovirus virus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV) and classical swine fever virus

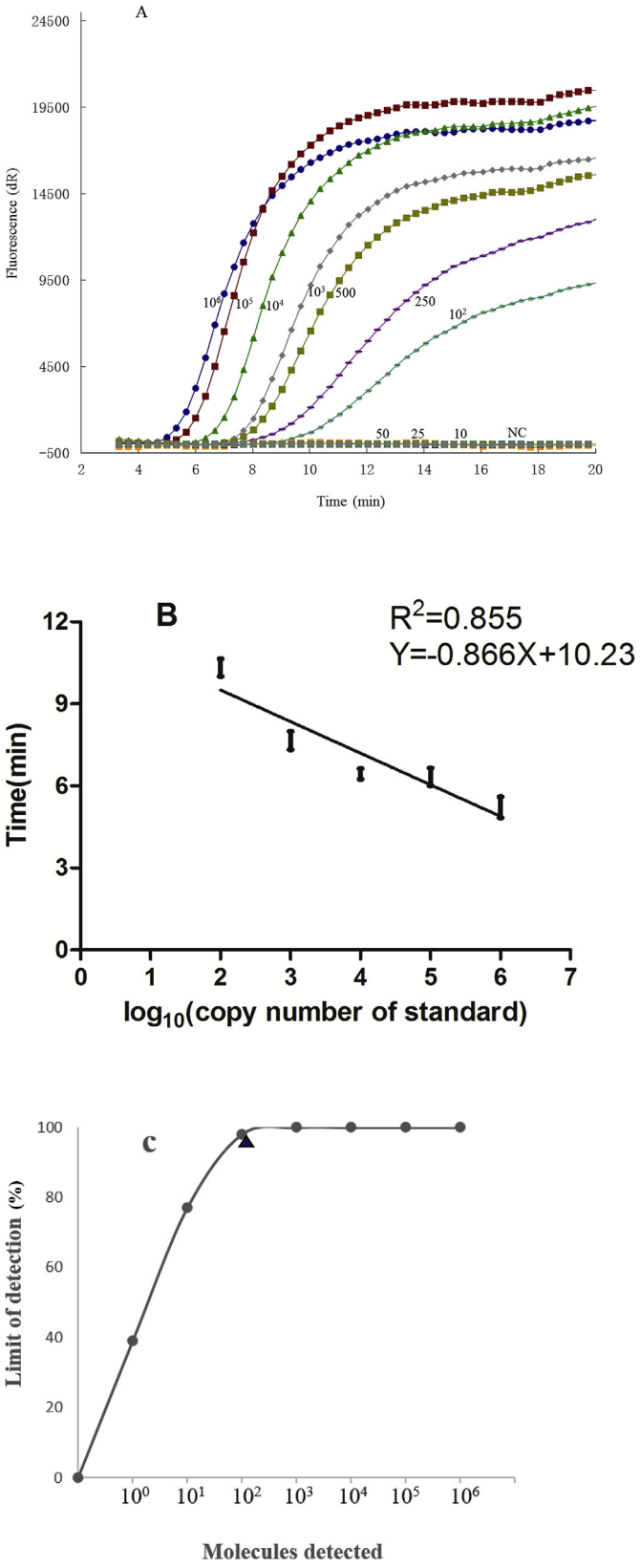

Fig. 1. Performance of the PRV real-time RPA assay (A) Fluorescence development of the PRV real-time RPA assay using a dilution range of pPRV/RPA DNA. NC represents negative control. (B) Reproducibility of the PRV real-time RPA assay was generated using PRISM 5.0 software (GraphPad Software, USA). (C) Probit regression analysis using statistics software. The limit of detection at 95% probability is depicted by a triangle.