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Rapid detection of transmissible gastroenteritis virus in swine small intestine samples using real-time reverse transcription recombinase polymerase amplification

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ABSTRACT

A rapid and specific real-time reverse-transcription recombinase polymerase amplification assay (RT-RPA) was developed to detect the transmissible gastroenteritis virus (TGEV) in this study. The primers and exo probe were designed to be specific for a portion of spike (S) gene conserved in TGEV, but absent in the closely related porcine respiratory coronavirus (PRCV). The amplification was performed at 40 °C for 20 min. The assay could only detect the TGEV, and there was no cross-reaction with other pathogens tested. Using the in vitro transcribed TGEV RNA as template, the limit of detection of the developed RT-RPA was 100 copies per reaction. The assay performance was evaluated by testing 76 clinical samples by RT-RPA and a real-time RT-PCR. Fourteen samples were TGEV RNA positive in RT-RPA (18.4%, 14/76), which were also positive in the real-time RT-PCR. The diagnostic agreement between the two assays was 100% (76/76). The R^2 value of RT-RPA and real-time RT-PCR was 0.959 by linear regression analysis. The developed RT-RPA assay provides a useful alternative tool for rapid, simple and reliable detection of TGEV in resource-limited diagnostic laboratories and on-site facilities.

Transmissible gastroenteritis (TGE) is an acute enteric viral disease of pigs resulting in vomiting and diarrhea in all ages of pigs and with high mortality rate in piglets (Garwes, 1988). The disease is caused by TGE virus (TGEV), which is an enveloped, single-stranded, positive-sense RNA virus belonging to the genus *Alphacoronavirus* of the family *Coronaviridae* (Saif and Wesley, 1999). TGE was first reported in 1933 in the United States, and TGEV was first isolated and identified in 1946 (Doyle and Hutchings, 1946). Since then, TGEV has spread throughout the world including America, Europe and Asia, and has caused significant economic loss in the pig industry (Kim et al., 2000a; Stevenson et al., 2013).

Since the mid 1980s, a variant respiratory form of the TGEV known as porcine respiratory coronavirus (PRCV) has become common in pigs (Laude et al., 1993). Compared to TGEV, PRCV has a deletion of between 672 and 681 nucleotides near the 5′ end of the spike (S) gene (Laude et al., 1993). Although PRCV replicates predominantly in the respiratory tract, some pigs infected with PRCV could also shed the virus in their feces (Costantini et al., 2004; Saif and Wesley, 1999). Therefore, any diagnostic assay for TGEV should be able to differentiate it from PRCV.

Rapid and specific detection of TGEV would be extremely important in the prevention and control of TGE. The conventional methods for detection of TGEV are time-consuming, laborious, and requiring well-trained technicians, such as virus isolation, immunofluorescence assay, electron microscopy and ELISA (Carman et al., 2002; Dulac et al., 1977; van Nieuwstadt et al., 1988). A series of developed RT-PCR assays for PEDV require the high-precision, sophisticated and expensive instruments, well-trained technicians and good laboratory circumstance, thus unsuitable for being applied in under-equipped laboratories and on-site applications, such as RT-PCR (Paton et al., 1997), nanoparticle-assisted RT-PCR (Zhu et al., 2017) and real-time RT-PCR (Vemulapalli et al., 2009). Recently, RT-LAMP assays have been developed for TGEV detection (Chen et al., 2010; Li and Ren, 2011). Six primers were needed in the RT-LAMP assays for TGEV, the reaction time was 30 or 60 min, and the results were visualized by agarose gel electrophoresis (Chen et al., 2010; Li and Ren, 2011).

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Recombinase polymerase amplification (RPA) is an isothermal gene amplification technique that has been demonstrated to be a rapid, specific, sensitive, and cost-effective molecular diagnostic method (Daher et al., 2016; Piepenburg et al., 2006). RPA employs three core enzymes: a recombinase, a single-stranded DNA-binding protein (SSB) and a strand-displacing polymerase. The recombinase forms a nucleoprotein filament with primers and probes. This filament scans the double-stranded DNA (dsDNA) target searching for homologous sequences and invades the dsDNA once homology is found. Then a D-loop structure is formed, which is a local separation of DNA strands in which the complementary strand is stabilized by SSB and the target strand is hybridized with primer. Recombinase disassembly from the nucleoprotein filament, the strand-displacing DNA polymerase adds bases to the 3′-end of the primer and extension occurs (Daher et al., 2016; Piepenburg et al., 2006). Real time detection of RPA amplicons could be performed through adding exonuclease III and exo probes to the reaction mixture. The exo probe typically consist of an oligonucleotide backbone that contains an abasic nucleotide analogue (tetrahydrofuran residue or THF) flanked by a dT-fluorophore and a corresponding dT-quencher group. In a double-stranded context the THF residue presents a substrate for Exonuclease III, which will cleave the probe at the THF position, thereby separating the fluorophore and the quencher and generating a fluorescent signal. Real-time RPA assays had been developed for the detection of a series of important viruses in swine (Wang et al., 2017a,b). In this study, we developed and evaluated the user-friendly on-site detection platform integrating the real-time RPA technology and a field-deployable device (Genie III tube scanner) for the rapid detection of TGEV.

The genomic RNA or DNA of TGEV (strain HB-YX), procine epidemic diarrhea virus (PEDV, strain JSCZ1601), porcine rotavirus (PoRV, strain HB-BD/2016), PRCV, porcine deltacoronavirus (PDCoV), *Lawsonia intracellularis* (strain LX5), porcine circovirus 2 (PCV2, strain HB-MC1) and porcine parvovirus (PPV, strain BJ-2) were kept in our laboratory. Seventy-six small intestinal samples from the piglets with signs of severe watery diarrhea, dehydration were collected from eleven pig farms in Hebei Province between April 2016 and February 2017. The piglets were 7–14 days old. The samples were homogenized with phosphate-buffered saline (PBS, pH 7.4) as a 10% (w/v) suspension and centrifuged for 10 min at 3000 g at 4 °C. Two hundreds microliter of the supernatant was used for RNA extraction using the Trizol Reagent. All the sample RNA were quantified using ND-2000c spectrophotometer (NanoDrop, Wilmington, USA), and were stored at −80 °C until use.

The 886bp RT-PCR product encompassing the TGEV-specific S gene was generated from viral genomic RNA extracted from strain HB-YX using F1122 and R1122 primers (Paton et al., 1997). The resulting fragment was ligated into a pGEM-T Easy vector and transformed into *E.coli* DH5α chemically competent cells according to standard procedures. The in vitro transcribed TGEV standard RNA was produced with RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, USA). The length from the T7 promoter region to the NdeI cut site of the pGEM-T Easy vector is 97 nucleotides, generating a total transcript of 983 nucleotides in length. The in vitro transcripts were quantified using ND-2000c and the copy number of RNA molecules was calculated by the following formula: Amount (copies/μL) = [RNA concentration (g/μL) /(transcript length in nucleotides × 340)] × 6.02×10^{23}. The in vitro transcribed RNA was diluted in ten-fold serial dilutions to achieve RNA concentrations ranging from 1.0×10^5 to $1.0 \times 10^{\circ}$ copies/μL, which were used as the standard RNA for TGEV RT-RPA assay.

According to the S gene nucleotides of different TGEVs (accession numbers: AF104420; AF179882; AF302263; AY335548; AF481360; AF494337; HQ462571; M21950; M94099; S51223; Z35758) and PRCVs (accession numbers: KR270796; M94103; M94102; M94098; M94097) available in Genbank, the RT-RPA primers and exo probe were designed to be specific to a portion of the S gene sequences conserved in TGEV but absent in PRCV. The forward primer was: 5′- TTC AGAGGCAAATTGTGGTAATATGCTGTATGGC-3′; the exo probe was: 5′- GCAGATGAGGTTGTTGCTTATTTACATGG(ROX-dT)G(THF)(BHQ2-dT)AGTTACCGTATTAG-C3spacer-3′; the reverse primer was 5′- ACG CATATCACCAAATGTGACAGTGCCAGACCA-3′. The primers and probe were synthesized by a commercial company (Sangon, Shanghai, China), and the length of the amplicon was 139 bp.

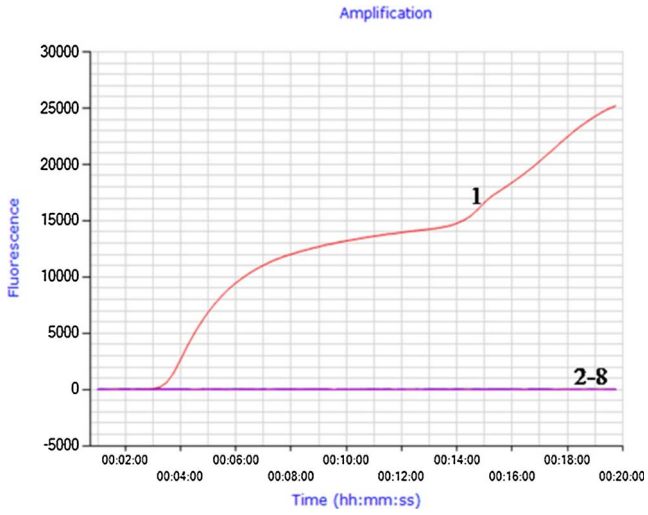

Fig. 1. Analytical specificity of the TGEV RT-RPA assay. RT-RPA was carried out at 40 °C for 20 min. The results showed RT-RPA only amplified the TGEV RNA, but not other pathogens tested (n = 5). lane 1,TGEV; lane 2, PEDV; lane 3, PDCoV; lane 4, PoRV; lane 5, PRCV; lane 6, PPV; lane 7, PCV2; lane 8,*L. intracellularis*.

RT-RPA reactions were performed in a total volume of 50 μL containing 29.5 μL rehydration buffer and 2.5 μL magnesium acetate (280 mM) from the TwistAmp™ RT exo kit (TwistDX, Cambridge, UK). Other components included 420 nM each RPA primer, 120 nM exo probe, and 1 μL of viral RNA or 5 μL sample RNA. The RT-RPA reactions were performed at 40 °C for 20 min in the Genie III scanner device. Ten nanograms of the different pathogen genomic RNA or DNA were used as template in the RT-RPA, and only the TGEV RNA were detected by RT-RPA while the other pathogens were not detected (Fig.1). No cross detections were observed in five independent reactions, demonstrating the high specificity of RT-RPA assay for the detection of TGEV. In the analytical sensitivity analysis, the limit of detection (LOD) of the RT-RPA was 1.0×10^2 copies per reaction, which was the same as the real-time RT-PCR (Fig. 2). The two-fold serial dilutions of the in vitro transcribed TGEV RNA were made from 1.0×10^3 to 6.25×10^1 copies, and from 1.0×10^2 to 1.25×10^1 copies, respectively. The above two-fold serial dilutions of the TGEV RNA, and 1.0×10^1 copies in vitro transcribed TGEV RNA were further used in the RT-RPA, and the LOD of the assay still was 100 copies per reaction. The threshold time (TT) values were 10.20 min and 10.42 min for 125 copies and 100 copies, while there were no fluorescence amplification curves for 62.5 copies and 50 copies. The RT-RPA assay was performed five times on the quantitative RNA with similar results.

For evaluating the potential applicability of the developed RT-RPA assay, 76 clinical samples had RNA extracted and were tested by the RT-RPA and real-time RT-PCR (Vemulapalli et al., 2009). Fourteen samples (18.4%, 14/76) were TGEV RNA positive in the RT-RPA, which were also positive in the real-time RT-PCR. The overall agreement between the RT-RPA and the real-time RT-PCR was 100% (76/76). The basic RPA assay were also performed for the 14 TGEV RNA positive samples using the TwistAmpTM RT Basic kit (TwistDX, Cambridge, UK), and the reaction system was the same as RT-RPA except the exo porbe was not used. The RPA amplification products were sequenced by a commercial company (Sangon, Shanghai, China), then were blasted in the GenBank. The blast results demonstrated that all the sequences showed more than 97.4% homology to TGEV strains in GenBank, which confirmed the good specificity of the RT-RPA assay for TGEV.