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Instrument-free, visual and direct detection of porcine reproductive and respiratory syndrome viruses in resource-limited settings

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Summary. – Porcine reproductive and respiratory syndrome (PRRS) caused by PRRS virus (PRRSV) is one of the most complicated and dangerous diseases in pigs with high mortality since it modulates the immune system of the lungs and has been closely associated with secondary infection of other lethal bacteria and viruses. The gold standard of molecular diagnosis for PRRSV, reverse transcription (RT)-PCR, is time-consuming, expensive and requires transportation of samples to a specialized laboratory. In this study, a direct colorimetric RT-loop-mediated isothermal amplification (RT-LAMP) method was developed to specifically and rapidly detect PRRSV. The RT-LAMP outcomes can be visualized by the naked eye after 45 min of incubation at 65 °C without any cross-reactivity recorded with the bacteria and other viruses tested. In particular, the mobile, non-instrumented, commercial pocket hand warmers were demonstrated to successfully provide constant temperature for consistent nucleic acid amplification throughout the RT-LAMP reactions. The limit of detection of the assay was defined as the genomic RNA concentration extracted from a known viral titer of $10^{-2.5}$ $TCID_{50}$/ml. The direct use of clinical serum samples required a simple dilution to maintain the performance of the colorimetric RT-LAMP assay. Therefore, the direct colorimetric RT-LAMP assay developed is well-qualified for producing a ready-to-use kit for PRRSV diagnosis in the field.

Keywords: porcine reproductive and respiratory syndrome; rapid testing; RT-LAMP; colorimetric; direct detection; instrument-free

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most severe and rapidly infectious diseases in pigs. The typical clinical signs of the disease include reproductive dysfunction in female pigs such as infertility, abortion or weakly-born offspring; and respiratory distress characterized by breathlessness, coughing, remarkable weight loss and sluggishness (Dietze *et al*., 2011). Porcine reproductive and respiratory syndrome virus (PRRSV) is a direct pathogen of PRRS. The virus belonging to the *Arteriviridae* family possesses a positive-sense single-stranded RNA of 14.9-15.5 kilobases in length as its genetic material (Kappes and Faaberg, 2015). Since the emergence of PRRS, outbreaks of this contagious and dangerous disease have become a substantial economic burden on the livestock industry in numerous regions, especially those in America, Europe, and Asia. There are two significant PRRSV genotypes so far: genotype 1, sometimes called the European (EU) type, and genotype 2, commonly known as the North American (NA) type. The type 2-related virulent PRRSV strain that emerged in China in 2006 is also the etiologic agent that has given

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Abbreviations: LAMP = loop-mediated isothermal amplification; LOD = limit of detection; PRRS = porcine reproductive and respiratory syndrome; PRRSV = PRRS virus; RT-LAMP = reverse-transcription LAMP; $TCID_{50}$ = Median Tissue Culture Infectious Dose

rise to PRRS and become predominant in Vietnam since 2007 (Tian *et al*., 2007; Metwally *et al*., 2010).

To date, PCR and real-time PCR are standard and effective diagnostic methods with high specificity and sensitivity. Nevertheless, these methods have several limitations, such as being time-consuming, and the necessity of a specialized laboratory with expensive equipment and experienced technicians. As a result, developing another simple, quick, and cost-effective method to respond to the disease and minimize its financial consequences is critical. Loop-mediated isothermal amplification (LAMP) is one of the isothermal nucleic acid amplification methods, first developed by Notomi and colleagues in 2000 (Notomi *et al*., 2000). Among various isothermal techniques, LAMP is considered the most common and efficacious diagnostic method, contributing to 60.7% of total publications in 2019 (Becherer *et al*., 2020). In general, LAMP can perform specific amplification with high sensitivity under a particular temperature, typically around 60–65˚C in less than one hour of incubation (Becherer *et al*., 2020). Moreover, based on the verified advantages over PCR, LAMP is a potential point-of-care technique for rapidly and effectively recognizing the infection by pathogens. Reverse transcription (RT)-LAMP has already been applied successfully for the detection of SARS coronavirus (Aoki *et al*., 2021; Jamwal *et al*., 2021; Tran *et al*., 2021; Huang *et al*., 2022; Promlek *et al*., 2022), influenza type A virus (Ahn *et al*., 2019; Chen *et al*., 2021; Golabi *et al*., 2021), and human papillomavirus, for which commercial kits have been approved as *in vitro* diagnostics (Mori *et al*., 2013).

LAMP reactions are usually performed in a laboratory setting using a heater or water bath set at a constant temperature, which is still dependent on the electrical supply. However, in order to demonstrate the isothermal technique's point-of-care feature, a number of devices have been constructed or installed as direct-to-infection alternatives, the majority of which rely on exothermal chemical processes. As a result, different works have been reported of applying those instruments for clinical diagnosis, such as man-made heaters using calcium oxide (CaO) and water to detect *Plasmodium falciparum* genomic DNA or HIV-1 (LaBarre *et al*., 2011; Curtis *et al*., 2012, 2012), disposable pocket warmers to diagnose *Bacillus anthracis* (Hatano *et al*., 2010) or *Mycobacterium ulcerans* (Ablordey *et al*., 2012). Nevertheless, these devices still require a certain degree of complexity and are not commercially available.

So far, several RT-LAMP assays have been established for the identification of PRRSV or its highly pathogenic variants (Chen *et al*., 2008; Li *et al*., 2009; Park *et al*., 2016). However, there have been no studies associated with PRRSV detection by RT-LAMP under limited settings. The ultimate goal of the study is to help farmers carry out diagnosis for PRRS easily and economically in the field; thus, there is no need to deliver samples to a laboratory, deterring the spread of PRRS in time to minimize financial loss. Therefore, in this work, we developed a rapid and colorimetric assay based on the RT-LAMP method to detect PRRSV specifically and sensitively. The performance of LAMP reactions detecting PRRSV using commercially available, inexpensive, convenient, disposable and instrument-free pocket hand warmers as well as crude specimens without sample extraction was assessed, raising the extremely high feasibility of the assay in reallife application.

Table 1. Bacterial and viral strains used in this study

Microorganisms	References
Salmonella enterica	ATCC14012
Staphylococcus aureus	ATCC6538
Pseudomonas aeruginosa	ATCC15442
L. monocytogenes	Laboratory collection
PRRSV EU (type 1)	MG251834
PRRSV NA (type 2)	KF699844
Classical swine fever virus (CSFV)	MH979232
Foot and mouth disease virus (FMDV)	MN379784
Porcine epidemic diarrhea virus (PEDV)	KT941120
African swine fever virus (ASFV)	MK554698

Materials and Methods

Sample collection and preparation. Serum samples of PRRS symptomatic pigs from domestic pig farms in Vietnam were collected. Viral genomic RNA was extracted from serum samples using the QIAamp Viral RNA Mini Kit (Qiagen, USA). The RNA of PRRSV extracted was identified using the VDx PRRSv qRT-PCR (NA/EU dual) Kit (Median Diagnostics Inc., Korea). The results of qRT-PCR are shown in Supplementary Table S1. For evaluation of the limit of detection (LOD), PRRSV genomic RNA was extracted from a known viral titer of $10^{5.5}$ Median Tissue Culture Infectious Dose per ml ($TCID_{50}$/ml). The extracted viral RNA samples obtained were stored at -20°C. The remaining portions of every serum specimen collected that were not subjected to the extraction procedure were preserved at -80˚C and subsequently used for the direct RT-LAMP assay. The bacterial cells (Table 1) were incubated at 37°C overnight with shaking at 180 rounds per minute. While other bacteria were cultured in Luria-Bertani medium (HiMedia Laboratories Pvt. Ltd, India), *Listeria monocytogenes* was inoculated in Brain Heart Infusion Broth (HiMedia Laboratories Pvt. Ltd, India). The DNA extraction of cultured bacteria used the cetrimonium bromide (CTAB) method (Wilson, 2001), which was modified to suit the

laboratory conditions. Extracted DNA samples were stored at -20°C for preservation until use.

Primer design. The membrane protein M (M gene) region of PRRSV was targeted due to the high level of conservation and specificity. The genome of the PRRSV SRV07 strain (GenBank Acc. No. JX512910.2) identified in Vietnam was used as the template for primer design (Guo *et al.*, 2013). PrimerExplorer V5 (http://primerexplorer.jp/lampv5e/index.html), a primer design tool, was employed to create two corresponding sets of six well-qualified primers, including F3, B3, FIP, BIP, LoopF and LoopR. The most qualified primer set was screened using primer similarity screening via NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool). The synthetic fragment (14543–14745) of the M gene of PRRSV (GenBank Acc. No. JX512910.2) was used as a positive control sample. The primers (Table 2) and synthetic DNA template were produced by Phusa Biochem (Can Tho, Vietnam).

Colorimetric RT-LAMP assay. The LAMP reaction (15 µl) contains 0.2 µM F3/B3, 1.6 µM FIP/BIP and 0.4 µM LoopF/LoopB, 7.5 µl of WarmStart® Colorimetric LAMP 2X Master Mix (DNA & RNA) (New England Biolabs, MA, USA), 5 µl of the template and nuclease-free H_2O. Nuclease-free H_2O was used as a negative control sample. The tubes were then incubated in BioSan Dry Block Thermostat Bio TDB-100. As for using thermopads, the reactions were placed in the middle of the sealed hand warmer. The amplification products were visualized by the color shifting from red to yellow/orange of the reaction, which is based on a pH-sensitive indicator as instructed by the manufacturer. After that, 1 µl of 100X SYBR Green I dye (Thermo Fisher Scientific, Massachusetts, USA) was added to the reaction tube, and the fluorescent signal was observed under blue light (470 nm, BlooK LED transilluminator, GeneDireX, Taiwan). The products were also analyzed by electrophoresis on a 2% agarose gel when needed.

Optimization of PRRSV colorimetric RT-LAMP assay. All optimization reactions used the genomic RNA of PRRSV extracted from a viral culture of $10^{2.5}$ $TCID_{50}$/ml concentration. According to the master mix manufacturer, temperature optimization was processed at 55°C, 60–65°C (1°C increment), and 70°C for 45 min. As for incubation time optimization, a wide range of time was investigated, from 15 to 60 min, with a 5-min interval at the optimal temperature. Regarding using the thermopads, the reactions were placed in the middle of the sealed hand warmer from 40 to 60 min (5-min increment).

Evaluation of primer specificity and limit of detection. The specificity of primers was tested among common bacterial strains and closely related viruses (Table 1). The genomic DNA/RNA samples examined were diluted to the same concentration (1 ng/µl) and then added into the LAMP reactions. The procedure was conducted according to the optimized conditions. Additionally, electronic LAMP (eLAMP), a virtual LAMP tool (Salinas and Little, 2012), was employed to intensively examine the specificity of the whole set of primers for each gene on sequences of common, closely related, and concurrently infected organisms. To identify the LOD value of the colorimetric RT-LAMP assay, a series of 10-fold dilutions of PRRSV genomic RNA samples ranging from $10^{1.5}$ to $10^{-6.5}$ $TCID_{50}$/ml were utilized as the template for the RT-LAMP reactions under optimal conditions.

Direct colorimetric RT-LAMP assay using crude samples. To determine the dilution factor of the crude sample, at which the LAMP amplicon could be formed, the extracted viral RNA corresponding to a PRRSV culture of $10^{4.5}$ $TCID_{50}$/ml concentration was spiked (1:10) into a commercial serum (Porcine serum, Sigma-Aldrich, Merck KGaA, Germany). And then the spiked serum was serially diluted by 1 to 200-fold in nuclease-free water. Next, 5 µl of simulated specimens were added to the RT-LAMP reactions.

Evaluation of direct colorimetric RT-LAMP assay. To evaluate the direct colorimetric RT-LAMP assay using clinical samples, fifty serum specimens were collected from domestic pigs suspected of being infected at local farms. The serum collected was first diluted 50-fold in nuclease-free water. Next, 5 µl of the diluted samples were added directly to the RT-LAMP reactions. To make comparisons, viral RNA was extracted from fifty serum specimens using the QIAamp RNA mini kit (Qiagen, USA), and the presence of PRRSV was confirmed using the VDx PRRSV qRT-PCR (NA/EU dual) Kit (Median Diagnostics Inc., Korea) (Supplementary Table S1).

Temperature monitoring of pocket hand warmers. Several types of commercial hot packs available on e-commercial platforms were purchased (Supplementary Fig. S1). Temperature tracking over time was measured by a portable digital thermometer placed in the closed setting of the thermopads. Measured temperatures were recorded every 5 min. The experiment was repeated several times, and the average temperature was plotted against time.

Results

Optimization of PRRSV colorimetric RT-LAMP assay

The RT-LAMP reactions were initially carried out to generally assess the amplification ability and accuracy of the primers designed. Regarding the positive control reactions using the synthetic DNA template at the con-

Table 2. Primer sequences used in this study

Name	Primer sequence (5'–3')
F3	TCAGCCATAGAAACCTGG
B3	AGGCTTTTCAACCCGG
FIB	ACTTTCGACGTGGTGGGCAG-CTCCAGATGCCGTTTGTGC
BIP	TCATCCGATTGCGGCAAATGA-TGCCGTTGACCGTAGTG
LoopF	AGAATGTACTTGCGGCCTAGCAA
LoopR	ACCACGCATTTGTCGTCCGG

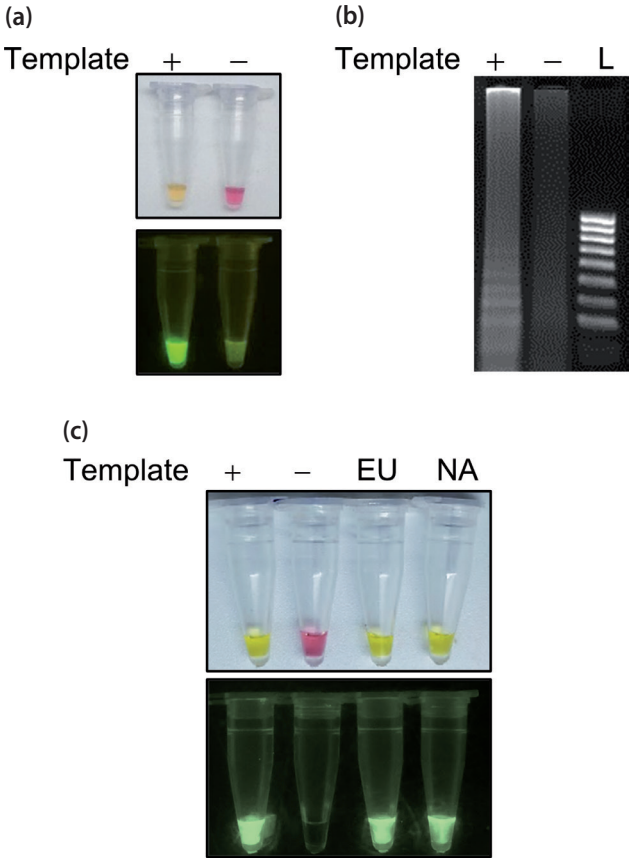

Fig. 1

Colorimetric RT-LAMP to detect the presence of PRRSV target gene

(a) The amplified product was visualized by the color change from red to yellow. The reactions containing 5 µl of a synthetic DNA template with 10^6 copies/µl were incubated at 65°C for 30 min. The reaction color was captured by the personal mobile phone (upper panel) and the SYBR Green I fluorescent signal was visualized by a blue light transilluminator (lower panel). **(b)** The LAMP product was analyzed by 2% agarose gel electrophoresis. **(c)** Colorimetric RT-LAMP to detect the presence of NA and EU genotypes of PRRSV. The reactions contain 5 µl of extracted genomic RNA corresponding to a PRRSV culture of $10^{2.5}$ $TCID_{50}$/ml concentration. L: 100 bp DNA ladder (NEB, USA).

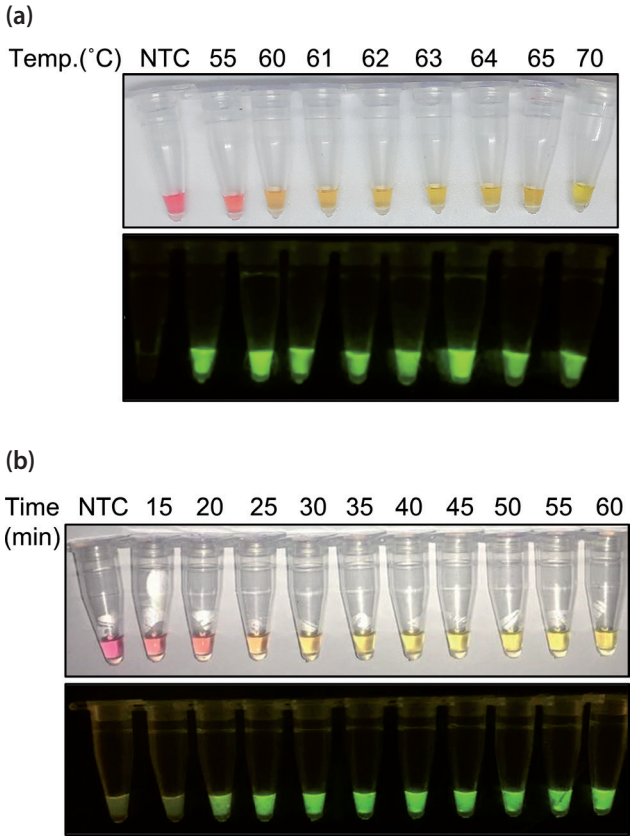

Fig. 2

Optimization of incubation temperature and time of the RT-LAMP reactions

The reactions contain 5 µl of genomic-RNA extracted from a PRRSV culture of $10^{2.5}$ $TCID_{50}$/ml concentration. **(a)** The reactions were incubated for 45 min at temperatures ranging from 55 to 70°C. **(b)** The reactions were incubated at 65°C for 15 to 60 min. The color was captured by the personal mobile phone (upper panel) and the SYBR Green I fluorescent signal was visualized by a blue light transilluminator (lower panel). NTC: negative control.

centration 10^6 copies/µl, the RT-LAMP reactions exhibited a color shift from pink to yellow after incubation at 65°C for 30 min (Fig. 1a). Meanwhile, the negative control reaction remained unchanged in color. The LAMP results were then reconfirmed by the fluorescent SYBR Green I dye. The positive results were luminous with a green glow under blue light, while the negative ones did not emit a green light signal. AGE was also performed to ensure the consistent and reliable results of the colorimetric LAMP assay. The positive reaction of LAMP appeared as a ladder-like pattern with numerous bands of varying sizes. In contrast, no band was detected in the negative control (Fig. 1b). Most importantly, the colorimetric LAMP assay with specific primers was able to detect both genotypes EU and NA of PRRSV, indicating the comprehensiveness of the method for the detection of PRRSV (Fig. 1c).

Next, the optimal temperature and incubation time for the detection of PRRSV by RT-LAMP were defined as described above. The results indicate that although the amplified products were formed within the range of 55 to 70°C (Fig. 2a, lower panel), 60°C was the minimum temperature for detecting the color change by the unaided eye (Fig. 2a, upper panel). To ensure the most distinctive color shift to yellow can be observed, 65°C was selected as the temperature to conduct subsequent RT-LAMP experiments. As for incubation time, although enough product was generated to provide a fluorescent signal after 20 min (Fig. 2b, lower panel), the colorimetric reaction required at least 25 min to exhibit the clear color shift (Fig. 2b, up-

per panel). Moreover, only after 40 min, reactions showed the brightest yellow color as well as strong fluorescent signals. To ensure the outcome signal of a sample with low template concentrations, the optimal reaction condition was set at 65°C for 45 min.

Primer specificity of PRRSV colorimetric RT-LAMP assay

Primer specificity is one of the crucial factors that significantly impacts the false-positive ratio. Thus, the LAMP reactions using the same concentration of genomic DNA/RNAs of different types of bacteria and viruses that are related to PRRSV (Table 1) were performed to evaluate the selectivity of designed primers. Among those bacterial and viral samples tested, except for PRRSV, no primer set designed showed amplified products; in other words, no cross-reactivity was observed (Fig. 3). Based on eLAMP results (Table 3), the RT-LAMP primer set used herein selectively detected the presence of PRRSV RNA. Consequently, the primers were highly specific for PRRSV under optimal parameters.

PRRSV colorimetric RT-LAMP reactions using pocket hand warmers

Temperature range plays a vital role in the utilization of pocket hand warmers in real-world applications.

Table 3. Primer specificity analysis by eLAMP

Genome information	Result
AP017922.1 *Staphylococcus aureus* DNA; complete genome; strain: JP080	0
AP018036.1 *Mycobacterium tuberculosis* DNA; complete genome; strain: HN-506	0
CP025256.1 *Streptococcus pneumoniae* Xen35; complete genome	0
CP039256.1 *Leptospira interrogans* strain FMAS_KW2 chromosome I; complete sequence	0
FJ009233.1 Porcine hemagglutinating encephalomyelitis virus strain HEV-JT06 membrane protein gene, complete cds	0
JX505432.1 H-1 parvovirus small nonstructural protein gene, complete cds, alternatively spliced; and major nonstructural protein, minor capsid protein, major capsid protein, and late nonstructural protein genes, complete cds	0
JX512910.2 Porcine reproductive and respiratory syndrome virus strain SRV07, complete genome	1
KT343756.1 Spheniscus humboldti poxvirus isolate 14PL151 4b core protein gene, partial cds	0
LC386157.1 Porcine teschovirus 15 JPN/Ishi-Ta2/2016/G new genomic RNA, nearly complete genomecomplete genome	0
LC386160.1 Porcine teschovirus 14 JPN/MoI2-2-2/2015/G new gene for polyprotein, partial cds	0
MG251834.1 Porcine reproductive and respiratory syndrome virus strain EuroViet-01, complete genome	1
NC_002204.1 Influenza B virus RNA 1; complete sequence	0
NC_002516.2 Pseudomonas aeruginosa PAO1; complete genome	0
NC_002657.1 Classical swine fever virus, complete genome	0
NC_003436 Porcine epidemic diarrhea virus, complete genome	0
NC_003985.1 Porcine teschovirus 1, complete genome	0
NC_003987.1 Porcine sapelovirus 1, complete genome	0
NC_004003.1 Goatpox virus Pellor, complete genome	0
NC_004461.1 *Staphylococcus epidermidis* ATCC 12228; complete sequence	0
NC_006151.1 Suid herpesvirus 1, complete genome	0
NC_007366 Influenza A virus (A/New York/392/2004(H3N2)) segment 4, complete sequence	0
NC_021283.1 *Mycoplasma hyopneumoniae* 168-L, complete genome	0
NC_035468.1 Murmansk poxvirus strain LEIV-11411, complete genome	0
NC_038861.1 Transmissible gastroenteritis virus complete genome, genomic RNA	0
NC_039210.1 Foot-and-mouth disease virus O isolate o6pirbright iso58, complete genome	0
NC_044956.1 African swine fever virus Benin 97/1 pathogenic isolate, complete genome	0
X55980.1 Porcine Respiratory Coronavirus mRNA for membrane protein, 5'end of the nucleoprotein and the products of ORF3(3'part) and ORF4	0
X72087.1 Aujeszky's disease virus RR1 and RR2 genes	0
X80797.1 Pseudorabies Virus genomic DNA	0

It is believed that if the working temperatures of the thermopads remained relatively stable and reached the favorable range of LAMP reactions, from 60 to 65°C, they could function as a thermal incubator. Here, three types of commercial hand warmers were purchased and compared for their heating performance. As it can be seen from Fig. 4a, all three kinds of thermopads required at least 15 min to reach their highest temperatures. However, type 3 performed the worst, reaching the lowest maximum temperature and rapidly losing heat over time. The two others could approach 63-65°C in 20 to 30 min and maintain the heat up to 70 min. It is clear that type 1 exhibited the best performance in all terms of maximum temperature reach, stability and heat maintenance. Additionally, multiple tracking times reveal that the type 1 thermopad can ensure the heating temperature and time required for the LAMP reactions (Fig. 4b). Consequently, the type 1 hand warmer was selected for further analysis.

Incubation time is also one of the essential parameters that needs to be defined when it comes to using pocket hand warmers. Because the type 1 thermopad only needed around 10 min to reach around 55°C (Fig. 4b), the LAMP reactions were placed in the pads at the beginning of the heating process. As was expected, after 50 min, the reaction tubes expressed the color shift from pink to yellow as well as strong fluorescent signals (Fig. 5). Thus, 55 min was chosen to conduct RT-LAMP reactions of PRRSV detection when using pocket hand warmers.

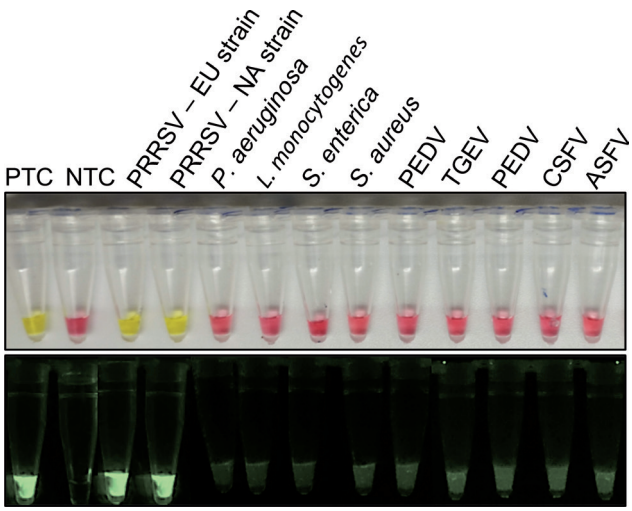

Fig. 3

Primer specificity of the RT-LAMP reactions
The reactions containing 5 µl of the same concentration (1 ng/µl) of extracted bacterial and viral DNA and RNA samples, respectively, were incubated at 65°C for 45 min. The color was captured by the personal mobile phone (upper panel), and the SYBR Green I fluorescent signal was visualized by a blue light transilluminator (lower panel). NTC: negative control, PTC: positive control.

LOD of PRRSV colorimetric RT-LAMP assay

The LOD is one of the most vital features of diagnostic assays that needs to be cautiously evaluated to provide information about sensitivity and superiority compared to other tests. The LOD of the RT-LAMP assay detecting PRRSV was thus determined using the genomic RNA sample ranging from $10^{1.5}$ to $10^{-6.5}$ $TCID_{50}$/ml concentrations. The results indicated that the colorimetric RT-LAMP reactions required at least the amount of RNA concentration extracted from the $10^{-2.5}$ $TCID_{50}$/ml viral culture to exhibit a clear color shift (Fig. 6). In particular, the PRRS colorimetric RT-LAMP assay using NA and EU templates showed the LOD values of $10^{-2.5}$ and $10^{-3.5}$ $TCID_{50}$/ml, respectively (Fig. 6a,b). It should be noted that the LOD values obtained were similar whether using the hand warmer or conventional heat block. The LOD value of $10^{-2.5}$ $TCID_{50}$/ml

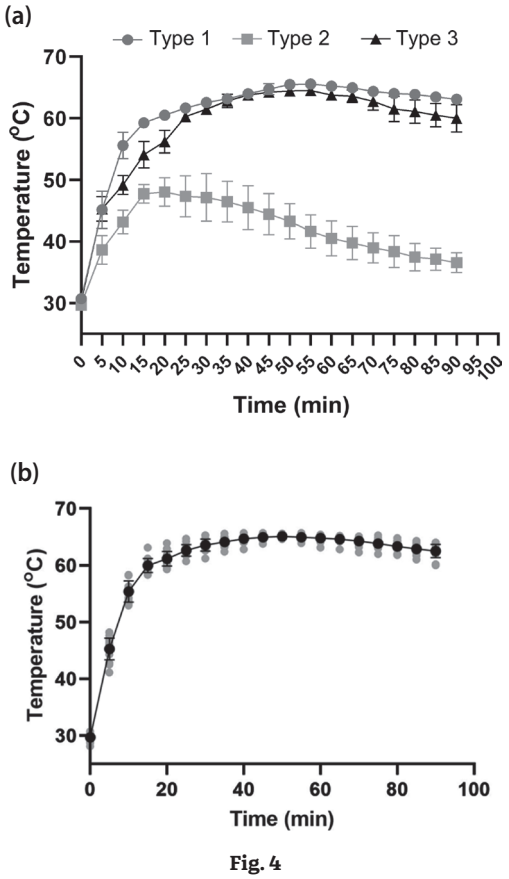

Fig. 4

Temperature monitoring of the thermopads
(a) The temperature was recorded every 5 min. The experiment was done three times independently, using three different pads of each type. **(b)** The temperature of the type 1 thermopad was recorded every 5 min after peeling the seal. The experiment was repeated 15 times independently, using 15 different pads contained in 5 different packing bags. The line represents the average values. Error bars indicate the standard deviation

is approximately 3 times more sensitive than the value of 10^{-2} $TCID_{50}$/ml claimed by the commercial VDx PRRSV qRT-PCR (NA/EU dual) Kit (Median Diagnostics Inc., Korea), as well as at least 10-fold more sensitive than the LOD numbers from 10^{-1} to 10^{4} $TCID_{50}$/ml identified by the RT-LAMP assays reported previously (Chen *et al.*, 2009; Li *et al.*, 2009; Rovira *et al.*, 2009; Gao *et al.*, 2012; Gou *et al.*, 2014). Therefore, the RT-LAMP assay developed herein should be sufficient for the routine diagnosis of PRRSV in real-life applications.

Direct PRRSV colorimetric RT-LAMP assay using crude samples

The goal of this work is to establish an alternative in-field method based on a newly designed colorimetric LAMP assay to diagnose PRRS in pigs grown in Vietnam. To reduce the time and cost of the assay, the direct use of crude clinical samples without the DNA extraction step can significantly benefit the diagnostic procedure. Indeed, LAMP reactions can resist different inhibitors, for example, trace amounts of whole blood, hemin, urine, saliva, mucus or feces (Kaneko *et al.*, 2007; Francois *et al.*, 2011; Dhama *et al.*, 2014), making LAMP an outstanding isothermal nucleic acid amplification method that is widely used in real-life today. Here, it was found that adding serum directly into the RT-LAMP reaction hindered the product formation (Fig. 7). So, serum samples should be diluted by a certain factor to allow amplification to occur. Dilution ensures that other components in clinical serum samples will not inhibit the colorimetric LAMP reactions. On the other hand, crude specimens should not be too diluted, otherwise, the low concentration of template could not be detected, leading to false-negative results. The finding reveals that the amplified product was generated starting from the 5-fold dilution (Fig. 7, lower panel). However, diluting samples by 50 folds was sufficient for the spiked viral-RNA in the commercial pig serum sample to be detected by the clear color shift (Fig. 7).

The sensitivity and specificity of the direct colorimetric RT-LAMP assay using pocket warmers for PRRSV detection were then evaluated using clinical samples. The performance of the direct LAMP assay was validated using a 50-fold dilution of the unextracted serum samples (Table 4). A parallel test was also conducted using the conventional RT-qPCR for comparison. The results showed that the direct LAMP performance was identical to the commercial real-time RT-PCR assay, with 20 positive and 30 negative samples, demonstrating 100% specificity and sensitivity (Table 4). The findings also suggest that the commercially available pocket warmer can be used as an

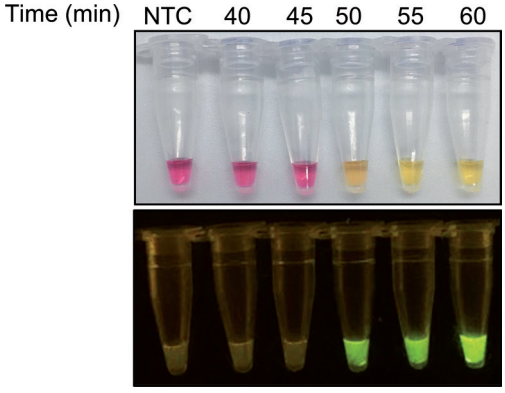

Fig. 5

Optimization of incubation time of the RT-LAMP reactions with heating pads
The reactions containing 5 µl of genomic-RNA extracted from a PRRSV culture of $10^{2.5}$ $TCID_{50}$/ml concentration were incubated in a heating pad for 40 to 60 min. The color was captured by the personal mobile phone (upper panel) and the SYBR Green I fluorescent signal was visualized by a blue light transilluminator (lower panel). NTC: negative control.

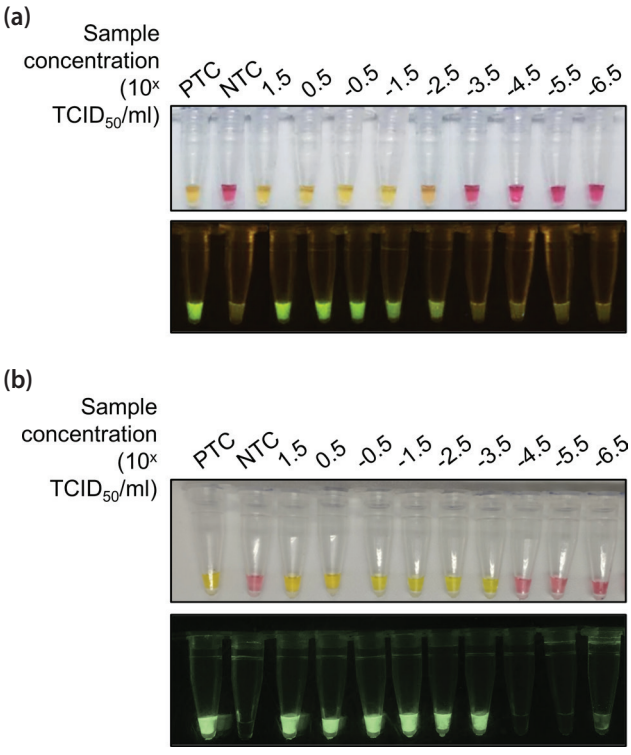

Fig. 6

LOD evaluation of the PRRS colorimetric RT-LAMP reactions
The reactions contain 5 µl of genomic-RNA extracted from a PRRSV culture of $10^{1.5}$ to $10^{-6.5}$ $TCID_{50}$/ml concentration. The color was captured by the personal mobile phone (upper panel), and the SYBR Green I fluorescent signal was visualized by a blue light transilluminator (lower panel). Genomic-RNA of (in A) NA and (in B) EU genotypes was used, respectively. Reactions were incubated for 55 min in thermopads. PTC: positive control, NTC: negative control.

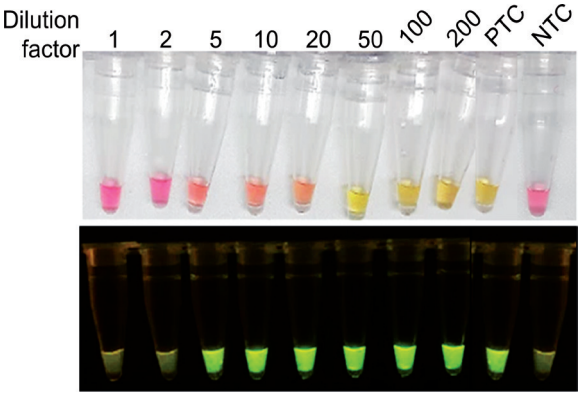

Fig. 7

Titration of the appropriate sample dilution rate for the direct RT-LAMP reaction

The extracted viral RNA corresponding to a PRRSV culture of $10^{4.5}$ $TCID_{50}$/ml concentration was spiked (1:10) into a commercial pig serum. The spiked serum was serially diluted into the nuclease-free water and 5 µl of each diluted sample was added to the RT-LAMP reactions. Reactions were incubated in thermopads for 55 min. The color was captured by the personal mobile phone (upper panel) and the SYBR Green I fluorescent signal was visualized by a blue light transilluminator (lower panel). PTC: positive control, NTC: negative control.

effective platform to perform the developed RT-LAMP assay for PRRSV detection, facilitating an electro-free diagnosis in a resource-limited field setting.

Discussion

Due to its isothermal property, LAMP has been considered superior to other conventional amplification methods such as PCR. In other words, a LAMP reaction can be operated at a particular temperature, within a range of 60–65°C. Taking advantage of this characteristic, a number of studies have developed portable devices called non-instrumented nucleic acid amplification (NINA) that could perform amplification in the field (Mori *et al.*, 2013). As a result, this improvement helps scientists take one step further toward the implementation of LAMP as a point-of-care diagnostic. In this study, we have succeeded in developing the direct colorimetric RT-LAMP test to quickly and accurately detect PRRSV in crude serum specimens. The LOD value of the assay is remarkable compared to the conventional real-time RT-PCR or RT-LAMP tests previously established for PRRSV detection. There was no cross-reactivity with closely related viruses or numerous bacterial genomic-DNAs, demonstrating that the newly designed RT-LAMP primers are highly specific. Furthermore, the use of crude clinical samples that did not go through the RNA extraction procedure did not hinder the colorimetric RT-LAMP assay, greatly lowering the time and cost of the testing process. Most importantly, commercial non-instrumented thermopads can be used to efficiently provide the heat required for RT-LAMP nucleic acid amplification. The results of the preliminary validation experiment using clinical specimens collected were promising, with 100% specificity and sensitivity compared to conventional real-time RT-PCR. The use of pocket hand warmers is obviously more convenient than previous man-made heating devices since they are disposable, cheap and can avoid mechanical errors or device maintenance.

Table 4. Sensitivity and specificity of direct PRRSV colorimetric LAMP assay using thermopads

VDx® PRRSV qRT - PCR	Direct RT-LAMP		Total
	Positive	Negative	
Positive	20	0	20
Negative	0	30	30
Total	20	30	50
Sensitivity: 100% Specificity: 100%			

According to the pocket warmers' manufacturer, each pad typically contains iron powder, salt, water, an absorbent material, and activated carbon (Klarzak *et al.*, 2018). The thermopad produces heat after peeling the airtight wrap due to the exothermal reaction between atmospheric oxygen and iron powder in the presence of salt and water, creating rust. The temperature range approaches the acceptable value for the LAMP reaction, which is around 60°C. Furthermore, the heat was quite stable, allowing the reaction to be completed in 55 min, which was only 10 min longer than when the heat block was used. Additionally, the incubation time defined is much faster than the results reported previously, ranging from 60 to 120 min (Hatano *et al.*, 2010; Ablordey *et al.*, 2012; Yu *et al.*, 2020). Nevertheless, the RT-LAMP reactions for PRRSV detection were more likely to observe the non-specific amplification if they were incubated for more than 60 min in the pocket warmer (data not shown). When using pocket hand warmers, it is suggested to perform the reaction in the open air or where there is good ventilation to promote air circulation or oxygen penetration into the pad to accelerate the oxidation reaction or to reach a higher temperature. Moreover, a needle can be used to puncture tiny holes on the outside surface to expand the contact area, thereby increasing the performance of the modified method that might become more comparable with the conventional incubation method using the heat block. However, there were uncontrollable variables such as the different quality of each pad, (diffe-

rent manufacturers) or different reacting environments. Therefore, a remarkable number of pocket hand warmers from different companies should be tested in different environments to validate the application of this device in clinical settings.

During sample collection and preparation, the pig blood was centrifuged, and serum was collected from the upper layer of the supernatant. For some samples, serum could not be collected as a clear liquid, which appeared as a red liquid that was still comprising red blood cells. However, this is not considered a problem since LAMP is known to be tolerant to sample matrix components such as red blood cells and salts, which are PCR inhibitors (Kaneko *et al*., 2005; Dhama *et al*., 2014). As a result, when it comes to clinical or patient samples, the application of PCR or real-time PCR requires the extraction of genetic materials. Thus, those specimens need to be transported to a qualified laboratory for testing. This step could be one of the contributory causes of false results if the preservation is done improperly and incautiously (Pan *et al*., 2021). Altogether, this characteristic makes LAMP a ready-to-use diagnostic in resource-limited field settings.

This is the first research to use and evaluate the direct colorimetric RT-LAMP test in the identification of PRRSV from infected domestic pigs in Vietnam utilizing field samples. In detecting PRRSV, the colorimetric RT-LAMP test developed was demonstrated to be as sensitive as the approved real-time RT-PCR assay. The combination of colorimetric assay as a visual readout and pocket hand warmers has extremely high potential for point-of-care diagnostics. Directly using clinical samples helped to gain better insights into the feasibility of the RT-LAMP method. The study has provided a clearer vision of the application of pocket hand warmers to detect pathogens on the infection site by directly using clinical specimens without extraction. Additionally, a higher number of clinical samples could be tested to provide a more general picture and higher reliability in the application of hand warmers in limited-resource and clinical settings.

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Supplementary information is available in the onlline version of the paper.

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SUPPLEMENTARY INFORMATION

Instrument-free, visual and direct detection of porcine reproductive and respiratory syndrome viruses in resource-limited settings

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Table S1. qRT-PCR results of PRRSV samples

Sample	Result	Ct	Genotype	Sample	Result	Sample	Result
1	Positive	31.15	NA	21	Negative	41	Negative
2	Positive	33.2	NA	22	Negative	42	Negative
3	Positive	21.41	NA	23	Negative	43	Negative
4	Positive	22.58	NA	24	Negative	44	Negative
5	Positive	24.65	NA	25	Negative	45	Negative
6	Positive	26.52	NA	26	Negative	46	Negative
7	Positive	27.96	NA	27	Negative	47	Negative
8	Positive	26.93	NA	28	Negative	48	Negative
9	Positive	25.13	NA	29	Negative	49	Negative
10	Positive	24.98	NA	30	Negative	50	Negative
11	Positive	24.55	NA	31	Negative		
12	Positive	25.15	NA	32	Negative		
13	Positive	20.68	NA	33	Negative		
14	Positive	20.46	NA	34	Negative		
15	Positive	22.39	NA	35	Negative		
16	Positive	35.88	NA	36	Negative		
17	Positive	10.56	NA	37	Negative		
18	Positive	10.01	NA	38	Negative		
19	Positive	21.89	NA	39	Negative		
20	Positive	33.98	NA	40	Negative		

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Fig. S1

Three types of commercial hot packs used in this study

Type 1 is produced by Iris Ohyama Inc. (Japan). Type 2 is manufactured by Shenzhen Xiandi Times Technology Co., Ltd (China). Type 3 is made by Kobayashi Pharmaceutical Co., Ltd (Japan).