

LAMP-based method for a rapid identification of *Legionella* spp. and *Legionella pneumophila*

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Abstract *Legionella pneumophila* is accounted for more than 80% of *Legionella* infection. However it is difficult to discriminate between the *L. pneumophila* and non-*L. pneumophila* species rapidly. In order to detect the *Legionella* spp. and distinguish *L. pneumophila* from *Legionella* spp., a real-time loop-mediated isothermal amplification (LAMP) platform that targets a specific sequence of the 16S rRNA gene was developed. LS-LAMP amplifies the fragment of the 16S rRNA gene to detect all species of *Legionella* genus. A specific sequence appears at the 16S rRNA gene of *L. pneumophila*, while non-*L. pneumophila* strains have a variable sequence in this site, which can be recognized by the primer of LP-LAMP. In the present study, 61 reference strains were used for the method verification. We found that the specificity was 100% for both LS-LAMP and LP-LAMP, and the sensitivity of LAMP assay for *L. pneumophila* detection was between 52 and 5.2 copies per reaction. In the environmental water samples detection, a total of 107 water samples were identified by the method. The culture and serological test were used as reference methods. The specificity of LS-LAMP and LP-LAMP for the samples detection were 91.59% (98/107) and 93.33% (56/60), respectively. The sensitivity of LS-LAMP and LP-LAMP were 100% (51/51) and 100% (18/18). The results suggest that real-time LAMP, as a new assay, provides a

specific and sensitive method for rapid detection and differentiation of *Legionella* spp. and *L. pneumophila* and should be utilized to test environmental water samples for increased rates of detection.

Keywords Loop-mediated isothermal amplification · *Legionella pneumophila* · Different diagnosis

Introduction

Legionella pneumophila, the agent of Legionnaires' disease and Pontiac fever, is ubiquitous in natural freshwater environments, and it is also present in man-made water systems (Dusserre et al. 2008; Steinert et al. 2007; Albert-Weissenberger et al. 2007). Due to the close relationship between the growth of *L. pneumophila* and human activities (Rowbotham 1980), infection is usually caused by inhalation of aerosols, produced by showers, air conditioning systems, and other aerosol-generating devices (Fields et al. 2002). Governments of various countries have recognized the harmfulness of this type of microorganism. For example, due to the positive result of *L. pneumophila* in the ventilation of public places, it has not been permitted since 2003 in China. Thus, the real-time monitoring of *L. pneumophila* from water systems, particularly in the public places such as hospitals and hotels, is essential for the prevention of legionellosis outbreaks (Aurell et al. 2004; Hilbi et al. 2010).

To date, at least 23 *Legionella* species can be recognized as human illness agents. However, more than 80% of *Legionella* infection is attributed to *L. pneumophila* (Yanez et al. 2005). Non-*L. pneumophila* species also have been reported to be infectious, but this happens at very low probability (Herwaldt et al. 1984; Gobin et al.

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2009). In fact, it is hard to distinguish *L. pneumophila* from other bacterial infections from clinical practice, because the symptoms are not typical (Diederer 2008). Therefore, it is quite evident that the identification of *L. pneumophila* from *Legionella* spp. and other bacteria is increasingly important.

Culture with buffered activated charcoal and yeast extract media (BCYE) provides definitive diagnosis and remains a reference standard for *Legionella* identification; however, this method takes at least 3–10 days under special conditions. Additional problems with culture detection include low sensitivity and the difficulty to distinguish *L. pneumophila* from non-*L. pneumophila*. Direct fluorescent antibody has a low sensitivity, and the urine antigen detection is limited to the identification of *L. pneumophila* serogroup 1; in addition, these two methods cannot detect non-*L. pneumophila* spp. (Zhan et al. 2010) All of these factors make *L. pneumophila* identification challenging. In recent years, molecular methods have been explored for the detection of *L. pneumophila*. The 16S rRNA gene sequencing was reported to be used for the identification of *L. pneumophila* and non-*L. pneumophila* (Cloud et al. 2000; Stolhaug and Bergh 2006), however the method is time consuming and difficult to used for a rapid diagnosis. Real-time PCR targeting the *mip* gene has been used for *L. pneumophila* identification, but the *mip* gene is variable, which makes probe designing difficult (Ratcliff et al. 1998) and requires a precise instrument. Most recently, a two-step method was developed for the identification of *L. pneumophila* and non-*L. pneumophila*, with which, the laborious postamplification procedures are needed (Zhan et al. 2010).

To address these deficiencies, we established a real-time loop-mediated isothermal amplification (LAMP) method for the detection of all *Legionella* spp. and the discrimination of *L. pneumophila* from other *Legionella* spp. without the need for sophisticated instruments and postamplification procedures. The LAMP technique is highly specific for the target sequence, which has been successfully used in differential diagnosis of microorganisms such as *Brettanomyces* and *Dekkera* sp. yeasts (Hayashi et al. 2007; Inacio et al. 2008) as well as fungi identification (Otori et al. 2006). Besides, the sensitivity of LAMP method is below 10 copies in one reaction according to a previous study (Notomi et al. 2000). Since the efficiency of the LAMP amplification can be conducted under isothermal condition (60–65°C), sophisticated instruments are not required during amplification (Notomi et al. 2000). The detection time could be shortened within 2 h according to our previous study (Wang et al. 2008). In the present study, the platform of two LAMP reactions was designed based on a specific sequence of 16S rRNA gene to identify *Legionella* spp. and *L. pneumophila*. It was evaluated by 23 *L. pneumophila*, 16 non-*L. pneumophila*, and 12 other strains.

Additionally, 107 environmental water samples were collected and analyzed by LAMP assay, which were compared with traditional culture method and serological test as well as a fatty acid analysis.

Materials and methods

Strains and growth conditions

A total of 61 bacterial strains, including 26 *L. pneumophila*, 23 non-*L. pneumophila*, and 12 environmental and food-borne strains were used as reference strains to test the specificity of the real-time LAMP method (Table 1). All *Legionella* spp. strains were grown on buffered activated charcoal and yeast extract containing 0.1% α -ketoglutarate, which was adjusted to pH 6.9 with KOH, and supplemented with 0.4 g L-cysteine and 0.25 g ferric pyrophosphate (BCYE) per liter for 48 h at 35°C with 5% carbon dioxide. For the isolation of *Legionella* from environmental samples, a GVPC medium was used. This medium is identical to BCYE except for 3 g of glycine, 1 mg of vancomycin, 50,000 IU of polymyxin B, and 80 mg of cycloheximide are added to 1 l of the BCYE medium.

Sampling and isolation of *Legionella* spp.

In all, 107 water samples were collected from the cooling towers, spray fountain, and artificial lakes with sterilized bottles from 2009 to 2010 in southern China. Isolation of *Legionella* from natural water samples was performed by culture according to International Standard method ISO 11731 with slight alteration. Briefly, 200 ml water sample was concentrated by filtration through 0.44 μ m-pore diameter polycarbonate membrane by microfilm filtration system (Millipore Company, France). After filtration, bacteria collected on the membranes were resuspended in 5 ml of the water to be analyzed by vortex for 1 min in order to release the cells from the membranes. The concentrated samples were subjected to acid treatments before being spread on GVPC agar medium.

Agglutination test

Serological agglutination was conducted by the *L. pneumophila* Latex Agglutination Kit (PRO-LAB) to identify *L. pneumophila* serogroups 1 to 14 based on the manufacturer's instruction. Briefly, as many suspected colonies as possible were picked from the BCYE medium and the colonies were suspended in about 1 ml of PBS (pH 7.4), and they should have an approximate turbidity

Table 1 Specificity of LAMP assay for identification of *L. pneumophila* and non-*L. pneumophila*

Species and serogroup ^a	Strains	No. of strains	LAMP results		Source
			LS	LP	
<i>L. pneumophila</i>		26			
1 ^b	ATCC 33153		+	+	Guangzhou CDC, China
6	ATCC 33215		+	+	Guangzhou Kingmed Center for Clinical Laboratory
8	ATCC 35096		+	+	Guangzhou Kingmed Center for Clinical Laboratory
2	ATCC 35849		+	+	Guangzhou Kingmed Center for Clinical Laboratory
1	LP1-1		+	+	Cooling tower, 2008, China
1	LP1-2		+	+	Cooling tower, 2008, China
1	LP1-3		+	+	Cooling tower, 2008, China
2–14	LP2-1		+	+	Cooling tower, 2009, China
2–14	LP2-2		+	+	Cooling tower, 2009, China
1	LP3		+	+	Condensation water from air conditioner, 2008, China
2–14	LP4		+	+	Condensation water from air conditioner, 2008, China
2–14	LP5		+	+	Condensation water from air conditioner, 2008, China
2–14	LP6		+	+	Condensation water from air conditioner, 2008, China
2–14	LP7		+	+	Condensation water from air conditioner, 2008, China
1	LP8		+	+	Condensation water from air conditioner, 2009, China
1	DA1		+	+	Fountain pool, 2009, China
2–14	DA2		+	+	Fountain pool, 2009, China
1	DA3		+	+	Artificial lake, 2010, China
1	DA4		+	+	Artificial lake, 2010, China
1	DA5		+	+	Artificial lake, 2010, China
2–14	DA6		+	+	Artificial lake, 2010, China
2–14	DA7		+	+	Artificial lake, 2010, China
2–14	DA8		+	+	Artificial lake, 2010, China
2–14	DA9		+	+	Artificial lake, 2010, China
2–14	DA10		+	+	Artificial lake, 2010, China
1	DA11		+	+	Artificial lake, 2010, China
Non- <i>L. pneumophila</i>		23			
<i>Legionella birminghamiensis</i>	ATCC 43702		+	–	Guangzhou Kingmed Center for Clinical Laboratory
<i>Legionella wadsworthii</i>	ATCC 33877		+	–	Guangzhou Kingmed Center for Clinical Laboratory
<i>Legionella bozemanii</i>	ATCC 33217		+	–	Guangzhou Kingmed Center for Clinical Laboratory
<i>Legionella feeleii</i>	ATCC 35072		+	–	Guangzhou Kingmed Center for Clinical Laboratory
<i>Legionella adelaidensis</i>	ATCC 49625		+	–	Guangzhou Kingmed Center for Clinical Laboratory
<i>Legionella gormanii</i>	ATCC 33342		+	–	Guangzhou Kingmed Center for Clinical Laboratory
<i>Legionella rubrilucens</i>	ATCC 35304		+	–	Guangzhou Kingmed Center for Clinical Laboratory
<i>L. longbeachae</i>	ATCC 33462		+	–	Guangzhou CDC, China
<i>L. feeleii</i>	LS1		+	–	Guangzhou CDC, China
<i>L. feeleii</i>	LS2		+	–	Guangzhou CDC, China
<i>L. rubrilucens</i>	LS3		+	–	Guangzhou CDC, China
<i>L. adelaidensis</i>	LS4		+	–	Guangzhou CDC, China
<i>Legionella steigerwaltii</i>	LS5		+	–	Guangzhou CDC, China
<i>L. rubrilucens</i>	NP1-1		+	–	Cooling tower, 2008, China
<i>L. rubrilucens</i>	NP1-2		+	–	Cooling tower, 2008, China
<i>L. longbeachae</i>	NP2		+	–	Cooling tower, 2009, China
<i>L. feeleii</i>	NP3-1		+	–	Condensation water from air conditioner, 2008, China
<i>L. feeleii</i>	NP3-2		+	–	Condensation water from air conditioner, 2008, China

Table 1 (continued)

Species and serogroup ^a	Strains	No. of strains	LAMP results		Source
			LS	LP	
<i>L. feeleii</i>	NP3-3		+	–	Condensation water from air conditioner, 2008, China
<i>L. steigerwaltii</i>	NP4		+	–	Artificial lake, 2010, China
<i>L. adelaidensis</i>	NP5		+	–	Artificial lake, 2010, China
<i>L. steigerwaltii</i>	NP6		+	–	Artificial lake, 2010, China
<i>L. steigerwaltii</i>	NP7		+	–	Artificial lake, 2010, China
Non- <i>Legionella</i> strains		12			
<i>Escherichia coli</i>	MG1655		–	–	Coli Genetics Stock Center, American
<i>Listeria monocytogenes</i>	ATCC 19115		–	–	South China University of technology, China
<i>Vibrio parahaemolyticus</i>	ATCC 17802		–	–	South China University of technology, China
<i>Salmonella enterica</i>	ATCC 14128		–	–	South China University of technology, China
<i>Staphylococcus aureus</i>	ATCC 29213		–	–	South China University of technology, China
<i>Staphylococcus epidermidis</i>	ATCC 29887		–	–	South China University of technology, China
<i>Pseudomonas aeruginosa</i>	ATCC 27853		–	–	South China University of technology, China
<i>Mycobacterium tuberculosis</i> H37RV	CMCC 93004		–	–	Shenzhen Chronic Disease Control Centre, China
<i>Mycobacterium avium</i>	CMCC 93026		–	–	Shenzhen Chronic Disease Control Centre, China
<i>Mycobacterium kansasii</i>	CMCC 93304		–	–	Shenzhen Chronic Disease Control Centre, China
<i>Francisella tularensis</i>	NP8		–	–	Cooling tower, 2010, China
<i>Brucella abortus</i>	LSB-1		–	–	Guangzhou CDC, China

ATCC American Type Culture Collection, CMCC National Center for Medical Culture Collections, + amplification was found within 60 min of incubation and Tt >0.1, – amplification was not found, amplification was found after 60 min or Tt <0.1

^a These strains were identified by fatty acid analysis, and serogroup was determined by agglutination test

^b The number means the serogroup

of 10^8 CFU ml⁻¹. The latex agglutination reagents were suspended by gentle agitation, and then, 1 drop of cell suspension and 1 drop of latex reagent were added, with a mixing stick, they were mixed and the card was gently rocked for 2 min. Finally results were read visually by examining the agglutination. If the organism is *L. pneumophila* serogroup 1 or others, the mixture will cause a visible agglutination. Positive and negative control was conducted in each run.

Fatty acid analysis

Fatty acid analysis was performed with the Sherlock microbial identification system (software version 6.0, MIDI; Microbial ID, Inc., Newark, DA) with the Agilent 7890 gas chromatograph. Each *Legionella* strain was performed by using the standardized protocol of MIDI. Briefly, after 72 h growth, about 40 mg of fresh colonies was harvested from BCYE. Fatty acids were extracted following the instructions of the MIS Sherlock operating manual. Gained FAME profiles could generally be compared in Aerobe Clin6 database, version 6.10.

DNA extraction

The DNA of the bacteria was extracted by using the Wizard Genomic DNA Purification kit (Promega, USA) according to the manufacturer's instruction. The extracted DNA was dissolved in double distilled water, tested with ultraviolet spectrophotometer at A260/A280, stored under –20°C before it was used.

The steps of DNA extraction from 107 natural water samples were as follows: The concentrated samples were heated for 15 min at 95°C and 10 min on ice. Finally the crude lysates were centrifuged at 12,000 rpm for 2 min at room temperature and the supernatant was used as templates for nucleic acid amplification.

Designing of primers for LAMP assay

LAMP primers were designed targeting the 226 bp of the 16S rRNA gene of the *Legionella* genus. This sequence was proved to be specified to all species of the genus *Legionella* (Zhan et al. 2010). The bioinformatics analysis found that the site between 178 and 182 bp of the fragment from the 16S

rRNA gene for *L. pneumophila* showed a specific consistent pattern: all strains had the sequence of ACNGT (N=A, T, G, C), while non-*L. pneumophila* strains had a variable base sequence in this region (Zhan et al. 2010). The primers of LS-LAMP were designed to prevent overlay in the sequence between 178 and 182 bp ACNGT (Fig. 1). As for the primers of LP-LAMP which specify to *L. pneumophila*, the sequence of ACNGT was covered by 3' region of primer B2c. The sequences of the primers of LS-LAMP specified for *Legionella* genus were as follows: LSFIP, 5'-CGATTAACGCTCG CACCCTCCGAAGCACCGGCTAACTCC-3'; LSBIP, 5'-ATTACTGGGCGTAAAGGGTGC GCCCAGGTTAAGCC CAGGAA-3'; LSF3, 5'-ACTGGACGTTACCCACAGAA-3'; and LSB3, 5'-CCTCTCCCATACTCGAGTCA-3'. The sequences of the primers of LP-LAMP specified for *L. pneumophila* were as follows: LPFIP, 5'-AGTAATTCGATTAACGCTCG CAACCGGCTAACTCCGTGC-3'; LPBIP, 5'-GGCGTAAAGGGTGC GTAGGTGACCAGTATATCT GACCGTCC-3'; LPF3, 5'-CGTTACCCACAGAAGAAGC-3'; and LPB3, 5'-ACCCTCTCCCATACTCGA-3'.

Fig. 1 Location of primers for LS-LAMP and LP-LAMP. Nucleotide sequences of 16S rRNA used for designing the primers. Recognition sequences of the primers are shown between the *bigger capital letters*. A right arrow indicates that a sense sequence is used for the primer. A left arrow indicates that a complementary sequence is used for the primers. LS-LAMP primers were designed for *Legionella* genus detection. LP-LAMP primers were designed for *L. pneumophila* detection, which can recognize the ACNGT only specify for *L. pneumophila* in this sequence

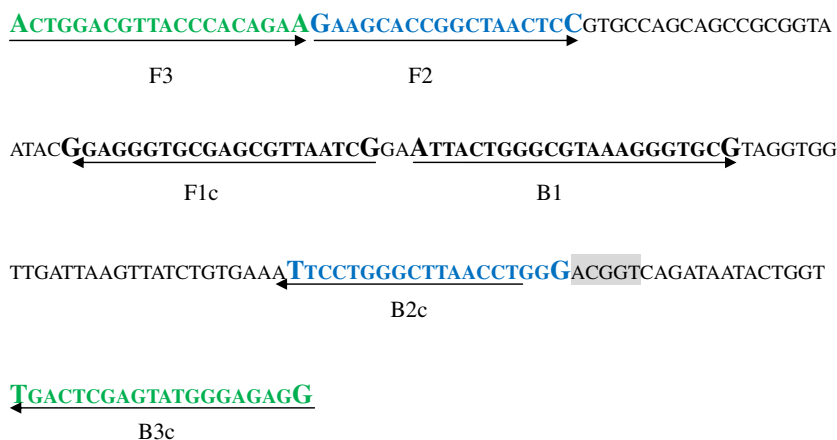
LAMP assay for *Legionella* detection

LAMP assay was performed with a Loopamp DNA amplification kit (Eiken Chemical). The final LAMP assay was comprised of 2 µl of template DNA, 1 µl of *Bst* DNA polymerase, 1.6 µmol l⁻¹ each of inner primers FIP and BIP, 0.2 µmol l⁻¹ each of outer primers F3 and B3, and 1× reaction mix (Eiken Chemical). The final volume was adjusted to 25 µl. All primers were synthesized by TaKaRa Co., Ltd. The reaction was considered to be positive when the turbidity reached 0.1 within 60 min of using a Loopamp real-time turbidimeter (LA-320; Teramecs, Kyoto, Japan). Turbidity visible with the naked eyes was also considered to indicate a successful LAMP procedure.

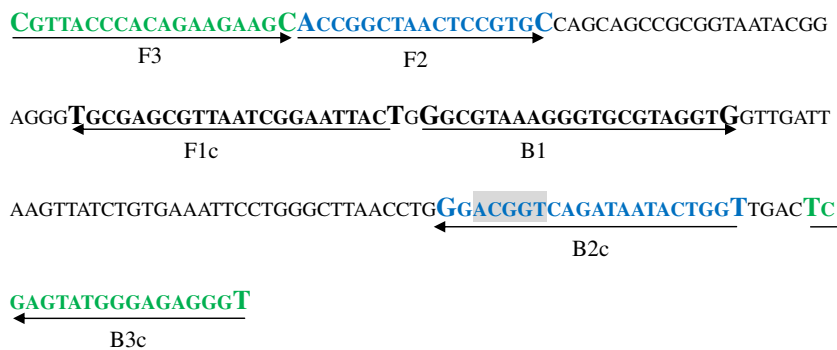
Real-time PCR assay

When a sample obtained a positive LAMP result and negative culture result, the real-time PCR method was used for verifying the inconsistent results. The PCR method was

LS-LAMP primer:



LP-LAMP primer:



performed according to our previous study (Mo et al. 2011). The specific primers and probes were designed according to the 16S rRNA gene of *Legionella* and *mip* gene of *L. pneumophila*. The probe of the 16S rRNA gene was labeled with FAM and the probe of the *mip* gene was labeled HEX respectively. The PCR amplification was carried out in a 25- μ l reaction volume with 2.5 μ l of the 10 \times buffer, 1.5 μ l (10 pmol/ μ l) two couples of primers and 0.6 μ l (10 pmol/ μ l) of probes, 4 μ l of dNTPs mixture (2.5 mM of each dNTPs), and 0.5 μ l (5 U/ μ l) of Taq DNA polymerase were mixed. The reaction was performed on BIO-RAD IQ5 Multicolor Real-Time PCR Detection System with the cycling conditions 95°C for 5 min, followed by 35 cycles of 95°C for 15 s and 60°C for 30 s. Positive and negative controls were included in each run.

Results

Evaluation of primer specificity for *Legionella* detection

LAMP is one of the nucleic acid amplification (NAA) methods with high sensitivity and specificity in pathogen detection. To determine the specificity of the primers, 61 reference strains including *L. pneumophila*, non-*L. pneumophila*, common environmental and food-borne strains were used in this experiment. Genomic DNA of the strains were extracted and amplified either for LS-LAMP or LP-LAMP method. The results from LS-LAMP, which specified for *Legionella* spp., showed that all of the 49 *Legionella* strains (including *L. pneumophila* and non-*L. pneumophila*) were positive, while other strains had negative results. LP-LAMP results showed 26 *L. pneumophila* strains out of 49 had positive results, while the rest of the 23 non-*L. pneumophila* and 12 other strains obtained negative results (Table 1.). To detect the specificity of LS-LAMP and LP-LAMP method, the genomic DNA of *L. pneumophila* ATCC 33153 and *Legionella longbeachae* ATCC 33462 was used. The results demonstrated that both strains had positive results by LS-LAMP assay, while the negative control of LS-LAMP was undetected as expected (Fig. 2a). For the LP-LAMP, a positive result was found in *L. pneumophila* detection, while negative results were obtained by *L. longbeachae* ATCC 33462 and the negative control (Fig. 2a, b).

Sensitivity results of LAMP for *Legionella* species and *L. pneumophila* detection

To accurately test the sensitivity of the LAMP assay targeting the 16S rRNA gene for *L. pneumophila* detection, the fragments of 226 bp of the 16S rRNA gene was cloned in pMD™ 18-T Vector. A representative sensitive result of

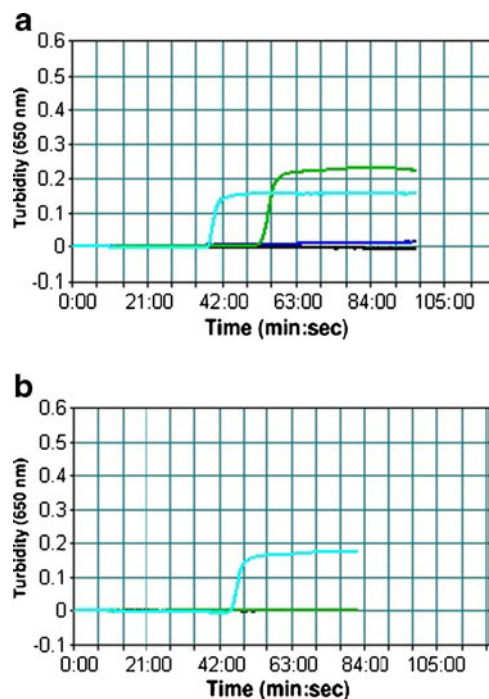


Fig. 2 The specificity of LAMP method when detecting *L. pneumophila* ATCC 33153 and *L. longbeachae* ATCC 33462 representative optic graphs generated using the real-time turbidimeter LA-320. Sky blue amplification curve of LS-LAMP, green amplification curve of LP-LAMP, dark blue negative control of LS-LAMP, black negative control of LP-LAMP. **a** The DNA of *L. pneumophila* ATCC 33153 was used as template. **b** *L. longbeachae* ATCC 33462 was used

the real-time turbidimeter for *L. pneumophila* detection was shown in Fig. 3a, b. The templates used ranging from 5.2×10^4 to 5.2×10^{-1} copies per reaction in concentration. Tt values of LS-LAMP were found between 26.32 and 59.11 min for templates ranging from 5.2×10^4 to 52 copies per reaction. Tt values of LP-LAMP ranged from 29.22 to 60.54 min for the same concentration of 5.2×10^4 to 52 copies per reaction. In two out of five repeats of both tests, 5.2 copies templates had a positive amplification. Therefore, the limit of real-time LAMP assay detecting either for *Legionella* species or *L. pneumophila* should be from 52 to 5.2 copies per reaction under the present study.

Detection and identification of *L. pneumophila* and non-*L. pneumophila* from water samples

A total of 107 water samples were collected and identified by this new method based on LAMP technique. The results from the traditional culture method and the agglutination identification were used as reference methods, fatty acid analysis was conducted as well. The LAMP assay was developed to detect the *Legionella* genus and to differentiate the *L. pneumophila* from the non-*L. pneumophila* by two reactions.

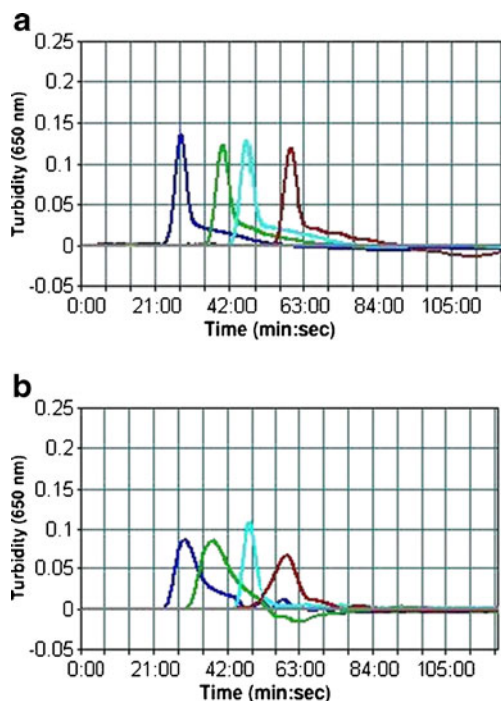


Fig. 3 The sensitivity of LAMP method for *L. pneumophila* ATCC 33153 and *L. longbeachae* ATCC 33462 detection. Black negative control, dark blue 5.2×10^4 copy, green 5.2×10^3 copy, sky blue 5.2×10^2 copy, red 5.2×10^1 copy, pink 5.2 copy, grey 5.2×10^{-1} copy. Ls-LAMP primer

After pretreatment, the water samples were first detected by LS-LAMP. There were 60 samples that had positive LS-LAMP results, while the other 47 samples had negative results. After that, the 60 LS-LAMP-positive samples were subject to a further detection by LP-LAMP. In this step, 22 samples had positive LP-LAMP results. At the same time, the culture and agglutination test were conducted. There were 98 samples with identical results to that of LAMP assay. The specificity of LS-LAMP was 91.59% (98/107) and LP-LAMP was 93.33% (56/60) in the water samples detection. Those nine inconsistent samples that obtained LAMP-positive (including four LP-LAMP-positive samples) and culture-negative results were further identified by a real-time PCR method. Out of these nine samples, there were still seven samples with positive PCR results.

The *Legionella* spp. was isolated from 51 samples after cultivation, and 18 were identified as *L. pneumophila*, while 33 were non-*L. pneumophila* (Table 2). For each of the 51

Legionella-positive water samples, one to three colonies from each sample were detected by agglutination test and fatty acid analysis. A total of 122 strains were identified. Out of all the total strains, 31 (25.41%) strains belonged to serogroup 1 and the remaining 91 strains (74.59%) belonged to serogroup 2–14. The results of the fatty acid analysis were identical to the agglutination test, except for two *L. pneumophila* isolates (serotype 14) detected as *Legionella rubrilucens*. This was most likely because the result of the fatty acid analysis was affected by growth condition or the cross reaction of serology may exist between *L. rubrilucens* and serotype 14 of *L. pneumophila*.

Discussion

Risk assessment and environmental monitoring for *Legionella* in air conditioning systems, potable water, and related sources are crucial to control the incidence of Legionnaires' disease. More significantly, *L. pneumophila* took up the majority of legionellosis cases (Yanez et al. 2005). Therefore there is an urgent need to develop a method of *L. pneumophila* identification, which can be used in sample detection with high sensitivity and rapidity.

In this study, two specific LAMP methods which target the specific sequence of the 16S rRNA gene of *Legionella* spp. have been developed successfully. The specificity and sensitivity of the LAMP methods have been tested and validated by 61 reference strains. All of the *Legionella* spp. strains had positive LS-LAMP results. *L. pneumophila* can be distinguished correctly from non-*L. pneumophila* and non-*Legionella* strains by LP-LAMP in 2 h without postamplification procedures. The specificity of two LAMP reactions was both 100%. In the sensitivity evaluation test, we found that the LAMP method was able to detect DNA as few as 52 to 5.2 copies per reaction. The results also showed that the newly developed LAMP methods can be used in *Legionella* spp. detection and *L. pneumophila* identification with high sensitivity and convenience.

In order to achieve the requirement of samples detection, 107 water samples were collected and detected by the LAMP assay. After comparing with culture and agglutination test, 98 samples obtained consistent results. The specificity of LS-LAMP and LP-LAMP were 91.59% (98/107) and 93.33% (56/60). However, after a further confirmation by real-time

Table 2 Result of detection and identification of *Legionella* species and *L. pneumophila* in 107 water samples with different methods

Methods	No. of positive LS samples/no. of total sample (%)	No. of positive LP samples/no. of total LS sample (%)
LAMP	60/107 (56.07)	22/60 (36.67)
Culture and agglutination test	51/107 (47.66)	18/51 (35.29)
Fatty acid analysis	51/107 (47.66)	16/51 (31.37)

PCR with inconsistent samples, the specificity seemed to be increased. The LS-LAMP and LP-LAMP were 98.13% (98+7/107) and 100% (56+4/60), respectively. The sensitivity of LS-LAMP and LP-LAMP were 100% (51/51) and 100% (18/18). In the present study, all of the culture-positive samples obtained LAMP-positive results.

From the data obtained in this study, the positive rate of the LAMP (56.07%, including seven real-time PCR-verified samples) was higher than that of the culture assay (47.66%). Although not all of the water samples were detected by real-time PCR, the positive rate of LAMP method is higher than that of classical culture. Furthermore, in order to make the pretreatment step more adaptive to rapid diagnosis, we attempted to use a 10-ml injector and 0.44- μ m-pore-sized filter instead of microfilm filtration system for the sample pretreatment. Two microliters of the filtrate was directly detected by LAMP assay without DNA extraction. Fifty water samples were pretreated by this method, and only four *Legionella*-positive samples escaped from detection. The results suggested that the LAMP was more sensitive and resulted in a higher positive rate. One of the probable reasons of the high positive rate was due to the characteristics of LAMP method. Other probable causes such as the viable but non-cultivable (VBNC) form and the strict requirement of culture condition for *Legionella* spp. also need to be considered. If the *Legionella* species changed into VBNC form, it would have escaped from the culture detection but would have had a minimal effect on NAA methods (Dusserre et al. 2008; Oliver 2005). At this point, the LAMP assay is more sensitive than the culture method in sample detection.

On the other hand, the current research showed that the positive rate of *Legionella* spp. and *L. pneumophila* in water samples from the environment were 47.66% (51/107) and 16.82% (18/107), especially the samples from cooling tower, 84.20%, which was higher than the former reports (Yaradou et al. 2007; Yanez et al. 2005). This might be due to the geographic location of Guangdong province in China, where the samples were collected. Guangdong is located in semitropical zone with high humidity and high average temperature year round. Additionally, *Legionella* was isolated from spray fountains in this study, which suggests the safety of the environment, such as pathogen should be paid more attention especially if it is found in public facilities, since the high positive rate indicates the high risk of infection.

Several assays have been developed to detect *L. pneumophila*. These include real-time PCR method (Yanez et al. 2005; Stolhaug and Bergh 2006), 16S rRNA gene sequencing (Cloud et al. 2000; Wilson et al. 2007), and the two-step scheme method (Zhan et al. 2010) for *L. pneumophila* detection effectively. With these methods, the precise instruments and complex operation are required. Fatty acid analysis which is certified by the FDA (Costa et al. 2005; Diogo et al. 1999) and even MALDI-TOF MS were

established for diagnosis in the isolates and clinical samples (Hilbi et al. 2010). Obviously, the water samples cannot be detected directly. So, it is a strong desire that a specific and rapid method to be determined for the identification of *L. pneumophila* and non-*L. pneumophila* species with convenient operation also meets the demand of field diagnostic. Apparently, our study demonstrates that the real-time LAMP assay makes it possible to take a high-throughput detection of environment and clinic samples.

Loop-mediated isothermal amplification is a popular nucleic acid detection method which was developed in recent years. This method is becoming accepted by researchers increasingly due to the following advantages: (1) high sensitivity—the affection caused by irrelevant and background DNA is less than that of PCR, and six copies of hepatitis B virus target can be detected under the presence of 100 ng of human genomic DNA (Notomi et al. 2000). In this study, we found the sensitivity of both LAMP reactions to range from 52 to 5.2 copies of target DNA. (2) High specificity—the LAMP method has been used in differential diagnosis of the pathogens with close genetic relationship (Bonizzoni et al. 2009; Ohori et al. 2006; Hayashi et al. 2007; Inacio et al. 2008). The four primers recognize the target by six independent sequences, which ensure the high specificity of LAMP amplification (Notomi et al. 2000). (3) Convenient operation and low cost—the most attractive characteristic of LAMP is the visual judgment of nucleic acid amplification (Notomi et al. 2000), which can be used not only in well-equipped hospitals or laboratories in developed countries but also in small-scale hospitals or even the field detection (Iwamoto et al. 2003). The risk of cross-contamination was greatly reduced after the invention of the turbidimeter. This equipment enables this technique developed from qualitative investigation to quantitative study possibility (Mori et al. 2001; Siyi and Beilei 2010).

In the present study, the LAMP method was designed based on the bioinformatics founding, the unique nature of the 16S rRNA gene between *L. pneumophila* and non-*L. pneumophila* (Zhan et al. 2010). There were five primer sets designed for the 16S rRNA gene of *Legionella* genus and *L. pneumophila*. All primer sets were evaluated for their amplification efficiency, specificity, and sensitivity (data not shown). The best sets were then selected and determined for both LS-LAMP and LP-LAMP respectively (Fig. 1). The sequence ACNGT specified for *L. pneumophila* was targeted again in the study. The biological significance of this conserved sequence has not been brought to light, but more attention should be paid during the detection related to *L. pneumophila* and non-*L. pneumophila* henceforth. Simultaneously using different genes such as *mip* and 16S rRNA gene for identification was not required. What is more, the *mip* gene is highly variable and likely to cause false-negative results (Ratcliff

et al. 1998; Stolhaug and Bergh 2006). As for the virulence and infection of *L. pneumophila*, a further study should be carried out for this specially conserved sequence.

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