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Point-of-care diagnostic assay for rapid detection of porcine deltacoronavirus using the recombinase polymerase amplification method

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Summary

Porcine deltacoronavirus (PDCoV) has emerged and spread throughout the porcine industry in many countries over the last 6 years. PDCoV caused watery diarrhoea, vomiting and dehydration in newborn piglets. A sensitive diagnostic method would be beneficial to the prevention and control of PDCoV infection. Recombinase polymerase amplification (RPA) is an isothermal amplification method which has been widely used for virus detection. A probe-based reverse transcription RPA (RT-RPA) assay was developed for real-time detection of PDCoV. The amplification can be finished in 20 min and fluorescence monitoring was performed by a portable device. The lowest detection limit of the PDCoV RT-RPA assay was 100 copies of RNA molecules per reaction; moreover, the RT-RPA assay had no cross-reaction with other common swine viruses. The clinical performance of the RT-RPA assay was evaluated using 108 clinical samples (54 intestine specimens and 54 faecal swab specimens). The coincidence rate of the detection results for clinical samples between RT-RPA and RT-qPCR was 97.2%. In summary, the real-time RT-RPA assay offers a promising alternative to RT-qPCR for point-of-care detection of PDCoV.

KEYWORDS
detection, porcine deltacoronavirus, recombinase polymerase amplification

1 | INTRODUCTION

Coronaviruses (CoVs) are a class of important viruses that could infect a variety of hosts, causing multiple diseases, including respiratory infections and enteric diseases, etc. (Coleman & Frieman, 2014; de Wit, van Doremalen, Falzarano, & Munster, 2016). Based on phylogenetic analysis, CoVs were tentatively classified as four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* (Woo et al., 2012). Several CoVs could infect swine and posed huge threat to the swine health and industry. Porcine transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhoea virus (PEDV), both belong to *alphacoronavirus*, are two commonly causative agents of the enteric diseases of pigs and have been well studied in previous studies (Coussement, Ducatelle, Debouck, & Hoorens, 1982; Doyle & Hutchings, 1946; Pensaert & de Bouck, 1978; Trapp, Sanger, & Stalnaker, 1966). Porcine deltacoronavirus (PDCoV), a new member of *Deltacoronavirus*, was firstly detected in pig faeces in the molecular investigation of CoV in Hong Kong in 2012

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(Woo et al., 2012). Up to now, PDCoV has been detected/isolated in pigs across the world, including USA, Mexico, Canada, South Korea, China and Thailand (Dong et al., 2016; Fu et al., 2018; Janetanakit et al., 2016; Jang et al., 2018; Jung, Hu, & Saif, 2016; Lee & Lee, 2018; Mai et al., 2018; Song et al., 2015; Wang, Byrum, & Zhang, 2014; Xu et al., 2018). PDCoV has become a reportable disease in the United States by United States Department of Agriculture, and an infection case is published weekly at www.aphis.usda.gov. The infection incidents were in wide range within the geographical scope in the affected countries. The piglets naturally infected with PDCoV exhibited diarrhoea, vomiting and dehydration together with histological lesions typical for multifocal to diffuse villous atrophy of the small intestines (Janetanakit et al., 2016; Song et al., 2015; Wang, Hayes, Sarver, Byrum, & Zhang, 2016). The characteristic clinical signs and microscopic intestinal lesions that were associated with PDCoV infection were evidenced by experimental infection studies (Chen, Gauger, et al., 2015; Jung et al., 2015).

The clinical symptoms of the pigs infected with PDCoV were characterized by watery diarrhoea, which were very similar to that caused by PEDV and TGEV. These similar symptoms caused by these CoVs made it difficult to clinically distinguish these viral pathogens. Rapid and sensitive assays for specific detection of PDCoV would be beneficial to the diagnosis and epidemiological investigation of the virus. At present, several diagnostic methods for rapid detection of PDCoV have been developed. Serological assays such as virus neutralization test, indirect fluorescent antibody assay and enzyme linked immunosorbent assay were reported to detect PDCoV-specific antibodies (Hu et al., 2015; Luo et al., 2017; Zhang, 2016). However, the serological methods were not suitable for the detection of antibodies during the early stage of virus infection as antibody generation required a few days. To rapid diagnosis of PDCoV infection, several molecular methods such as reverse transcription-polymerase chain reaction (RT-PCR) and RT-quantitative PCR (RT-qPCR) assays have been established for direct detection of the PDCoV nucleic acids in the swine samples (Marthaler et al., 2014; Song et al., 2015; Wang et al., 2014). However, these diagnostic methods do have some drawbacks, such as labour-intensive and time consuming. The loop-mediated isothermal amplification (LAMP) assay, which required shorter run time compared with PCR/qPCR, was reported for the detection of PDCoV (Hanaki et al., 2014). In comparison to LAMP assay, the recombinase polymerase amplification (RPA) assay, which required fewer primers (two primers) and shorter run time (20–30 min), has been widely reported in pathogen detection (Lillis et al., 2016; Ma, Zeng, Huang, et al., 2018; Ma, Cong, et al., 2018; Yang et al., 2016; Yang, Qin, Sun, et al., 2017). The isothermal amplification of the RPA assay depends on three enzymes: a recombinase polymerase, a single-stranded binding protein and a DNA polymerase (Piepenburg, Williams, Stemple, & Armes, 2006). RPA amplicons can be detected by gel electrophoresis or directly visualized by lateral flow dipstick (LFD) (Yang, Qin, Song, et al., 2017); real-time detection of the amplification can be achieved by adding a probe to the reaction (Ma, Zeng, Huang, et al., 2018).

Previous studies have reported the real-time RT-RPA assays for rapid detection of PEDV and TGEV respectively (Wang, Wang, et al., 2018; Wang, Zhang, et al., 2018). Up to now, there is no study about the RPA method for rapid detection of PDCoV. The aim of our study was to develop a probe-based RT-RPA assay for rapid detection of PDCoV and evaluate the performance of the assay on diagnosing clinical samples.

2 | MATERIALS AND METHODS

2.1 | Viruses

Porcine deltacoronavirus strain CHN-GD16-02, swine acute diarrhoea syndrome CoV (SADS-CoV), porcine reproductive respiratory syndrome virus (PRRSV) strain JXA1, classical swine fever virus (CSFV) strain C virus, TGEV of swine, PEDV, porcine rotavirus (RV) NX, pseudorabies virus (PRV) strain HB-98, porcine parvovirus (PPV) CP-99 killed vaccine, porcine circovirus type 2 (PCV2), pig foot-and-mouth disease virus (FMDV) and swine influenza virus (SIV) was preserved in our laboratory.

2.2 | Generation of MNV RNA standard

The sequence of N gene was highly conserved within the whole PDCoV genome, thus N gene was chose for preparing the RNA standard in this study. The in vitro transcribed RNA standard was prepared following a previously reported method (Ma, Zeng, Huang, et al., 2018). Nucleic acids were extracted from the cells infected with PDCoV strain CHN-GD16-03 (accession no: KY363867) using a TIANamp virus DNA/RNA Kit (Tiangen Biotech, China), and used as the template in the RT-PCR assay with a one-step RT-PCR Kit (Takara, China). A primers pair (PDCoV-F: 5′-ACGCTGCTGATTCCTGCT-3′, PDCoV-R: 5′-GCTACTCATCCTCAGTTTCGTG-3′) were used for the amplification of the whole N gene for ligation into the TA cloning vector pGEM-T.

2.3 | Primer and probe design

In this study, RPA primers were designed according to the recommendations from TwistDx Co. Ltd (https://www.twistdx.co.uk/en/support/rpa-assay-design-2). The primer set with the highest amplification efficiency determined by basic RT-RPA assay was used in the following assay. A probe was designed to compatible to the primer set with the highest amplification efficiency.

2.4 | RT-RPA assay

Basic RT-RPA reaction was performed using the TwistAmp Basic RT kit (TwistDx, UK) according to the manufacturer's instruction. The reagents in the kit except the rehydration buffer and magnesium acetate were supplied in a dried enzyme pellet which was distributed into each 0.2 ml tube. The RPA reaction in a 50 μl volume included 30.1 μl of rehydration buffer, 2.1 μl of each primer (10 μM), 1 μl of RNA template, 12.2 μl of distilled water and 2.5 μl of magnesium acetate (280 mM). The microtubes were placed in a heating block. After incubating at 39°C for 20 min, the products were purified by a DNA clean-up kit (Beyotime, China) and subjected to agrose gel electrophoresis.

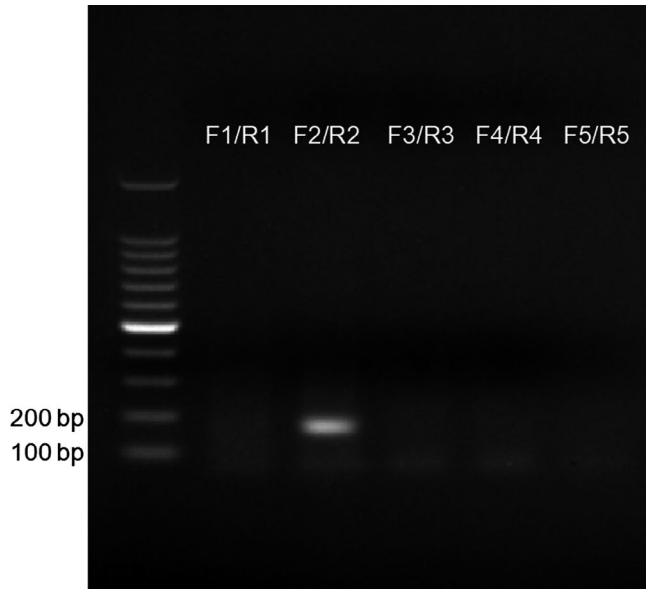

FIGURE 1 Primer sets screening. The products of RT-RPA by the basic RPA kit using five primer sets were subjected to electrophoresis on a 2% agarose gel respectively

Real-time RT-RPA assay was performed using the TwistAmp exo RT kit. The reaction procedure of real-time RT-RPA was performed as that of the basic RT-RPA assay with minor modification: 0.6 μl of distilled water was replaced by 0.6 μl of the probe (10 μM). The RT-RPA reaction was carried out at 39°C in the Deaou-308C tubescanner (DEAOU Biotechnology, China) for 20 min with a mixing and centrifugation step after the first 4 min recommended by TwistDx (Lillis et al., 2016). A sample was considered positive when it generated an exponential amplification curve above the threshold of the negative control.

2.5 | Sensitivity of the RT-RPA assay

To determine the analytical sensitivity of the RT-RPA assay for the detection of PDCoV, the in vitro transcribed RNA was serially 10-fold diluted and tested by the assay in eight replicates. A semi-log regression was performed by plotting the threshold time (TT) against the $\log_{10}$ RNA copy numbers using Prism 5.0 software (GraphPad).

2.6 | Specificity of the RT-RPA assay

The specificity of the PDCoV RT-RPA assay was evaluated by testing a panel of swine viruses including SADS-CoV, TGEV, PEDV, PRRSV, PRV, CSFV, PCV2, RV, PPV, FMDV and SIV. Genome extraction and real-time RT-RPA were performed as the above procedure. Distilled water was served as negative control.

2.7 | Detection of PDCoV in clinical samples

Clinical samples were obtained from the piglets with diarrhoea symptoms in a commercial swine farm in Guangdong province, China. A total of 108 swine clinical samples including 54 faecal swab specimens and 54 intestine specimens were collected. 10% (wt/vol) suspensions of the samples were prepared with Dulbecco's modified eagle medium. The supernatant fluids of the homogenates after passing through the 0.45 μm filter membrane were harvested. The internal control (IC) containing a lambda phage DNA segment was prepared following the armoured RNA technique and preserved in our laboratory (Pasloske, Walkerpeach, Obermoeller, Winkler, & DuBois, 1998; Yuan et al., 2015). Twenty μl IC was mixed with 180 μl supernatant fluids to monitor the process of nucleic acid extraction. Total nucleic acids were manually extracted from the mixtures using the innuPREP MP basic kit A (Jena Analytik, Jena, Germany) in accordance with the manufacturer's instruction and detected by RT-RPA and RT-qPCR respectively.

The primer-probe sets for RT-qPCR assays for the detection of PDCoV and IC were previously reported (Marthaler et al., 2014; Yuan et al., 2015). The probe for PDCoV was labelled with FAM, the probe for IC was labelled with JOE. These two primer-probe sets were included in one reaction tube for simultaneous detection of PDCoV and IC. The sample was considered positive by RT-qPCR when it generated two fluorescent signals for PDCoV and IC (Figure S4). The association between RT-RPA TT and RT-qPCR cycle threshold (C_t) values was analysed by Prism 5.0 software (GraphPad).

3 | RESULTS

3.1 | Screening of the primer sets

The relative performances of candidate primer sets have to be evaluated and compared. Previous studies have demonstrated that the RT-RPA assay can amplify the target gene in a wide range temperature of 30–42°C in 15–30 min, thus the reaction temperature of 39°C and reaction time of 20 min were set up in this study. Using the in vitro transcribed RNA as the template, the performances of the candidate primer pairs were assessed by the basic RT-RPA assay. As shown in Figure 1, the primer set F2/R2 successfully amplified the target region. Then, a probe was design which was located within the amplicon defined by the primer set F2/R2. The conservation of the sequences of the primer-probe set was evaluated by alignment of 26 PDCoV strains (Figure 2). Most of the sequences of the primer-probe set were identical, although one point mutation of two strains was on the reverse primer, we have the ground to state that the primer-probe set could detect a broadly range of PDCoV strains based on the previous studies showing that up to nine mismatches were tolerated and did not influence the sensitivity of the RPA assay (Boyle et al., 2013; Ma, Cong, et al., 2018). The sequences of primer candidates and probe used in this study were included in Table 1.

3.2 | PDCoV real-time RT-RPA Assay Sensitivity and Specificity

A dilution range of 10^1–10^6 copies per μl RNA molecules was used to assess the minimal detection limit of the real-time RT-RPA assay. The detection limit of the basic RT-RPA assay was firstly evaluated.

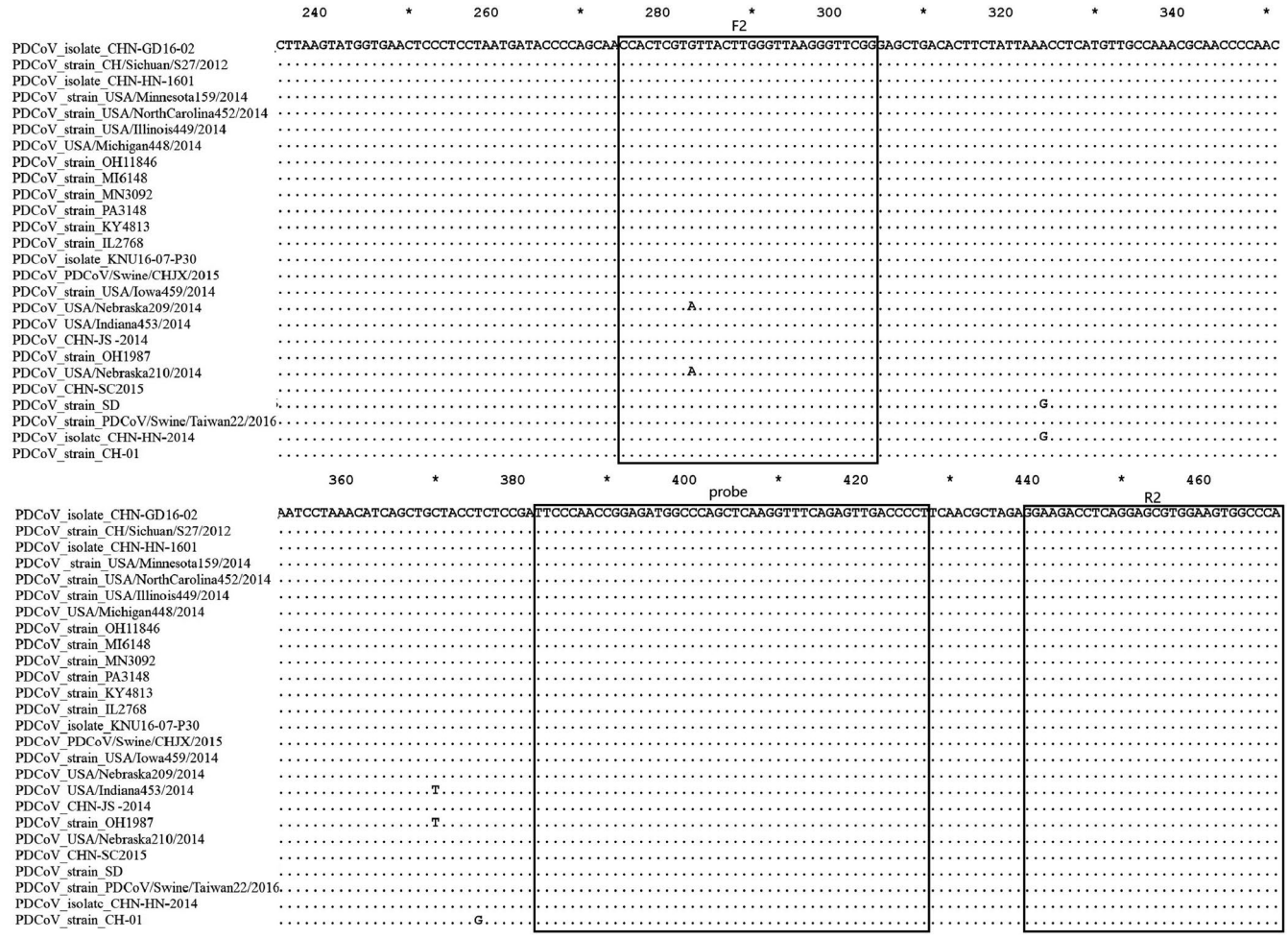

FIGURE 2 Alignment of the sequences of the primer-probe set used in the real-time RT-RPA assay. The forward primer (F2) and probe were used as shown while the reverse primer (R2) was used as antisense oligonucleotides. Nucleotide sequences of the primers (F2/R2) and probe are shown at the top of the frames while the corresponding nucleotide sequences of other 25 PDCoV strains are shown at the bottom. Dots represent that nucleotides are identical to that of PDCoV strain CHN-GD16-02

TABLE 1 Sequences of primers and probe for the real-time RT-RPA assay

Name	Sequence (5′–3′)	Genome position
RPA-F1	CTTCTCTTACTCAATCACAGTGAAGGAGGG	24763–24792
RPA-R1	GAGTTACCTTTTTAGGTTTCTTCTGCTGTTT	24936–24907
RPA-F2	CCACTCGTGTTACTTGGGTTAAGGGTTCGG	24258–24287
RPA-R2	TGGGCCACTTCCACGCTCCTGAGGTCTTCC	24451–24422
RPA-F3	TTCTCCTGACTATGAGAGACTTAAGGATG	24793–24821
RPA-R3	TTTTAGGTTTCTTCTGCTGTTTGGGTTTAG	24935–24906
RPA-F4	TTACTTGGGTTAAGGGTTCGGGAGCTGACA	24267–24296
RPA-R4	GATTGAGATCTTGGGCCACTTCCACGCTCC	24462–24433
RPA-F5	TTAAGGGTTCGGGAGCTGACACTTCTATTA	24276–24305
RPA-R5	CCACTTCCACGCTCCTGAGGTCTTCCTCTA	24447–24418
RPA-probe	TTCCCAACCGGAGATGGCCCAGCTCAAGG(FAM-dT)(THF)(BHQ1-dT)CAGAGTTGACCCCT(C3 spacer)	24365–24410

The basic RT-RPA assay using the primer set F2/R2 could detect as few as 10^2 copies RNA (Figure S1). When the serial dilutions of RNA standard were tested by the real-time RT-RPA assay, it produced fluorescence signals for a wide range of RNA molecule input levels. As illustrated in Figure 3a, RNA copy number over a range of 2 log10 RNA copies (LRC) to 6 LRC per reaction produced positive

fluorescence signals within 2.5–12.5 min. To evaluate the reproducibility of the real-time RT-RPA assay, serial dilutions of RNA standard were tested for eight replicates. Eight out of 8 (8/8) runs produced positive signals when RNA input was 10^6–10^2 copies per reaction, and 0/8 positive when 10 copies RNA per reaction were used. Semi-logarithmic regression analysis was performed using the data from the eight runs (Figure 4a). The result showed that the real-time RT-RPA assay can detect PDCoV as few as 10^2 copies per μl molecular RNA. Two replicates of the sensitivity test were supplied in Figure S2.

The specificity of the real-time RT-RPA assay was assessed by testing the genome extractions from relevant swine viral pathogens including SADS-CoV, TGEV, PEDV, PRRSV, PRV, CSFV, PCV2, RV, PPV, FMDV and SIV. As shown in Figure 3b, only PDCoV produced positive signal; no fluorescence signals were observed for the other viruses and distilled water throughout the whole reaction time. Three replicates of specificity test were performed in three independent day, another two replicates were supplied in Figure S3.

3.3 | Assay performance on clinical samples

For use in field, extraction of the virus genome from clinical samples was performed using the magnetic bead-based kit instead of the column extraction kit which required the unwieldy centrifugal machine. Eighteen of 54 faecal swab specimens and 21 of 54 intestine specimens were determined to be positive by RT-RPA. When RT-qPCR was applied to detect the samples, all the negative faecal swab samples by RT-RPA was also negative; 16 of 18 PDCoV-positive faecal swab samples determined by RT-RPA were positive, another two PDCoV-positive faecal swab samples were negative. No fluorescent signals were observed for the IC of these two samples, the other 106 samples all generated fluorescent signals of IC in RT-qPCR. So the extractions of these two samples were then purified using TRIzol reagent (Invitrogen) and tested again by the RT-qPCR. The result demonstrated that these two samples were positive for PDCoV, suggesting the extractions of these two samples by the magnetic bead-based kit may contain amplification inhibitors which were tolerated by the RT-RPA assay.

All the 21 PDCoV-positive intestine samples by RT-RPA were also positive by RT-qPCR. One intestine sample which was negative by RT-RPA was positive by RT-qPCR. The viral load in the intestine sample was 55 copies determined by RT-qPCR, which was lower than the detection limit of the RT-RPA assay. The coincidence rate of the detection results between RT-qPCR and RT-RPA was 97.2% (Table 2). Linear regression analysis showed a poor correlation between TT values and C_T values (Figure 4b), implying the RT-RPA assay could not be used to quantitative detection of PDCoV. To confirm the results of RT-RPA, amplicons of 20 positive samples were obtained by conventional RT-PCR (Ma, Zeng, Cong, et al., 2018) and sequenced. The generated sequences were 99% identical to that of N gene of PDCoV (data not shown).

4 | DISCUSSION

Since the first case of PDCoV isolation in Hong Kong in 2012, PDCoV infection has become prevalent in the pig herds across the world and caused huge economic losses to swine industry (Hu et al., 2015; Jung et al., 2015; Song et al., 2015; Wang et al., 2014; Xu et al., 2018; Zhang, 2016). Notably, the clinical sighs caused by PDCoV are very similar to that associated with other viral diarrhoea pathogens such as PEDV and TGEV, which makes it difficult to differential diagnosis. Once diarrhoea disease was occurred in the pig herd, scientists

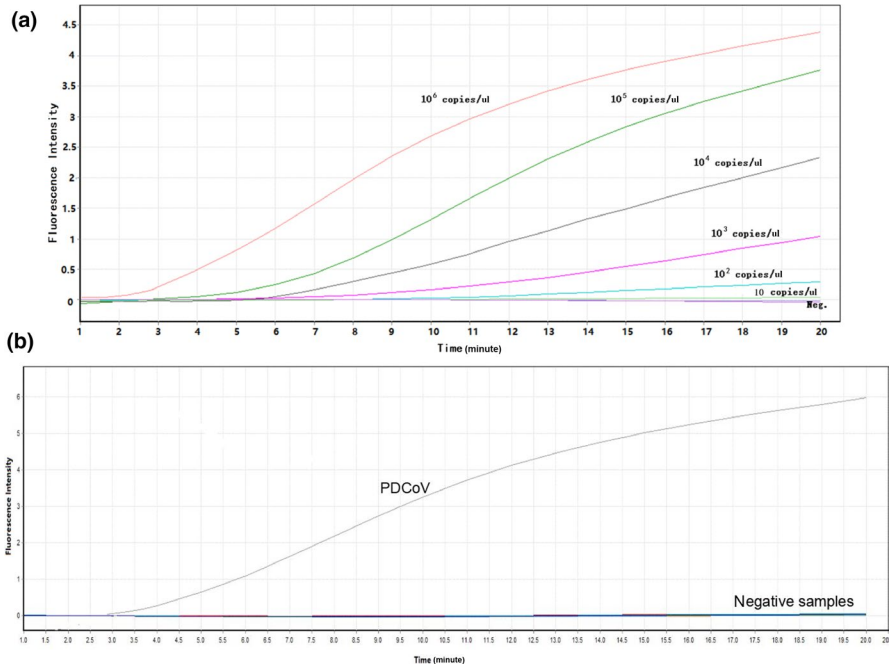

FIGURE 3 Sensitivity and specificity of the real-time RT-RPA assay. (a) The sensitivity of the real-time RT-RPA assay. (b) The specificity of the real-time RT-RPA assay. SADS-CoV, TGEV, PEDV, PRRSV, PRV, CSFV, PCV2, RV, PPV, FMDV, SIV and distilled water were the negative samples [Colour figure can be viewed at wileyonlinelibrary.com]

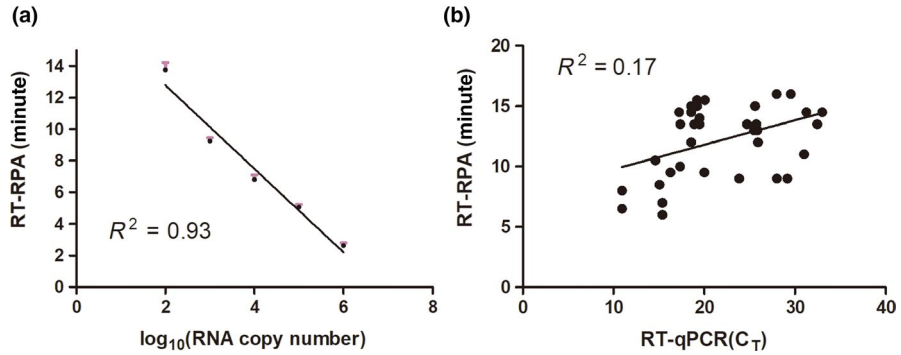

FIGURE 4 Performance of the RT-RPA assay. (a) Semi-logarithmic regression of the data collected from eight runs using the RNA standard analyzed by GraphPad Prism 5.0. (b) Linear regression analysis of RT-RPA threshold time (TT, *y* axis) and RT-PCR cycle threshold (CT) values (*x* axis) were determined by Prism software. R^2 value was 0.11 [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Coincidence rate of RT-RPA and RT-qPCR

		RT-qPCR			
		Positive	Negative	Total	CR
RT-RPA	Positive	37	2	39	
	Negative	1	68	69	97.2%
	Total	38	70	108	

Notes. CR: coincidence rate. CR = (37 + 68)/108*100%.

commonly suspected the causative agent of the diarrhoea cases was PEDV rather than PDCoV, which would delay the confirmation of suspected clinical cases (Wang et al., 2014). High positive rate of PDCoV infection has been demonstrated in several studies (Chen, Zhu, et al., 2015; Mai et al., 2018; Song et al., 2015). A rapid and sensitive method would be great helpful to the diagnosis of PDCoV infection in a timely manner. In this study, a real-time RT-RPA assay was successfully established and could detect PDCoV in less than 20 min. The RT-RPA assay had a detection limit of 100 copies molecule and no cross reaction with other common swine pathogens. The critical step of genome extraction from clinical samples was carried out using a magnetic bead-based kit. In addition, the real-time RT-RPA assay was performed by a portable device. All equipments and reagents for performing the real-time RPA assay could be put into a suitcase and took away from the centralized laboratory to the field or resource-limited settings, enabling the RT-RPA assay used for point-of-care diagnostic detection for the PDCoV.

Currently, molecular methods, especially RT-PCR and real-time RT-PCR, were routinely employed to confirm PDCoV in suspected cases associated with diarrhoea disease (Song et al., 2015; Wang et al., 2014). However, these assays required expensive and complex equipments and couldn't be performed outside of a well-equipped laboratory. In addition, these assays regularly required 90–120 min to produce detectable amplification product. RT-PCR and real-time RT-PCR are therefore not appropriate for point-of-care detection of PDCoV. Recently, a RT-LAMP assay was established for the detection of PDCoV (Zhang et al., 2017). The LAMP assay was performed at 63°C for 70 min in a water bath and required five primers. The amplification product was visualized by adding SYBR Green I dye to the reaction tube which did not require expensive equipment (Zhang et al., 2017). RPA, another isothermal amplification assay, requires only two primers, is carried out at a lower temperature (39°C) and takes shorter time (<30 min). Due to its advantages compared to other isothermal amplification methods, the RPA method has been widely used for rapid detection of multiple pathogens that infected with human beings and animals (Chen et al., 2018; Ma, Zeng, Huang, et al., 2018; Moore & Jaykus, 2017; Vasileva Wand, Bonney, Watson, Graham, & Hewson, 2018; Wang, Zhao, et al., 2018). For point-of-care diagnostics in remote area, RPA method is of great interest due to its portability, short turnaround time and potential for construction of a mobile laboratory. A RPA assay has been well studied for dengue virus detection in field (Abd El Wahed et al., 2015). Abd El Wahed et al. reported a mobile laboratory which included the magnetic beads-based extraction kit, the master mix, the sample mix and the tubescanner. Field trial in the Kedougou and Senegal demonstrated that the clinical sensitivity of the dengue virus RT-RPA assay was 98% compared to RT-qPCR (Abd El Wahed et al., 2015).

In our study, the analytical sensitivity of the PDCoV RT-RPA assay was 100 copies molecules, which was 10-fold lower sensitivity compared with RT-qPCR (Ma, Zeng, Cong, et al., 2018). The relative low sensitivity of the RT-RPA assay was confirmed by the results of intestine samples, in which one sample containing low RNA titre (55 RNA copies) was tested negative by RT-RPA. However, the nucleic acids extracted from two faecal swab samples by the magnetic bead-based kit were negative by RT-qPCR and positive by RT-RPA. No fluorescent signals of IC were observed for the extracts of the two faecal swab samples, suggesting the samples had high levels of inhibitors. One of the advantages of RPA over PCR was tolerance for amplification inhibitors, which has been proved by two previous studies (Krolov et al., 2014; Moore & Jaykus, 2017). In most of the studies regarding the RPA assay, researches commonly used the column kit to extract the genome which required a centrifuge, limiting the application of the RPA assay in field. In this study, the manual kit was used to extract the genome from the clinical samples and avoided the use of a centrifuge. Therefore, the platform combining magnetic bead-based extraction method and RPA shows promising advantages, such as

convenient operation, short run time, tube-closed reaction, less well-trained personnel and sophisticated equipments requirement, enabling the RPA assay to apply in field to detect clinical samples. The RPA products also could be visualized through the LFD. Although the LFD-RPA assay did not require a device, it is needed to open the tube lid and to add the RPA product to LFD, which made this assay potential of contamination (Zhang et al., 2017). In addition, it also increases the turnaround time. In comparison to the LFD-RPA assay, the tube-closed RT-RPA assay can real-time monitor the fluorescence of the amplicons, which reduces the run time and potential of contamination.

Collectively, a point of care method for rapid detection of PDCoV was established based on RPA. The RT-RPA assay had several advantages over RT-qPCR, including: (a) a portable device is sufficient; (b) shorter run time; (c) the use of enzyme pellet in one tube simplifies the operation procedures and reduces the potential of contamination; (d) the great potential of detection of PDCoV in the clinical samples in field. Thus, the RT-RPA assay provides a potential for point of care detection of PDCoV infection which is a worldwide epidemic in the pig herds across the world.

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AVAILABILITY OF DATA AND MATERIALS

The datasets contained in this study are available from the corresponding author upon request.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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