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Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay for the detection of porcine pegivirus

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ABSTRACT

A simple and accurate reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay was developed and evaluated for the detection of porcine pegivirus (PPgV). The specific RT-LAMP primers targeting the conserved regions of NS5A genes were designed and used to detect PPgV. The optimal reaction parameter for RT-LAMP assay was 63°C for 60 min. The detection limit of the RT-LAMP assay was 10 copies of PPgV genome, which was 100 times more sensitive than that of the conventional RT-PCR and comparable to nested RT-PCR and quantitative RT-PCR (qRT-PCR). There was no cross amplification with other related RNA viruses. In the clinical evaluation, the RT-LAMP assay exhibited a similar sensitivity with nested RT-PCR and qRT-PCR. The results indicated that RT-LAMP assay developed in this study could be a highly specific, sensitive, and cost-effective alternative for a rapid detection of PPgV in field settings.

Porcine pegiviruses (PPgV) are small enveloped viruses with a single-stranded, positive-sense RNA genome that have recently been assigned as the *Pegivirus* genus in the *Flaviviridae* family (Berg et al., 2015). PPgV genome contains a single large open reading frame (ORF) encoding a polyprotein, which is cleaved into individual proteins including E1, E2, X, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Pegiviruses are detected in diverse mammalian hosts including pigs, humans, horses, chimpanzees, bats, monkeys, and rodents (Yang et al., 2018). All known Pegiviruses have been classified into 11 species (A–K), and PPgV is a member of *Pegivirus* K. PPgV was first discovered in Germany in 2016 (Baechlein et al., 2016), and then reported in the United States in 2018 (Yang et al., 2018). In China, PPgV was first identified in Guangdong province (Lei et al., 2019), where vesicular diseases broke out in a swine farm in 2017. The serum samples were submitted to the Veterinary Diagnostic Laboratory of Jiangxi Agriculture University, China for diagnosis. The tested results indicated that foot-and-mouth disease virus (FMDV) and Seneca Valley virus (SVV) were negative but positive for PPgV by a RT-PCR assay. Although it remained yet unknown about the association between PPgV infection and the disease, more epidemiological studies are needed to address the clinical effect of PPgV on swine health. Currently, there are some methods available for PPgV diagnosis, including conventional RT-PCR, nested RT-PCR, and semi-quantitative RT-PCR (Yang et al., 2018; Lei et al., 2019; Chen et al., 2019). Therefore, a rapid and accurate method to detect PPgV is urgently needed for monitoring its presence.

In the past decade, loop-mediated isothermal amplification (LAMP) has become an effective technique, which exhibits high sensitivity and specificity for diagnosing important pathogens in medicine and veterinary medicine (Notomi et al., 2015). LAMP is a nucleic acid amplification-based method that generally requires a group of four specific external and internal primers (Notomi et al., 2000). Besides, additional loop primers can be used to further accelerate the reaction (Nagamine et al., 2002). In this study, a reverse transcription LAMP (RT-LAMP) assay was established and evaluated for surveying the prevalence of PPgV in porcine serum samples. A set of three specific primers pairs was designed to amplify the NS5A gene, which is suitable for RT-LAMP and relatively conserved in PPgV genome. The developed RT-LAMP assay showed high sensitivity and specificity for detection of the PPgV. The results tested by RT-LAMP assay on clinical samples were 100% correlated to that of nested RT-PCR and qRT-PCR, indicating the assay could be used for the surveillances of PPgV infection.

In this study, serum samples (N = 90) of 20-week-old pigs with a vesicular disease from Guangdong Province, China in 2017 were collected. Total RNA was extracted by using MiniBEST Viral RNA/DNA

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Table 1
Primers used in this study.

Test	Primer	Type	Length (bp)	Sequence 5'-3'	Position[a]	Remarks
RT-LAMP	F3	Forward outer	18	CTTCCGTTTGGCGCCTCG	7572-7589	F3 and B3 were used for nested RT-PCR (inner primers) of 217 bp products.
	B3	Reverse outer	19	GCCCGCAATCCAGGACTAC	7787-7769	
	FIP	Forward	45	AKYARCTCACCMACGTTRGCWSTTT-TYTGCTGTGGTGARGACMGW		
	F2	inner	19	YTGCTGTGGTGARGACMGW	7597-7615	
	F1c		22	AKYARCTCACCMACGTTRGCWS	7662-7641	
	BIP	Reverse	46	TCTGGTTGGAAGTTYASTGCYATTTT-AHGWMCCRAGYRCCTCCGTC		
	B2	Inner	20	AHGWMCCRAGYRCCTCCGTC	7748-7729	
	B1c		22	TCTGGTTGGAAGTTYASTGCYA	7670-7691	
	LF	Forward loop	25	WATGAGGTAGAKWCAMRCTAAAGGA	7640-7616	
	LB	Reverse loop	25	AYCAYRTTCCYTTCATYGAYGGRGA	7788-7769	
RT-PCR	F4	Forward	20	TGGCGTCTACTACTGCTACC	6900-6919	F4 and R4 were used as outer primers for nested RT-PCR and conventional RT-PCR (the expected size of the amplicon is 889 bp)
	R4	Reverse	20	GCCCGCAATCCAGGACTACT	7788-7769	
qRT-PCR	F5	Forward	20	TGACTCGGGTAGGACTTTGG	6921-6940	
	R5	Reverse	20	AAAGTACCGGGCTCCTCAGT	7158-7139	

[a] Genome position according to PPgV_GD/CH/2017 (GenBank: MG874672.1).

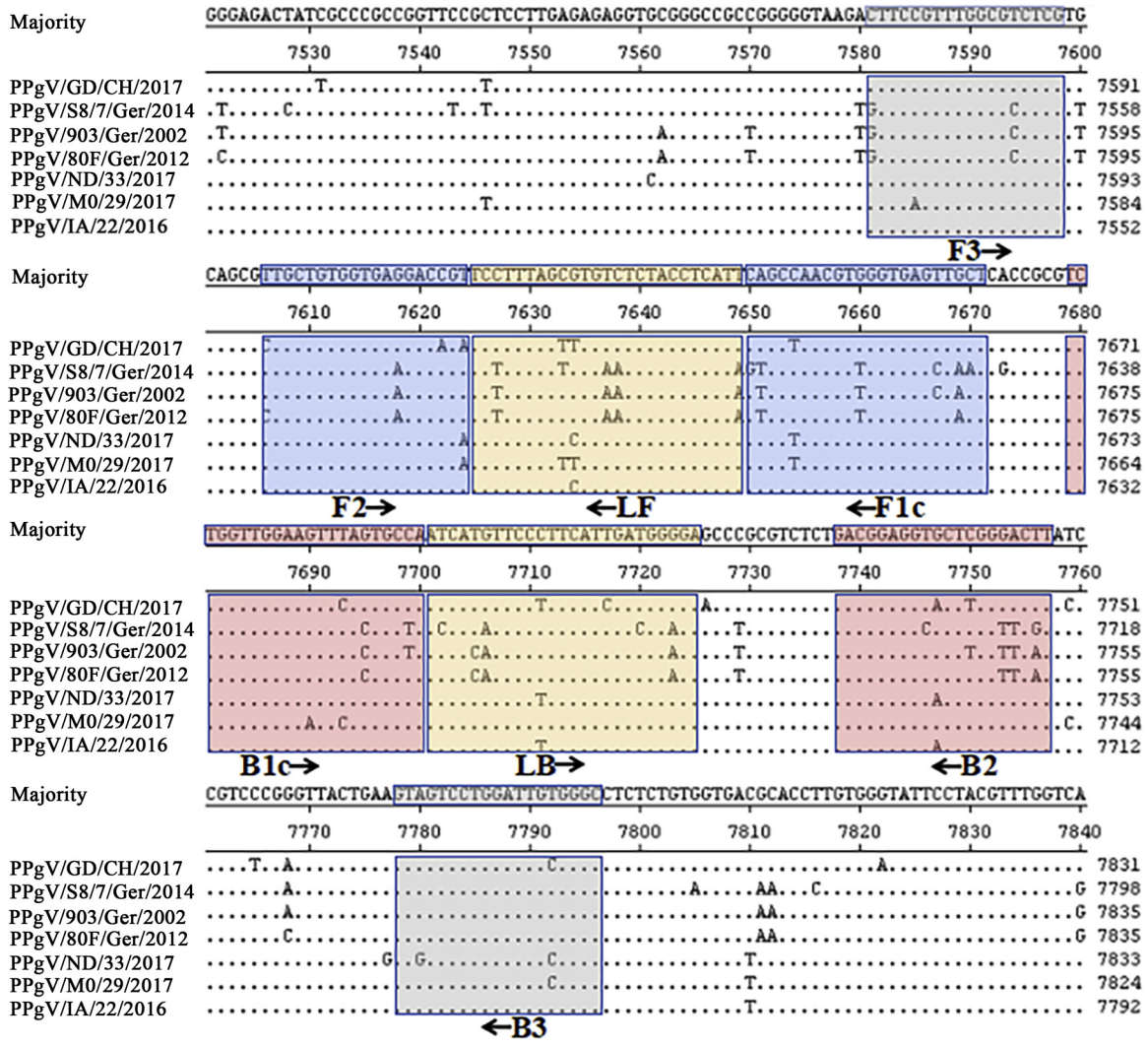

Fig. 1. Schematic representation of primer design of RT-LAMP assay located at NS5A gene of PPgV. The dots denote nucleotide identity among 7 reference genomes of PPgV isolates.

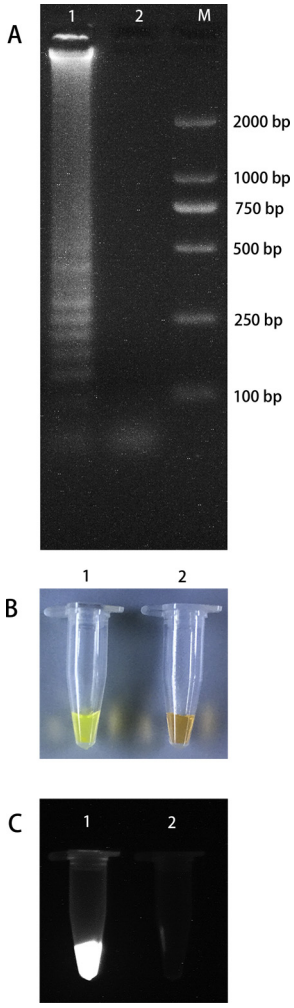

Fig. 2. RT-LAMP results: PPgV RNA was used as a template and six primers targeting the PPgV NS5A gene were used in the PPgV NS5A-based RT-LAMP assay performed at 63℃ for 60 min. The agarose electrophoresis results are shown in panel A: lane M 2000 bp DNA marker, lane 1 RT-LAMP products, lane 2 negative control. The direct observations by adding SYBR Green I are displayed in panels B (in daylight) and C (in UV light): tube 1 positive reaction, tube 2 negative reaction (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Extraction Kit (TaKaRa, China), and then stored at -80℃ until use. The extracted RNA was converted to the first strand of cDNA using PrimeScript RT Reagent Kit (TaKaRa, China) following the manufacturer's instructions. For the construction of plasmid standards, an 889-bp fragment of the NS5A gene of PPgV was initially amplified using a pair of primers (Table 1) with the 1st strand of cDNA as template. Afterwards, the purified fragment was cloned into the pMD19-T vector (TaKaRa, China) according to the manufacturer's protocol. To assess the successful construction of the plasmid, designated as pMD-PPgV, DNA sequencing was performed by Sangon Biotech Company (Shanghai, China). The concentration of pMD-PPgV plasmid was measured by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The copy number of the cloned gene was quantified as follows: [copy/μL = plasmid concentration (g/μL) / [(plasmid length) ×660) × (6.022×10^{23})] (Park et al., 2018). Serial dilutions of plasmid standards (1×10^{6}-1×10^{0} copies) were used as templates for the determination of detection limit of PCR, which would be used as a positive control in the RT-LAMP assay system.

The three pairs of RT-LAMP primers targeting the conserved regions of NS5A genes were designed based on the sequences of the reference strain (PPgV_GD/CH/2017, GenBank: MG874672.1; PPgV_S8/7/Ger/2014, GenBank: KU351671.1; PPgV_903/Ger/2013, GenBank: NC_034442.1; PPgV_80 F/Ger/2012, GenBank: KU351670.1; PPgV_33/ND/2017, GenBank: MG595747; PPgV_29/MO/2017, GenBank: MG595746; PPgV_22/IA/2016, GenBank: MG595745) using the online software Primer Explorer V4 (http://primerexplorer.jp/e/), which included an outer pair (F3 and B3), an inner pair (FIP and BIP), and a loop pair (LF and LB). The inner primers FIP and BIP contained F2 and B2 sequences with the complementary sequences of F1 and B1 (F1c and B1c) in their 5′-terminal, respectively (Fig. 1). A pair of primers (F4 and R4) was used for amplifying the NS5A gene in conventional RT-PCR and meanwhile served as outer primers for the nested RT-PCR (Table 1). The primer pair of F3 and B3 was used as inner primers for nested PCR. A pair of primers (F5 and R5) was used for qRT-PCR targeting the NS5A gene which was designed using the online software Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/). The specificity of the primers was then examined by a basic local alignment search tool (BLAST) search (http://www.ncbi.nlm.nih.gov/Blast) against the NCBI database. The results demonstrated that there was no hits, that is, no nonspecific primer binding site was observed.

The final volume of 25 μl reaction mixtures for RT-LAMP was prepared, which contained 1 μl of Bst DNA polymerase (NEB, USA) (8000 U/ml), 2.5 μl of 10 × Isothermal Amplification Buffer, 5 μl of Betaine (5 M), 1 μl of $MgSO_4$ (100 mM), 5 μl of dNTP (2.5 mM), 2 μl of each inner primers FIP and BIP (10 μmol), 0.25 μl of each outer primers F3 and B3 (10 μmol), 0.25 μl of each loop primers LF and LB (10 μmol), 1.25 μl of AMV reverse transcriptase (TaKaRa, China) (40 U/μl), 2 μl of RNA template, and the sterile distilled water was set as a negative control template. To determine the optimal reaction conditions, the RT-LAMP reactions were incubated at different temperatures of 60, 61, 62, 63, 64, and 65℃ for 30, 40, 50, 60, 70, and 80 min, respectively. Finally, the reaction was terminated by heat inactivation at 85℃ for 10 min. The amplified DNA products from the RT-LAMP were analyzed by 1.5% agarose gel electrophoresis with the addition of 0.01% ethidium bromide using a gel electrophoresis system (Liuyi, China). The results were also observed directly by color change from orange to green with staining due to the presence of SYBR Green I (Thermo Scientific, USA). After the amplification was completed, 1 μl of coloring agent was added to each reaction tube, and the results were then examined visually. In RT-LAMP assay with PPgV RNA as template, the amplified DNA products showed characteristic ladders of multiple bands on an agarose gel, indicating that the final products were a mixture of stem-loop DNA with different stem lengths (Khan et al., 2012). The results of the RT-LAMP reaction were also determined by direct visualization of the color change under daylight and UV light (Zheng et al., 2018) as shown in Fig. 2. In contrast, LAMP-specific DNA bands and color changes were not observed in the negative control. Meanwhile, the DNA products of RT-LAMP showed the highest intensity when the reaction was carried out for 60 min (Fig. 3). In regard to temperature, the optimal reaction temperature of RT-LAMP was 63℃. Therefore, the optimized parameter for the established RT-LAMP was 63℃ for 60 min.

To determine the detection limit of the RT-LAMP, 10-fold serially diluted plasmid standards of pMD-PPgV (1×10^{6}-1×10^{0} copies) were used as templates in 25 μl RT-LAMP reaction system under optimized conditions. The sterile distilled water was set as a negative control template. Nested RT-PCR was performed based on the protocol established in our laboratory (Lei et al., 2019). For the PCR step of the nested RT-PCR, the primer sets of F4/R4 and F3/B3 respectively served as outer (1st set) and inner primers (2nd set) (Table 1). The reaction system contained 1 μl of plasmid standards of pMD-PPgV (1×10^{6}-1×10^{0} copies) or 1 μl of the first PCR product, 2.5 μl of 10 × PCR buffer (TaKaRa, China), 0.3 μl of rTaq (5 U/μl), 2 μl of MgCl (25 mM), 1 μl of dNTP (2.5 mM), and 1 μl of each primer (10 μmol). Two rounds of nested PCRs were performed under the following conditions: 94℃ for 5 min; 35 cycles at 94℃ for 30 s, 55℃ for 1 min, and 72℃ for 1 min; and a final extension of 72℃ for 10 min (Wang et al., 2017). The

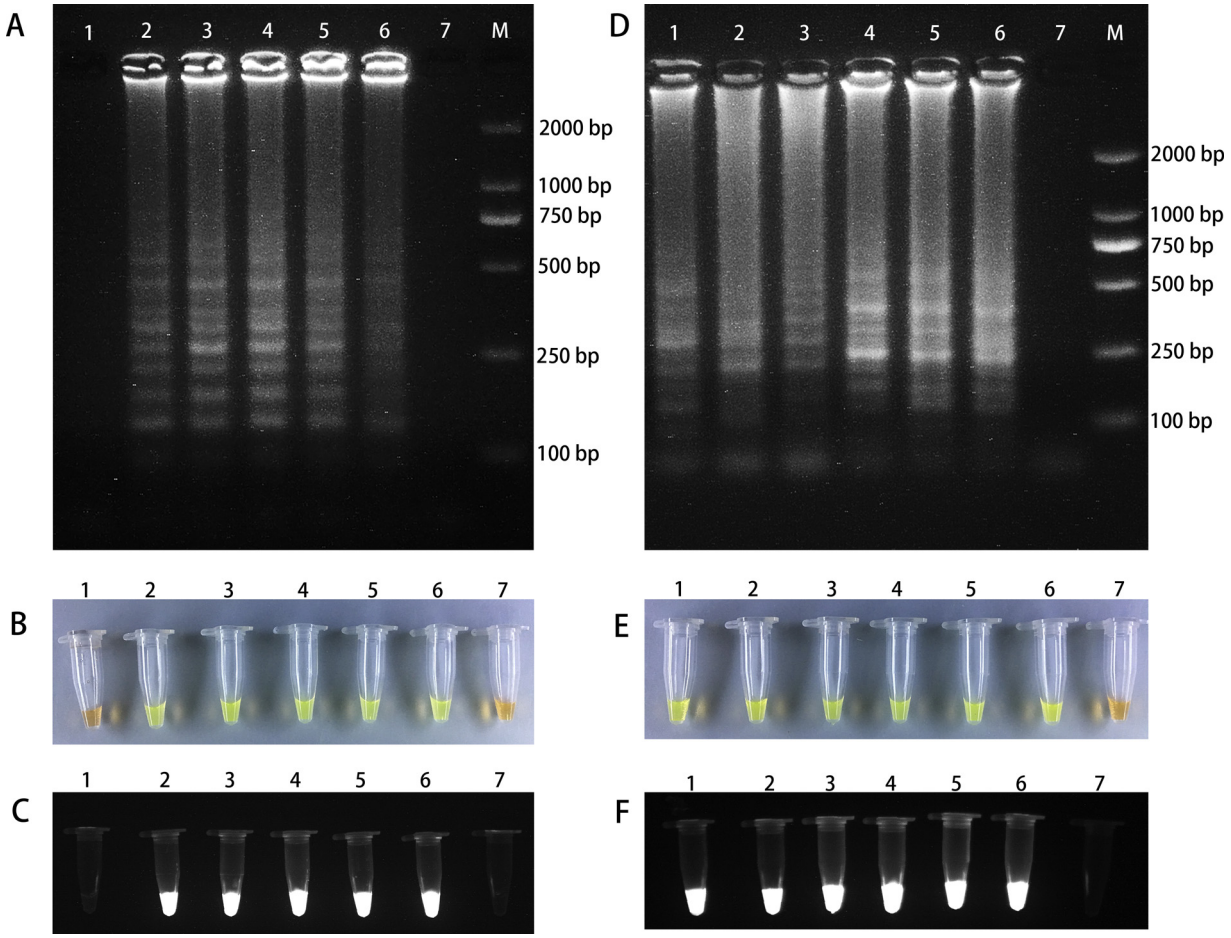

Fig. 3. Optimization results of RT-LAMP assay for amplification of PPgV RNA template at different reaction times (A, B, and C) and temperatures (D, E, and F). A. electrophoretic detection, B. visualization in daylight and C. in UV light of the RT-LAMP results. Tubes and lanes 1–6, different reaction times of 30, 40, 50, 60, 70, or 80 min. respectively; tube and lane 7, negative control; lane M, 2000 bp DNA marker; D. electrophoretic detection, visualization, E. in daylight and F. in UV light of the RT-LAMP results: tubes and lanes 1–6, at different reaction temperatures of 60, 61, 62, 63, 64, or 65°C, respectively; tube and lane 7, negative control and lane M, 2000 bp DNA marker.

conventional RT-PCR was carried out under the same reaction condition as above in nested RT-PCR using the primer pair of F4 and R4 based on the procedure described previously (Lei et al., 2019). The products of nested RT-PCR and conventional RT-PCR were analyzed by 1.5% agarose gel electrophoresis with 0.01% ethidium bromide. For qRT-PCR, the primer set of F5 and R5, targeting conserved region of the NS5A gene of PPgV, was used (Table 1). The qRT-PCR assay was performed by using a commercial qPCR kit (TaKaRa, China) based on the manufacturer's procedure. Each 20 µl reaction mixture consists of 10 µl of TB Green Fast qPCR mix, 1 µl of plasmid standards of pMD-PPgV (1×10^6-1×10^0 copies), 0.8 µl of (10 µmol) each forward and reverse primers and 0.4 µl of ROX. The amplification parameters included an initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 5 s and 60°C for 1 min. The melting curve analysis was measured using the software supplied with ABI7500 Fast Real-time PCR System (Applied Biosystems, USA). The results demonstrated that the detection limit of the developed RT-LAMP was 10 copies/µl, which was much higher than conventional RT-PCR (10^3 copies/µl), while it was comparable to that of the nested RT-PCR (10 copies/µl) and qRT-PCR (10 copies/µl), respectively (Fig. 4).

To assess the specificity of RT-LAMP for PPgV, cDNAs of Seneca Valley virus (SVV), food and mouth disease virus (FMDV), porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus (PDCoV), and swine acute diarrhea syndrome coronavirus (SADS-CoV) were used, and the plasmid standards of pMD-PPgV was used as the positive control. The amplified products were analyzed by 1.5% agarose gel electrophoresis with 0.01% ethidium bromide and addition of SYBR Green I staining solution. The results indicated that there was no cross amplification of the RT-LAMP assay for these control viruses. Using PPgV RNA as a template, positive DNA bands were amplified as expected (Fig. 5), indicating that the RT-LAMP assay developed was specific for PPgV.

To further evaluate the developed RT-LAMP assay, serum samples (n = 299) from finishing pigs and sows collected in Guangdong and Jiangxi province in China during October 2017 to January 2018 were tested (Lei et al., 2019). Total RNA was extracted and used as a template for conventional RT-PCR, nested RT-PCR, qRT-PCR, and RT-LAMP according to aforementioned methods. As shown in Table 2, the positive sample rates of conventional RT-PCR, nested RT-PCR, qRT-PCR, and RT-LAMP were 9.03% (27/299), 10.37% (31/299), 10.37% (31/299), 10.37% (31/299), respectively, suggesting these data were consistent with our previous findings (Lei et al., 2019). The detailed information is listed in Table S1. The results showed the RT-LAMP had a similar sensitivity to nested RT-PCR and qRT-PCR, which was superior to conventional RT-PCR.

Pegiviruses have a broad host range and infect diverse animal species. PPgV is recently proposed as a novel species in the *Pegivirus* genus. PPgV epidemics have been confirmed in Germany in 2017, the United States in 2018, and China in 2018. Although several pigs positive for PPgV exhibited vesicle and lameness (Yang et al., 2018), there is limited evidence that infections were associated with a clinical disease. Therefore, the clinical significance and epidemiological investigation of

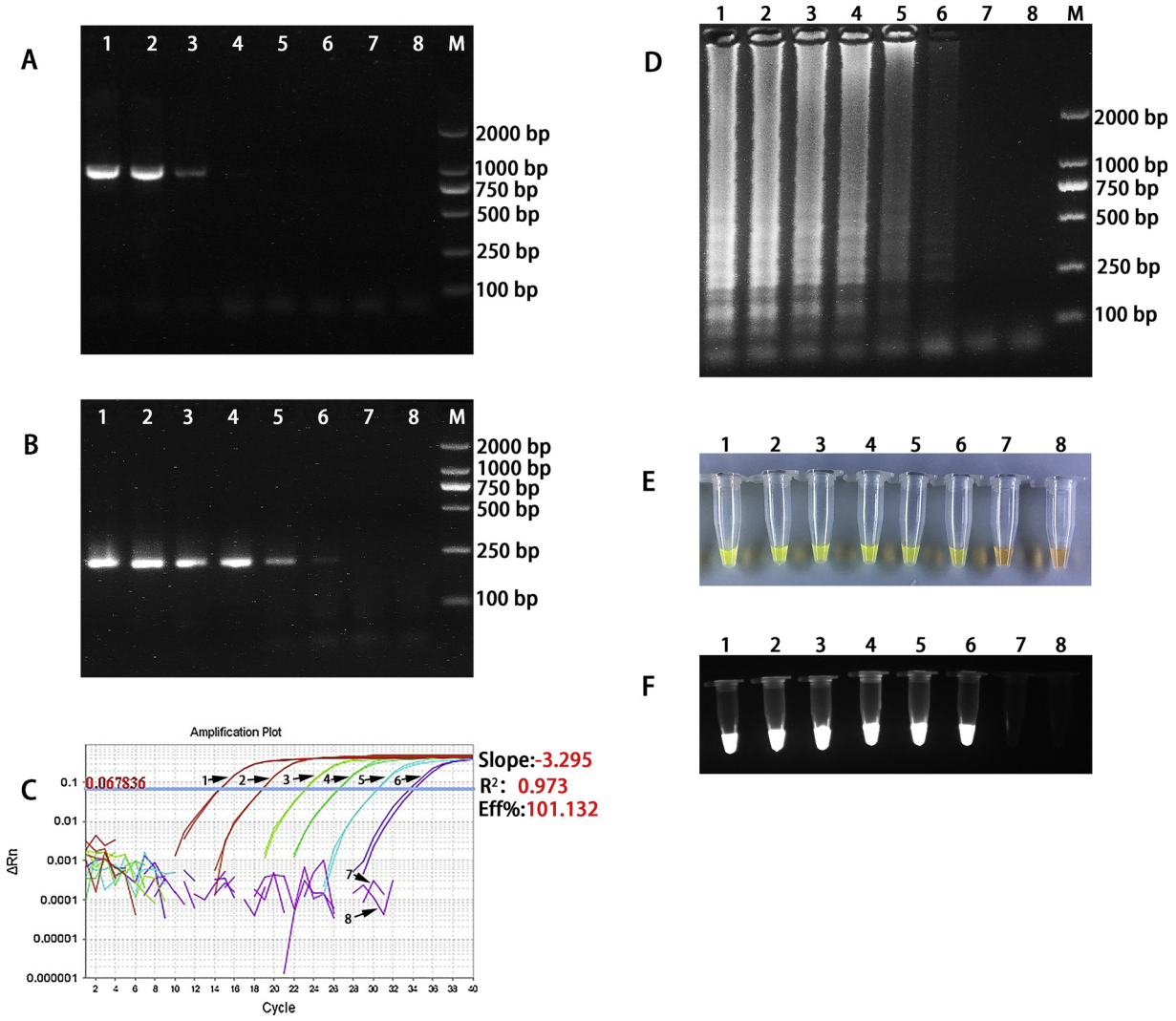

Fig. 4. Comparison of sensitivities of the conventional RT-PCR, nested RT-PCR, qRT-PCR, and RT-LAMP assays. A. detection limit of the conventional RT-PCR assay, B. nested RT-PCR, C. qRT-PCR and RT-LAMP assays. (D, E, and F): D. electrophoretic detection, E. visualization in daylight, and F. in UV light for the amplification of the plasmid pMD-PPgV (1×10^6-1×10^0 copies). Tubes and lanes 1–7, 10-fold serial dilutions (from 1×10^6-1×10^0 copies) of the plasmid standards of pMD-PPgV. Tube and lane 8, negative control; lane M, 2000 bp DNA marker.

PPgV infection should be further studied. Nevertheless, the establishment of a sensitive, cost-effective, and rapid assay for detecting PPgV is urgently needed. In this study, a RT-LAMP assay was developed to monitor PPgV in pig populations. Compared to other diagnostic methods, such as conventional RT-PCR, nested RT-PCR, and qRT-PCR (Baechlein et al., 2016), RT-LAMP assay may eliminate a need for complex procedures and expensive instruments, which makes it suitable for poorly equipped laboratories and clinics (Lopez-Jimena et al., 2018).

RT-LAMP is considered to be an alternative diagnostic tool in terms of its ability to rapidly amplify target nucleotide sequence(s) under isothermal conditions. LAMP has been widely used to detect a variety of important swine pathogens, including PDCoV (Zhang et al., 2017) and PEDV (Mai et al., 2018). In the present study, the RT-LAMP reaction conditions were optimized in terms of reaction time and temperature. The detection limit of RT-LAMP assay established is 100 times higher than that of conventional RT-PCR, and had a similar sensitivity to nested RT-PCR and qRT-PCR. RT-LAMP developed in the study showed a high specificity as it did not cross react with other related porcine viruses. In the clinical evaluation, the positive rate of RT-LAMP were consistent with that of nested RT-PCR and qRT-PCR, indicating the PPgV-specific RT-LAMP assay was a valuable tool for the rapid detection in field settings.

In summary, we developed a rapid, simple, accurate, and cost-effective RT-LAMP assay for detection of PPgV, and the assay could be used as an alternative for the clinical diagnosis of PPgV infection and epidemiological investigation.

Declarations of interest

None.

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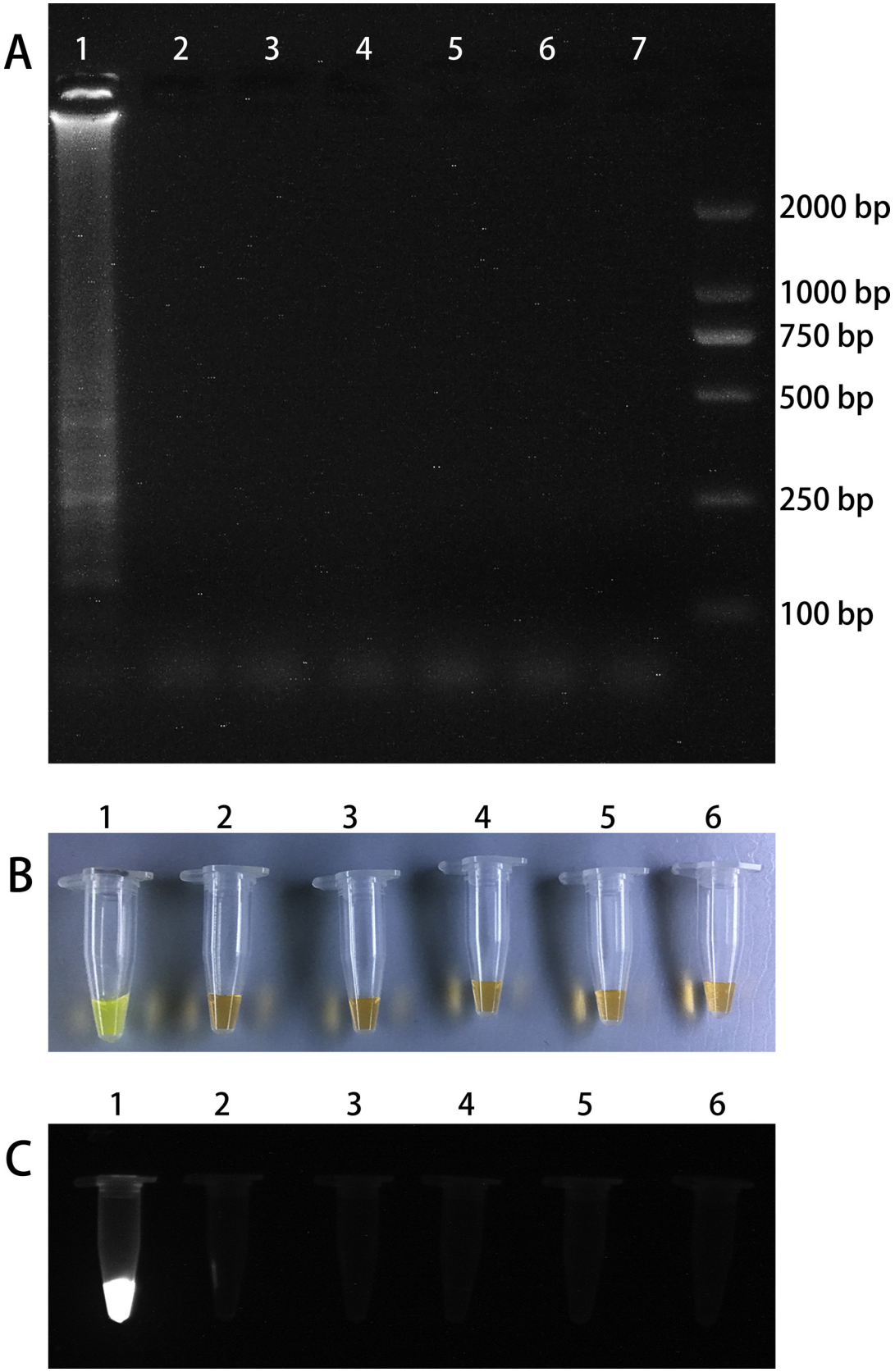

Fig. 5. Specificity of the RT-LAMP assay. PPgV (plasmid standards of pMD-PPgV of 1×10^5 copies) and other related swine pathogenic RNA viruses were used as templates and subjected to RT-LAMP performed at 63℃ for 60 min. Tube and lane 1–6 are RT-LAMP results from templates of PPgV, SVV, FMDV, PEDV, PDCoV, and SADS-CoV, tube and lane 7, negative control, and lane M, 2000 bp DNA marker.

Table 2
Detection results of PPgV in clinical samples by conventional RT-PCR, nested RT-PCR, qRT-PCR, and RT-LAMP.

Samples type	Total number of samples	Positive rate of PPgV			
		conventional RT-PCR	nested RT-PCR	qRT-PCR	RT-LAMP
Serum	299	9.03% (27/299)	10.37% (31/299)	10.37% (31/299)	10.37% (31/299)

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.04.019.

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