

## Comparison of PCR and Culture for Detection of *Legionella pneumophila* in Bronchoalveolar Fluid Samples

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Atypical pneumonia due to *Legionella pneumophila* is termed Legionnaires' disease. Clinical manifestations are often undistinguishable from other causes of pneumonia. Laboratory test such as culture, serological test and molecular detection facilitated diagnosis. The aim of the present study was use of polymerase chain reaction with primers that amplify a 654bp pair segment of the coding region of the 16S rRNA gene of *L. pneumophila* in bronchoalveolar lavage fluid (BALF) specimens and evaluated results with Culture. A total of fifty archived specimens from patients were evaluated. 8% cases are positive for the PCR. 4% of specimens were culture positive for legionella. 4% positive cases in PCR method had a negative result in culture. After sequencing, specificity and sensitivity for PCR in this study was 100%. Detection of legionella DNA in samples is valuable method for rapid diagnosis. Legionella, PCR, culture, Bronchoalveolar lavage.

**Key words:** PCR, Atypical pneumonia, *Legionella pneumophila*, Legionnaires' disease.

*Legionella* spp. are ubiquitous in natural and artificial aquatic environments. These bacteria are causing infections range from a severe multisystem disease including an asymptomatic infection to pneumonia. Atypical pneumonia due to *Legionella pneumophila* is termed Legionnaires' disease. Worldwide, *L. pneumophila* serogroup 1 accounts for 70-90% of the cases<sup>1,2</sup>.

Since wide range of clinical symptoms cause in Legionella infection it is not possible to that distinguish patients with atypical pneumonia due to Legionella from other forms of pneumonia by radiological tests or clinical. Also identification

of the causative agent is essential for initiation of effective treatment. Therefore laboratory confirmation is necessary for diagnosis<sup>3</sup>.

There are different methods for diagnosis of legionnaire's disease including of serological testing, culture, detection of urinary antigen and detection of Legionella DNA<sup>4-7</sup>.

Diagnosis of *L. pneumophila* is commonly made by culture, but culture is costly since it requires special media and expertise. Furthermore, this bacterium is fastidious and slow growing (often taking five days or more to culture). The serological detection of Legionella is non-sensitive due to antigenic cross-reactivity with other bacteria and yeasts<sup>8,9</sup>.

Legionella spp. can be detected in four hours by nucleic acid amplification techniques. In the present study, PCR was revolutionizing diagnosis for the organisms that were difficult to

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cultivate, this method has been used for detection of all species and serogroups in various samples including environmental specimens, serum, throat swabs, urine and bronchoalveolar lavage specimens. Various studies have shown that PCR have sensitivity equal to or greater than culture<sup>10-12</sup>. The aim of the present study was to compare culture and PCR methods for detection of *L. pneumophila* in bronchoalveolar lavage (BAL) specimens.

## MATERIALS AND METHODS

### Clinical specimens

Specimens of were collected from a total of 50 patients with clinical signs referred to hospital in Tehran by bronchoscope. These patients suffered from pneumonia. Patients were first visited by specialist and then BAL fluid samples were collected. These samples immediately were transported to laboratory on ice.

### Culture

In laboratory, the samples were first centrifuged at 2500 rpm for 15 min and the top suspension was removed. The remaining pellets were mixed and inoculated into a selective medium which was Buffered Charcoal Yeast Extract supplemented with polymyxin B (39600/IU), cycloheximide (40 mg) and vancomycin (0.5 mg). All the inoculated culture media were incubated in an atmosphere with 2% CO<sub>2</sub> and 70-90% moisture at 37°C for up to two weeks. Thereafter, grown colonies on the selective medium were checked in regard to morphology and biochemical features. Doubtful colonies were moved to blood agar medium on which *Legionella* is unable to grow on. Media that did not develop typical colonies within 20 days were considered as negative.

### Sample preparation for Polymerase Chain Reaction amplification

To eliminate inhibitors in PCR, samples were washed three times with phosphate buffered saline and centrifuged at 3000g for five minutes. Afterwards, the remaining concentrate was mixed and used for the following examination. 100 µl of these homogenized samples moved to microtubes and left at 95°C for 20 min. 5µl of proteinase K was used for the samples that had a lot of proteins and were stood at 55°C for three hours and at 95°C in hot plate for 20 min.

Normal saline contain ten to hundred *L. pneumophila* ATCC33152 bacteria was used as positive control in PCR. Samples with negative result in culture and PCR were used as negative controls.

Oligonucleotide primers which were chosen from the published nucleotide sequences of *16S rRNA* gene with sequences (13):

Leg225- (5'-AAGATTAGCCTGCGTCCGAT-3')

Leg 858- (5'-GTCAACTTATCGCGTTTGCT-3')

PCR reaction included 5µl of bacterial genomic DNA (containing 100 ng), 0.15 mM dNTP, 40 Pmol of each forward and reverse primer (Cinna Gen, Iran), 1.5 mM MgCl<sub>2</sub>, 1X PCR buffer, 1.25 U Taq DNA polymerase and sterile distilled water up to 50 µL as the final volume.

PCR was performed in a GenAmp PCR system (Corbett, Germany) according to the following program: pre-denaturation for five-minutes at 95°C, followed by 30 cycles each containing denaturation at 94°C for 30 sec, annealing at 64°C for 20 second and extension at 72°C for 20 second, followed by a final extension at 72°C for five minutes.

### Analysis of PCR product

The PCR products were electrophoresed on 1.5% agarose gel for one hour at 85 V and 25mA, stained by SYBER Green and visualized under UV transilluminator. The amplification products were further evaluated by sequencing and restriction digestion procedure.

Statistical analysis was conducted to determine the number of positive samples for *L. pneumophila*. Perspective analyses were performed and data in the form rounded numerical values (percentage) was documented. Positive and negative values were calculated. Statistical analysis was using SPSS 10.0 for windows statistical package program.

## RESULTS

In this study, 50 BAL samples were collected as described in materials and methods that 40%<sup>20</sup> cases of them were bedridden in hospital and had medical document. 50% of the patients were 35 to 45 years old and the remaining 70 to 80. The review of patient's records show that all the patient were positive for *Legionella* had breath shortness and sodium concentration of in

their serum were equal or lower than 130 mq/L.

The specimens DNA were extracted and analyzed by PCR (figure1) and the amplification products were confirmed by sequencing. Of the 50 patients that were surveyed, 2 were culture positive (4%) and 4 were PCR positive (8%) for *L. pneumophila*. That cause of two cases wasn't recognized by hospital laboratory. On basis of results of hospital laboratory one of case was positive for pneumococcus and another one was positive for mycobacterium that in the study in one case, Legionella and pneumococcus and another case, Legionella and mycobacterium simultaneously were diagnosed. Patients that bacterium has been isolated, all were bedridden in hospital. The two specimens that were culture positive for *L. pneumophila* were PCR positive too. The numbers of positive cases were equal in women and men.

## DISCUSSION

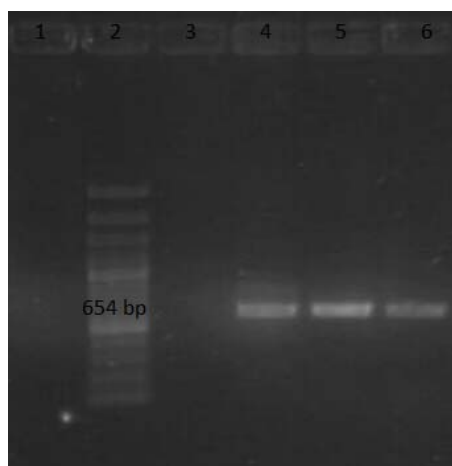
The accurate diagnosis of *L. pneumophila* is important for selection of an effective treatment. Because of Legionella strains are intracellular pathogen and many antibiotics commonly used to treat bacterial pneumonia are

ineffective against it. Therefore, there is an essential need for a rapid diagnosis of Legionella infection. In this study, methods of PCR and bacterial culture were used for detection of *L. pneumophila* in clinical samples of BAL and together were compared.

Bacterial culture is generally considered as the gold standard detection method of *L. pneumophila* in clinical samples. However the results of this study as well as those of other investigations particularly Cloud, Huang (9, 10, 14), have indicated that PCR has much higher sensitivity rate than culture. In the present study, the detection rate for *L. pneumophila* by culture was approximately 4%. Whereas, the detection rate by PCR was 8%, respectively. Some of studies have shown that PCR has more ability than of culture that our study proved this object too<sup>15</sup>.

The lower extent of culture detection may in part be attributed to intracellular bacteria that find in alveolar space and respiratory secretory as well as loss of viability during specimen collection and transport. Organisms that die before incubation, of course cannot be cultured, whereas the DNA of dead organisms can still be detected by PCR<sup>16</sup>. On the other hand, bacterial culture can take 5 days or more. *L. pneumophila* not only requires special culture media to grow, but also technical expertise in crucial for successful cultivation; whereas, PCR is rapid (<8 hours) and cost effective for detection of it in a single amplification reaction. In addition, unexpected pathogens such as *Blastomyces dermatitidis* and *Nocardia* species are able to grow on BCYE medium. Therefore, specific PCR for *L. pneumophila* is a good option.

In this study, no of the PCR -negative cases was not culture-positive. So PCR reaction results are not affected to the presence of Taq polymerase and reaction inhibitors, traces of blood and antibiotics in clinical specimens. Sequence variability and genome degradation are alternative source for potential negative PCR results. Analysis of PCR amplification products by sequencing indicated that all bacterial species belonged to one type. Furthermore, no sequence divergence has been detected for all the PCR amplified products. Therefore, targeting the 16S rRNA gene for detection of Legionella DNA is a suitable target for laboratory probing of *L. pneumophila*.



**Fig. 1.** Analysis of PCR product on gel agarose. Lane 1, Negative control, lane 2, DNA size marker (100bp DNA ladder, SM#333), lane 3, Negative sample without product. Lane4. 654 bp *L. pneumophila* amplification product in sample. lane 5, positive control (*L. pneumophila* ATCC33152), lane 6, 654bp *L. pneumophila* amplification product in sample of patient. (this sample was also positive)

In summary, the PCR assay developed in this study using only one primer pair has proven to be a simple, rapid, and more sensitive method for detection of *L. pneumophila*. Rapid detection of *L. pneumophila* is clinically very significant, particularly in the management of pneumonia due to these bacteria that may be cause of death. We therefore recommend that this PCR assay may be used, instead of bacterial culture, for rapid diagnosis of *L. pneumophila* in clinical samples especially BAL.

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