



Research article

Development of a loop-mediated isothermal amplification assay for rapid detection of African swine fever

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Abstract

Since the first African swine fever (ASF) outbreak was reported in China in 2018, the disease has spread rapidly to several countries in Asia. The early detection of this disease is essential for the ASF control strategy to be effective. Loop-mediated isothermal amplification (LAMP) is a nucleic acid detection assay that is rapid, simple, cost-effective and field-friendly. In this study, we have developed a colorimetric assay of LAMP to detect ASF virus (ASFV). A set of LAMP primers was designed to target the conserved region of the VP72 gene. The conditions of LAMP were optimized. Sensitivity and specificity of the assay were demonstrated in comparison with the OIE-recommended real-time PCR. A total of 211 samples including 121 confiscated pork products and 90 spiked clinical specimens were tested. Optimal amplification of ASFV DNA by LAMP was incubated at 60 °C for 90 min. The amplification products were easily detected by the naked eye using hydroxynaphthol blue (HNB). The positive LAMP reaction generated a violet to sky blue color change. The analytical sensitivity of ASFV LAMP assay was at least 368 plasmid DNA copies/ μ l without cross-reactivity with other swine pathogens. The diagnostic sensitivity and specificity of LAMP were 88% and 100%, respectively. There was almost perfect agreement between LAMP and real-time PCR assays (Kappa value=0.84). This novel LAMP assay is deemed to be a rapid, simple, sensitive, specific diagnostic tool and suitable for early detection of ASF to minimize the likelihood of ASF spread nationwide.

Keywords: African swine fever, LAMP assay, Rapid diagnosis, VP72 gene

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INTRODUCTION

African swine fever (ASF) is a highly contagious hemorrhagic disease of domestic pigs and wild boars of all ages. The disease leads to huge economic losses in the pig production sector due to the high fatality rate and also the trade restrictions. Since 2016, there has been a significant increase in the number of ASF outbreaks in Africa, Europe, and Asia. The highest impact in terms of animal losses have been reported in Asia (OIE, 2019) and the first ASF outbreak in Asia was reported in China in 2018. Then, the disease has been spread among Asian countries including Vietnam, Cambodia, Laos, and Myanmar. Although ASF has never been reported in Thailand, the disease entry could be inevitable due to many risk factors for instance the movement of live pigs and contaminated meat products such as sausage, fermented pork and ham. The control measure of ASF is a challenge because effective treatment or vaccine is not yet available. Early detection of the disease is therefore one of the essential tools to prevent ASF spread (Gallardo et al., 2019).

ASF is caused by African swine fever virus (ASFV); family *Asfarviridae*, genus *Asfivirus*, which is a double-stranded DNA virus with large genome ranging in size between 170 and 194 kbp (Alonso et al., 2018). The genetic variability of ASFV is as high as 24 different genotypes based on the VP72 gene (also termed B646L and VP73) (Quembo et al., 2018), which are not related to ASFV virulence (Arias et al., 2018). Pigs affected with peracute and acute forms of ASF, hemorrhages in the skin and some visceral organs are present with high fever, loss of appetite, and eventually death. The disease characteristics of ASF could often be clinically misdiagnosed with some other swine diseases especially classical swine fever (OIE, 2020). Therefore, laboratory diagnosis is crucial to differentiate these implicated pathogens.

Rapid diagnostic tests are designated to promptly detect ASF to control the spread of diseases. Real-time polymerase chain reaction (Real-time PCR) is a recommended assay for identifying the ASFV genome in cases of ASF suspicion because of its rapidness, sensitivity and specificity (OIE, 2020). However, it requires expensive equipment and considerable operator skill. Since loop-mediated isothermal amplification (LAMP) was first developed by Notomi et al. (2000), it is well recognized as a simple, rapid, sensitive and specific assay. For detection of ASFV, a LAMP was studied targeting Topoisomerase II gene by James et al. (2010). This LAMP protocol was applied in Uganda and provided a high sensitivity but low specificity (Atuhaire et al., 2014). Recently, a study of LAMP based on K205R gene of ASFV established more sensitive results than conventional PCR. However, the products of LAMP were observed as the fluorescent dye changed color under a UV illuminator or by agarose gel electrophoresis (Wu et al., 2016).

Hydroxynaphthol blue (HNB), a new colorimetric indicator for LAMP reaction, was first applied by Goto et al. (2009). Later, it has been widely used in the detection of many pathogens providing the obvious results (Das et al., 2012; Duan et al., 2014; Zhang et al., 2019). HNB can develop a violet color in the presence of Mg^{2+} ion and change violet to sky blue in the LAMP positive reaction. Thus, LAMP amplicons could be easily observed by the naked eye without further equipment requirement. In this study for the first time, we developed a novel LAMP for detection of ASFV based on VP72 gene integrated with the application of HNB as a colorimetric indicator.

MATERIALS and METHODS

ASFV reference control samples

The three strains of ASFV were used as the reference samples in this study. The first strain was acquired from ASFV-contaminated pork products at the animal quarantine stations and the international ports of Thailand during 2018-2019. These samples were tested for ASFV at the National Institute of Animal Health (NIAH). The nucleotide sequences of ASFV were homologous with the strain ASFV-SY18 reported in China, which belongs to genotype II (Songkasupa et al., 2020). The ASFV-SY18-like strain from these samples was used as the positive control for LAMP optimization and validation. The other two strains comprising of BA71V (genotype I) and Georgia 2007/1 (genotype II) strains, received from the Australian Centre for Disease Preparedness (formerly Australian Animal Health Laboratory), were used to determine the specificity of novel LAMP assay.

Extraction and quantification of genomic DNA

Genomic DNA of the ASFV samples were extracted using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Germany) following the manufacture protocol. The concentration and purity of the extracted DNA were measured as a ratio of absorbance at 260 and 280 nm (A₂₆₀/A₂₈₀) using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). DNA extracts were stored at -20 °C until use.

Design of primers for LAMP assay

A set of primers specific to ASFV was designed based on the alignment of 73 complete sequences of the VP72 gene available in GenBank. The alignment of DNA sequences was generated using Geneious Prime version 2019.2.1. A consensus sequence was used to design primers for LAMP assay using PrimerExplorer V5 software (<https://primerexplorer.jp/e/>). The positions and sequences of LAMP primers on their complementary target DNA were shown in figure 1. The primer set included two inner primers (FIP and BIP), two outer primers (F3 and B3), and one loop primer (FLoop). Further details of the primers are shown in table 1.

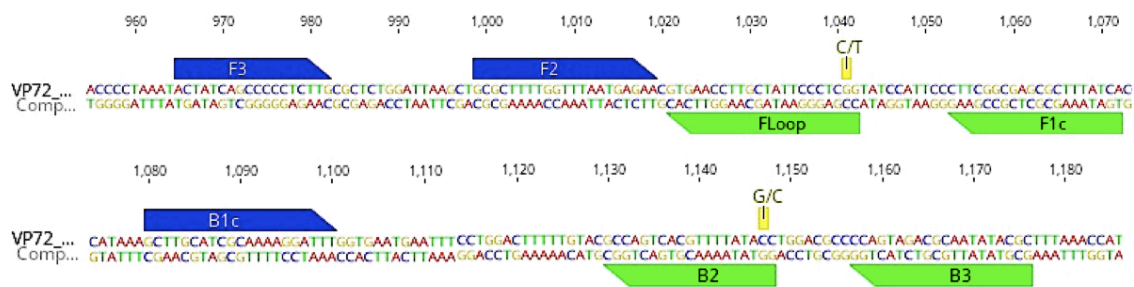


Figure 1 Schematic diagram of positions and sequences of LAMP primers targeting VP72 gene of ASFV. The conserved region of the VP72 gene used to design inner primers (F2, F1c, B1c and B2), outer primers (F3 and B3), and loop forward primer (FLoop). Numbers on the top of each sequence corresponds to the position in VP72 gene. The direction of primer sequences is indicated by color of boxes; blue represents forward primers; and green represents backward primers.

Table 1 Positions and sequences of LAMP primers targeting VP72 gene of ASFV.

Primer name	Type	Length (bp)	VP72 gene position	Sequence (5'-3')
FIP	Forward inner (F1c + F2)	41	F1c: 1072-1053 F2: 999-1019	TGATAAAGCGCTCGCCGAAG-GCGCT-TTTGGTTTAATGAGAA
BIP	Backward inner (B1c + B2)	40	B1c: 1080-1100 B2: 1130-1148	GCTTGCATCGCAAAGGATTT-GC-TATAAACGTGACTGGC
F3	Forward outer	18	965-982	ACTATCAGCCCCCTCTTG
B3	Backward outer	20	1176-1157	GCGTATATTGCGTCTACTGG
FLoop	Forward loop	22	1042-1021	CTGAGGGAATAGCAAGGTTAC

Optimization of LAMP assay

To develop a novel visualization LAMP assay, the parameters of LAMP reaction were optimized including amplification temperature, incubation time and concentrations of LAMP components. LAMP reaction mixture was performed in a total volume of 25 µl containing 2.5 µl of 10x isothermal amplification buffer with 5 µl of template DNA. The genomic DNA of ASFV-SY18-like strain was used as a template, and deionized water (diH₂O) was used as a negative control. For optimization of reagents, concentrations of *Bst* 2.0 WarmStart DNA Polymerase (New England Biolabs, UK) 0.128-0.64 U/µl, dNTPs (New England Biolabs, UK) 0.4-1.6 mM, MgSO₄ (New England Biolabs, UK) 4-10 mM, inner primers FIP and BIP 0.4-1.6 µM, outer primers F3 and B3 0.2-1.0 µM, loop forward primer (Integrated DNA Technologies, USA) 0.2-1.0 µM, betaine (Sigma-Aldrich, USA) 0.6-1.0 M, and HNB (Honeywell Fluka, China) 120-150 µM were evaluated. The rest of the reaction volume was filled with diH₂O. The reaction mixture was incubated in a heating block at 56-66 °C with increments of 2.0 °C for 30, 40, 50, 60, 70, 80 and 90 min and then heated at 80 °C for 2 min to terminate the reaction. The LAMP products were visually inspected and confirmed using 2% agarose gel electrophoresis prestained with Safe-Green.

Real-time PCR

To assess LAMP efficiency, the OIE-recommended real-time PCR using primers targeting the VP72 gene was performed as the reference method according to [Fernandez-Pinero et al. \(2013\)](#) except that the DNA template volume was 5 µl along with the standard probe recommended by World Organisation for Animal Health or OIE ([OIE, 2020](#)).

Plasmid preparation

A recombinant plasmid containing the complete VP72 gene of ASFV was produced by Gene Universal Inc. (Delaware, USA). A 1,941 bp DNA fragment corresponding to the sequence of the full VP72 gene of ASFV-SY18 strain (GenBank accession no. MH713612) was synthesized and cloned into pGEM-T easy vector system (Promega, USA) (~5 Kb, the vector plus target gene). The lyophilized VP72 standard plasmid was diluted in Salmon Sperm DNA solution (Invitrogen, USA) prior to the determination of DNA concentration using a NanoDrop ND-1000 spectrophotometer. The concentration of plasmid is converted to copy the number based on the equation described previously ([Whelan et al., 2003](#)).

Analytical sensitivity and specificity

To evaluate the analytical sensitivity of LAMP assay, a 10-fold serial dilution of aforementioned plasmid was prepared in the range of 3.68×10^{-1} and 3.68×10^9 copies/µl. The analytical sensitivity of LAMP was determined in comparison to the reference method real-time PCR. Each dilution of standard plasmid was tested in triplicate by both LAMP and real-time PCR.

The analytical specificity of LAMP was assessed by performing LAMP reaction with classical swine fever virus (CSFV), porcine respiratory and reproductive syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV), swine influenza virus (SIV), transmissible gastroenteritis (TGE) virus, foot-and-mouth disease virus (FMDV), and porcine circovirus types II (PCV-II). These swine viruses were derived from the archival virus collection held in the NIAH laboratory. Both genomic DNA and RNA were extracted using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies Sdn Bhd, Malaysia). The nucleic acid of these pathogens had been previously confirmed by the molecular techniques ([Table 2](#)) and the amplified products were shown in [figure 2](#).

Table 2 Reference porcine viruses confirmed by molecular techniques to determine LAMP assay specificity.

Porcine virus	Method	Target gene	Product size (bp)	Reference primers
CSFV strain ALD	RT-PCR	E2	271	Lowings et al. (1996)
PRRSV strain				
-North American genotype 2 (VR-2332)	Nested RT-PCR	ORF7	432	Drew et al. (1997) and Choi et al. (2013)
-European genotype 1 (Lelystad)	Nested RT-PCR	ORF7	398	(modified primer)
PEDV	RT-PCR	M	377	Kweon et al. (1997)
SIV	RT-PCR	M	1,027	Hoffmann et al. (2001)
TGE	RT-PCR	S	886	Paton et al. (1997)
FMDV	RT-PCR	UTR	328	Reid et al. (2000)
PCV-II	PCR	ORF2	481	Ellis et al. (1999)

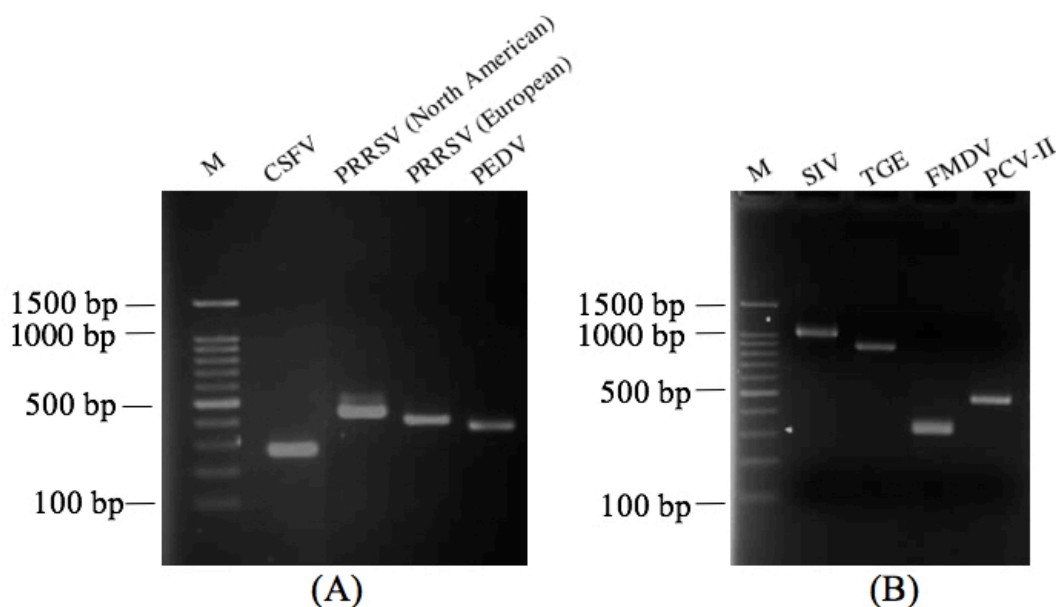


Figure 2 The reference porcine viruses confirmed by molecular techniques using specific primers. The amplified products were electrophoresed on 1.5% agarose gel followed by ethidium bromide staining. (A) The expected PCR products of CSFV, PRRSV strain North American, PRRSV strain European and PEDV were detected at 271, 432, 398, and 377 bp, respectively. (B) The expected PCR products of SIV, TGE virus, FMDV, and PCV-II were detected at 1,027, 886, 328, and 481 bp, respectively. M=100 bp DNA marker.

Diagnostic sensitivity and specificity

A total of 211 samples including 121 of confiscated pork products and 90 ASFV-spiked clinical specimens were tested for diagnostic sensitivity and specificity of LAMP. To prepare the ASFV-spiked clinical specimens, both porcine whole blood and tissue homogenates of visceral organs (e.g. spleen, lymph nodes, kidney and lung) which had been negative for ASFV by real-time PCR, were spiked with different concentrations of ASFV genomic DNA. The diagnostic sensitivity and specificity were determined by LAMP in comparison with the reference assay, OIE-recommended real-time PCR. The results of LAMP and real-time PCR were entered in a 2x2 contingency table and the overall percentage agreement between the two assays analyzed (concordance) by Kappa coefficient (*k*) statistics. The *k* value was evaluated using MedCalc Statistical Software version 19.1.5 (MedCalc Software Ltd, Ostend, Belgium).

RESULTS and DISCUSSION

Optimal novel LAMP reaction and visualization

LAMP assay using a set of five VP72-specific primers for ASFV detection yielded positive results from a reference strain ASFV-SY18-like. The optimized LAMP assay in a total reaction volume of 25 µl consisted of 1x isothermal amplification buffer, 0.64 U/µl of *Bst* 2.0 WarmStart DNA Polymerase, 0.8 mM dNTPs, 0.9 M betaine, 6 mM MgSO₄, 1.6 µM of FIP and BIP each, 0.2 µM of F3 and B3 each, 0.4 µM FLoop, 120 µM HNB with 5 µl of template DNA and 1.4 µl of diH₂O. During optimization, LAMP reactions were performed in a heating block which is a user-friendly equipment and more affordable than a thermal cycler for PCR technology. The optimal conditions were determined to be ranging from 56-66 °C for 30-90 min. Generally, the amplification time of LAMP varies from 60-120 min (Francois et al., 2011). The previous LAMP studies for ASFV detection revealed that the optimal incubation time is 60 min (Atuhaire et al., 2014; Wu et al., 2016). For this novel LAMP, the amplification was observed at a temperature of 60 °C as early as 50 min. However, the optimal conditions were selected at 60 °C for 90 min because of its capability to provide the highest yield of LAMP products.

The color of the positive reaction using the HNB indicator changed from violet to sky blue while the negative reaction remained violet (Figure 3A). The LAMP amplified products were confirmed by using gel electrophoresis (Figure 3B). HNB dye has several advantages such as user-friendliness, inexpensiveness and reliability. HNB-based LAMP can be easily visualized as color change with the naked eye. The cost of HNB is cheaper than other colorimetric indicators for instance calcein and SYBR green. HNB-based LAMP is a closed-tube method that reduces the likelihood of cross-contamination because opening the tubes after LAMP amplification is not required. Furthermore, the sensitivity of LAMP assay using HNB is supposed to be equivalent to the use of SYBR green dye, although it is higher than calcein (Goto et al., 2009).

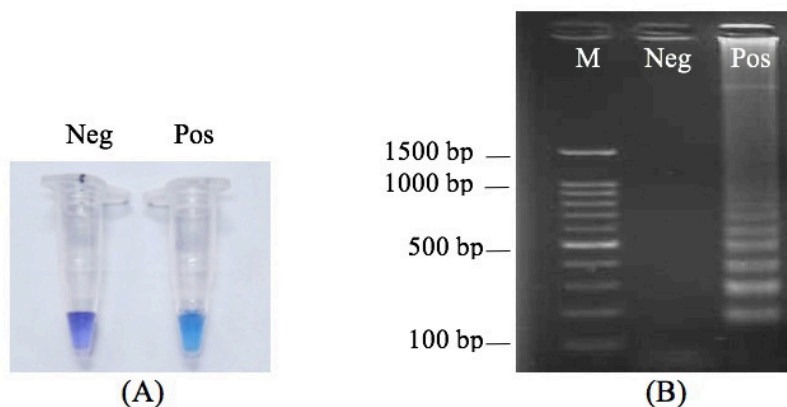


Figure 3 LAMP assay visualization for ASFV detection based on VP72 gene. Genomic DNA of ASFV-SY18-like strain was used as template while deionized water was used as negative control. (A) The reactions of colorimetric ASFV-LAMP using HNB dye inspected by the naked eye immediately after incubation. Violet=negative reaction; sky blue=positive reaction. (B) The amplified LAMP product was also verified by 2% agarose gel electrophoresis. The negative reaction of LAMP did not show any product bands, while the positive reaction presented the bands. bp=base pair; M=100 bp DNA marker; Neg=negative reaction; Pos=positive reaction.

Analytical sensitivity of LAMP

A 10-fold serial dilutions of VP72 standard plasmid of ASFV-SY18 was evaluated for the analytical sensitivity of LAMP assay in comparison to the reference method real-time PCR. Initially, real-time PCR experiments were carried out in triplicates for each dilution. To ensure the standard DNA amounts were diluted properly, a standard curve was generated across nine concentrations of standard DNA ranging from 3.68×10^0 to 3.68×10^8 copies/ μ l. The threshold cycle (Ct) was plotted against the log values of the initial DNA template quantity. A log-linear regression curve indicated that the amplification time (or Ct value) and corresponding standard DNA concentration were highly correlated with coefficient of determination (R^2) equal to 0.9992 (Figure 4).

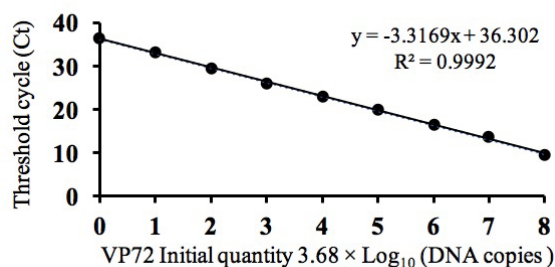


Figure 4 A log-linear regression curve of the real-time PCR amplification time and corresponding standard DNA concentration. The curve was constructed by analyzing triplicates of 10-fold serial dilutions of the quantified VP72 standard plasmid. The Ct value of each dilution corresponded to the mean value of three replicates.

The analytical sensitivity of LAMP assay then was performed in triplicate in parallel to the real-time PCR. The results of both the HNB-based method and gel electrophoresis showed that LAMP assay could visually detect the standard DNA from the dilution 10^{-2} to 10^{-8} (Figure 5). The detection limit of LAMP assay was 368 copies/ μ l of ASFV standard DNA which was 2 logs lower than that of the real-time PCR (Table 3). This indicated that the LAMP assay was capable of detecting DNA copies of ASFV but less sensitive than the real-time PCR which has been shown to exhibit the highest sensitivity for detection of ASFV DNA (OIE, 2020).

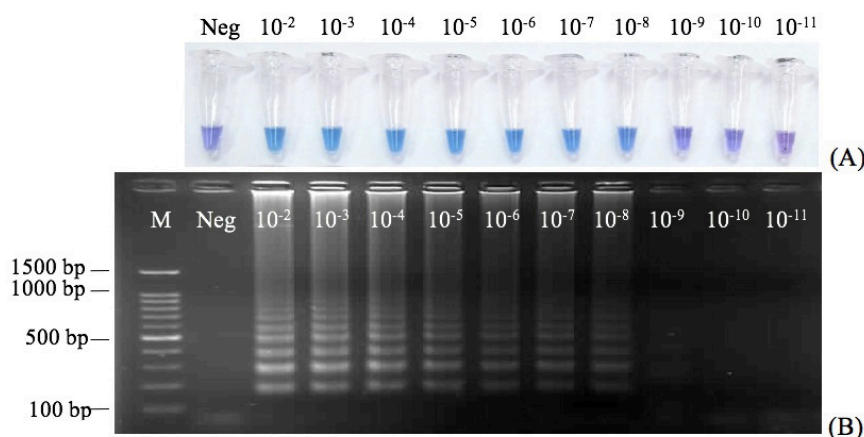


Figure 5 Analytical sensitivity of LAMP assay for detection of ASFV based on VP72 gene. The 10-fold serial dilutions of standard plasmid DNA starting from the dilution 10^{-2} to 10^{-11} (3.68×10^8 to 0.368 DNA copies/ μ l) were used as template, while deionized water was used as negative control. (A) Colorimetric LAMP using HNB indicator. Violet=negative reaction; sky blue=positive reaction. (B) The amplified LAMP products were confirmed by 2% agarose gel electrophoresis. The dilutions 10^{-2} to 10^{-8} presenting amplified LAMP product bands, but from the dilutions 10^{-9} to 10^{-11} did not. bp= base pair; M=100 bp DNA marker; Neg=negative control.

Table 3 Analytical sensitivity of LAMP for ASFV detection compared to the reference real-time PCR. Quantified DNA plasmid containing VP72 gene of ASFV was used as template.

Dilution	Standard DNA (copies/ μ l)	Real-time PCR Ct (Mean \pm SD)	Triplicate LAMP ^a
10^{-2}	3.68×10^8	9.55 ± 0.55	+++
10^{-3}	3.68×10^7	13.45 ± 0.95	+++
10^{-4}	3.68×10^6	16.51 ± 1.00	+++
10^{-5}	3.68×10^5	19.82 ± 0.98	+++
10^{-6}	3.68×10^4	22.91 ± 1.38	+++
10^{-7}	3.68×10^3	25.96 ± 1.07	+++
10^{-8}	3.68×10^2	29.42 ± 1.03	+++
10^{-9}	3.68×10^1	33.21 ± 1.54	--+
10^{-10}	3.68×10^0	36.49 ± 2.16	---
10^{-11}	3.68×10^{-1}	No Ct	---

^a + = detected, - = non-detected.

Analytical sensitivity of LAMP assay may be affected by different factors such as the target gene selection and the detection system. In this study, for the first time, the VP72 gene has been proven in its application in ASFV LAMP detection. This gene has previously been successfully applied to the conventional and real-time PCR to detect ASFV (Aguero et al., 2003; Fernandez-Pinero et al., 2013; King et al., 2003). The analytical sensitivity of our LAMP assay is relatively low, compared to the real-time PCR, possibly because of the detection system. To determine the results of LAMP reaction, the amplification could be evaluated by many detecting systems such as turbidity, fluorescence or colorimetric dyes, lateral flow, and real-time turbidimetry or colorimetric detection (Dehghan Esmatabadi et al., 2015). However, because monitoring the signal of real-time LAMP requires a well designed thermocycler, the colorimetric-based LAMP using HNB developed in this study is considered to be a fairly sensitive and easily performed. It is a suitable approach not only for the laboratory detection, but also for the field diagnosis.

Analytical specificity of LAMP

The analytical specificity of LAMP assay was tested by detecting DNA of different ASFV strains and also non-ASFV, which were the template DNA/RNA from eight reference porcine viruses (CSFV strain ALD, PRRSV strains North American and European, PEDV, TGE virus, PCV-II, FMDV and SIV). As shown in figure 6, no positive amplification was observed for other reference porcine viruses using both HNB visualization and gel electrophoresis. The LAMP assay showed positive reactions only for ASFV.

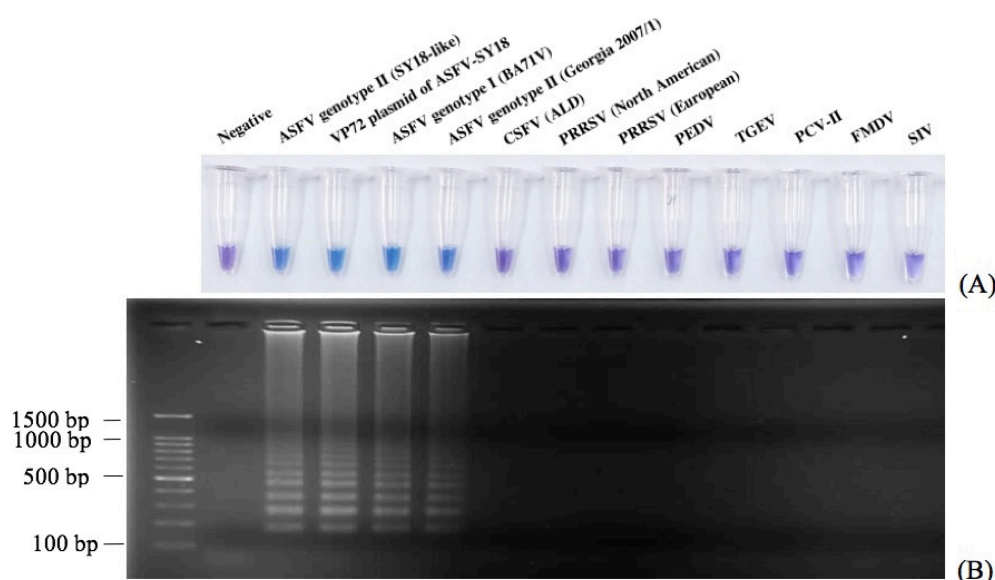


Figure 6 Analytical specificity of LAMP assay for ASFV detection. The nucleic acid of ASFV and eight other porcine viruses were used as template, while deionized water was used as negative control. (A) LAMP detection using HNB as a visual indicator. Positive reactions turned sky blue; negative reactions remained violet. (B) Agarose gel electrophoresis of amplified LAMP products. The positive reactions manifested bands on 2% agarose gel. The negative reaction did not present the product bands. bp=base pair.

This novel LAMP method was able to detect the DNA of ASFV genotype II, strains ASFV-SY18-like and the plasmid of ASFV-SY18, which has been reported in China (Zhou et al., 2018). Likewise, the LAMP could also detect the representative ASFV isolates belonging to genotype I, BA71V and Georgia 2007/1. The assay could correctly identify ASFV without cross-reactivity with any other porcine viral pathogens. This indicated that the LAMP assay is highly specific meaning it can be used to accurately detect ASFV. The LAMP exhibited high specificity because it used five primers to recognize seven distinct regions on the target sequence. LAMP hardly produces non-specific products (Notomi et al., 2000). The specificity of LAMP was also reported that higher than PCR-based assays (Khan et al., 2017; Wang et al., 2014).

Diagnostic sensitivity and specificity of LAMP assay

The parallel testing of samples of LAMP and real-time PCR was evaluated in ASFV-contaminated pork products, and ASFV-spiked whole blood and tissue homogenates of visceral organs of pigs. The results showed that 120 of 136 positive samples were detected ASFV genome by both LAMP and real-time PCR assays (Table 4). The 16 of weak positive real-time PCR samples, which had a range of cycle threshold (Ct) values between 33 and 37, were negative by LAMP assay. The diagnostic sensitivity of novel LAMP for detection of ASFV was 88% (95% CI: 81.6-100), while the diagnostic specificity was 100% (95% CI: 95.2-100). The *k* value for the level of agreement between real-time PCR and LAMP assay was 0.842 (Table 5), which indicated an almost perfect agreement between the two assays.

Table 4 Number of positive and negative by both OIE-recommended real-time PCR and the LAMP assay for detection of ASFV in different sample types.

Sample	Real-time PCR		LAMP		Total
	Positive	Negative	Positive	Negative	
Pork products	83	38	73	48	121
Spiked clinical sample					
▫ Whole blood	30	20	27	23	50
▫ Visceral organ	23	17	20	20	40
Subtotal	136	75	120	91	211

Table 5 The diagnostic sensitivity and specificity of LAMP assay in comparison to OIE-recommended real-time PCR for detection of ASFV.

		Real-time PCR (OIE recommended)		Total
		Positive	Negative	
LAMP	Positive	120	0	120
	Negative	16	75	91
	Total	136	75	211

Diagnostic sensitivity (DSe) = 88% (120/136); 95% CI 81.6% to 93.12%

Diagnostic specificity (DSp) = 100% (75/75); 95% CI 95.2% to 100%

Kappa value (*k*) = 0.842 “almost perfect agreement” (Landis and Koch, 1977)

In this study for the first time, the efficiency of LAMP assay has been validated with the most sensitive protocol currently of OIE-recommended real-time PCR for ASFV genome detection (OIE, 2020). This study proved that a novel HNB-based LAMP targeting VP72 gene could detect ASFV DNA with high sensitivity and specificity among various kinds of samples. Although the sensitivity of novel LAMP was slightly lower than real-time PCR, it is likely to be sensitive enough to detect the large amounts of ASFV present in infected pigs (alive and dead), particularly in contaminated pork products which is one of risk factors for spreading the disease.

CONCLUSION

This study has shown the successful development of LAMP assay for the detection of ASFV. The LAMP does offer a rapid, simple, cost-effective, sensitive and specific diagnostic method for ASF. The sensitivity of LAMP could be improved even more in the future through changing to a more effective dye indicator or inspection system. This novel LAMP is reliable enough to be used as an alternative method for the early detection of ASF to reduce the risk of epidemics especially in the countries with limited resources.

CONFLICT of INTEREST

The authors of this research certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript. All authors have seen and agree with the contents of the manuscript. They certify that the submission is original work and is not under review at any other publication.

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AUTHOR CONTRIBUTION

Dokphut, A. conceived and designed of the study. Dokphut, A., Boonpornprasert, P. and Songkasupa, T. contributed to materials and samples preparation. Dokphut, A. and Tangdee, S. performed the experiments; assay optimization and validation. Dokphut, A. analyzed and interpreted the data with assisted from all authors. Dokphut, A. wrote the manuscript in consultation with Boonpornprasert, P. and Songkasupa, T.

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