

Loop-mediated isothermal amplification for detection of *Legionella pneumophila* in respiratory specimens of hospitalized patients in Ahvaz, southwest Iran

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Background: Legionnaires' disease is an important public health problem that can cause substantial mortality and morbidity. Legionnaires' disease-risk estimation may be compromised by uncertainties in *Legionella*-detection methods. The aim of this study was the detection of *L. pneumophila* in respiratory specimens of hospitalized patients with respiratory symptoms by culture, PCR, and loop-mediated isothermal amplification (LAMP) methods.

Methods: Sputum and bronchoalveolar lavage samples were obtained from patients with pneumonia admitted to teaching hospitals in Ahvaz, Iran from June 2016 to March 2017. Isolation of *Legionella* spp. was done by culturing the samples directly onto buffered charcoal-yeast extract and modified Wadowsky-Yee agar medium. Then, PCR and LAMP assays were performed for detection of *L. pneumophila* via its *mip* gene in respiratory specimens.

Results: A total of 100 respiratory specimens were collected. Our results showed that 1% of the samples were culture positive for *Legionella* spp., and 3% and 7% of samples were positive for *L. pneumophila* using the *mip* gene on PCR and LAMP assays, respectively.

Conclusion: Legionnaires' disease should be considered in the diagnosis of pulmonary infectious diseases. Also, the LAMP assay is a faster method with higher sensitivity and specificity than conventional methods, such as PCR and culture, for laboratory diagnosis of Legionnaires' disease.

Keywords: *Legionella pneumophila*, LAMP, PCR, *mip* gene

Introduction

Legionnaires' disease is an important public health problem that causes substantial morbidity and mortality. This bacterial infection is caused primarily by the Gram-negative bacterial genus *Legionella*.¹ *Legionella* spp. are found in the environment and can enter man-made hot-water systems, air conditioners, and cooling towers of defined facilities, such as hotels, hospitals, and whirlpool spas.² These bacteria cause community-, travel-, and hospital-acquired pneumonia in humans, usually via inhalation of contaminated aerosols.³ Even with antibiotic therapy, Legionnaires' disease causes high mortality rates (15%–25%), with elderly and immunocompromised patients being most susceptible.^{2,4} Approximately 80%–90% of reported cases of Legionnaires' disease are attributed to *L. pneumophila*; however, all species may cause infection, especially in immunocompromised hosts.⁵ Legionnaires' disease can be potentially fatal if treatment is not undertaken in time, so clinical cases suspected of Legionnaires' disease should

be reported immediately to epidemiological centers to help confirmation of their laboratory diagnoses.⁶ Over time, the prevalence of Legionnaires' disease has risen, but the exact incidence of this disease remains unknown, as a result of nonspecific signs and symptoms and variation in diagnostic and surveillance systems among involved countries.^{1,2}

Diagnosis of Legionnaires' disease is based on culture, serological testing, antigen detection in urine, and nucleic acid–amplification techniques.² The gold-standard method for identification of *Legionella* is culture; however, this method has low sensitivity and is time-consuming. Also, direct fluorescent antibody has low sensitivity, and urine-antigen detection is limited to identification of *L. pneumophila* serogroup 1.⁷ These limitations have led to the development of rapid molecular methods for detection of *Legionella*. The current gold standard in molecular diagnosis is based on detection of the *mip* gene specific for *L. pneumophila*.⁸ This gene encodes the peptidylprolyl *cis/trans* isomerase, and was one of the first genes associated with the ability of *Legionella* to replicate in eukaryotic cells. *mip* stands for “macrophage infectivity potentiator”, and *mip* mutants of *L. pneumophila* are defective for replication in macrophages, epithelial cells, and amoebae and attenuated in infection.⁹

Molecular techniques, such as PCR or loop-mediated isothermal amplification (LAMP), can potentially detect *Legionella* within a few hours in clinical specimens.¹⁰ LAMP rapidly amplifies a few DNA molecules with high specificity and efficiency.¹¹ This method has attracted a lot of attention as a potentially simple, accurate, and cost-effective novel nucleic acid–amplification method that has the ability to detect of a wide range of *Legionella* spp. with high specificity, sensitivity, and rapidity.^{12,13} The aim of this study was the identification of *L. pneumophila* from patients with pneumonia symptoms by PCR and LAMP assays as a rapid diagnostic method in comparison with the culture method for laboratory diagnosis of Legionnaires' disease.

Methods

Ethical considerations

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1395.620). Written informed consent was obtained from all patients.

Clinical specimens

Respiratory specimens, including sputum and bronchoalveolar lavage (BAL), were taken by infectious disease specialists

from patients with clinical and radiological signs (cough, fever, chill, shortness of breath) suspected of atypical pneumonia who were admitted to teaching hospitals in Ahvaz in the southwest of Iran from June 2016 to March 2017. Samples were transferred to the microbiology laboratory of the School of Medicine in <2 hours in a cold condition.

Isolation of *Legionella* species

Two respiratory specimens were collected from each patient: one sample inoculated onto the culture media and the other stored at -70°C until molecular assay. Sputum specimens were decontaminated by heat treatment (56°C for 30 minutes), and BAL samples were concentrated by centrifugation. The prepared specimens were cultured onto an unselective medium – buffered charcoal–yeast extract (BCYE) agar (Oxoid, Basingstoke, UK) – and a selective medium, modified Wadowsky–Yee agar (Oxoid) supplemented with L-cysteine. The plates were incubated in candle jars (3%–5% CO_2) at 35°C in a humidified atmosphere and inspected for 4–14 days for the presence of *Legionella* spp. colonies. Colonies showing characteristics of *Legionella* spp., such as grayish-white, shiny colonies were selected. Gram staining was done to show the thin, faintly stained filamentous Gram-negative morphology. Suspected colonies were subcultured on BCYE agar with and without L-cysteine and unselective media, such as sheep-blood agar and MacConkey agar, for verification. Isolates that grew on BCYE agar with L-cysteine, but not on the other media, were considered *Legionella*. Strains unable to grow on media without L-cysteine were further identified by PCR and LAMP for the *mip* gene.¹⁴

DNA extraction

Extraction of DNA from cultivated strains, sputum, and BAL samples was performed by High Pure PCR template preparation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The concentration and purity of DNA were determined by absorbance at 260 and 280 nm using NanoDrop spectrophotometry (Thermo Scientific, Waltham, MA, USA) and agarose-gel electrophoresis, respectively.

PCR amplification of *mip* gene

PCR was employed for the amplification of a 159-bp fragment of the *mip* gene using specific primers (Table 1).¹⁵ The PCR assay was carried out thus: an initial denaturation at 95°C for 4 minutes, followed by 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. PCR amplification was

Table 1 Primers used in LAMP and PCR for detection of *Legionella pneumophila*

Methods	Primer	Sequence (5'→3')
LAMP	F3	ACCCGGAAAATCGGATAC
	B3	GTGGGCCATATGCAAGAC
	FIP	TACCAGTTTTTCGGTACTGTACTGTCAAACACTGTCTGAATATACTGGTCG
	BIP	CAACGTTCAGGTTTCACAAGTTATAAATTTCCCAAGTTGATCCA
PCR	<i>mip</i> -F	GCAATGTCAACAGCAA
	<i>mip</i> -R	CATAGCGTCTTGCATG

Abbreviation: LAMP, loop-mediated isothermal amplification.

performed on an Eppendorf thermocycler 5,530 (Roche). In our study, *L. pneumophila* serogroup 1 (ATCC 33152) was used as the positive control. Amplicons were separated on 1.5% agarose gel prepared in TAE (Tris–acetate–EDTA) buffer and visualized using the gel-documentation system (ProteinSimple, San Jose, CA, USA) after staining with 0.5 µg/mL ethidium bromide (SinaClon, Tehran, Iran).

Primer design for LAMP assay

To design *mip*-specific LAMP primers, sequences of the *mip* gene of *Legionella pneumophila* subsp. *pneumophila* Philadelphia 1 strain with gene ID 19832357 was downloaded from the National Center for Biotechnology Information (NCBI) GenBank database. LAMP primers were designed using PrimerExplorer V4 software (<http://primerexplorer.jp/e>) based on software default settings. Primers included two inner primers, FIP and BIP, and two outer primers, F3 and B3 (Table 1). The specificity of the primers for the *mip* gene was confirmed using BLAST (basic local alignment search tool) on the NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The primers were synthesized commercially by Pishgam (Tehran, Iran).

Optimization of LAMP reaction

Optimization of the LAMP assay was performed in a 25 µL reaction mixture containing 2.5 µL 10× Bst buffer (New England Biolabs, Ipswich, MA, USA), 0.2 pmol each of F3 and B3, 0.8 pmol each of FIP and BIP, different concentrations of dNTPs (0.4, 0.6, 0.8, 1, and 1.2 mM), different concentrations of MgSO₄ (3, 4, 5 and 6 mM), 8 U Bst DNA polymerase (New England Biolabs), and different concentrations of DNA template. The reaction mixture was incubated at 60°C–65°C for different times and heated to 95°C for 5 minutes to stop Bst DNA-polymerase activity in the reaction. *L. pneumophila* serogroup 1 (ATCC 33152) was used as the positive control.

Finally, LAMP-reaction products were electrophoresed on 2% agarose gel for detection of best LAMP product. After optimization of the LAMP assay, the amplified products were detected by adding 1 µL 1:10 SYBR green I (Thermo Fisher Scientific) into a reaction microtube. The reaction was considered positive if its color turned from orange to green under ultraviolet light.¹⁶

Specificity of LAMP and PCR amplification

The specificity of LAMP and PCR assays was evaluated using different *Legionella* spp.: *L. dumoffii*, *L. worsleienensis*, and *L. fairfieldensis*. The product was analyzed by gel electrophoresis.

Determination of detection sensitivity in LAMP and PCR assays

For determination of the detection limit, PCR and LAMP reactions were performed using serially diluted DNA templates of *L. pneumophila* strains, and amplicons were detected by agarose-gel electrophoresis and naked-eye inspection.

Application of LAMP to clinical samples

DNA extracted from 100 clinical respiratory samples was subjected to LAMP and PCR detection for the *mip* gene. The results of LAMP and PCR assays with the culture were analyzed in comparison.

Statistical analysis

All data were analyzed with SPSS 22. Comparisons between culture, PCR, and LAMP were performed with χ^2 using Fisher's exact test. Independent *t*-tests were used for analysis of quantitative data between *Legionella*-positive and -negative groups. Values of $P < 0.05$ were accepted as being statistically significant.

Results

Bacterial isolates by culture method

In this study, 94 sputum samples and 6 BAL samples were collected from patients with pneumonia symptoms. The age of the patients was 15–93 years (average 54 years), including 41 females and 59 males. Results of the culture method showed that one of the sputum samples (1%) was positive for *Legionella* spp, then confirmed as *L. pneumophila* by *mip*-gene PCR. None of the BAL specimens was positive for the presence of *Legionella* spp. by the culture method.

PCR assay of *mip* gene

Results of *mip* PCR revealed that three (3%) sputum samples were positive for 159 bp amplicons. Furthermore, the sample that was positive on culture showed a positive result on PCR. None of the BAL specimens was positive for the presence of *mip* amplicons on PCR.

Most appropriate factors for LAMP optimization

The optimized LAMP assay was carried out thus: a 25 μ L reaction mixture containing 2.5 μ L 10 \times Bst buffer, 3 mM MgSO₄, 1 μ L Bst DNA polymerase (8 IU), 0.8 mM dNTPs, 0.2 pmol each of F3 and B3, 0.8 pmol each of FIP and BIP, 3 μ L DNA template, and 9.5 μ L distilled deionized water. The best temperature was established as 62°C for 60 minutes. The results of electrophoresis of LAMP-amplification products are shown in Figure 1.

LAMP assay for *mip* gene

L. pneumophila strains were detected in seven sputum samples (7%) using LAMP (Figure 2). Also, samples that were positive on culture and PCR showed positive results on LAMP assay. *L. pneumophila* was not found in BAL samples with LAMP. The mean age of patients with positive sputa for *L. pneumophila* was 57.5 years, with three and four females and males, respectively. These results showed a significant difference in the detection of *L. pneumophila* by culture and PCR ($P=0.031$) and also between LAMP and PCR methods ($P=0.001$). No significant correlation was found among age, sex, and Legionnaires' disease ($P>0.05$).

Specificity of LAMP and PCR assays

The specificity of LAMP and PCR assays was tested using DNA samples from different *Legionella* spp. other than *L. pneumophila*. The results showed no non-specific

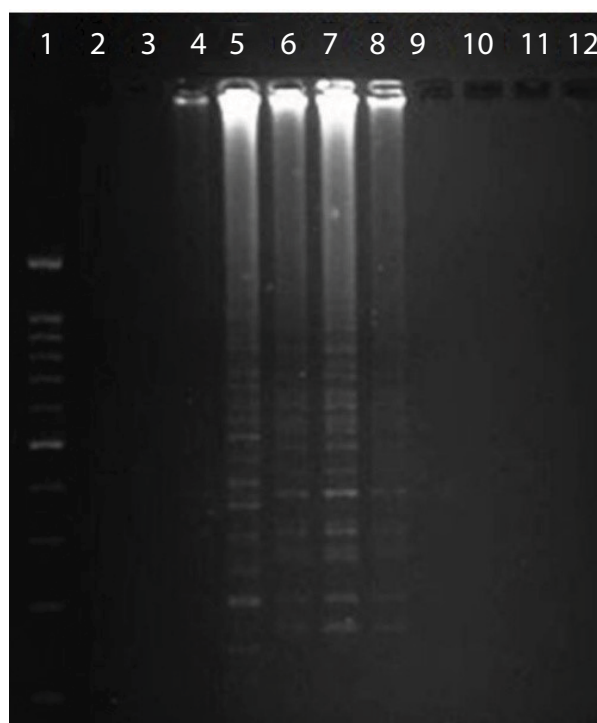


Figure 1 Electrophoresis of loop-mediated isothermal amplification products.
Notes: Lane 1, DNA ladder; lanes 2 and 3, negative controls, distilled water; lane 4, negative sample; lane 5, positive control – *Legionella pneumophila* (ATCC 33152); lanes 6–8, positive samples; lanes 9–12, negative samples.

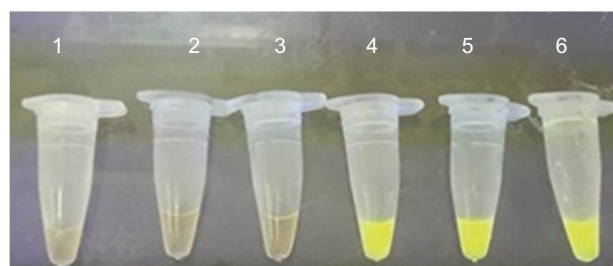


Figure 2 Loop-mediated isothermal amplification for detection of *Legionella pneumophila* in sputum samples with SYBR green added.
Notes: Lane 1, negative control – distilled water; lanes 2 and 3, negative samples; lane 4, positive control – *L. pneumophila* (ATCC 33152); lanes 5 and 6, positive samples.

amplifications of the *mip* gene when using DNA from the aforementioned species.

Detection limit of LAMP and conventional PCR

To determine the detection limit of the LAMP and PCR methods, serially diluted DNA templates of *L. pneumophila* with DNA concentrations ranging from 115 ng/ μ L to 0.115 pg/ μ L were provided. SYBR green I (1 μ L) was added to the LAMP reaction mixture. Positive reactions would turn green, while

negative ones remained orange. Agarose-gel electrophoresis and naked-eye inspection were used to detect the amplified production of PCR and LAMP, respectively. Electrophoresis results showed that the detection limit of PCR for the *mip* gene was <11.5 pg/μL. The results of naked-eye inspection of LAMP reaction showed that the detection limit of LAMP for *mip* was <1.15 pg/μL. These results suggested that detection sensitivity of LAMP for *mip* was tenfold that of PCR.

Discussion

In the present study, three methods were used for detection of *Legionella* in patients with pneumonia symptoms. We demonstrated that the LAMP assay is a potential diagnostic tool for *L. pneumophila* detection in sputum specimens. The rate of *Legionella* spp. detected by culture and PCR methods was 1% and 3%, respectively, while the ability of LAMP was more than the other two methods – 7%. Therefore, LAMP can be useful in specific detection of *Legionella*.

When comparing PCR to culture, PCR demonstrated more sensitivity (63%). In accordance with our study, Peci et al reported that PCR is a better diagnostic method than urinary antigen and culture for detection of *Legionella* from BAL, urine, and sputum specimens. They identified *L. pneumophila* in 3% of BAL fluid and sputum samples by PCR.¹⁷

In several studies from Western Europe and North America, 1%–13% of all pneumonia cases were related to *Legionella*, but because of the difficulty in distinguishing Legionnaires' disease, many cases probably go unreported.⁴ Consistently with our results, Cloud et al detected 5.6% *Legionella* spp. from respiratory specimens by PCR, and stated that the PCR method is a more sensitive and specific method than the culture method for the detection of *Legionella* in respiratory samples.¹⁸ In Weir et al, *L. pneumophila* was identified in 3.17% of sputum and BAL specimens by PCR. Molecular techniques based on direct extraction and amplification of DNA from respiratory specimens may be useful for the timely diagnosis of Legionnaires' disease.¹⁹

Many studies have shown that the specificity and sensitivity of the LAMP method is greater than that of PCR and culture and other laboratory diagnostic methods.^{20–22} Initially, we evaluated the specificity of the LAMP assays for these organisms. Four specific primers can recognize the six conserved regions of the target gene, ensuring the high specificity and sensitivity of LAMP. The target DNA fragments can be amplified 10^9 – 10^{10} times within 1 hour under isothermal conditions, indicating that the LAMP assay is stable, rapid, and efficient. Similar to our study, Furuhashi et al compared culture and LAMP methods for detection of

Legionella spp. in hot-water-bath samples. *Legionella* spp. were detected in 32% and 60.8% of samples via culture and LAMP, respectively. They indicated that the positive rate in the LAMP test was higher than that in the culture test.²⁰

In a study by Inoue et al, LAMP, PCR, and culture were compared for the rapid detection of *Legionella* in bathwater samples. A total of 71, 66, and 49 samples were *Legionella* -positive by the LAMP, PCR, and plate-culture methods, respectively. From these results, it is considered that the LAMP and PCR would be very effective methods for the rapid detection of *Legionella*.²³

Rapid laboratory diagnosis and prompt initiation of appropriate antibiotics for Legionnaires' disease (community-acquired or hospital-acquired pneumonia) is a crucial approach for management of the disease.²⁴ Therefore, the development of highly sensitive and rapid methods for *L. pneumophila* detection directly from respiratory samples without the need for sophisticated equipment can improve the management of patients with Legionnaires' disease in regions with limited diagnostic resources. One of these methods is LAMP.⁷

In comparison of LAMP with PCR and culture, with LAMP as the reference method, LAMP detected four and six *L. pneumophila*-positive specimens that were not identified by PCR and culture, respectively. This may be attributed to false-negative results of culture and PCR, due to the fastidious nature of *Legionella* in culture, PCR inhibition, primer mismatch, and the presence of *Legionella* target below the detection rate.¹⁷ LAMP is a powerful amplification assay with a higher detection rate than that of general PCR and other methods.^{25,26} Furthermore, this cost-effective assay can be performed with limited equipment, even without needing a thermal cycler. According to our results and another study, PCR is a better method than culture, but the LAMP assay is faster and more reliable than both methods for screening of *Legionella*.

This study had some limitations. The actual prevalence of Legionnaires' disease cases may be higher than we reported, as we were investigating only *L. pneumophila* using PCR and LAMP due to its high environmental distribution, while other *Legionella* spp. can also contribute to Legionnaires' disease. Additionally, if the urine-antigen method had been used, we probably could have had more ideal comparisons. In this study, due to the fact that the control strains of all *Legionella* spp. were not available, it was not possible to measure the specificity of the LAMP assay with these strains. As such, more *Legionella* spp. will help for specificity testing of *L. pneumophila* PCR and LAMP methods. Furthermore, due to a shortage of

funding, we were not able to perform DNA sequencing of PCR and LAMP products for validation of molecular identification, in order to avoid false-positive results.

Conclusion

This study showed that Legionnaires' disease should be considered in the diagnosis of respiratory infectious diseases. We also concluded that the LAMP assay is a faster method with high sensitivity and specificity than conventional methods, such as PCR and culture, for laboratory diagnosis of Legionnaires' disease. The LAMP assay can be employed in medical centers with limited equipment without the need for a thermocycling apparatus.

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Disclosure

The authors report no conflicts of interest in this work.

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