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A rapid and sensitive loop-mediated isothermal amplification procedure (LAMP) for *Mycoplasma hyopneumoniae* detection based on the *p36* gene

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ABSTRACT. The aim of this study was to establish a method for sensitive and rapid diagnosis of *Mycoplasma hyopneumoniae* in clinical specimens. To this effect, we employed three sets of primers specifically designed for amplification of nucleic acids under isothermal conditions. After optimization of reaction conditions, *M. hyopneumoniae* could be successfully detected at 63°C in 45 min through use of the loop-

mediated isothermal amplification (LAMP) assay. A positive reaction was identified visually as white precipitate and confirmed by gel electrophoresis. The detection limit for this assay was 10 copies/μL, as observed by electrophoretic analysis. The accuracy of the LAMP reaction was confirmed by restriction endonuclease digestion as well as by direct sequencing of the amplified product. This method can specifically detect *M. hyopneumoniae*; other species with high homology and other bacterial and virus strains gave negative results. To test the utility of this procedure, the LAMP assay was applied to 40 clinical samples collected from swine lung tissues experimentally challenged with *M. hyopneumoniae* isolates, and compared to the results from a real-time polymerase chain reaction (PCR) assay. A concordance of 100% was observed between the two assays. In conclusion, the results from our study demonstrated that the LAMP assay provided a rapid reaction and was inexpensive to perform, with no need of complex instruments or systems such as Geneamp PCR. The LAMP assay may therefore be applied in routine diagnosis in the clinical laboratory and for in-field detection of *M. hyopneumoniae* infection.

Key words: *p36* gene; Loop-mediated isothermal amplification; *Mycoplasma hyopneumoniae*

INTRODUCTION

A high proportion of farms with respiratory problems among their stock are infected with *Mycoplasma hyopneumoniae*, which is the primary agent of enzootic pneumonia of swine, a worldwide chronic respiratory disease in pig farms characterized by high morbidity and low mortality (Kobisch and Friis, 1996; Maes et al., 1996). The economic impact of mycoplasma pneumonia of swine is considerable, due mainly to reduced growth and feeding efficiency (Straw, 1990; Paisley et al., 1993), and an increased susceptibility of pigs to infection by other organisms (Kobisch and Friis, 1996). Currently, clinical examination is usually considered to be of low value for diagnosis, in particular when compared to the direct detection of *M. hyopneumoniae* in lung lesions by polymerase chain reaction (PCR) amplification. In fattening pigs, a quantitative assessment of the onset of coughing (typically dry and non-productive) improves the diagnosis of enzootic pneumonia and can occasionally substitute for the detection of *M. hyopneumoniae* by PCR (Nathues et al., 2012). *M. hyopneumoniae* culture is laborious, time-consuming, and requires several weeks to produce results; frequently, the culture media can become overgrown with *M. hyorhinis* or *M. flocculare* as well (Dorigo-Zetsma et al., 1999). Serological methods are insufficiently sensitive and cannot distinguish between vaccinated and *M. hyopneumoniae*-infected pigs in field screening (Moitinho-Silva et al., 2012; Simionatto et al., 2012). More rapid, higher sensitivity methods were therefore developed, one of them being the PCR technique for *M. hyopneumoniae* DNA detection (Dorigo-Zetsma et al., 1999; Lu et al., 2010; Shen et al., 2011). In the last few years, real-time PCR methods for the diagnosis of *M. hyopneumoniae* have been described (Dubosson et al., 2004). The advantage of this application, compared to conventional PCR methods, is its higher speed and reduced requirement in handling of PCR products such as needed for

electrophoretic analysis. However, owing to the expensive systems required, this application is still not very common in hospital-based laboratories.

Notomi et al. (2000) reported a novel nucleic acid amplification method called loop-mediated isothermal amplification (LAMP), which is capable of amplifying DNA under isothermal conditions with high specificity, efficiency, and speed. The most significant advantage of LAMP is the ability to amplify specific sequences of DNA under isothermal conditions between 60° and 65°C, thereby obviating the need for a thermal cycler (Notomi et al., 2000). Moreover, the LAMP assay synthesizes a large amount of by-product, an insoluble white precipitate of magnesium pyrophosphate, which can be judged visually through the appearance of a white precipitate, or by fluorescence when observed under ultraviolet light following the addition of SYBR Green dye. The LAMP reaction does not require a complex thermocycler; rather, a simple water bath is sufficient for the entire process (Yamazaki et al., 2008; Fu et al., 2011).

The aim of the current study was to develop a LAMP assay specific for *M. hyopneumoniae* in order to facilitate its rapid, sensitive, and reliable detection and/or identification from clinical samples. Such an assay would be an invaluable tool for both animal health studies and pathogen detection.

MATERIAL AND METHODS

Bacterial strains and clinical specimens

For the evaluation of primer specificity, bacteria and virus were prepared from 12 reference strains: *M. hyopneumoniae* 168 strain, *M. hyorhinis*, *M. flocculare*, *M. hyosynoviae*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Haemophilus parasuis*, swine *Pasteurella multocida*, classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine pseudorabies virus (PRV), preserved by the Key Laboratory of Animal Diseases Diagnostic and Immunology of Ministry of Agriculture, Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences.

Reagents and materials

The TIANamp DNA Kit was a produced by the Tiangen Biotech Co., Ltd. (Beijing, China). *Bst* DNA polymerase and betaine were purchased from New England Biolabs (Ipswich, MA, USA) and Sigma-Aldrich Chemical Company (Hattiesburg, MS, USA), respectively. *Mob*I restriction endonuclease was a product of TaKaRa BioTechnology Co., Ltd. (Dalian, China). SYBR Green I was obtained from Invitrogen Corporation (Carlsbad, CA, USA).

Design of primers for LAMP

Three sets of oligonucleotide primers were used for the *M. hyopneumoniae* LAMP assay and were designed based upon the *M. hyopneumoniae p36* conserved region using the LAMP primer design software Premier 5.0 (http://primerexplorer.jp/elamp4.0.0/index.html; Eiken Chemical Co. Ltd.; Taito-ku, Japan). Each set consisted of four primers: an outer (F3 and B3) and an inner (FIP and BIP) primer pair. Primer sequences are shown in Table 1.

Table 1. Three sets of primers for detection of *Mycoplasma hyopneumoniae* in loop-mediated isothermal amplification assays.

Primers	Sequences (5'-3')
361F3	TGC ATC CGA GTA TGG AAT T
361B3	TTC AAG CCG AGT TTC ACC
361FIP	GCA AAG AAG CTG AGG CAT CTT CGA TAT TAA TCC TGA TTT TGC CGA T
361BIP	CCG ATT AGT GTC TCC CGT TAT GAT TTT TGT GGT CTT CCC GC
362F3	AGT ATT ATT GTT GCT AAT CCT GT
362B3	GTT CAC CCA TCA CGT AGG
362FIP	CAC TAC CGA TAA CTT TTT GAT CGG ATG ATA TAA TTA CAA GGG CTT ACC
362BIP	GAA CTG TTT TAG ATA CAG CAA GGC TTG AAC CGA ATT AGG CGA TAC
363F3	CTG ATA ACA TCC GAA TTA TCC G
363B3	CCT GAA CCG AAT TAG GCG
363FIP	CCG GTA AGC CCT TGT AAT TAT ATC ATG CAC TAA AAG TCA AAG AAA GT
363BIP	CAT CTG GAT TTT CCG ATC AAA AAG TCG ATT GCA AAT TGA AGC CT

DNA and cDNA from *M. hyopneumoniae* and other strains

The amplified *p36* PCR product was purified and cloned into the vector pET-28a (+) for the construction of a *p36* expression vector [pET-28a (+)-p36]. Purified plasmid DNA, and genomic DNA and cDNA from *M. hyopneumoniae* and other strains were extracted from bacterial cultures with a commercial DNA/RNA extraction kit (Tiangen), following the manufacturer instructions, for detection of LAMP assay specificity. *M. hyopneumoniae* genomic DNA from lung tissues was prepared via the boiling method. The resulting supernatant was used as a template for determining the clinical application of the LAMP assay *vs* real-time PCR.

LAMP reaction

pET-28a (+)-p36 plasmid DNA was used for the LAMP assay as follows: the reaction mixture consisted of a final volume of 25 µL containing ddH_2O, 10X ThermoPol buffer (2.5 µL), 4 mM $MgSO_4$, 0.2 µM of each primer F3 and B3, 0.8 µM of each primer FIP and BIP, 0.48 M betaine, 1.2 mM dNTP mixture, 12 U *Bst* DNA polymerase, and 1 µL template DNA. LAMP primers were optimized with 361, 362, and 363 primers by incubating the mixture at 61°, 63°, and 65°C for 45 min, respectively. The reaction was terminated via heating at 80°C for 5 min. A negative control (replacement of the template with H_2O) was included in each run. The reaction mixture was visually checked for a white precipitate, followed by electrophoresis of the LAMP products (5 µL) in 1.5% agarose gels containing SYBR Green I to determine the optimal reaction conditions. All experiments were repeated at least twice. The LAMP product was identified by digestion with the restriction endonuclease *Mob*I. The restriction digestion system consisted of 25 µL LAMP products, 4 µL 10X K Buffer, 4 µL *Mob*I, and 7 µL ddH_2O. The length of the digested restriction fragment was 188 bp.

Real-time PCR amplification

A 1-µL DNA aliquot from the targeted organism was used for real-time PCR in a 25-µL final volume containing 2X Premix (12.5 µL) [TaKaRa Biotechnology (Dalian) Co., Ltd.] 0.05 µM probes, 50X Rox 0.5 µL, and 0.4 µM of each primers F and R. The reaction included an initial PCR activation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C

for 20 s and combined annealing/extension at 60°C for 1 min. The PCR products were detected with 7500 instrument (Applied Biosystems). Subsequent data analysis was performed using the CFX manager software.

Sensitivity of the LAMP assay

To determine the sensitivity of the LAMP and real-time PCR assays, serial dilutions of plasmid pET-28a (+)-p36, ranging from 10^6 to 10^{-1} copies/μL were amplified. The LAMP sensitivity test was conducted under the optimal reaction conditions containing 1 μL diluted DNA templates. The LAMP reaction was performed at 63°C for 45 min. Negative and positive controls were performed using sterile water and genomic DNA template, respectively. For detection of LAMP products, the reaction tubes were observed both visually and under ultraviolet illumination, and the final reaction mixtures (5 μL) run on a 1.5 % agarose gel were visualized under ultraviolet light illumination as well following SYBR Green I staining.

Specificity of the LAMP assay

The specificity of the LAMP assay was first determined with *M. hyopneumoniae* DNA templates. A negative control was included in each run using ddH_2O as the template. In addition, other species with high homology to *M. hyopneumoniae*, including *M. hyorhinis*, *M. flocculare*, *M. hyosynoviae*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Haemophilus parasuis*, swine *Pasteurella multocida*, CSFV, PCV2, PRRSV, and PRV were tested to further assess the specificity of the LAMP assay. The LAMP reactions were performed at 63°C for 45 min. The LAMP products was electrophoresed on 1.5% agarose gels containing SYBR Green I, and detected as described above.

Application of LAMP assays on clinical samples

The applicability of the LAMP assay to detect *M. hyopneumoniae* was assessed by comparison of the detection results between LAMP and real-time PCR using field samples. Pigs were obtained from a source known to be negative for *M. hyopneumoniae*. All animal procedures were approved by the Animal Ethics Committee of Jiangsu Academy of Agricultural Sciences. The experiments were performed in Jiangsu Academy of Agricultural Sciences. Fifty pigs were employed for the study and divided into five groups. Forty pigs were challenged by delivering 5 mL 10^{-2} to 10^{-5}-diluted *M. hyopneumoniae* NJ strain culture by intratracheal injection. As a control group, 10 pigs were injected with phosphate-buffered saline into the trachea. Pigs were monitored twice daily for a minimum of 15 min for clinical signs of illness. Clinical signs and lung lesions were observed 25 days post challenge. Eight pigs from each group were randomly slaughtered and the lungs were collected for LAMP and PCR assays. *M. hyopneumoniae* genomic DNA from pig lung tissues was prepared by boiling for 10 min. After centrifugation, the resulting supernatant was used as DNA templates.

RESULTS

Optimization of LAMP assay conditions

The specific amplification generated many ladder-like patterned bands on the agarose

gel. Using the 361 and 362 primers, amplification was initially detected at 61°, 63°, and 65°C, and showed higher levels of DNA resolution at 63°C when compared to other temperatures. However, no amplification could be detected from the 363 primers. Clearer ladder-like patterned bands (Figure 1A) and a white precipitate (Figure 1B) were observed at 63°C with the 362 primers when compared to other temperatures and primer sets.

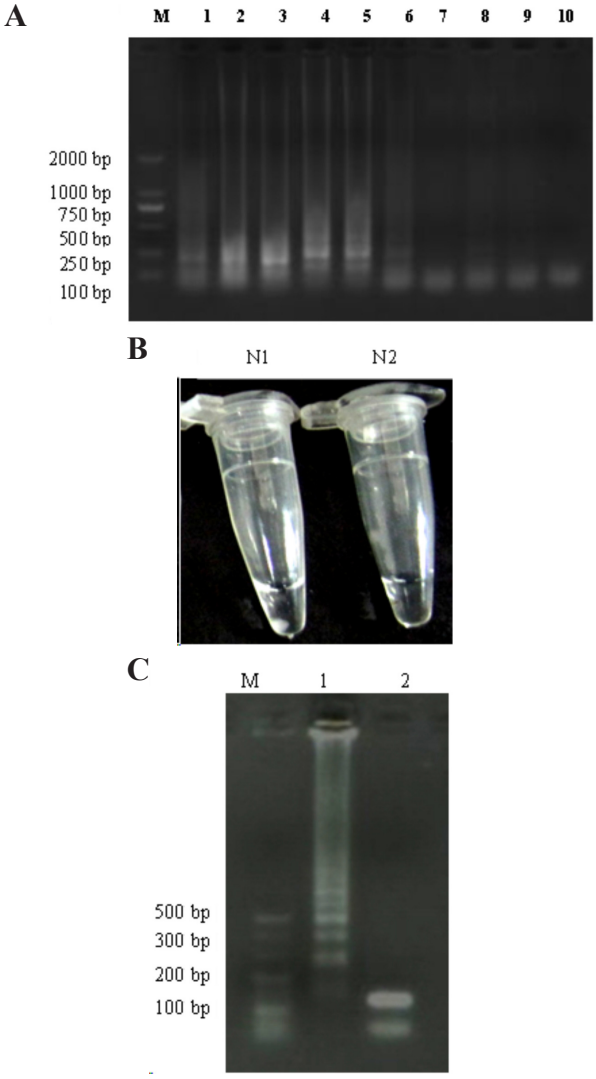

Figure 1. Optimization of the loop-mediated isothermal amplification (LAMP) procedure. **A.** Electropherogram determination of optimal LAMP primer pairs and reaction temperature. *Lane M* = DL2000 DNA marker; *lanes 1-3* = 361 primers, at 61°, 63°, and 65°C, respectively; *lanes 4-6* = 362 primers, at 61°, 63°, and 65°C, respectively; *lanes 7-9* = 363 primers, at 61°, 63°, and 65°C, respectively; *lane 10* = negative control. **B.** White precipitate of the optimal LAMP reaction products. N1 = *Mycoplasma hyopneumoniae* genomic DNA template; N2 = negative control. **C.** Electropherogram of endonuclease digestion identification of the LAMP-amplified product. *Lane M* = 20-bp ladder DNA marker; *lane 1* = LAMP product from genomic DNA of *M. hyopneumoniae*; *lane 2* = LAMP product from lane 1 after digestion.

The accuracy of the LAMP reaction was confirmed by digestion with the restriction enzyme *Mob*I to ensure that the LAMP products had the sequence corresponding to the *p36* gene of *M*. *hyopneumoniae*. The product of *Mob*I digestion was 188 bp in size, a finding consistent with the predicted size (Figure 1C).

Sensitivity of the LAMP assays

The detection limits of the LAMP assay were found to be 10 copies/μL for a 45 min reaction at 63°C and analysis by agarose gel electrophoresis (Figure 2A) as well as for visualization of a white precipitate (Figure 2B). Real-time PCR demonstrated detection limits of 10 copies (Figure 2C) requiring over 60 min in the thermocycler. No amplification was apparent in the LAMP reaction when the sample tube lacked target DNA.

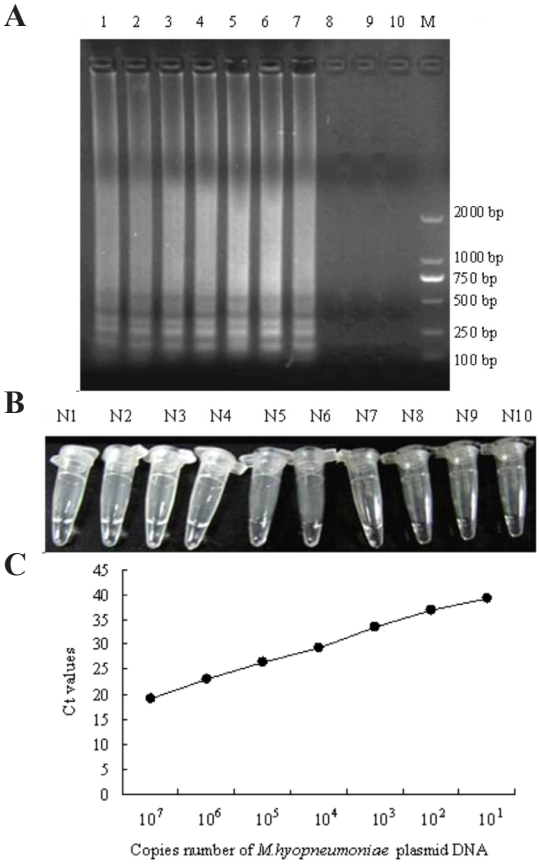

Figure 2. Sensitivity determination of loop-mediated isothermal amplification (LAMP) and real-time polymerase chain reaction (PCR) assays. **A.** Electropherogram determination of LAMP assay sensitivity. *Lane M* = DL2000 DNA marker; *lane 1* = *Mycoplamsa hyopneumoniae* genomic DNA template; *lanes 2-9* = 10^X copies (1 μL); *lane 2* = 10^6; *lane 3* = 10^5; *lane 4* = 10^4; *lane 5* = 10^3; *lane 6* = 10^2; *lane 7* = 10^1; *lane 8* = 10^0; *lane 9* = 10^{-1}; *lane 10* = negative control. **B.** White precipitates of LAMP amplification products. N1 = *M*. *hyopneumoniae* genomic DNA template; N2-9 = 10^X copies (1 μL); N2 = 10^6; N3 = 10^5; N4 = 10^4; N5 = 10^3; N6 = 10^2; N7 = 10^1; N8 = 10^0; N9 = 10^{-1}; N10 = negative control. **C.** Relationship between Ct values and copy number of *M. hyopneumoniae* plasmid DNA in the real-time PCR assay. Copy numbers were from 10^7-10^1 copies/μL.

Specificity of the LAMP assays

The LAMP assay specifically amplified DNA from *M. hyopneumoniae* but not from any of the other *Mycoplasma* species or other bacterial or viral species. No false-positive amplification was observed, indicating the high specificity of the established LAMP assays in electrophoretic analysis (Figure 3).

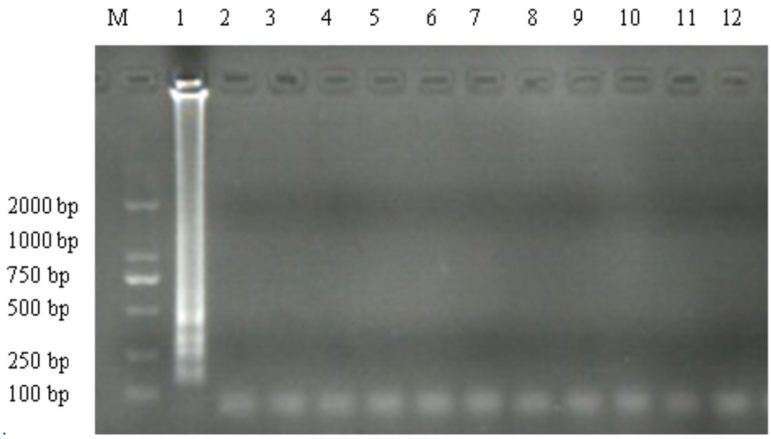

Figure 3. Electropherogram determination of loop-mediated isothermal amplification (LAMP) assay specificity. *Lane M* = DL2000 DNA marker; *lane 1 = Mycoplasma hyopneumoniae* genomic DNA template; *lane 2* = negative control; *lane 3 = Haemophilus parasuis* DNA template; *lane 4 = Actinobacillus pleuropneumoniae* DNA template; *lane 5 = Streptococcus suis* DNA template; *lane 6* = swine *Pasteurella multocida* DNA template; *lane 7 = Mycoplasma hyosynoviae* DNA template; *lane 8 = Mycoplasma hyorhinis* DNA template; *lane 9 = PRRSV* cDNA template; *lane 10 = PCV2* DNA template; *lane 11 = CSFV* cDNA template; *lane 12 = PRV* DNA template. PRRSV = porcine reproductive and respiratory syndrome virus; PCV2 = porcine circovirus type 2; CSFV = classical swine fever virus; PRV = porcine pseudorabies virus.

Application of LAMP assays on clinical samples

For both LAMP and real-time PCR assays, 35 clinical samples tested positive for *M. hyopneumoniae*. Thus, the concordance between the two assays was 100%. The positive predictive value was determined to be 100% for both LAMP and real-time PCR assays, with no false positives observed (Table 2). In comparison with real-time PCR methods, LAMP showed a more rapid reaction and a lower cost.

Table 2. Loop-mediated isothermal amplification assay results from *Mycoplasma hyopneumoniae* clinical samples.

Samples (total number)	LAMP detection rate (%)/N positive samples	Real-time polymerase chain reaction
Swine lung tissues (N = 40)	100/35	100/35

DISCUSSION

Although major efforts have been made to control *M. hyopneumoniae* infection and its detrimental effects, significant economic losses remain in pig production worldwide due

to enzootic pneumonia (Thacker, 2006). Effective control requires a number of actions, including establishing and monitoring the extent of disease, reducing transmission of infection, and developing an appropriate vaccination strategy. Establishing and monitoring the extent of enzootic pneumonia in pig herds now typically incorporate enzyme-linked immunosorbent assay, PCR, and slaughter surveillance methods. These technologies, however, have limitations. Furthermore, these molecular diagnostic techniques require either a precise instrument for amplification or an elaborate method for detection of the amplified products. In the present study, we evaluated the LAMP assay for detection of *M. hyopneumoniae* in either culture isolates or lung tissues. This assay is a simple diagnostic tool in which the reaction takes place in a single tube. In evaluating detection methodologies for epidemiological purposes, a series of attributes should be considered and assessed, including specificity, sensitivity, simplicity, expense, and time. LAMP has been known as a rapid, specific, sensitive, cost-effective, and easy-to perform alternative for diagnosis of pancreatic necrosis virus and porcine circovirus type 1 (Suebsing et al., 2011; Wang et al., 2011), and has been applied to the direct detection of the *Mycobacterium tuberculosis* complex, *Mycobacterium avium* and intracellular *Mycobacterium*, and *M. pneumoniae* (Iwamoto et al., 2003), and of the severe acute respiratory syndrome coronavirus (Hong et al., 2004).

The genome of *M. hyopneumoniae* encodes several immunodominant proteins, including a cytosolic protein (P36), three membranous proteins (P46, P65, and P74), and an adhesion protein (P97). Cross-reactions with *M. flocculare* and *M. hyorhinis* reduce the specificity of conventional serological detection methods. However, certain antigenic determinants of the P36 protein have been shown to be specific for *M. hyopneumoniae* (Caron et al., 2000; Cheikh Saad et al., 2003). The extreme sensitivity provided by P36 may therefore be of substantial diagnostic value in swine mycoplasma pneumonia. To capitalize on this potential, it is very important to find a conserved nucleic acid fragment for designing specific LAMP primers and develop an efficient, accurate LAMP assay (Notomi et al., 2000; Fu et al., 2011). In the present study, two sets of *M. hyopneumoniae*-specific primers were designed with inner and outer primer pairs recognizing six distinct regions, and which amplified only *M. hyopneumoniae* DNA. High specificity was illustrated through lack of false-positive observations for reference strains and 100% concordance during application. Due to its powerful amplification efficiency, LAMP was characterized by high sensitivity and low detection limits at 10 copies for a 45-min reaction at 63°C, and could be performed in a laboratory with limited technology. In comparison, although the real-time PCR detection limit was also 10 copies, this technique commonly requires a costly instrument and nearly 2 h for amplification reactions alone.

As the reaction was performed under isothermal conditions without a thermal cycler, only simple equipment like a heat block or water baths were needed for the performance of LAMP assays at low expense. Detection time was no more than 1.2 h for one sample. Therefore, both simplicity and sensitivity make the LAMP assay a simple, rapid, and cost-effective alternative to the current rapid detection methodologies utilized in epidemiologic studies.

In conclusion, this assay was highly specific and showed a sensitivity of 10 copies of *M. hyopneumoniae* in a 45-min reaction, as determined by visualization of a white precipitation as well as by electrophoretic analysis. Together with its ease of operation, rapid reaction, and inexpensive system, the LAMP assay is therefore more appropriate than the real-time PCR assay for clinical detection of diseases, especially in field conditions and at quarantine inspection stations.

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