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Full Length Research Paper

Development and application of a loop-mediated isothermal amplification method for rapid detection of *Haemophilus parasuis*

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***Haemophilus parasuis* is the causative agent of Glässer's disease that has received much attention recently, due to the increasing economic losses this disease inflicts upon the pig industry worldwide. In this study, loop-mediated isothermal amplification method (LAMP) methodology was designed for diagnosing *H. parasuis* infections and tested against 56 clinical samples. Two sets of primers for LAMP were designed based on the *H. parasuis inf* B gene sequence. Target DNA was amplified and visualized on agarose gels after 50 min incubation at 63°C. The LAMP amplicon was also directly visualized in the reaction tubes by the naked eye following the addition of SYBR green I. The detection limit of the *inf* B-LAMP method was 10 cfu mL^{-1}, that was 10 times more sensitive than conventional PCR. Furthermore, positive rates of *H. parasuis* detection using *inf* B-LAMP were higher (46.4%, 26/56) than the rates obtained with conventional PCR (33.9%, 19/56). *inf* B-LAMP specificity analysis demonstrated no cross-reactivity with any other swine pathogens. In conclusion, *inf* B-LAMP was more sensitive and faster and could be carried out in the absence of expensive equipment. Furthermore, the visual readout demonstrated great potential for the use of *inf* B-LAMP in the clinical detection of *H. parasuis*.**

Key words: Glässer's disease, *Haemophilus parasuis*, *inf* B, PCR, LAMP

INTRODUCTION

Haemophilus parasuis is a commensal bacterium that colonizes the upper respiratory tract of domestic pigs and is the causative agent of Glässer's disease. Under appropriate conditions, *H. parasuis* can invade and cause severe systemic disease, characterized by fibrinous polyserositis, arthritis and meningitis (Del Rio et al., 2006; Oliveira et al., 2004). The diseases caused by *H. parasuis* have drawn significant attention worldwide (Blackall et al.,1997), with infection rates reaching 50 to 70% and mortality rates over 10%, leading to substantial economic losses (Baumann et al., 2002). During the past decade, even swine farms with high sanitary standards have experienced a significant increase in the incidence, morbidity and mortality associated with Glässer's disease (Rapp-Gabrielson et al., 2006).

Detection of *H. parasuis* using conventional methods, including culture, biochemical and immunological assays is time-consuming and laborious, typically requiring more than 3 days, although, PCR-based assays have markedly improved the sensitivity and rapidity of *H. parasuis* detection from clinical samples (Ferri et al., 2000). Oliveira

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Abbreviations: LAMP, Loop-mediated isothermal amplification method; **PCR,** polymerase chain reaction; **TSA,** tryptic soy agar; **EDTA,** ethylene diamine tetraacetic acid; **NAD,** nicotinamide adenine dinucleotide.

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et al. (2001) established a PCR test based on amplifying the 16S rRNA sequence and evaluated its efficacy on a number of clinical samples and reference strains. Detection of *H. parasuis* by PCR was shown to be more sensitive than culture-based techniques, however, Turni et al. (2009) demonstrated that, real-time PCR designed to amplify 16S rRNA sequence could not differentiate *Pasteurella mairii* from *H. parasuis*. Fortunately, the *inf* B gene is a potential alternative gene target to the 16S rRNA gene that was shown to be a useful genetic marker for carrying out phylogenetic studies (Hedegaard et al., 2000). This observation was also true for *H. parasuis,* since the *inf* B gene present in this organism has been used to identify *H. parasuis* from all other closely related species (Turni et al., 2009). In addition, the high cost of the instruments required to carry out real-time PCR restricts its use to financially stable clinics and laboratories. Therefore, an affordable, rapid, sensitive and specific *H. parasuis* detection method needs to be developed that will allow a broader range of clinics/laboratories to carry out *H. parasuis* diagnosis. A promising candidate that could full fill these requirements is loop-mediated isothermal amplification (LAMP) (Nagamine et al., 2002a; Notomi et al., 2002b) that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. The LAMP assay is faster, more specific and easier to perform than conventional PCR (Nagamine et al., 2002b; Goto et al., 2007; Hara-Kudo et al., 2005) and LAMP results in the synthesis of large amounts of DNA that can be analyzed by agarose gel electrophoresis (Mori et al., 2004; Mori et al., 2001). The successful development of LAMP procedures has been reported for many different clinical applications, including the diagnosis of viral and bacterial infections (Iwamoto et al., 2003; Minami et al., 2006; Song et al., 2005; Wang et al., 2009) and for diagnosis of protozoal diseases, including trypanosomiasis (Kuboki et al., 2003; Thekisoe et al., 2005) and canine and equine piroplasmosis (Alhassan et al., 2007; Ikadai et al., 2004). Although, two LAMP methods have been designed for *H. parasuis* detection, there exist significant differences between them. The LAMP assay developed by Wang et al. (2010) targets the16S rRNA gene, however, the 16S rRNA gene is not species-specific (Turni et al., 2009). For this reason, we choose *inf* B as the target gene to established the *inf* B-LAMP method for the detection of *H. parasuis*. The four specific primers used in our study recognized 6 distinct *inf* B target sequences different from those described by Chen et al. (2010). Furthermore, the results of our *inf* B-LAMP method can be evaluated by visualizing color changes with the naked eye after the addition of SYBR green I (Parida et al., 2006), that is, the positive reaction turns green while negative reactions remain orange. Therefore, using the *inf* B-LAMP assay would increase the probability of diagnosing *H. parasuis* infections in laboratories not equipped with the technology or equipment needed to carry out more labor intensive methodologies such as instrument for PCR or where gel electrophoresis cannot be carried out.

In this study, a specific, sensitive, rapid and simple *inf* B-LAMP method targeting the *inf* B gene of *H. parasuis* was developed. The assay was validated using 56 clinical samples harvested from pigs in herds in south China. This assay provided a powerful *H. parasuis* diagnostic tool which can be applied easily in less well-equipped laboratories, including field conditions.

MATERIALS AND METHODS

Bacterial strains

Fifteen (15) reference strains kindly provided by Dr. Chen (Huazhong Agricultural University, China) were used to standardize the LAMP method in this study. Additional strains including *Actinobacillus pleuropneumoniae* (Serovar 5), *A. pleuropneumoniae* (Serovar 9), two strains *Bordetella bronchiseptica* strains, *Pasteurella multocida*, *Streptococcus suis*, *Salmonella typhimurium* and *Escherichia coli* were used to determine the *inf* B-LAMP specificity.

DNA extraction

Template DNA used for *inf* B-LAMP and PCR were prepared by resuspending individual colonies of respective bacteria grown on tryptic soy agar (TSA) plates in distilled water to a concentration of approximately 1 x 10^7 cfu mL^{-1} and genomic DNA isolated as previously described (Sambrook and Russell, 2001). Bacterial cells were boiled in water for 10 min, centrifuged at 14,000 rpm for 10 min and supernatants then, used as template DNA.

Design of *inf* B-specific LAMP primers

To obtain a specific *H. parasuis* gene marker, genome and BLAST analyses were carried out using the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/). Results showed that, the *H. parasuis* isolate WB24/06-1 translation initiation factor IF-2 (*inf* B) gene (Gene ID: EF424388) sequence was specific and highly conserved in *H. parasuis* isolates indicating that, *inf* B could serve as a potential new candidate gene for the detection of *H. parasuis*. Four primers were designed based on the *inf* B gene sequence for use in the LAMP assay and included two outer primers (F3 and B3), one forward inner primer (FIP) and one reverse inner primer (BIP) (Table 1) as previously described (Notomi et al., 2000; Tomita et al., 2008). These primers specifically recognized 6 distinct regions on target DNA (Figure 1). FIP is comprised of the F1c sequence complementary to F1, a TTT linker and the F2 sequence; BIP is comprised of the B1c sequence complementary to B1, a TTT linker and the B2 sequence.

Optimization of the *inf* B-LAMP conditions

Final reaction mixtures consisted of 0.8 µM each FIP and BIP primers, 0.2 µM of each F3 and B3 outer primers, 400 µM of each dNTP, 1 M betaine (Sigma, St. Louis, MO, USA), 1X thermopol buffer (New England Biolabs, Ipswich, MA, USA) 2 mM $MgSO_4$, 8 U Bst DNA polymerase large fragment (New England Biolabs) and 1 µl genomic DNA (~100 ng). The optimum temperature and time needed for *inf* B-LAMP amplification was determined by carrying out reactions in a water bath at 61, 62, 63, 64 or 65°C for 20, 30, 40, 50 or 60 min. The reaction was terminated by increasing the

Table 1. PCR and LAMP primers designed for of the amplification of 16S rRNA or *info* B gene sequences from *H. parasuis*.

Primer	Sequence (5’-3’)
P1	5’-GTG ATG AGG AAG GGT GGTGT-3’
P2	5’-GGC TTC GTC ACC CTC TGT-3’
F3	5’- CGATTGTGGTTGCGGTAAAC-3’
B3	5’- TAAGATTCGATAACCACACCGC-3’
FIP (F1c+ F2)	5’-GAACATCACCACCGAATTTCTCAGAA TTTTAAACCAGAAGCAAACCCAGAGC-3’
BIP (B1c+ B2)	5’-ATGGGGATTGACGACTTACTTGAAGCTTTCATACCCTCTTTCACTGCACTTAATTC-3’

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1   AAGCGAAAGT AGCAGCAGGT GAAGCTGGCG GTATTACTCA GCATATCGGT GCATATCACG TTGAAACCGA CGACGGTAAG ATGATTACCT TCTTAGATAC
101 ACCAGGACAC GCGGCATTTA CCTCAATGCG TGCGCGTGGT GCGAAAGCAA CGGATATCGT TGTTCTTGTA GTAGCAGCTG ACGATGGCGT AATGCCACAA
201 ACCATTGAAG CAATCCAACA CGCGAAAGCA GCTGGTGCGC CGATTGTGGT TGCGGTAAAC AAAATTGATA AACCAGAAGC AAACCCAGAG CGTGTAGAGC
                                              F3                               F2
301 AAGAGTTATT ACAACACGAA GTGATTTCTG AGAAATTCGG TGGTGATGTT CAATTTGTTC CTGTTTCAGC GAAAAAAGGA ATGGGGATTG ACGACTTACT
                              F1                                                            B1c
401 TGAAGCCATT CTTCTTCAAT CGGAAGTATT AGAATTAAGT GCAGTGAAAG AGGGTATGGC AAGCGGTGTG GTTATCGAAT CTTACCTCGA TAAAGGTCGT
                                         B2c                          B3
501 G
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Figure 1. *H. parasuis inf* B gene nucleotide sequence (Gene ID: EF424388) used for primer design. Locations of target primer sequences are underlined.

temperature to 80°C for 4 min. A positive control (purified *H. parasuis* DNA) and a negative control (distilled water) were included during each run. LAMP products (3 µl) were electrophoresed on 2% molecular-grade agarose gel prepared in 0.5× tris-borate-EDTA buffer stained with 0.5 g mL^{-1} ethidium bromide. In addition, the amplification products were visualized following the addition of 1 µlof SYBR green I dye to the reaction tube.

PCR conditions

Conventional PCR designed to target nuclear 16S rRNA gene sequence described by Oliveira et al. (2001) was performed on all bacterial species described for this study. PCR reactions were carried out as follows: 2.5 µl 10x Ex Taq buffer, 0.2 mM of each dNTP, 0.2 µM of each primer (P1 and P2), 0.625 U Ex T*aq* DNA polymerase (TaKaRa, Dalian, China) and 1 µl of genomic DNA template adjusted to a final volume of 25 µl with ddH_2O. Amplification conditions consisted of an initial denaturation at 94°C for 4 min followed by 30 cycles of amplification for 30 s at 94°C, 30 s at 59°C, 1 min at 72°C and a final extension for 8 min at 72°C. PCR products were subjected to electrophoresis on a 2% agarose gel.

Sensitivity and specificity of *inf* B-LAMP and PCR

To determine the sensitivity of the LAMP assay, 10-fold serial dilutions were made from 1×10^7 cfu mL^{-1} *H. parasuis* stock solutions and compared with PCR results obtained using similar templates at identical concentrations. To determine the specificity of *inf* B-LAMP, 15 *H. parasuis* reference strains as well as 8 different bacterial strains were tested.

Evaluation of the *inf* B-LAMP assay using clinical samples

Fifty-six (56) samples obtained from tonsil, lung and pericardium tissues or from nasal swabs were collected from pigs of 10 farms in south China. Samples were excised and placed in sterile tubes containing 5 ml TSB (containing 10 mg/ml NAD and 5% bovine serum) and incubated for 8 h at 37°C with agitation. Genomic DNA was isolated as described previously. DNA templates (1 µl) from each sample were then, used for LAMP and PCR.

RESULTS

Validation of *inf* B-LAMP

LAMP products were detected at 5 different temperatures (61, 62, 63, 64 and 65°C) and the best results were obtained when the reaction temperature was maintained at 63°C (Figure 2a). To determine the optimum duration

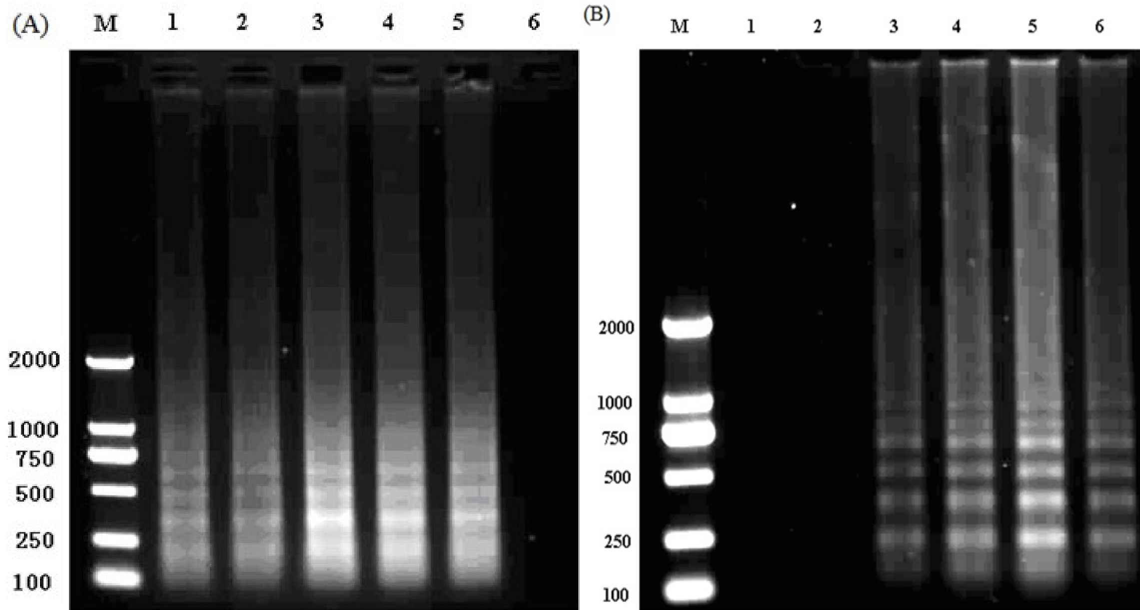

Figure 2. Determination of LAMP conditions at different temperatures (A) or times (B) using 100 ng DNA extracted from *H. parasuis*. (A) Lane M, DNA marker 2000; lanes 1 to 5, reactions carried out at 61, 62, 63, 64 or 65℃ for 50 min, respectively; lane 6, negative control. (B) Lane M, DNA marker 2000; lane 1, negative control; lanes 2 to 6, reactions carried out at 63℃ for 20, 30, 40, 50, or 60 min, respectively.

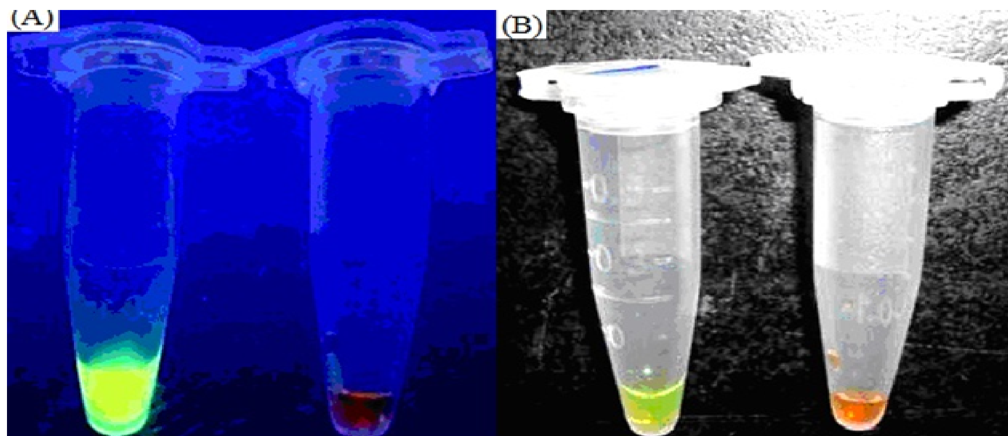

Figure 3. Visual detection of *H. parasuis inf* B gene LAMP reaction using SYBR green I under UV light (A) or white light (B). Plus (+) sign denotes positive reactions (with target DNA) and minus (-) signs denote negative reactions (without target DNA).

of the LAMP assay, LAMP reactions were set up for 5 different incubation times including 20, 30, 40, 50 and 60 min. As shown in Figure 2b, the LAMP amplicon accumulation was observed at five of these times; however, for complete amplification, the 50 min reaction time was optimal.

Detection of *inf* B-LAMP and PCR products

Electrophoresis of LAMP products demonstrated that, all positive LAMP reactions produced the characteristic multiple band ladder and the sizes of the multiple bands were of the anticipated sizes. Furthermore, LAMP results could be evaluated with the naked eye under natural or UV light (Figure 3). Under UV light or daylight, color changes could be visualized with the naked eye following the addition of SYBR green I to the reaction tubes (the positive reaction turned green whereas, the negative one remained orange). These results supported the electrophoresis data. Electrophoresis of PCR products generated an expected single band of 821 base pairs. The sequence

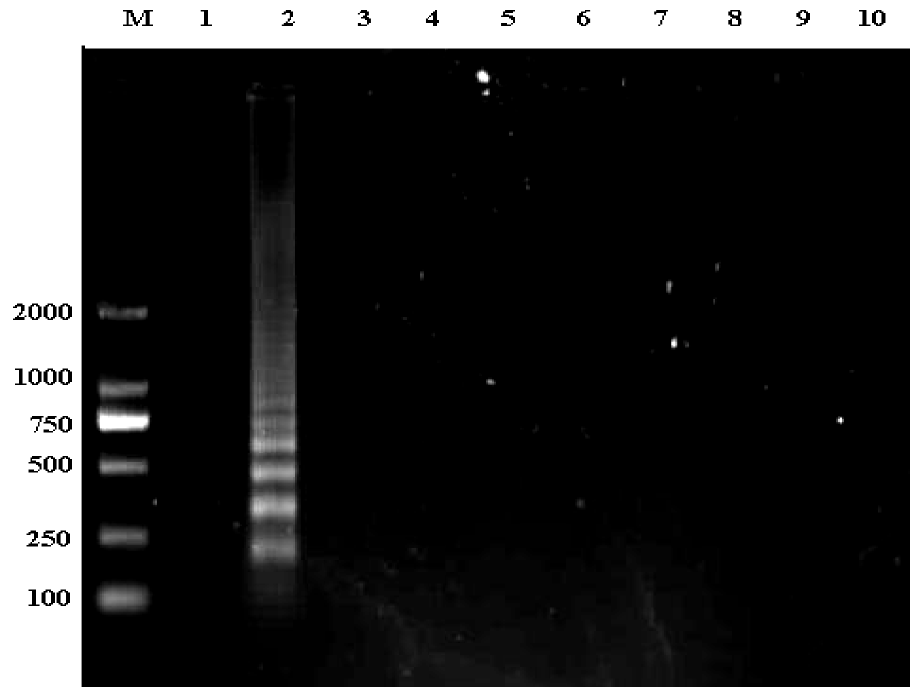

Figure 4. LAMP specificity was confirmed using template DNA extracted from related pathogens. Lane M, DL2000 molecular marker; lane 1, negative control; lane 2, *H. parasuis*; lane 3, *A. pleuropneumoniae* (Serovar 5); lane 4, *A. pleuropneumoniae* (Serovar 9); lane 5, *B. bronchiseptica* strain 1; lane 6, *B. bronchiseptica* strain 2; lane 7, *P. multocida*; lane 8, *S. suis*; lane 9, *S. typhimurium*; lane 10, *E. coli*.

of the PCR product showed 100% identity with the target sequence of 16S rRNA gene.

Specificity and sensitivity of *inf* B-LAMP and PCR

inf B-specific LAMP accurately detected all 15 reference *H. parasuis* strains but not other bacterial species or the negative (no template) reaction control (Figure 4). The limit of the LAMP assay was 10 cfu mL^{-1} compared with the 100 cfu mL^{-1} PCR assay detection limit (Figure 5) demonstrating that, *inf* B-LAMP was more sensitive than conventional PCR for detecting *H. parasuis*.

Evaluation of the *inf* B-specific LAMP assay using clinical samples

Clinical samples (n = 56) were obtained from pigs of 10 farms in south China. Thirteen (13) samples were positive for *H. parasuis* by bacterial isolation, 19 and 26 samples tested positive by PCR and LAMP, respectively. All samples defined as positive by bacterial isolation also tested positive by both PCR and LAMP. All PCR-positive samples were also identified by LAMP (Table 2), however, the LAMP assay demonstrated higher sensitivity than PCR suggesting that, the high specificity and sensitivity of the *inf* B-LAMP assay was effective in detecting *H. parasuis* from clinical samples.

DISCUSSION

H. parasuis is a non-motile, NAD-dependent, gram-negative bacteria belonging to the family Pasteurellaceae. The fastidious nature of *H. parasuis* hinders its isolation from clinical samples (Oliveira and Pijoan, 2004). In order to reduce the time needed to diagnose *H. parasuis* infections, new techniques will need to be developed. In this study, LAMP was evaluated as a means of diagnosing *H. parasuis* infections using primers designed to target the *H. parasuis inf* B gene as a means of establishing a sensitive, rapid and specific method for diagnosing *H. parasuis* infections.

The *inf* B gene was chosen for this analysis since the 16S rRNA gene sequence is not species-specific, that is, real-time PCR-based technologies designed to amplify *H. parasuis* 16S rRNA were unable to differentiate *H. parasusis* sequences from *P. mairii* (Turni et al., 2009). Hedegaard et al. (2000) reported that, the *inf* B gene was a useful genetic marker for phylogenetic studies and this observation was also true for *H. parasuis*. Furthermore, Turni et al. (2009) indicated that the *inf* B gene was a suitable target for real-time PCR designed to amplify

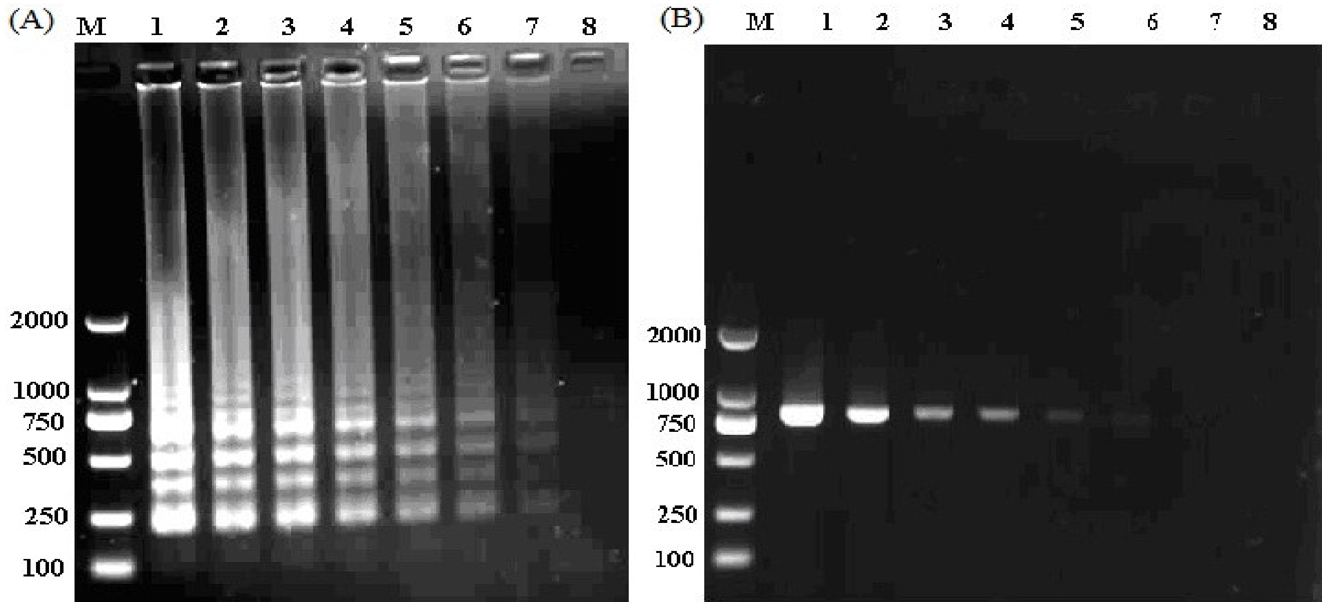

Figure 5. Analytical sensitivity of LAMP and PCR. (A) Sensitivity of LAMP under UV light. Lane M, DL 2000 bp marker; lane 1 to 7, 1 x 10^7, 1 x 10^6, 1 x 10^5, 1 x 10^4, 1 x 10^3, 1 x 10^2 and 1 x 10^1 cfu mL^{-1} of *H. parasuis* as a template for LAMP (A) or PCR (B) reactions, respectively. Lane 8, negative control.

Table 2. *H. parasuis* detection using LAMP, PCR or culture methodologies.

Result	Number of positive or negative sample (% positive or negative)*		
	LAMP	PCR	Bacterial isolation
Positive	26 (46.4)	19 (34)	13 (23.2)
Negative	30 (53.6)	37 (66)	43 (76.8)

*The number in parentheses indicates the percent positive or negative *H. parasuis* isolates identified using the various methodologies (n = 56).

H. parasuis-specific sequences since it distinguished *H. parasuis* from other closely related species. This observation was confirmed by comparing the *H. parasuis inf* B gene sequence (Gene ID: EF424388) derived from NCBI BLAST with other *inf* B sequences, revealing that *H. parasuis inf* B was significantly different from the *inf* B sequence of other species. This demonstrated that, the *H. parasuis inf* B gene was unique and could serve as a genetic marker for phylogenetic studies. In this study, the specificity of *inf* B-LAMP was examined by demonstrating that, *H. parasuis inf* B sequences did not cross react with *inf* B sequences from *A. pleuropneumoniae*, *B. bronchiseptica*, *P. multocida*, *S. suis*, *S. typhimurium* or *E. coli*. This analysis demonstrated that, the *H. parasuis inf* B sequence was non-cross reactive with any sequences associated with related pathogens.

H. parasuis is a slow-growing, fastidious organism with specific nutritional requirements (Ferri et al., 2000; Oliveira et al., 2004) making its isolation from clinical samples difficult. Therefore, culture methodologies are not sensitive enough for identifying *H. parasuis.* Recently, however, PCR-based methodologies have become attractive diagnostic alternatives. A caveat associated with PCR-based technologies, however, is that amplification of the *H. parasuis* 16S rRNA PCR can result in a significant number of false positive results due to the high level of similarity between the *H. parasuis* 16S rRNA sequence and the 16S rRNA sequence of related pathogens (Oliveira et al., 2001; Angena et al., 2007). The *inf* B-LAMP method described here was *H. parasuis* specific and did not produce false positive reactions when tested against the 8 non-target species. According to the sensitivity assay data, *inf* B-LAMP demonstrated a sensitivity of 10 cfu mL^{-1} for DNA extracted using the boiling water method. The LAMP reaction was carried out with two sets of specific primers that recognized six distinct sequences on the target *inf* B gene. Therefore, it was expected that these primers would amplify the target sequence with high specificity (Notomi et al., 2000; Nagamine et al., 2002a, b). Moreover, the LAMP assay could be performed on-site, using simple and inexpensive experimental set-ups such as a water bath or heat block that provide a constant temperature. In addition, LAMP reaction products can be evaluated with the naked eye by

examining the color changes following the addition of SYBR green I to the reaction mixture. The positive rate of *H. parasuis* detection using *inf* B-LAMP was higher than the rates obtained with either PCR or culture methods. Overall, these data indicated that, the *inf* B-LAMP assay was more sensitive than conventional PCR assays.

In this study, the bacteriologic culture method was not more accurate than conventional PCR; however, *inf* B-LAMP was significantly more sensitive than PCR-based technologies designed to amplify 16S rRNA-PCR from clinical samples. Data presented in this report suggested that, *inf* B-LAMP is likely to enhance the diagnosis efficiency of *H. parasuis* infections, particularly since this technique can be carried out in the field or laboratories unable to carry out more labor intensive and expensive methodologies. Moreover, using *inf* B-LAMP, we were able to detect *H. parasuis* in different tissues (lung, pericardium, nasal swabs) from clinically infected pigs, further validating the specificity of this method.

The *inf* B-LAMP protocol described in this study is a sensitive, specific and rapid diagnostic tool designed for diagnosing *H. parasuis* infections. This protocol described will be useful in the detection of *H. parasuis* in low concentrations in tissues biopsied from infected pigs. *inf* B-LAMP can also be used to confirm the early stages of *H. parasuis* infection when bacterial burdens are relatively low. We recommend that, this technique would be applied routinely in the diagnosis of infections and in *H. parasuis* surveillance on swine farms. This approach will facilitate the identification of bacteria-carrying swine at the early stages of infection allowing containment and treatment protocols to be implemented.

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REFERENCES

Alhassan A, Thekisoe OMM, Yokoyama N, Inoue N, Motloang M Y, Mbati P A, Yin H, Katayama Y, Anzai T, Sugimoto C and Igarashi I (2007). Development of loop-mediated isothermal amplification (LAMP) method for diagnosis of equine piroplasmosis. Vet. Parasitol. 143: 155-160.

Angena O, Oliveira S, Ahrens P, Svensmark B, Leser TD (2007). Development of an improved species specific PCR test for detection of Haemophilus parasuis. Vet. Microbiol. 119: 266–276.

Baumann G, Bilkei G (2002). Effect of vaccinating sows and their piglets on the development of Glässer's disease induced by a virulent strain of *Haemophilus parasuis* serovar 5. Vet. Rec. 151: 18–21.

Blackall PJ, Trott DJ, Rapp-Gabrielson V, Hampson DJ (1997). Analysis of *Haemophilus parasuis* by multilocus enzyme electrophoresis. Vet. Microbiol. 56: 125-134.

Chen HT, Chu YF, Liu YS, Zhang J, Lu ZX (2010). Loop-mediated isothermal amplification for the rapid detection of *Haemophilus parasuis*, FEMS. Immunol. Med. Microbiol. 60: 283–285

Del Rio ML, Navas J, Martin AJ, Gutierrez CB, Rodríguez-Barbosa JI, Rodríguez Ferri EF (2006). Molecular characterization of Haemophilus parasuis ferric hydroxamate uptake (fhu) genes and constitutive expression of the FhuA receptor. Vet. Res. 37: 49–59.

Ferri EFR, Gutierrez CB, De La Puente VA, Garcia del Blanco N, Navas J, Paniagua ML, del Rio ML, Monter JL (2000). Bacterial meningitis in pigs: Glässer's disease. Porci, 59: 43–60.

Goto M, Hayashidani H, Takatori K, Hara-Kudo Y (2007). Rapid detection of enterotoxigenic *Staphylococcus aureus* harbouring genes for four classical enterotoxins, SEA, SEB, SEC and SED, by loop-mediated isothermal amplification assay. Lett. Appl. Microbiol. 45:100-107.

Hara-Kudo Y, Yoshino M, Kojima T, Ikedo M (2005). Loop-mediated isothermal amplification for the rapid detection of *Salmonella*. FEMS Microbiol. Lett. 253:155-161.

Hedegaard J, Hauge M, Fage-Larsen J, Mortensen K, Kilian M, Sperling-Petersen U, Pousen K (2000). Investigation of the translation-initiation factor IF2 gene, *inf* B, as a tool to study the population structure of *Streptococcus agalactiae*. Microbiology 146: 1661–1670

Ikadai H, Tanaka H, Shibahara N, Matsuu A, Uechi M, Itoh N, Oshiro S, Kudo N, Igarashi I, Oyamada T (2004). Molecular evidence of infections with Babesia gibsoni parasites in Japan and evaluation of the diagnostic potential of a loop-mediated isothermal amplification method. J. Clin. Microbiol. 42: 2465–2469.

Iwamoto T, Sonobe T, Hayashi K (2003). Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J. Clin. Microbiol. 41: 2616-2622.

Kuboki N, Inoue N, Sakurai T, Di Cello F, Grab DJ, Suzuki H, Sugimoto C, Igarashi I (2003). Loop-mediated isothermal amplification for detection of African trypanosomes. J. Clin. Microbiol. 4: 5517-5524.

Minami M, Ohta M, Ohkura T, Ando T, Torii K, Hasegawa T, Goto H (2006). Use of a combination of brushing technique and the loop-mediated isothermal amplification method as a novel, rapid, and safe system for detection of *Helicobacter pylori*. J. Clin. Microbiol. 44: 4032-4037.

Mori Y, Kitoa M, Tamita N, Notomi T (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA. J. Biochem. Biophys. Methods 59: 45–157.

Mori Y, Nagamine K, Tomita N and Notomi T (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289: 150–154.

Nagamine K, Hase T, Notomi T (2002a). Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell Probes, 16: 223–229.

Nagamine K, Kuzuhara Y, Notomi T (2002b). Isolation of single-stranded DNA from loop-mediated isothermal amplification products. Biochem. Biophys. Res. Commun. 290: 1195–1198.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28: 63.

Oliveira S, Galina L, Pijoan C (2001). Development of a PCR test to diagnose *Haemophilus parasuis* infections. J. Vet. Diagn. Invest. 13: 495–501

Oliveira S, Pijoan C (2004). *Haemophilus parasuis*: new trends on diagnosis, epidermiology and control. Vet. Microbiol. 99: 1–12.

Parida MM, Santhosh SR, Dash PK, Tripathi NK, Saxena P, Ambuj S, Sahni AK, Lakshmana Rao PV, Kouichi Morita (2006). Development and Evaluation of Reverse Transcription–Loop-Mediated Isothermal Amplification Assay for Rapid and Real-Time Detection of Japanese Encephalitis Virus. J.Clin. Microbiol. 44: 4172–4178.

Rapp-Gabrielson V, Oliveira SR, Pijoan C (2006). *Haemophilus parasuis*. In: Straw, B., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine. Blackwell Publishing, Williston, FL, USA, pp. 681–690.

Sambrook J, Russell D (2001). Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Song T, Toma C, Nakasone N, Iwanaga M (2005). Sensitive and rapid detection of Shigella and enteroinvasive *Escherichia coli* by a loop-mediated isothermal amplification method. FEMS Microbiol. Lett. 243: 259-263.

Thekisoe OMM, Inoue N, Kuboki N, Tuntasuvan D, Bunnoy W, Borisutsuwan S, Igarashi I, Sugimoto C (2005). Evaluation of loop-mediated isothermal amplification (LAMP), PCR and parasitological tests for detection of *Trypanosoma evansi* in experimentally infected pigs. Vet. Parasitol. 130: 327–330.

Tomita N, Mori Y, Kanda H, Notomi T (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protoc. 3: 877–882.

Turni C, Pyke M, Blackall PJ (2009). Validation of a real-time PCR for *Haemophilus parasuis.* J. Appl. Microbiol. 108: 1323-1331.

Wang Y, Chen P, Guo HB, Chen Y, Liu H, He QG (2009). Loop-mediated is othermal amplification targeting the apxIVA gene for detection of *Actinobacillus* pleuropneumoniae. FEMS. Microbiol. Lett. 300:83-89.

Wang Y, Fang Y, Liu YY, Chen P, Li WT, Liu SQ, Zou HY, He QG (2010). Development and evaluation of loop-mediated isothermal amplification for rapid detection of *Haemophilus parasuis*. FEMS. Microbiol. Lett. 333: 54-60.