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Development of recombinase polymerase amplification assays for the rapid detection of peste des petits ruminants virus

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ABSTRACT

Peste des petits ruminants (PPR) is a severe infectious disease of small ruminants caused by PPR virus (PPRV). Rapid and sensitive detection of PPRV is critical for controlling PPR. This report describes the development and evaluation of a conventional reverse transcription recombinase polymerase amplification (RT-RPA) assay and a real-time RT-RPA assay, targeting the PPRV N gene. Sensitivity analysis revealed that the conventional RT-RPA assay could detect 852 copies of standard PPRV RNA per reaction at 95% probability within 20 min at 41 °C, and the real-time RT-RPA assay could detect 103 copies of RNA molecules per reaction at 95% probability. Specificity analysis showed that both assays have no cross-reactivity with nucleic acid templates prepared from other selected viruses or common pathogens. Clinical evaluation using 162 ovine and hircine serum and nasal swab samples showed that the performance of both the real-time RT-RPA assay and the conventional RT-RPA assay were comparable to that of real-time RT-PCR. The overall agreements between real-time RT-PCR and real-time RT-RPA, and conventional RT-RPA were 99.4% (161/162) and 98.8% (160/162), respectively. The R^2 value of real-time RT-RPA and real-time RT-PCR was 0.900 by linear regression analysis. Our results suggest that both RT-RPA assays have a potential application in the rapid, sensitive and specific detection of PPRV.

Peste des petits ruminants (PPR) is an acute and highly contagious disease that is notifiable to the World Organisation for Animal Health, which primarily infects domestic small ruminants such as goats and sheep (Mariner et al., 2016; Parida et al., 2015). Clinically, the disease is characterized by pyrexia, conjunctivitis, mucopurulent ocular and nasal discharge, erosive-ulcerative stomatitis, pneumonia and severe diarrhea that ultimately results in high mortalities (Parida et al., 2015). These symptoms are clinically indistinguishable from those of foot-and-mouth disease, bluetongue and capripox, which make initial diagnosis quite difficult, particularly in regions where these diseases are endemic (Balamurugan et al., 2014; Mariner et al., 2016). Peste des petits ruminants virus (PPRV), the causative agent of PPR, is classified in the *Morbillivirus* genus of the *Paramyxoviridae* family (Parida et al., 2015). The virus has a linear, non-segmented, single-stranded, negative-sense RNA genome that encodes six structural (N, P, M, F, H and L) and two non-structural (C and V) proteins (Parida et al., 2015). Although only one serotype of PPRV is known to exist, PPRV isolates can be divided into four genetically distinct lineages (I, II, III and IV) (Parida et al., 2015). The four lineages exhibit distinct patterns in terms of geographic distribution, with lineages I and II mainly restricted to western and central Africa, lineage III to eastern Africa and the Arabian peninsula, and lineage IV to the Middle East, Asia, and more recently some parts of Africa (Parida et al., 2015).

Currently, a variety of diagnostic methods targeting PPRV-antigen, -nucleic acid and -antibody have been developed for the diagnosis of PPR (Balamurugan et al., 2014; Santhamani et al., 2016). Among the nucleic acid-based molecular methods, RT-PCR, real-time RT-PCR and loop-mediated isothermal amplification have been widely used to detect PPRV (Balamurugan et al., 2014; Santhamani et al., 2016). However, these techniques have certain intrinsic shortcomings, such as requiring high-precision instruments, specialized personnel, being time-consuming, or difficult to design proper primers or probes. These restrictions constrain their widespread use in the diagnosis of PPR, especially in remote rural areas. As a novel isothermal nucleic acid

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amplification technology, recombinase polymerase amplification (RPA) shows several comparative advantages over its counterparts (Daher et al., 2016). For example, RPA is fast, sensitive, specific, temperature- and sample-tolerant, and does not require sophisticated equipment. To date, RPA has been successfully employed for the detection of a variety of human and animal pathogens including bacteria, viruses, fungi and parasites (Daher et al., 2016; James and Macdonald, 2015). In the present study, a conventional and a real-time RT-RPA assay for the detection of PPRV were developed and evaluated.

PPRV Nigeria 75/1 vaccine strain (lineage II) was propagated in Vero cells as previously described (Zhang et al., 2013). Bovine viral diarrhea virus (BVDV), infectious bovine rhinotracheitis virus (IBRV), canine distemper virus (CDV), Newcastle disease virus (NDV) LaSota vaccine strain, and *Pasteurella multocida* were preserved in our laboratory. Inactivated foot-and-mouth disease virus (FMDV, serotype O) was obtained from a commercial Liquid-phase Blocking ELISA Kit (Lanzhou Veterinary Research Institute, Lanzhou, China) (Wang et al., 2016b; Wang et al., 2017). Thirty serum and 17 nasal swab samples of sheep and goats showing pyrexia or nasal discharge were collected from Tibet, China (Wang et al., 2016a). In addition, 115 serum samples were taken from sheep and goats imported from Australia in 2013 (Supplemental Table 1). Viral RNA of Nigeria 75/1 strain and the total RNA of each serum and nasal swab sample was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Genomic DNA of *Pasteurella multocida* was extracted using a Rapid Bacterial Genomic DNA Isolation Kit (Sangon Biotech [Shanghai] Co., Ltd., China).

Fourteen published PPRV N gene sequences of representative strains of all four lineages (Supplemental Fig. 1) were used for the design of the RPA primers; PPRV-RPA-F: 5′-CTCGTCCATCATTACCCGTTCAAGACTG CTC-3′; PPRV-RPA-R: 5′-GGACCTAGTACTTTGAACTACCTCAACA AGG-3′, and the RPA exo probe, PPRV-RPA-Probe: 5′-AGCTGACCGGG GTGATGATCAGCATGTTATC/FAM-dT/THF/BHQ1-dT/GTTCGTAGAG TCACCCG-C3 spacer-3′. The location of the RPA primers and the exo probe are indicated in Supplemental Fig. 1. PPRV N genes were amplified by RT-PCR/PCR using PPRV RNA (Nigeria 75/1 strain), the genomic cDNA (China/BJ/2014 strain of lineage IV [KP260624], a kind gift from Dr. Hongxuan He at the Institute of Zoology, Chinese Academy of Sciences, Beijing, China) or the synthesized N genes of lineage I (EU267273) and lineage III (KJ867543) as templates along with forward primer PPRV-N-F (5′-ATGGCGACTCTCCTTAAAAGCTTAG-3′) and reverse primer PPRV-N-R (5′-TTAGCCGAGGAGATCCTTGTC-3′). The resulting fragments were then transcribed into four standard RNA molecules of about 1578 bp in length (data not shown), using an *in vitro* transcription system as previously described (Wang et al., 2017).

Conventional RT-RPA assays were performed at 41 °C in a water bath using the TwistAmp® Basic RT Kit (TwistDX, Cambridge, UK). The 50-μL reaction system contained 29.5 μL of rehydration buffer, 2.4 μL of each PPRV-RPA-F/R primer (10 μM), 2.5 μL of magnesium acetate (280 mM), 0.5 μL of RNase inhibitor, 11.7 μL of nuclease-free water, and 1.0 μL of viral or sample RNA. To optimize the reaction time, 10^5 copies of standard RNA of PPRV N were amplified for 10, 20, 30 and 40 min, respectively. RPA amplicons were purified using the TIANGel Midi Purification Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to manufacturer's instructions. Five μL of the purified products were analyzed using 2.0% (w/v) agarose gel electrophoresis and visualized under UV light after Goldview staining. As shown in Fig. 1, a clear DNA band of the expected size (~205 bp) was visualized shortly after a 10-min reaction. Densitometric analysis of the DNA bands revealed that the DNA yield after a 20-min reaction was ~3 times that generated by a 10-min reaction, whereas no significant difference was observed among products at 20, 30 and 40 min. Similar results were also obtained from the other three lineages of PPRV standard RNA (data not shown). Therefore, 20 min was set as the reaction time for all conventional RT-RPA assays performed in the subsequent study.

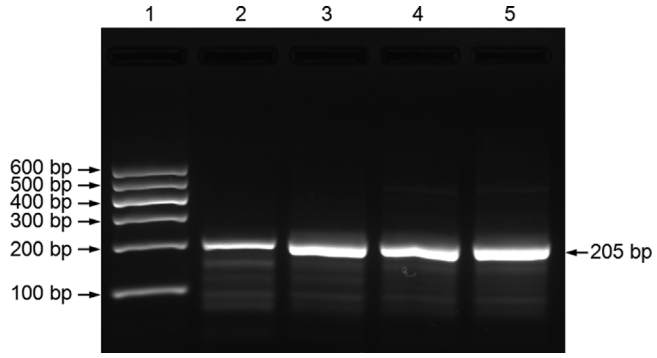

Fig. 1. Optimization of the reaction time for the conventional RT-RPA assay. A total of 10^5 copies of standard RNA of lineage IV PPRV were used as the template, and the amplicons at different time points were analyzed using agarose gel electrophoresis. A clear DNA band with an expected size (~205 bp) could be visualized after a 10-min reaction at 41 °C. Lane 1, Marker I; lanes 2–5, amplicons with a reaction time of 10, 20, 30 and 40 min, respectively.

Real-time RT-RPA assays were carried out in a 50-μL volume using the TwistAmp® exo RT Kit (TwistDX). The reaction mixture included 420 nM of each PPRV-RPA-F/R primer, 120 nM PPRV-RPA-Probe, 14 mM magnesium acetate, and 1.0 μL of viral or sample RNA. After adding the relevant reagents and RNA templates in the same way as previously described (Wang et al., 2016b; Wang et al., 2017), the reaction tubes were immediately placed in a Genie III Fluorimeter Instrument (OptiGene Ltd., West Sussex, UK) and incubated at 39 °C for 20 min. The fluorescence signal was monitored in real-time. Samples that produced an exponential amplification curve above the threshold of the negative control within 20 min were considered to be positive. The threshold time was calculated based on the "fluorescence increase above threshold" by the Genie Explorer software when the RPA reaction was completed.

For sensitivity analysis, 10-fold serial dilutions of each of the *in vitro* transcribed RNA molecules ranging from 10^6 to 10^0 copies per reaction were simultaneously detected by the two RT-RPA assays and an N-gene based real-time RT-PCR assay developed by Bao and colleagues (Bao et al., 2008). As shown in Fig. 2A–C, all three assays have an approximate detection limit of around 10^2 copies of PPRV standard RNA. Similar results were obtained from the other three lineages of PPRV (data not shown). To analyze the detection limit more accurately, eight independent runs for each assay were performed using the serially diluted (10^6–10^0) RNA standards, and the data were used for probit regression analysis using IBM's Statistical Product and Service Solutions (SPSS) software (Chicago, IL, USA). As shown in Fig. 2D–F, conventional RT-RPA, real-time RT-RPA and real-time RT-PCR assays could detect 852, 103 and 700 copies of RNA molecules per reaction at 95% probability, respectively. The sensitivity of our developed real-time RT-RPA assay was consistent with a recently reported PPRV real-time RT-RPA assay (Yang et al., 2017), but superior to their lateral-flow strip based RT-RPA assay, which had a sensitivity of 150 copies of RNA molecules per reaction. To assess the intra-assay repeatability and inter-assay reproducibility of our developed real-time RT-RPA assay, three different concentrations (high, medium, and low, i.e., 10^6, 10^4, and 10^2 copies/μL) of each standard RNA were tested in triplicate in one run or in three independent runs on different days, and the coefficient of variation (CV) for the threshold times was calculated. As shown in Supplemental Table 2, the intra-assay CV ranged from 0.29% to 2.75% while the inter-assay CV ranged from 3.38% to 6.52%, indicating good repeatability and reproducibility for the real-time RT-RPA assay.

For specificity analysis, nucleic acid templates prepared from other pathogens, including BVDV, IBRV, CDV, NDV, FMDV and *Pasteurella multocida*, were detected by the conventional and real-time RT-RPA assays. As shown in Fig. 3, only PPRV RNAs of all four lineages were positively amplified by both conventional and real-time RT-RPA assays. No cross detection was observed among the selected pathogens. These