

Original Article

IDENTIFICATION OF *LEGIONELLA PNEUMOPHILA* IN BRONCHOALVEOLAR LAVAGE FLUID SPECIMENS BY PCR

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Background – Among the members of *Legionellaceae*, *Legionella pneumophila* is involved in more than 95% of cases of severe pneumonia. Isolation of the causative agent from bronchoalveolar lavage (BAL) fluid specimen is a delicate process and also time-consuming. Moreover, it has been shown that some *Legionella* strains may be viable but cannot be cultured. The serological diagnosis, which is usually determined by the immunofluorescent assay, may also be hindered, due to the delayed rise in *Legionella* antibody levels with the onset of illness. The aim of this study was to apply PCR method for specific identification of the *L. pneumophila* in the BAL fluid specimens.

Method – In this study, 46 BAL fluid specimens were collected from patients suspected of having Legionnaires' disease. These samples were kindly provided from educational hospitals of Hamadan and cultured on selective buffered charcoal-yeast extract agar (BCYE) and then tested with specific *L. pneumophila* PCR. In order to prepare the *Legionella* DNA, specimens were first treated by proteinase K. DNA was then extracted using the phenol-chloroform method. Specific primers used in this study were those targeting macrophage infectivity potentiator gene of the *L. pneumophila*.

Results – The *Legionella* DNA was extracted from known strains and then PCR was optimized. The *Legionella* PCR sensitivity test showed 120 copies of chromosome DNA as a final detection limit. The specificity test did not produce a cross reaction for a range of respiratory pathogenic organisms except for *L. pneumophila*. Forty-six BAL fluid specimens were cultured on BCYE medium to isolate the organisms and these were also tested by the PCR.

Conclusion – Analysis of the results showed one positive for the culture and four for the specific *Legionella* PCR. The PCR set that was used showed that it is sensitive enough for identification of *L. pneumophila* to apply on BAL fluid specimens for the tested samples.

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Introduction

Legionella pneumophila is recognized as an important cause of atypical pneumonia.¹ Although *L. pneumophila* usually causes a small number of community-acquired respiratory infections, the number of hospital-acquired cases is usually higher.² Traditional method is culture, but it is tedious and

time consuming. It is reported that recovery of the organism requires up to 2 weeks of recommended incubation conditions.³ The maximum sensitivity of culture is 50 – 60%,⁴ and it has been also shown that some *Legionella* strains may be viable but cannot be cultured.⁵ The serological detection of *Legionella* shows false positive reactions due to antigenic cross-reactivity.⁶ Direct detection of organisms is by the use of the immunofluorescent method, which produces the culture more rapidly, but its sensitivity has been reported to be relatively poor.³ DNA probe has been used, reported sensitivity is approximately 10³ cells with 75% sensitivity.⁷

Polymerase chain reaction (PCR) methodology has been used primarily against the 5S and 16S

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rRNA genes^{7 - 9} and against the macrophage infectivity potentiator (MIP) gene of *L. pneumophila*. The latter amplification assays have been utilized for the detection of *Legionella* species in environmental specimens, serum, urine, throat swabs, and bronchoalveolar lavage (BAL) specimens.^{10 - 13} These reported PCR protocols have been able to provide the basis for the required degree of specificity and sensitivity.¹⁴ It is essential for each test to have a high detection limit that has been amplified and that a false positive does not occur due to nonspecific amplification of nontarget sequences.¹⁵ On the other hand, the role of different inhibitors remaining after DNA extraction that cause false negative must be noted.

The aim of this research was to study the reliability of specifically detecting a positive signal from the used target gene for identification of *L. pneumophila* in bronchoalveolar lavage fluid specimens.

Patients and Methods

Bronchoalveolar lavage and culture procedure

The 46 BAL fluid specimens were kindly provided from patients from Mobasher and Ekbatan Hospitals of Hamadan. These samples were first centrifuged for 15 min at 1,200 g and the top suspension was removed. The remaining cell concentrate was mixed and used for culture. Culture of *Legionella* was performed on selective BCYE α with polymyxin B, anisomycin, and vancomycin; the plates were incubated at 35°C for up to 2 weeks.³

Processing BAL fluid specimens for DNA amplification

Two μ L of each BAL fluid specimen obtained from each patient was mixed with an equal volume of phosphate-buffered saline and was centrifuged for 15 min at 3,500 g. This wash step was repeated once. The pellet was treated with 50 μ g of proteinase K, 0.5% Nonidet p-40 and 0.5% Tween 80 in 500 μ L of 10 mM trise-HCl (pH 8)-50 mM KCl - 50 mM MgCl₂. DNA was then purified by the phenol: chloroform and finally suspended in 50 μ L TE buffer,¹⁶ and 1 μ L was used for PCR.

Bacterial strains and culture

A range of microorganisms was provided from National Culture Type Collection including: *L. pneumophila*, *M. pneumoniae*, *S. pneumoniae*, *H.*

influenzae, *H. parainfluenzae*, *N. meningitides*, *K. pneumoniae*, *C. pneumoniae*, *C. trachomatis*, and avian *C. psitaci*. Human DNA was prepared from lung fibroblast cells. *Legionella* known strain (11192) and BAL fluid specimens were cultured on BCYE α medium.¹

Extraction of *Legionella* DNA from culture

Purified known strains of *Legionella* (11192) were centrifuged at 20,000 g for 30 min, and the pellet resuspended in 500 μ L TE buffer (10 mM tris/HCl pH 8.0, 1 mM EDTA) containing 250 μ g/mL proteinase K for 2 hr at 56°C. DNA was extracted using phenol: chloroform and precipitated with ethanol.¹⁶ DNA was finally suspended in 50 μ L TE buffer. Extracted DNA was used for the preliminary run and sensitivity test.

Quantitation methods

Extracted chromosomal DNA was measured by spectrophotometry. The optical density (OD) of pure DNA was determined at wavelengths of 260 and 280 nm. The ratio OD_{260/280} gives an estimation of DNA purity, the value being 1.8 for pure preparations. An OD of 1 measured at 260 nm corresponds to approximately 50 μ g/mL for double stranded DNA¹⁶ since the number of each molecule in 1 g is 6.023×10^{23} (Avogadro's Law). Number of extracted molecules of *Legionella* DNA can be calculated in measured purified DNA considering its specific genome size that is approximately 2.5×10^9 daltons.¹⁷

PCR amplification and electrophoresis

To detect *L. pneumophila* the Lmp1 - 2 primers described by Jaulhac were chosen.⁸ This pair of primers targets the MIP gene. PCR mixes were prepared in a total volume of 50 μ L containing 0.2 mM of each dNTP, 0.3 μ M of each primer, 2 mM MgCl₂, 0.5 U of Taq DNA polymerase, and PCR buffer (10 mM tris HCL pH 8.3, 50 mM KCl). After amplification (Table 1), 10 μ L of the products were analyzed by agarose gel electrophoresis in (tris-borate-EDTA) TBE* buffer, and DNA was stained with ethidium bromide.¹⁶

Results

Purified DNA from the culturing of known

* 1 \times TBE buffer: 89 mM tris base, 89mM boric acid, and 4 mM EDTA Dissolved in 1000 mL of double distilled water.

Table 1. Amplification conditions used in the PCR protocol of *Legionella pneumophila* DNA extracted from bronchoalveolar lavage fluid specimens.

Amplification condition	<i>Legionella</i> PCR
Reaction mixture	
Primer concentration	0.3 μ M
MgCl ₂ concentration	2 mM
dNTP concentration	0.2 mM
taq polymerase	0.5 U
Amplification program	
Denaturation	92°C for 1.5 min
Annealing	62°C for 1.5 min
Extension	72°C for 1.5 min
Cycle number	40

μ M = micromole, mM = milimole, U = unit.

strains was firstly tested to ensure proper working *Legionella* PCR protocol. The purified DNA was examined by BAL fluid specimens once sensitivity and specificity tests were performed.

Results of *Legionella* PCR

Preliminary runs, using the reaction conditions given in Table 1, yielded a PCR product with the expected size of 632 base pair.

Sensitivity test

The measured extracted chromosomal DNA by spectrophotometry contained 12×10^4 copied DNA, titrated in ten fold dilution using TE buffer to determine the lowest detection rate of the test. Mixture reactions were prepared and PCR tests were performed separately for each dilution. Analysis electrophoresis revealed that the lowest dilution of the *Legionella* DNA, capable of detection by PCR, was 120 chromosomal copies as a final detection limit (Figure 1).

Specificity test

To undertake the specificity test, PCR was checked with the purified DNA of a range of

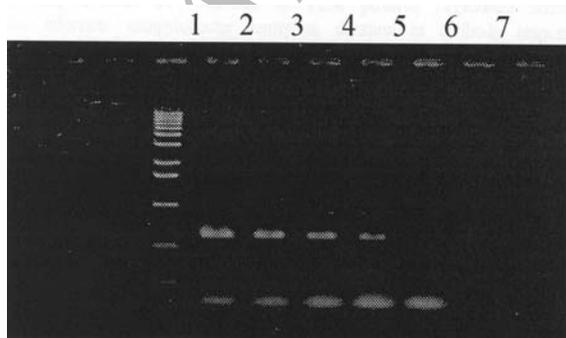


Figure 1. Sensitivity test. Lanes 2, 3, 4, and 5 contain 12×10^4 , 12×10^3 , 12×10^2 , and 120 copies DNA; lane 1 is 1k base ladder.

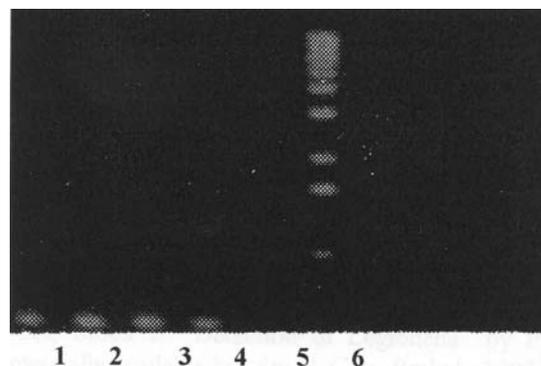


Figure 2. Results of gel electrophoresis obtained from patient's samples PCR. Lanes 1, 2, 3, and 4 are patient's samples. Lane 5 is negative control and lane 6 is 1k base ladder.

pathogenic organisms encountered in respiratory tract infections: *M. pneumoniae*, *S. pneumoniae*, *H. influenzae*, *H. parainfluenzae*, *N. meningitides*, *K. pneumoniae*, *C. pneumoniae*, *C. trachomatis*, and avian *C. psitaci*, and also against human DNA extracted from lung fibroblast cells. No amplified products were detected.

Results of BAL fluid specimens

Forty-six BAL specimens were used to identify *Legionella* by culture and PCR. Amongst those specimens tested, only one sample was positive by the culture and four by PCR (Figure 2).

Discussion

The accurate diagnosis of *Legionella pneumophila* has an important implication for the treatment of the infection. Many first-line antibiotics commonly used to treat typical bacterial pneumonias (i.e., beta-lactams) are ineffective against *Legionella* species. This is at least partially due to the fact that *Legionella* strains are intracellular pathogens, hence nonculturable *Legionella* are frequently reported.⁵ There is a great need for a rapid diagnosis of *Legionella* infection. While direct fluorescent antibody (DFA) is rapid, its sensitivity is poor and it may take several days for results.

Therefore, specific *Legionella* PCR can be a good option. The *Legionella* PCR assay used in this study to target the MIP gene showed to be a rapid and sensitive test. Our experiments demonstrated that 120 copies of chromosomal DNA is a final detection limit for the test when PCR is performed on pure *Legionella* DNA extracted from the culture. The number of total

positive BAL samples was 4, while only 1 sample was isolated on the culture media. It is frequently reported that the sensitivity of the culture is low and it is also highlighted that some *Legionella* strains may be viable in samples but nonculturable.¹⁸

Successive application of specific *L. pneumoniae* PCR in BAL fluid samples have been frequently reported.¹⁹ Cloud et al¹⁸ reported a number of PCR-positive specimens in a comparison study, while their culture results were negative. The true positive results of PCR were proved after sequencing confirmation.

Looking closer at these three PCR-positive and culture-negative patients showed that two of them had hyponatremia (serum sodium less than 130 meq/mL), abdominal pain, and hematuria symptoms, strongly suggesting Legionnaires disease.²⁰

Genetic targets other than MIP genes have been used to diagnose the *Legionella* DNA such as 16S rRNA¹⁶ and intergenic 16S-23S ribosomal spacer region.²¹ In the present study, the primers used appear to be sensitive and specific enough to detect *Legionella* DNA in clinical specimens. Lower sensitivity of the culture method might be due to different reasons:

- collection of specimens in saline lowers the sensitivity of the culture because saline inhibits growth of *Legionella*; and
- samples may be collected from patients currently being treated with antibiotics.

Therefore, a PCR test is likely to overcome the mentioned complications, because of its high efficiency. The current clinical investigation on *Legionella* demonstrates that the required time for the transportation of patient's sample could be reduced. Our findings show that a *Legionella*-specific PCR can be performed in 6 to 8 hours by ordinary thermocycler or less than one hour with lightcycler PCR²² with high sensitivity and high specificity of results.

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