



Detection of *Haemophilus parasuis* isolates from South China by loop-mediated isothermal amplification and isolate characterisation

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Haemophilus parasuis is the etiological agent of Glässer's disease, which is characterised by fibrinous polyserositis, meningitis and polyarthritis, causing severe economic losses to the swine industry. In this study, a loop-mediated isothermal amplification (LAMP) test was developed to improve the specificity, facility and speed of diagnosis of *H. parasuis* isolates. The LAMP assay rapidly amplified the target gene within 50 min incubation at 63 °C in a laboratory water bath. The LAMP amplicon could be visualised directly in the reaction tubes following the addition of SYBR Green I dye. The detection limit of this LAMP method was 10 CFU/mL, which was 10 times more sensitive than the earlier 16S rRNA polymerase chain reaction (PCR) test conducted by Oliveira, Galina and Pijoan (2001), and no cross-reactivity was observed from other non-*H. parasuis* strains. This LAMP test was evaluated further on 187 clinical specimens from pigs suspected of being infected with *H. parasuis*. Forty-three were found positive by bacterial isolation of *H. parasuis*, as well as by the 16S rRNA PCR and LAMP tests. The 43 *H. parasuis* isolates were classified into 9 serovars and had 37 genetic patterns when analysed by pulsed-field gel electrophoresis (PFGE). This displayed that various *H. parasuis* serovars and genotypes were widely distributed in South China. Therefore, the speed, specificity and sensitivity of the LAMP test, the lack of a need for expensive equipment, and the visual readout showed great potential for a correct clinical diagnosis of *H. parasuis* in favour of controlling Glässer's disease.

Introduction

Haemophilus parasuis is the causative agent of Glässer's disease in swine, a disease characterised by fibrinous polyserositis, meningitis and polyarthritis (Li *et al.* 2009; Olvera, Segalés & Aragón 2007). In recent years, diseases in swine caused by *H. parasuis* have attracted worldwide attention (Baumann & Bilkei 2002). It is therefore necessary to carry out timely and effective monitoring of *H. parasuis* to identify strains circulating between farm animals as a means of defining the epidemiology and types of *H. parasuis* strains present in respective areas in order to control and prevent outbreaks.

Haemophilus parasuis is a slow-growing, fastidious organism with specific nutritional requirements, making its isolation difficult (Oliveira, Galina & Pijoan 2001). Moreover, detection of *H. parasuis* using conventional methods, including culture, biochemical or immunological assays is not effective and requires several days to complete (Angen *et al.* 2007). The polymerase chain reaction (PCR) provided a major advance in the diagnosis of *H. parasuis* infections (Oliveira *et al.* 2001), including nested PCR (Jung *et al.* 2004) and real-time PCR assays (Turni, Pyke & Blackall 2009). However, PCR assays are limited by the equipment required to carry out the reactions and the need for skilled personnel. Fortunately, a promising candidate for a new detection method is loop-mediated isothermal amplification (LAMP), which amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. It is faster, more specific and easier to perform than conventional PCR (Nagamine, Hase & Notomi 2002; Notomi *et al.* 2000). The development of LAMP procedures has been reported for many different clinical applications: the diagnosis of viral and bacterial infections (Iwamoto, Sonobe & Hayashi 2003; Minami *et al.* 2006; Song *et al.* 2005; Wang *et al.* 2009), for diagnosis of protozoal diseases (Kuboki *et al.* 2003; Thekisoe *et al.* 2005) and for canine and equine piroplasmiasis (Alhassan *et al.* 2007; Ikadai *et al.* 2004). The *infB* gene of *H. parasuis* has been proven to be useful as a genetic marker for phylogenetic studies (Turni *et al.* 2009), making it a better alternate gene to the 16S rRNA gene in establishing the LAMP method.

To date, 15 *H. parasuis* serovars have been recognised using immunodiffusion tests with heat-stable antigens. However, about 25% of isolates are non-typable using this methodology (Vanier *et al.* 2006). In view of the fact that serotyping does not provide enough discrimination of *H. parasuis*



isolates, an optimised and standardised pulsed-field gel electrophoresis (PFGE) method established by our research team was used to characterise the *H. parasuis* isolates. This method provides a higher discriminatory power, allowing for the typing of previously undefined isolates (Zhang *et al.* 2011).

In the present study, 15 serovar reference strains were used to develop the LAMP assay of *H. parasuis*. In addition, the *H. parasuis* strains isolated from clinical specimens collected from South China, were serotyped and characterised by PFGE patterns.

Materials and methods

Bacterial strains

The test was optimised using the 15 reference strains for the different serovars of *H. parasuis* and the field isolates of *Actinobacillus pleuropneumoniae* (serovar 5), *A. pleuropneumoniae* (serovar 9), two strains of *Bordetella bronchiseptica*, *Pasteurella multocida*, *Streptococcus suis*, *Salmonella typhimurium* and *Escherichia coli*.

Clinical samples

From 2008 to 2010, 187 specimens, including lung, nasal swab, synovia and heart from pigs suspected of being infected with *H. parasuis* were collected in South China.

Bacterial isolation

All 187 specimens were cultured on tryptic soy agar (OXOID Inc., Basingstoke, UK) containing 10 mg/mL nicotinamide adenine dinucleotide (NAD, Sigma-Aldrich, St. Louis, USA) and 5% bovine serum (Gibco®, Invitrogen, Carlsbad, USA) for isolation of *H. parasuis* strains. Plates were incubated at 37 °C for 24 h – 72 h and presumptive small and translucent bacterial colonies were selected for further characterisation by PCR, LAMP and biochemical testing.

DNA extraction

Template DNA used for LAMP and 16S rRNA PCR were prepared as follows: bacterial cells of each strain from colonies on Trypticase Soy agar (TSA) were re-suspended in double-distilled water (ddH₂O) to achieve a concentration of approximately 10⁶ CFU/mL. In order to examine the detection limit for LAMP and 16S rRNA PCR, a 10-fold dilution series of *H. parasuis* of serovar 5 with ddH₂O was made. The cells were heat-treated in a boiling water bath for 10 min and were centrifuged at 12 000 rpm/min for 10 min. The resulting supernatant was used as the template for the LAMP and 16S rRNA PCR.

Development of the loop-mediated isothermal amplification and polymerase chain reaction methods

Four primers corresponding to FIP, BIP, F3 and B3 (Table 1) were used for the LAMP assay and designed based on the

conserved regions of the *infB* gene sequence (GeneBank accession numbers: EF424388), according to criteria established previously (Notomi *et al.* 2000; Tomita *et al.* 2008). The reaction mixture consisted of 0.8 μM of each FIP and BIP primers, 0.2 μM of each F3 and B3 outer primers, 400 μM of each dNTP (Takara Bio Inc., Shiga, Japan), 1 M betaine (Sigma-Aldrich, St. Louis, USA), 1X thermopol buffer (New England Biolabs Inc., Ipswich, USA), 2 mM MgSO₄, 8 U of Bst DNA polymerase large fragment (New England Biolabs Inc., Ipswich, USA) and 2 μL of genomic DNA. The reaction mixture was incubated at 61 °C, 62 °C, 63 °C, 64 °C or 65 °C using a heat block for 20 min, 30 min, 40 min, 50 min or 60 min, respectively. Reactions were terminated by increasing the temperature to 80 °C for 4 min. To determine the sensitivity of the LAMP assay, different dilutions of *H. parasuis* from 1 × 10⁶ CFU/mL to 1 × 10¹ CFU/mL (i.e. a series of 10-fold dilutions) was prepared and compared with PCR by using the same templates at identical concentrations. The details of the primers (Table 1) and conditions for the PCR assay for the detection of *H. parasuis* were carried out as described previously (Oliveira *et al.* 2001). Cross-reactivity between the primers used in LAMP PCR with DNA isolated from *A. pleuropneumoniae*, *B. bronchiseptica*, *P. multocida*, *S. suis*, *S. typhimurium* and *E. coli* was also carried out. Genomic DNA extracted from the 15 *H. parasuis* reference strains were used as positive controls and distilled water was used as a negative control.

Detection of *Haemophilus parasuis* using loop-mediated isothermal amplification and polymerase chain reaction

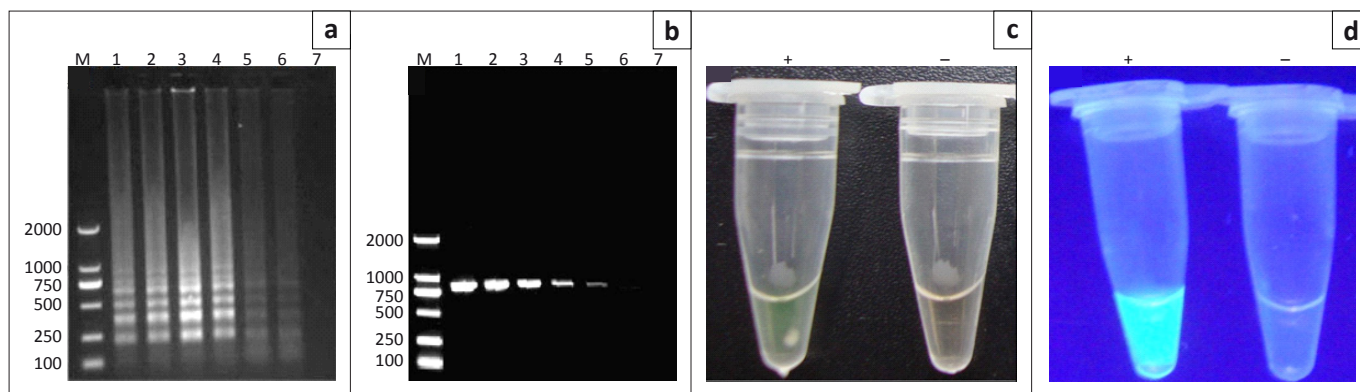
Detection of the LAMP reaction products was performed using 2% agarose gel electrophoresis prepared in 0.5X tris-borate-EDTA buffer stained with 0.5 g/mL ethidium bromide. Banding patterns were examined visually under natural or UV light following the addition of 1 μL SYBR Green I dye to the reaction tube. PCR products were subjected to electrophoresis on a 2% agarose gel and visualised as above.

Serotyping of *Haemophilus parasuis* isolates

Serotyping of *H. parasuis* isolates using gel diffusion (GD) and indirect hemagglutination assays (IHA) were performed as described previously (Cai *et al.* 2005; Tadjine *et al.* 2004) and all isolates were tested using GD first. If a definitive serotype could not be determined, respective isolates were then examined by IHA testing. Isolates that could not be identified by either test were defined as non-typable (serovar NT).

TABLE 1: Loop-mediated isothermal amplification (LAMP) primers designed for detection of the *Haemophilus parasuis infB* gene.

Primer	Sequence (5'-3')
P1	5'-GTG ATG AGG AAG GGT GGTGT-3'
P2	5'-GGC TTC GTC ACC CTC TGT-3'
F3	5'-CGATTGTGGTTGCGGTAAC-3'
B3	5'-TAAGATTGATAACACACCGC-3'
FIP (F1c + F2)	5'-GAACATCACCACCGAATTTCTCAGAA TTTTAAACCAGAAGCAAACCCAGAGC-3'
BIP (B1c + B2)	5'-ATGGGGATTGACGACTTACTTGAAGCTTT ATACCCCTTTCACTGCACCTAATTC-3'



Lane M, DNA marker; Lanes 1–6, 1×10^6 CFU/mL, 1×10^5 CFU/mL, 1×10^4 CFU/mL, 1×10^3 CFU/mL, 1×10^2 CFU/mL and 1×10^1 CFU/mL of *Haemophilus parasuis* used as a reaction template, respectively; Lane 7, negative control; +, positive reaction; –, negative reaction.

FIGURE 1: Detection of (a) *infB* loop-mediated isothermal amplification (LAMP) and (b) polymerase chain reaction products, as well as visual detection of *infB*-LAMP products under (c) daylight and (d) ultraviolet light.

TABLE 2: Comparative analysis of *Haemophilus parasuis* detection by either loop-mediated isothermal amplification, polymerase chain reaction or bacterial isolation.

Result	LAMP		PCR		Bacterial isolation	
	N	%	N	%	N	%
Positive	89	47.6	64	34.2	43	23.0
Negative	98	52.4	123	65.8	144	76.8

LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction; N, number of samples.

Genotyping of *Haemophilus parasuis* by the pulsed-field gel electrophoresis method

Haemophilus parasuis field isolates were genotyped using the PFGE method established by our research team to determine the number of different genotypes (Zhang *et al.* 2011). A dendrogram illustrating the genetic relatedness between identified *H. parasuis* isolates was generated using Bionumerics 4.0 software (Applied Maths, Inc., Austin, USA), with a 1.5% tolerance for fragment shifts.

Ethical considerations

All animal experiments conducted for the purposes of this study conform to the Chinese national laws regarding the use of animals in biomedical research.

Results

Rapid, sensitive and specific detection of *Haemophilus parasuis* using loop-mediated isothermal amplification

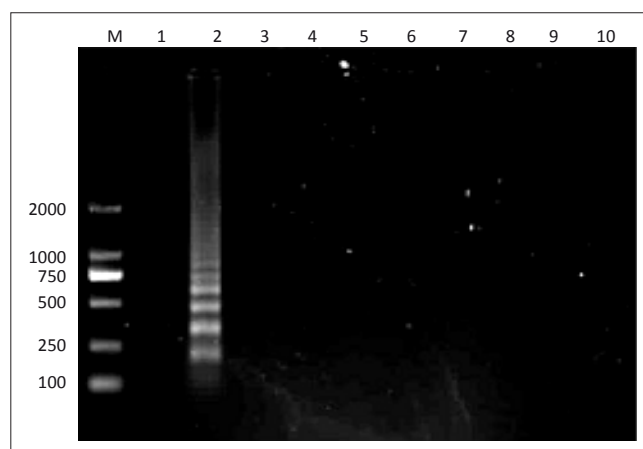
The LAMP method described in this study was performed in a simple water bath at 63 °C for 50 min. All positive LAMP reactions produced a characteristic ladder of multiple bands and the results could also be seen after staining with SYBR Green I (Figure 1). The detection limit of LAMP was 10 CFU/mL, which was 10 times more sensitive than 16S rRNA PCR (Figure 1). LAMP accurately detected all 15 *H. parasuis* reference strains and no amplification products were detected when either DNA from the other swine bacterial pathogens, or the negative (no template) reaction control was used (Figure 2).

Detection and isolation of *Haemophilus parasuis*

A total of 187 clinical specimens were obtained from pigs in South China that were suspected of being infected with *H. parasuis*. Eighty-nine (47.6%) and 64 (34.2%) samples tested positive for *H. parasuis* by LAMP and PCR, respectively. Forty-three samples (23.0%) were positive for *H. parasuis* by bacterial isolation and all the 43 samples tested positive for *H. parasuis* by PCR and LAMP (Table 2).

Serotyping and genotyping of *Haemophilus parasuis* isolates

Nine of the fifteen recognised serovars were identified amongst the isolates collected from South China. Serovar 5



Lane M, DL2000 molecular marker; Lane 1, negative control; Lane 2, *Haemophilus parasuis*; Lane 3, *Actinobacillus pleuropneumoniae* of serovar 5; Lane 4, *A. pleuropneumoniae* of serovar 9; Lane 5, *Bordetella bronchiseptica* strain-1; Lane 6, *B. bronchiseptica* strain-2; Lane 7, *Pasteurella multocida*; Lane 8, *Streptococcus suis*; Lane 9, *Salmonella typhimurium*; Lane 10, *Escherichia coli*.

FIGURE 2: Specificity tests of loop-mediated isothermal amplification in agarose gel under ultraviolet light for *Haemophilus parasuis* detection.

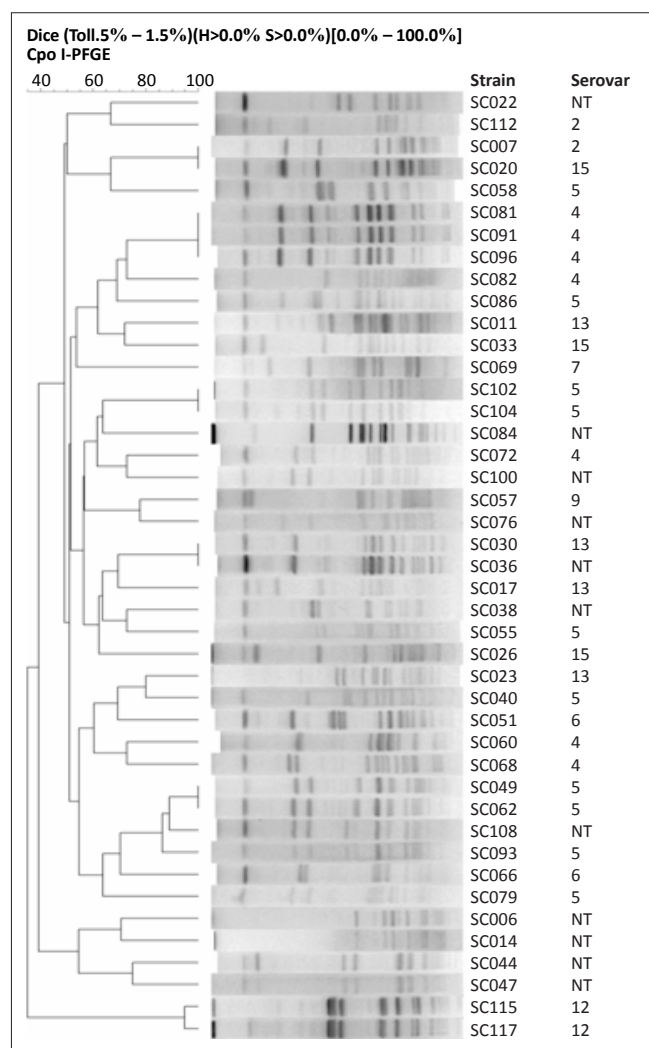


FIGURE 3: Dendrogram of pulsed-field gel electrophoresis subtype patterns of all 43 *Haemophilus parasuis* field strains identified.

was the most prevalent (23.3%), followed by serovar 4 (16.3%), whilst 25.6% of strains were non-typable by either GD or IHA. Serovars 2, 6, 7, 9, 12, 13 and 15 were represented by a small number of strains only. All 43 *H. parasuis* isolates were typed by PFGE and great genetic diversity was observed. A total of 37 different PFGE patterns were observed from the 43 *H. parasuis* isolates (Figure 3 and Table 3). In addition, strains that could not be typed by GD or IHA tests were genotyped by PFGE and displayed unique PFGE patterns. Genotyping analysis demonstrated that the *H. parasuis* population was heterogeneous and no dominant clones predominated.

Discussion

Haemophilus parasuis is a commensal of the upper respiratory tract in domestic pigs and is also the etiological agent of Glässer's disease (Oliveira *et al.* 2001). However, the bacterium can cause acute disease when introduced into immunologically naive herds (Little 1970). Laboratory diagnosis of *H. parasuis* using conventional methods such as culture, biochemical and immunological assays is time-consuming and laborious. *Haemophilus parasuis* is a fastidious bacterium and its isolation in pure culture from

TABLE 3: Characterisation of 43 *Haemophilus parasuis* field isolates from South China.

Isolate code	Isolation site	Serovar	PFGE type	LAMP	PCR
SC006	Lung	NT	A1	+	+
SC007	Lung	2	A2	+	+
SC020	Lung	15	A2	+	+
SC011	Nasal	13	A3	+	+
SC014	Lung	NT	A4	+	+
SC017	Synovia	13	A5	+	+
SC022	Lung	NT	A6	+	+
SC023	Synovia	13	A7	+	+
SC026	Synovia	15	A8	+	+
SC030	Lung	13	A9	+	+
SC036	Nasal	NT	A9	+	+
SC033	Synovia	15	A10	+	+
SC038	Lung	NT	A11	+	+
SC040	Heart	5	A12	+	+
SC044	Lung	NT	A13	+	+
SC047	Lung	NT	A14	+	+
SC049	Lung	5	A15	+	+
SC062	Heart	5	A15	+	+
SC051	Lung	6	A16	+	+
SC055	Synovia	5	A17	+	+
SC057	Lung	9	A18	+	+
SC058	Lung	5	A19	+	+
SC060	Synovia	4	A20	+	+
SC066	Synovia	6	A21	+	+
SC068	Lung	4	A22	+	+
SC069	Lung	7	A23	+	+
SC072	Lung	4	A24	+	+
SC076	Heart	NT	A25	+	+
SC079	Nasal	5	A26	+	+
SC081	Lung	4	A27	+	+
SC091	Lung	4	A27	+	+
SC096	Synovia	4	A27	+	+
SC082	Lung	4	A28	+	+
SC084	Lung	NT	A29	+	+
SC086	Synovia	5	A30	+	+
SC093	Lung	5	A31	+	+
SC100	Lung	NT	A32	+	+
SC102	Synovia	5	A33	+	+
SC104	Nasal	5	A33	+	+
SC108	Lung	NT	A34	+	+
SC112	Lung	2	A35	+	+
SC115	Lung	12	A36	+	+
SC117	Lung	12	A37	+	+

PFGE, pulsed-field gel electrophoresis; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction.
+, Denotes positive reaction.

diseased animals is usually difficult; therefore, rapid and accurate identification of *H. parasuis* isolates is essential for the diagnosis of Glässer's disease and a key element for controlling the disease (Aarestrup, Seyfarth & Angen 2004; Oliveira & Pijoan 2004). A fast and accurate LAMP test was developed to improve the diagnosis of *H. parasuis* infections. This LAMP test can be used to detect *H. parasuis* in both pure culture and clinical samples.

The LAMP method can amplify DNA with high specificity, efficiency and speed under isothermal conditions (Nagamine *et al.* 2002; Tomita *et al.* 2008). The LAMP assay also can be performed on-site, using a simple and inexpensive experimental set-up, such as a water bath or heat block that



provides a constant temperature. In addition, the LAMP reaction products can be evaluated by a visual inspection of the colour change of the mixture when SYBR Green I dye is added. After isolation of *H. parasuis* from the clinical specimens, the LAMP can be performed as a substitute for 16S rRNA PCR tests for bacterial identification, eliminating the need for time-consuming electrophoresis and costly specialised equipment, as well as reducing the time for the diagnosis of *H. parasuis* infection considerably.

It has been reported that the 16S rRNA gene is not species-specific to enable *H. parasuis* to be distinguished from other closely related species when the short amplification sequence of real-time PCR was used (Turni *et al.* 2009). Recently, researchers have reported that the *infB* gene is useful as a genetic marker for phylogenetic studies. This observation is also true for *H. parasuis*, in that the *infB* gene be used to distinguish *H. parasuis* from all other closely related species, such as *Actinobacillus suis* and *A. pleuropneumoniae* (Hedegaard *et al.* 2000; Turni *et al.* 2009). In this study, we evaluated the applicability and efficacy of *infB*-LAMP for the identification of *H. parasuis* from 187 different specimens. Analysis of these samples by *infB*-LAMP resulted in the positive identification of 89 *H. parasuis* samples, compared to only 64 positive samples identified by PCR. The results of this study also indicate that *H. parasuis* is widely distributed in South China. Moreover, the *infB*-LAMP technique has the potential to be applied in clinical settings in which 16S rRNA-PCR is too sophisticated to be implemented. In addition, it is a suitable tool for investigating the epidemiology of *H. parasuis* in a clinic.

Detection and isolation of *H. parasuis* is important in understanding the epidemiology of *H. parasuis* infections and can provide critical information used in the design of vaccines specific to strains endemic to certain geographic locations. In this study, the *H. parasuis* serovars present in South China were determined by GD and IHA. These results showed that 32 (74.4%) *H. parasuis* isolates could be assigned accurately to a specific serovar, whilst nine (20.9%) *H. parasuis* serovars were identified and 11 (25.6%) isolates are typically non-typable. So, the PFGE method was used to analyse the genetic diversity of *H. parasuis* isolates collected from South China and a computer analysis program was used to determine similarities between isolates, thereby providing a more objective assessment of clonality (in conjunction with epidemiologic data) that can be used to establish *H. parasuis* as the etiologic agent of animal disease in this region.

Conclusion

In this study, our results indicate that *infB*-LAMP can be used in the diagnosis of *H. parasuis* infections, which is essential in defining the exact prevalence of Glässer's disease in South China. Combining this assay with traditional serotyping methods and a highly discriminatory PFGE genotyping technique will provide comprehensive information regarding the epidemiology of *H. parasuis* in South China. Knowledge of the *H. parasuis* strains present in this region can be used

in the rational design of vaccines against these isolates, in addition to providing information useful to epidemiologic research.

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Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this article.

Authors' contributions

C.X. (South China Agricultural University) was the project leader, H.S. (South China Agricultural University) and J.Z. (South China Agricultural University) were responsible for experimental and project design and M.L. (South China Agricultural University), S.F. (South China Agricultural University) and B.Z. (South China Agricultural University) were in charge of clinical samples collection. L.G. (South China Agricultural University) and J.L. (South China Agricultural University) performed most of the bacterial isolation and PCR detection, whilst H.F. (South China Agricultural University), J.C. (South China Agricultural University) and T.R. (South China Agricultural University) prepared the DNA of the samples, in order to optimise the reaction condition and establish the LAMP.

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