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Development of a ladder-shape melting temperature isothermal amplification (LMTIA) assay for detection of African swine fever virus (ASFV)

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ABSTRACT

Background: Due to the unavailability of an effective vaccine or antiviral drug against the African swine fever virus (ASFV), rapid diagnosis methods are needed to prevent highly contagious African swine fever.

Objectives: The objective of this study was to establish the ladder-shape melting temperature isothermal amplification (LMTIA) assay for the detection of ASFV.

Methods: LMTIA primers were designed with the p72 gene of ASFV as the target, and plasmid pUC57 was used to clone the gene. The LMTIA reaction system was optimized with the plasmid as the positive control, and the performance of the LMTIA assay was compared with that of the commercial real-time polymerase chain reaction (PCR) kit in terms of sensitivity and detection rate using 200 serum samples.

Results: Our results showed that the LMTIA assay could detect the 10⁴ dilution of DNA extracted from the positive reference serum sample, which was the same as that of the commercial real-time PCR kit. The coincidence rate between the two assays was 100%.

Conclusions: The LMTIA assay had high sensitivity, good detection, and simple operation. Thus, it is suitable for facilitating preliminary and cost-effective surveillance for the prevention and control of ASFV.

Keywords: African swine fever (ASF); African swine fever virus (ASFV); ladder-shape melting temperature isothermal amplification (LMTIA); commercial real-time PCR kit

INTRODUCTION

African swine fever (ASF) is caused by the African swine fever virus (ASFV) with a high fatality rate, high fever, and reticuloendothelial system hemorrhage [1]. The high incidence and fatality rate of ASF cause huge economic losses to the global swine industry [2]. Due to the shortage of effective vaccines or antiviral drugs, the prevention and control of ASF rely on culling the infected animals. Strict sanitary measures, rapid diagnosis techniques, and rapid and reliable detection methods of ASFV are important for the timely diagnosis and prevention of ASF [3].

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Conflict of Interest

The authors declare no conflicts of interest.

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Many modern molecular biology techniques have been used for ASFV detection. The assays based on immunology include colloidal gold test strip assay [4], time-resolved fluorescence immunoassay [5], fluorescent immunochromatography test strip [6], amplified luminescent proximity homogeneous assay (AlphaLISA) [7], triplex bead-based assay for the simultaneous detection of antibodies [8], Eu (III) chelate microparticle-based lateral flow test strip and blocking enzyme-linked immunosorbent assay [2,9]. Many nucleic acid amplification methods have been improved or modified for the detection of ASFV, such as multiplex reverse transcription-polymerase chain reaction (RT-PCR) assay [10], triplex real-time PCR assay [11], real-time loop-mediated isothermal amplification (LAMP) assay [12,13], LAMP combined with lateral flow dipstick [14], semi-quantitative colorimetric LAMP [15], recombinase polymerase amplification (RPA) combined with lateral flow [16-18], PCR with a lateral flow strip [19], improved real-time PCR system with probe modification [20], CRISPR-Cas12a system coupled with PCR or LAMP [21], recombinase aided amplification combined with CRISPR/Cas12a [22], RPA-CRISPR-based nucleic acid detection method [23] and Crispr/cas12a technology combined with immunochromatographic strips [24]. The microfluidic-circular fluorescent probe-mediated isothermal nucleic acid amplification (CFPA) system has been newly developed for the detection of ASFV [25], and advanced nanopore sequencing has also been used for the diagnosis of ASFV [26]. These assays are very specific and sensitive and require time, highly sophisticated equipment, and qualified personnel [17]. In addition, these assays do not meet the World Health Organization (WHO) criteria for the ideal diagnostic assay, which are cost-effectivity, accuracy, and applicability for on-site practice with little or no requirement for specialized equipment or technical assistance [27]. Thus, a new diagnostic assay for ASFV is urgently needed.

Ladder-shape melting temperature isothermal amplification (LMTIA) is a newly developed nucleic acid amplification technique. In the LMTIA, single-strand DNA template supply was used via the melting temperature (T_m) difference between the primer and its target sequence rather than thermal denaturation or enzyme catalysis. The melting temperature curve of a target sequence in the LMTIA reaction is ladder-shaped. The whole reaction is divided into the initial structure formation stage and the exponential amplification stage. The initial structure formation stage is composed of the cycle reactions of annealing, strand displacement, and synthesis. The exponential amplification stage is the length doubling process composed of the cycle reactions of annealing, strand displacement and synthesis, and strand displacement [28]. This technique has the following advantages: simple mechanism, easy primer design, quick turn-around time, high specificity, high sensitivity, short target sequence, and wide application range of single-stranded DNA and double-stranded DNA or RNA (for reverse transcriptase activity of Bst DNA Polymerase) as its target [28]. Thus, the objective of this study was to develop the LMTIA assay for rapid, sensitive, specific, and on-site detection of ASFV, which may facilitate the prevention and control of ASF.

MATERIALS AND METHODS

Target sequence selection and LMTIA primer design

According to the principle for the target sequence selection of LMTIA, the sequence with a ladder-shaped melting temperature curve was selected as a target from the p72 gene of ASFV (GenBank accession No.: MN393476.1) using the software Oligo 7 (Molecular Biology Insights, Inc., USA). The p72 gene was selected as a target gene because of its high specificity to ASFV [7,8,11,16,19,21,22]. The length of the selected target was 118 nt, and its GC content

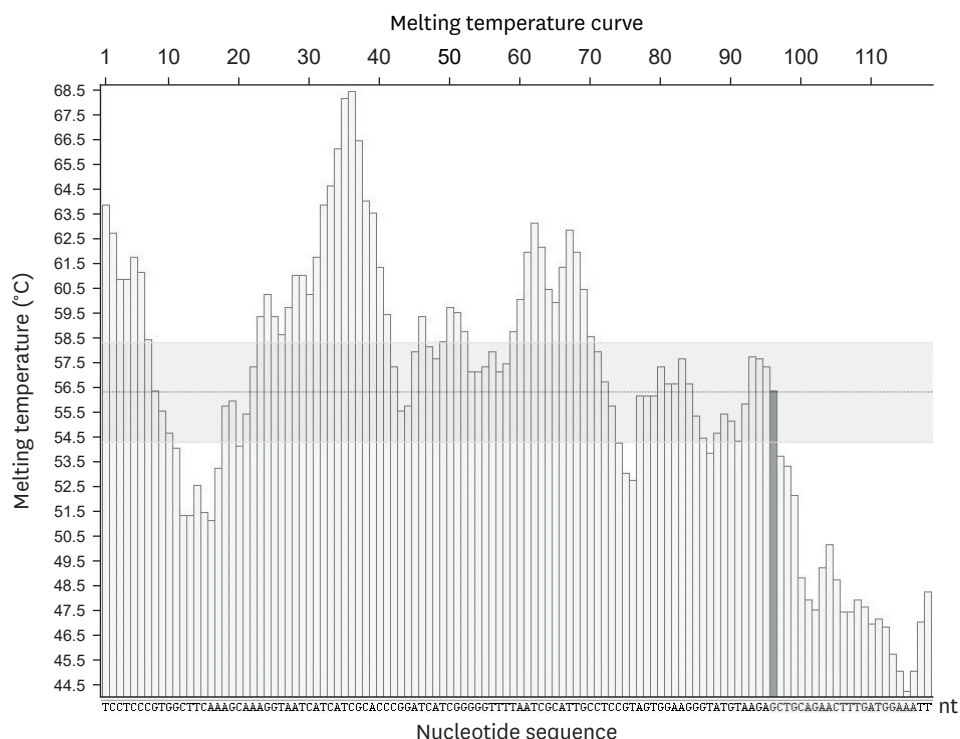


Fig. 1. Ladder-type melting temperature curve of target sequences.

Table 1. LMTIA primers from the p72 gene of ASFV

Primer	Sequence (5'-3')
P	CCCGATGATCCGGTTTTCTCCCGTGGCTTCAAAG
D	TTTAATCGCATTGCCTTTTTAATTCCATCAAAGTTCTGCAGC

LMTIA, ladder-shape melting temperature isothermal amplification; ASFV, African swine fever virus.

was 48.3%. The melting temperature curve of the selected sequence is shown in Fig. 1. The LMTIA primers were designed with the onlinesoftwarePrimer3Plus (<http://www.primer3plus.com/>), and a 118 nt sequence was used as a target. The parameters of primers were set as described in the previous report [28]. The primer sequences are listed in Table 1.

DNA preparation

The 368 nt fragment (GenBank accession No.: MN393476.1) of ASFV p72 gene was chemically synthesized and cloned to pUC57 plasmid (hereinafter referred to as pUC57-p72 DNA) by General Biosystems Co. Ltd., China, which contained the amplification target of the above-mentioned LMTIA primers (Fig. 2). The pUC57-p72 DNA was used as the template for optimization of the LMTIA system and determination of the sensitivity, as a standard ASFV plasmid was used in previous reports [5,18]. Genomic DNAs (gDNAs) extracted from 200 swine serum samples were provided by Henan Tuodao Biotechnology Co., Ltd., China, and these DNAs were used for comparison of the LMTIA assay and the commercial real-time PCR kit (Zhengzhou Zhongdao Biotechnology Co., Ltd., China).

LMTIA reaction temperature optimization

The LMTIA reaction temperature was optimized with 10 µL reaction mixture containing 1.3 µM of primer P and primer D, 1× General LMTIA Reaction Mix 2.0 (1.0 mM dNTPs, 20 mM Tris-HCl [pH 8.8], 10 mM KCl, 10 mM [NH₄]₂SO₄, 6 mM MgSO₄, 0.1% Triton X-100,

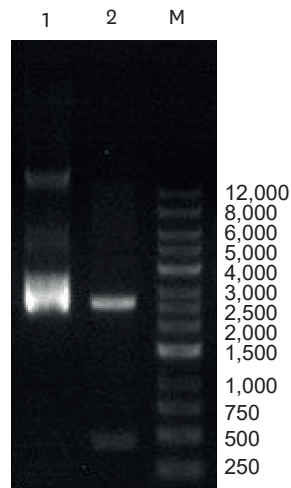


Fig. 2. Restriction digestion map of pUC57 plasmid containing 376 nt fragment of ASFV p72 gene. Note: Lane 1: Plasmid; Lane 2: Plasmid digested with EcoRI-Sall; Lane M: 1 kb DNA Marker.

1×EvaGreen and 0.32 U/μL Bst DNA polymerase) (Merit Biotech Co., Ltd., China), and 1 pg pUC57-p72 DNA, and 20 μL of liquid paraffin was added to avoid aerosol contamination after preparing the reaction mixture [28]. The LMTIA reaction mixture was heated at 54°C, 55°C, and 56°C in an Automatic PCR Analysis System (Xi'an Tianlong Science and Technology Co., Ltd., China). This system was also used to collect fluorescent signals with 23-sec intervals, and 40 signals were collected.

Sensitivity determination of the LMTIA assay and comparison with the real-time PCR assay kit

The sensitivity of the newly developed LMTIA assay was determined with pUC57-p72 DNA ranging from 1 pg to 0.01 fg, and the reaction mixtures were heated at the selected temperature in the Automatic PCR Analysis System. Fluorescent signals were collected with 23-sec intervals, and 40 signals were collected using this system.

The gDNA extracted from the ASFV-positive serum sample was diluted to 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} . These dilutions were determined with the newly developed LMTIA assay and the commercial real-time PCR kit for sensitivity comparison, which was approved by the Ministry of Agriculture and Rural Affairs of the People's Republic of China (Veterinary Drugs Production License No. 163668871). The detection limit of the real-time PCR kit was 0.5 copy/μL.

Evaluation of the developed LMTIA assay

The LMTIA assay was evaluated using the gDNAs extracted from 200 swine serum samples, compared with the commercial real-time PCR kit.

RESULTS

Selection of LMTIA reaction temperature

As shown in **Fig. 3**, the results of all positive controls (with pUC57-p72 DNA as template) were positive for p72 gene of ASFV, and the results of all negative controls (DNA template substituted by ddH₂O) were negative at 54°C, 55°C, and 56°C. The highest amplification

efficiency of the LMTIA reaction was observed at 55°C, and 55°C was temporarily selected as the reaction temperature of the LMTIA assay for the subsequent experiments.

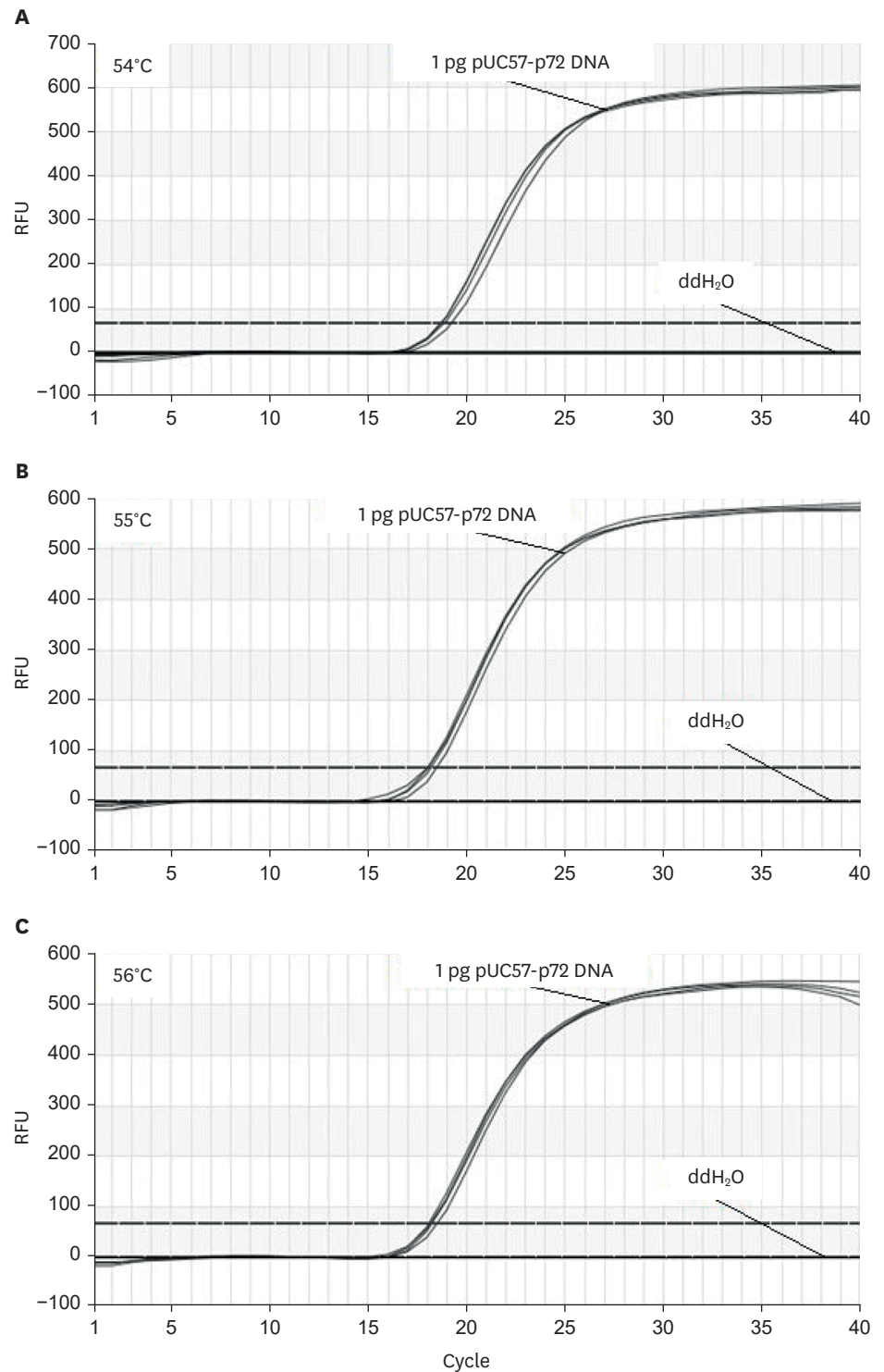


Fig. 3. Amplification plot of the LMTIA reaction at 54°C, 55°C, and 56°C. LMTIA, ladder-shape melting temperature isothermal amplification; RFU, relative fluorescence units.

Detection limit of the LMTIA assay and comparison with the real-time PCR kit

The sensitivity of the LMTIA assay was determined with pUC57-p72 DNA ranging from 1 pg to 0.01 fg at 55°C in the Automatic PCR Analysis System. This system was also used to collect fluorescent signals with 23-sec intervals, and a total of 40 signals were collected. The detection limit was 0.1 fg pUC57-p72 DNA (**Fig. 4**).

The detection limit of the LMTIA assay was 10^{-4} dilution of gDNA extracted from ASFV-positive serum sample, which was the same as that of the commercial real-time PCR kit, and the amplification efficiency of the LMTIA assay was relatively higher than that of the real-time PCR kit, as shown in **Figs. 5 and 6**.

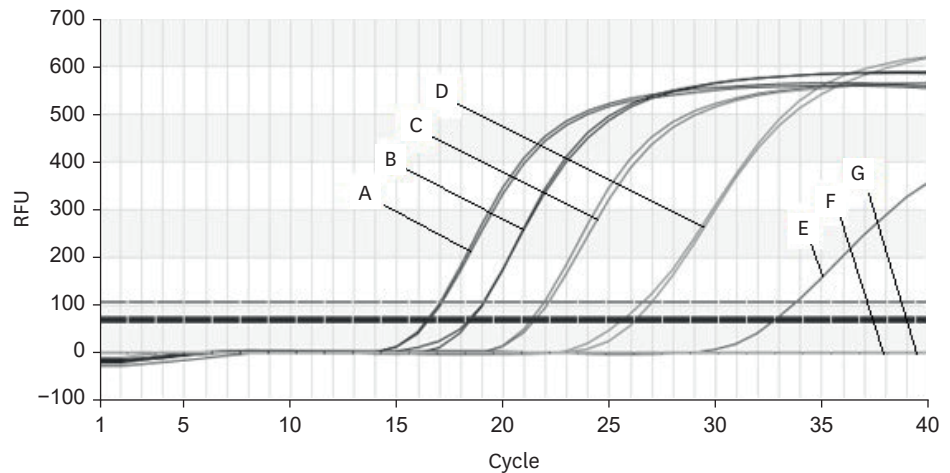


Fig. 4. Sensitivity of the developed LMTIA assay with pUC57-p72 DNA as template. Note: A: 1 pg pUC57-p72 DNA; B: 100 fg pUC57-p72 DNA; C: 10 fg pUC57-p72 DNA; D: 1 fg pUC57-p72 DNA; E: 0.1 fg pUC57-p72 DNA; F: 0.01 fg pUC57-p72; G: ddH₂O. LMTIA, ladder-shape melting temperature isothermal amplification; RFU, relative fluorescence units.

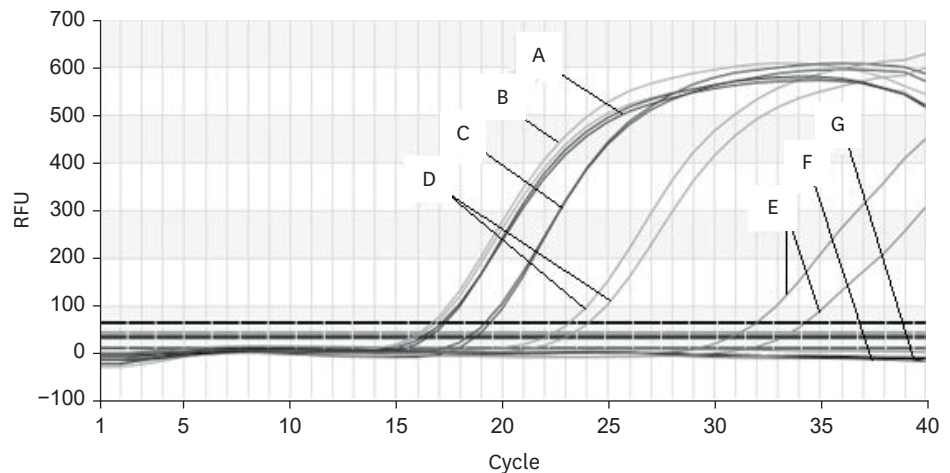


Fig. 5. Sensitivity of the developed LMTIA assay for detection of the genomic DNA of ASFV. Note: A: Positive controls (1 pg pUC57-p72 DNA); B: 10^{-1} dilution of genomic DNA extracted from ASFV-positive serum sample; C: 10^{-2} dilution of genomic DNA extracted from ASFV-positive serum sample; D: 10^{-3} dilution of genomic DNA extracted from ASFV-positive serum sample; E: 10^{-4} dilution of genomic DNA extracted from ASFV-positive serum sample; F: 10^{-5} dilution of genomic DNA extracted from ASFV-positive serum sample; G: ddH₂O. LMTIA, ladder-shape melting temperature isothermal amplification; ASFV, African swine fever virus; RFU, relative fluorescence units.

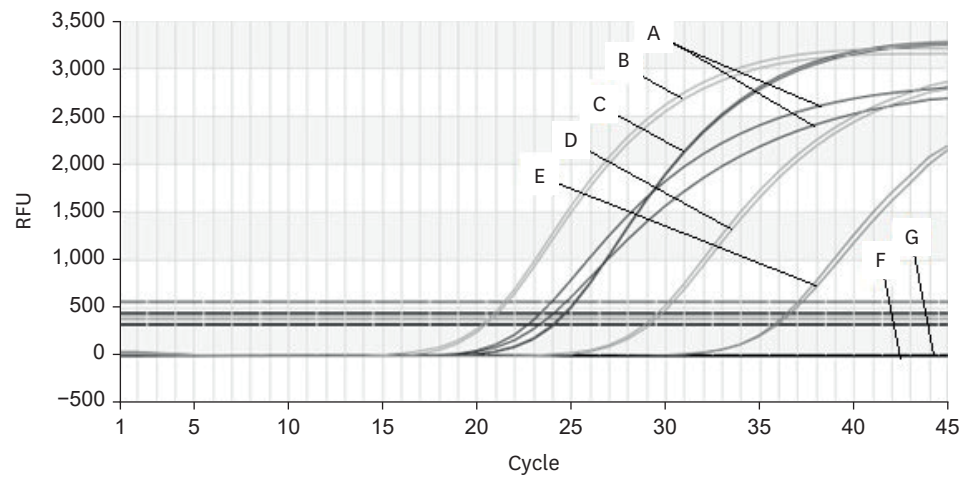


Fig. 6. Sensitivity of the commercial Real-time PCR Kit for detection of the genomic DNA of ASFV. Note: A: Positive controls (1 pg pUC57-p72 DNA); B: 10^{-1} dilution of genomic DNA extracted from ASFV-positive serum sample; C: 10^{-2} dilution of genomic DNA extracted from ASFV-positive serum sample; D: 10^{-3} dilution of genomic DNA extracted from ASFV-positive serum sample; E: 10^{-4} dilution of genomic DNA extracted from ASFV-positive serum sample; F: 10^{-5} dilution of genomic DNA extracted from ASFV-positive serum sample; G: ddH₂O. PCR, polymerase chain reaction; ASFV, African swine fever virus; RFU, relative fluorescence units.

Table 2. ASFV detection with the LMTIA assay and the commercial Real-time PCR kit of ASFV

Number of samples	LMTIA assay		Commercial real-time PCR kit	
	Positive	Negative	Positive	Negative
24	24	0	24	0
176	0	176	0	176

ASFV, African swine fever virus; LMTIA, ladder-shape melting temperature isothermal amplification; PCR, polymerase chain reaction.

Performance of the developed LMTIA assay

Of 200 swine serum samples, 24 swine serum samples tested by LMTIA assay contained ASFV, which was the same as that of the commercial real-time PCR kit. The coincidence rate between the two assays was 100% (Table 2).

DISCUSSION

The LMTIA technique is the recently developed nucleic acid isothermal amplification upon modification of LAMP [28]. The isothermal amplification of PCR reaction was used in this technique via T_m difference between primers and their target sequences. It shortens the reaction time from more than 1 hr to less than 25 min using the primer design method of LAMP as a reference, which has a clear single-strand template generation mechanism, high specificity, and sensitivity and is rapid and suitable for short target sequence amplification over LAMP. In this study, the LMTIA primers were designed using a 118 nt sequence on the p72 gene of ASFV as a target, and the LMTIA assay for detection of ASFV was established. As shown by experiments, the technique of LMTIA is reproducible.

When the plasmid containing the target sequence was used as a template, the detection limit of the LMTIA assay was 0.1 fg pUC57-p72 DNA. The detection limit of real-time LAMP was 0.1 fg pUC57-p10 DNA, which is equivalent to 30 copies/μL [12]. The sensitivity of LMTIA was the same as that of the real-time LAMP. When the gDNA extracted from the ASFV-positive serum sample was used for sensitivity determination, the detection limit of LMTIA was 10^{-4}

dilution of gDNA extracted from the ASFV-positive serum sample, which was the same as that of the commercial real-time PCR kit. The sensitivity of the developed assay was 0.5 copy/ μ L of the gDNA of ASFV, and the amplification efficiency of the LMTIA assay was significantly higher than that of the real-time PCR kit. The reaction time of the LMTIA assay was 15 min. The plasmid DNA template of pUC57-p72 DNA is a closed circular structure, and the gDNA template of ASFV is opened linear structure, which causes sensitivity difference. Also, the primers of LMTIA bind more easily with the DNA template of opened linear structure.

In this study, the specificity of the LMTIA assay was not determined with several virus strains as in the traditional practice [12], but the LMTIA assay could be directly compared with the approved commercial real-time PCR kit. The test results of 200 swine serum samples indicate that the coincidence rate between the two assays is 100%, and the LMTIA is highly specific [28].

For the LMTIA assay, the Bst DNA polymerase (large fragment) should be modified to improve the catalytic efficiency and shorten the amplification time to less than 10 min, and the Bst DNA polymerase (large fragment) should be modified compatible with the UNG-dUTP system to reduce the product pollution. Moreover, the fluorescent probe should be designed to overcome the shortcomings of the absence of internal amplification controls and limitations of quantitative assessments and multiplexing [29].

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