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Real-time reverse transcription recombinase polymerase amplification assay for rapid detection of porcine epidemic diarrhea virus

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ABSTRACT

Porcine epidemic diarrhea (PED), which is caused by porcine epidemic diarrhea virus (PEDV), is an acute, highly contagious enteric disease characterized by severe watery diarrhea, vomiting, dehydration, and high mortality in suckling piglets. A real-time reverse-transcription recombinase polymerase amplification assay (RT-RPA) was developed based on the nucleocapsid gene of PEDV. RT-RPA assay was performed at 40 °C for 20 min. The assay could detect both the classical and variant PEDV strains, and there was no cross-reaction with other pathogens tested. Using the in vitro transcribed PEDV RNA as template, the analytical sensitivity was 23 copies per reaction. The assay performance was evaluated by testing 76 clinical samples. PEDV RNA positive rate was 55.3% (42/76) by RT-RPA and 59.2% (45/76) by real-time RT-PCR. The diagnostic agreement between the two assays was 96.1% (73/76), and the R^2 value of the two assays was 0.903 by linear regression analysis. The developed RT-RPA assay provides a useful alternative tool for simple, rapid and reliable detection of PEDV in resource-limited diagnostic laboratories and on-site facilities.

Porcine epidemic diarrhea (PED) is an acute, highly contagious enteric disease characterized by severe watery diarrhea, vomiting, dehydration, and high mortality in suckling piglets (Pensaert and de Bouck, 1978). PED is caused by porcine epidemic diarrhea virus (PEDV), which is an enveloped, single-stranded, positive-sense RNA virus belonging to the genus *Alphacoronavirus* of the family *Coronaviridae* (Pensaert and de Bouck, 1978). PED was first reported in England in the 1971, and has since spread to other European and Asian countries (Song and Park, 2012). In China, PED first occurred in 1973 as a sporadic viral enteric disease in pig herds (Li et al., 2012). Since 2010, a highly virulent PEDV strain emerged in China causing significant loss in the swine industry (Sun et al., 2012). In 2013, PED first emerged in the United States, subsequently spread rapidly across the country and led to enormous economic losses (Chen et al., 2014). In 2014, the first case of PED was reported in Canada (Pasick et al., 2014). Currently, PED remains a significant threat to the global swine industry.

Presently, the control of PED primarily depends on the rapid and accurate diagnosis to prevent the further spread of the virus. For PED diagnosis, RT-PCR, nanoparticle-assisted RT-PCR and real-time RT-PCR have been reported (Kim et al., 2007; Liu and Wang, 2016; Yuan et al., 2015). All these assays require high-precision, sophisticated and expensive instruments and unsuitable for under-equipped laboratories and on-site applications. Recently, several isothermal amplification assays, including the insulated isothermal PCR, RT-LAMP, and reverse transcription cross-priming amplification (RT-CPA) were developed for PEDV detection (Ren and Li, 2011; Wang et al., 2016; Zhang et al., 2016). Although these assays do not require specialized equipment, they are difficult to design as six primers are needed in the RT-LAMP, and three primers and two probes are needed in the RT-CPA. Furthermore, the results are usually produced within 45–60 min for the above methods. Therefore, a simple, rapid, and sensitive method is still needed for detection of PEDV for those without access to real-time PCR instrumentation.

Recombinase polymerase amplification (RPA) is an isothermal gene amplification technique that has been demonstrated to be a simple, rapid, 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

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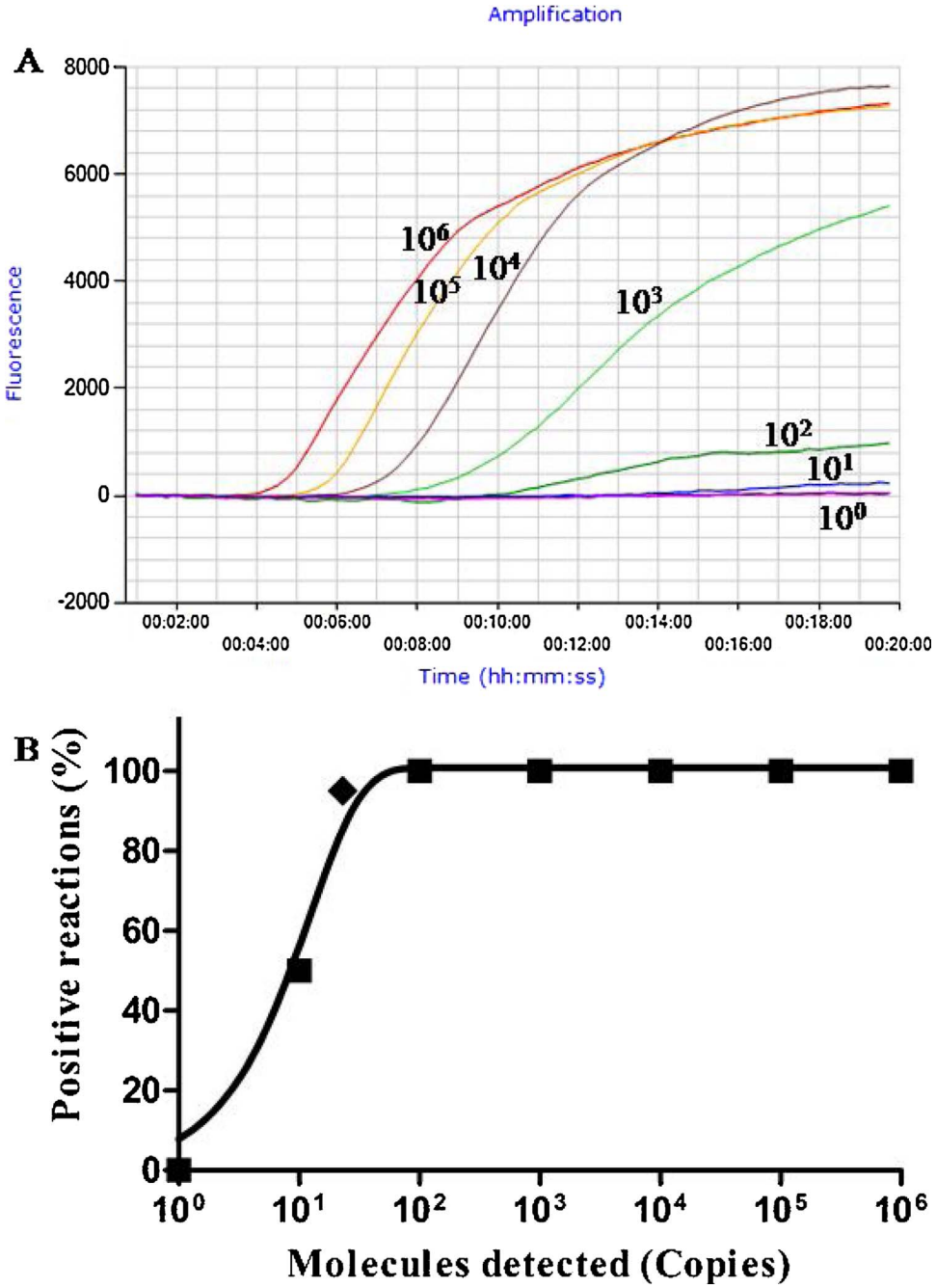

Fig. 1. Performance of the PEDV RT-RPA. (A) Fluorescence development over time using a dilution range of 1.0×10^6–1.0×10^0 copies of the PEDV standard RNA. (B) Probit regression analysis using SPSS software on data of eight PEDV RT-RPA tests on the standard RNA. The limit of detection of the assay at 95% probability (23 copies) is depicted by a rhomboid.

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 the RPA amplification depends on the exo or nfo probe and the florescence signal is measured in real-time via a tube scanner. Real-time RPA assays had been developed for on-site detection of influenza virus and dengue virus (Abd El Wahed et al., 2015). In this study, the user-friendly on-site detection platform integrating the real-time RPA technology and a field-deployable Genie III device (OptiGene, West Sussex, UK) was developed for the rapid detection of PEDV.

PEDV (classical strain CV777 and variant strain JSCZ1601), transmissible gastroenteritis virus (TGEV, attenuated H strain), porcine rotavirus (PoRV, strain HB-BD/2016), *Lawsonia intracellularis* (strain LX5), classical swine fever virus (CSFV, strain AV1412), porcine parvovirus (PPV, strain BJ-2), and the viral RNA of porcine deltacoronavirus (PDCoV) were kept in our laboratory. PEDV, TEGV, PoRV and CSFV viral RNA was extracted using Trizol Reagent (Invitrogen, Waltham, USA) according to manufacturer's instructions. PPV and *L. intracellularis* DNA was extracted using the TIANamp Virus DNA kit (Tiangen, Beijing, China) according to manufacturer's instructions. Seventy-six small intestinal samples from the piglets with signs of severe watery diarrhea, dehydration were collected from 11 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