Hospital acquired pneumonia: Comparison of culture and real-time PCR assays for detection of Legionella pneumophila from respiratory specimens at Tehran Hospitals

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HOSPITAL ACQUIRED PNEUMONIA: COMPARISON OF CULTURE AND REAL TIME PCR ASSAYS FOR DETECTION OF *LEGIONELLA PNEUMOPHILA* FROM RESPIRATORY SPECIMENS AT TEHRAN HOSPITALS

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Legionella pneumophila is an important etiological agent in both hospital and community acquired pneumonia. The sensitivity of culture for isolation of *L. pneumophila* from clinical specimens is low and time consuming. Similar problem also exists when the method of direct immunofluorescence is used. To detect this organism quantitatively from respiratory specimens, a Taq Man based real time PCR targeting the *mip* sequence was developed. Both real time PCR and culture methods were applied on 262 respiratory specimens from 262 ICU patients with pneumonia admitted to 5 different hospitals in Tehran. The results of real time PCR were compared with those obtained by culture. Real time PCR and culture found 12 and 4 specimens, respectively, as positive for *L. pneumophila*. Its technical specificity (100%) was checked against a panel of microorganisms consisting of both gram positive and gram negative bacteria. Our real time PCR assay showed high sensitivity (100%) and specificity (96.9%) and could detect 200 organisms per ml from respiratory specimens. Using real time PCR as a screening method, the frequency of nosocomial pneumonia with *L. pneumophila* at Tehran hospitals was estimated as 4.58%.

Keywords: hospital acquired pneumonia, *Legionella pneumophila,* bronchoalveolar lavage, real time PCR

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Introduction

Leginella pneumophila, particularly strains within serogroups 1 and 6, is known as causative of both community acquired (CAP) and hospital associated pneumonia (HAP) [1–3]. Transmission to humans occurs through inhalation of aerosols generated from an environmental source such as cooling towers, shower heads, whirlpools, and other devices [1]. While, the health standards and economic status have affected the prevalence of CAP with *Legionella* spp. in the population, the case fatality rate for legionellosis ranges from 10% to 15% [4]. However, the mortality can increase to 80% in hospital-acquired legionellosis, particularly in immunosuppressed patients who did not receive appropriate antibiotics. Risk factors for mortality include virulence of the involved strain, delay in antibiotic therapy, and degree of immunosuppression [5].

Rapid and correct diagnosis is crucial for correct prescription of antibiotics within 4 to 6 hours of admission to inhibit the progression of infection [2]. Death occurs through progressive pneumonia, respiratory failure and/or shock, acute kidney and multi-organ failure within 2–10 days of incubation period [6]. Clinical symptoms can not differentiate legionellosis from pneumonia caused by other organisms. Dissemination of this organism through the circulatory system may cause infections in other organs including kidneys, liver, heart, central nervous system, lymph nodes, spleen, bone marrow and skin. Bacteraemia, renal failure, liver function abnormalities, watery diarrhoea, nausea, vomiting, headache, confusion, lethargy, and other central nervous system abnormalities have been associated with these infections [6].

Diagnosis of infection by conventional culture techniques requires a selective medium such as BCYE with antibiotics and supplements. The culture takes five to seven days to yield visible colonies since in vitro growth of *Legionella* spp. is slow [1, 2, 7]. Of 3,254 cases of legionellosis reported by Centres of Disease Control in a 10 year period, 37.5% were confirmed by culture [3]. Moreover, certain strain described as *Legionella* like amoebal pathogens (LLAPs) cannot grow on *Legionella* selective culture media [1].

Serological assay used in medical centres also have limitations, i.e. immunofluorescence assay (FDA) may be impeded due to the delayed rise in *Legionella* antibody levels with the onset of infection. Detection of *Legionella* antigen from urine specimens can be used only for serogroup 1 and has low sensitivity for legionella other than *L. pneumophila* serogroup 1 [1, 7, 8]. To increase the sensitivity and specificity, PCR based methods have been described for detection of *Legionella* DNA from sputum, urine, BAL and blood. The PCR target is based

on the sequences of the gene encoding macrophage infectivity potentiator (mip), 16S rRNA and 5S rRNA genes [9–11].

Prior to this study, limited data were available on the role of *L. pneumophila* as causative agent of HAP in Iran. The objective of this study was to develop a real time PCR to screen the patients with respiratory tract infection for this pathogen and to determine the cultured – confirmed cases of legionellosis at Tehran Hospitals. To our knowledge, this is the first study in which both conventional culture techniques and a molecular assay is used to determine the prevalence of HAP with Legionella in Tehran.

Materials and Methods

Clinical specimens and culture

A total of 262 clinical specimens including sputum (n = 31), bronchus aspirates (n = 7) and bronchoalveolar lavages (n = 224) were received from hospitals A (n=53), B (n = 48), C (n = 49), D (n = 69) and E (n = 43) in 2009–2010. These specimens had been collected from impaired respiratory patients admitted to ICU wards.

Patients who stayed in hospital more than 96 hours and suffered from previously absent respiratory dysfunction and showed signs of pneumonia in chest X-ray were included in this study. The outpatients with pneumonia were excluded from the study. Patients were selected under supervision of pulmonary specialist.

The specimens were processed for bacterial culture and real time PCR assay. From every collected respiratory tract specimen, 1 ml was taken and treated by heating at 50ºC for 30 min to inactivate the organisms other than *Legionella* spp. [12]. After treatment, 100 µl of each specimen was inoculated on a BCYE (Becton, Dickinson and Company, MD, USA) medium supplemented with glycine, vancomycin, cyclohexamide and polymyxin B (GVPC) [6, 12].The plates were incubated under microaerophilic conditions at 35 C for 4–7 days. The Gram negative rods or coccobacilli were identified as *Legionella* by failure to grow on blood agar and positivity for catalase and oxidase reaction [13, 14]. *Legionella* spp. have an absolute growth requirement for L-cysteine and without this amino acid they are unable to grow, colonies with typical morphology on selective media are subcultured onto BCYE and BCYE without L-cysteine. Those isolates that grow on BCYE but fail to grow on this media may be presumptively identified as *Legionella* spp. [6] . Direct fluorescent antibody kit (Prolab, Texas,

USA) was used to determine the serogroup 1 of *L. pneumophila* as instructed by the supplier.

The susceptibility of positive culture isolates to antimicrobial agents was assessed using disk diffusion test. Specimens that gave positive results for *Legionella* in real time PCR and culture were also cultured on Blood agar and EMB agar to detect other possibly involved organisms.

Primer design

The sequence of *mip* gene (macrophage infectivity potentiator), was selected to design primers and probe using Primer 3 plus (http://www.bioinformatics.nl/cgi-bin/primer 3 plus/ primer 3 plus.cgi). The *mip* gene encodes a 24 kDa protein that promotes the entry of bacteria into macrophages and amoeba [9]. It has enough conserved sequence to be used for discrimination of *L. pneumophila* from other species of this genus [9–11, 15, 16]. The primers and probes of mip gene used in real time PCR was shown bellow as described previously [17].

F: GTATCCGATTTTCCGGGTTT
R: TTTGATGGCAAAGCGTACTG
P: JOE_CAACGCCTGGCTTGTTTTTG-BHQ1

DNA extraction

We used phenol chloroform method for DNA extraction [17, 18]. In our real time PCR assay, 5 µl of extracted DNA solution was used as template.

Standards, sensitivity and specificity

L. pneumophila type strain NCTC 11192 (purchased from the Health Protection Agency, London, UK) was used as positive control in all experiments. Suspended cells were adjusted to the turbidity of 0.5 McFarland. The approximate number of cells at this turbidity is 1.5×10^8 CFU/ml. The *L. pneumophila* culture was serially diluted from 10^{10} CFU/ml to 10^2 CFU/ml in 0.85% NaCl to draw the standard curve and to determine the sensitivity of RT-PCR. This range of dilution is significant and reflects the linearity of standard curve due to limitation of JOE

dye excitation at this range. At concentration of over 10^{11} cells, the curve does not follow linearity. The optical density of dilutions was also measured at 600 nm using spectrophotometer and the results were compared with the results of real time PCR for determination of sensitivity of reaction [17, 18].

To control the quality of the assays, each dilution of standard was run in triplicate. After optimization and qualification of standards curves, the dilutions series were run along with every test series.

DNA from all clinical specimens were extracted and subjected to real time PCR in duplicate and the mean number of target copies was recorded. The efficacy of real-time PCR was determined by slope of standard curve: $E = 10^{(-1/\text{slope})} - 1$ [17, 18]. The correlation coefficient was considered equal or higher than 0.99.

Real time PCR was run every standard strain of DNA and samples had good correlation cycle threshold value (Ct) of real time PCR and number of CFU/ml [17].

Real-time PCR analysis and quantification

Optimization

The protocol for DNA extraction from respiratory specimens and the control strain has been described previously [17, 18].

To optimize the amplification conditions, the parameters that affect the real-time PCR including buffer, probe and primer concentrations, $MgCl_2$ concentration, and annealing temperature were checked [19]. The final optimized PCR reaction consists of 0.4 µM of each primer and probe, 3 µL dNTP (10 mM), 3 mM $MgCl_2$, 1 unit Taq polymerase (Metabion, Martinsried, Germany), 5 µM PCR buffer, and 5 µL of DNA template in total volume of 45 µl with double distilled water. The cycling program was adjusted as follows: initial denaturation at 95ºC for 5 min followed by 40 cycles of 95ºC for 15 s, 60ºC for 35 s.

Setting the threshold

The point at which threshold met the product's detected fluorescence was regarded C_T value [20]. Whenever the CT of 10^{10}, 10^8 and 10^6 copies number were 13.1 ± 0.6, 19.2 ± 0.5 and 25.4 ± 0.8, respectively, the results of our study was acceptable according to the following formula:

$2^{(CT1-CT2)}$ = fold difference in the amount of starting target [20].

Verification of specificity

To check the technical specificity of our primers and probes, DNA from *E. coli* (NTCC 21157), *S. aureus* (ATCC 29213), *E. feacalis* (ATCC 29212), *P. aeruginosa* (ATCC 49189), *A. baumannii* (NTCC 12156), *Stenotrophomonas malthophilia* (NTCC10257), *K. pneumoniae* (NTCC 5056), *Salmonella enterica* (ATCC 10708), *Mycoplasma pneumoniae* (NTCC 10119).

L. pneumophila NCTC 11192 and water were used as positive and negative control, respectively. Beta actin gene (primer and probe) was used as internal control with all samples to detect PCR inhibitor as recommended by Mehndiratta et al. [21]. The human white blood cell DNA was used as external control since *mip* primers and probe do not react with human genome.

Results

The male to female ratio was 1.30 (148 versus 114) and medium age of patients was 58 years (range, 39–85 years). Of 262 respiratory tract specimens, 12 were positive in real time PCR assay and 4 yielded growths in BCYE agar. The results of culturing confirmed the identity of the isolated organisms as *L. pneumophila*. The number of CFUs for *L. pneumophila* in positive culture were $>10^5$ CFU/ml and real time PCR showed these specimens contained $>10^9$ copies/ml (Table I).

Three isolates (A3, A4, and D4) belonged to serogroup 1 as determined by direct fluorescent antibody. The serogroup of the 4th isolate (D2) was not identified as serogroup1.

The number of CFUs for *L. pneumophila* in positive culture were$>10^5$ CFU/ml and real time PCR showed these specimens contained $>10^6$ copies/ml (Table I).

Patients A3, A4, D2, and D4 were positive in culture. These patients were infected with high number of bacteria. The results cultures were confirmed quantitatively by real time PCR.

Patients at hospitals A and C were mostly treated with new macrolides such as azithromycin (500 mg orally or intravenously every 24 h). At hospitals B and D, the antibiotic therapy for *Legionella* included ciprofloxacin (400 mg intravenously every 8 h) or azithromycin (500 mg orally or intravenously every 24 h). The lung transplanted patients D2 and A4 exceptionally were treated with ciprofloxacin (400 mg intravenously every 8 h) and rifampin (300 mg orally or in-

Table I

The results of quantitative culture for 12 patients who were positive in real time PCR

Patients*	Gender	Age years	Culture CFU/ml	qPCR Copies/ml	Sample	Polymicrobial conditions	Underlying
A1	M	64	NG	1.84 E + 05	BAL	–	Heart disease
A2	M	41	NG	8.1 E + 03	BAL	*S. aureus*	Chemotherapy
A3	M	50	>100000	8.84 E + 11	BAL		Lung cancer
A4	M	41	>100000	3.24 E + 07	BAL		Lung transplant
A5	M	58	NG	6.01 E + 03	BAL		Kidney transplant / diabetes
B1	M	82	NG	4.23 E + 09	BAL	α-haemolytic Streptococcus	Ageing
B2	F	57	NG	8.20 E + 02	Sputum		Colon cancer
C1	M	81	NG	2.33 E + 03	BAL		Ageing and heavy smoking
D1	M	53	NG	1.87 E + 03	Sputum		Tuberculosis
D2	M	77	>100000	5.30 E + 08	BAL		Lung transplant
D3	M	51	NG	2.41 E + 02	BAL		Lung cancer and heavy smoking
D4	M	50	>100000	3.58 E + 06	BAL		HIV^+, HBS^+

* Letters (A–D) shows the study hospitals, NG: No growth. M: male, F: female, BAL: bronchoalveolar lavages

travenously every12 h). D2, the 77 years old patient died due to severe pulmonary infection at ICU. Drug susceptibility testing showed that all 4 isolates were susceptible to azithromycin (20 μg), clarithromycin (15 μg) and tetracycline (15 μg). They showed resistance to amikacin (30 μg), neomycin (30 μg) and streptomycin (10 μg). Based on the results of real time PCR assay, the prevalence of infection with *L. pneumophila* among the patients with HAP is estimated to be 4.58%.

Of 12 specimens that were positive in real time PCR, 2 were co-infected with *Staphylococcus aureus* and streptococci. Of 12 cases detected by real time PCR, 11 were male and the twelfth was a 57 year old female who had colon cancer (patient B2). The results of bacteriology and real time PCR for all patients who gave positive results is shown in Table 1. At concentration of over 10^{11} cells, the curve doses not follow linearity. Standard curve of every dilution demonstrated a slope of –3.34 ± 0.1 and R2 > 0.99 with SD 0.1. The efficacy of PCR was >99.2 ($E = 10^{(-1/slope)} - 1$).

All bacterial DNA other than *L. pneumophila* used as controls in this study gave negative results in real time PCR.

Discusion

Early diagnosis followed by correct antibiotic therapy and tracing the source of the nosocomial infections are very important to save the lives of patients infected with *L. pneumophila*. Our patients had history of respiratory and cardiac dysfunction, solid organ transplant, heavy smoking, chemotherapy for cancer and corticotherapy.

Therefore, rapid and sensitive methods are required for diagnosis of *L. pneumophila* in patients with HAP [10, 22]. Culture has been recognized as golden standard for diagnosis but it takes 5–7 days to yield result and its sensitivity is lower than molecular techniques. One factor that affects the sensitivity of culture is the phenomenon of viability but non culturable state found among the *L. pneumophila* strains even before initiation of antibiotic therapy [1]. Strains of *L. pneumophila* may not grow after initiation of antibiotic therapy too [1]. Therefore, a technique such as real time PCR would help to resolve this problem and can be a good alternative. Real time PCR can be also useful for surveillance study in the hospital and clinical setting to monitoring the presence of *Legionella* spp. in water supply and central heating warm water. Its rapid detection is economical to eradication of the pathogenic bacteria sources before harming to inpatients of hospital. Depend on geographic location; the prevalence of legionellosis is different. This variation may be due to differences in definitions, diagnostic methods, surveillance systems and data presentation. It is also been attributed to publication bias, e.g. in 10 small studies (<100 patients) 13.2% of patients with pneumonia had Legionnaires' disease, but in 5 large studies (≥500 patients) the figure was 3.6% [23].

Little information is available on prevalence of HAP caused by *L. pneumophila* in countries around Iran. Prior to this study, information concerning the prevalence of *L. pneumophila* among the hospitalized Iranians was rare. To get reliable data on the prevalence of HAP caused by *L. pneumophila,* we increased both the number of hospitals (n = 5) and patients (n = 262).

When samples from the lower respiratory tract are tested, PCR has repeatedly been shown to have sensitivity equal to or greater than culture. Most diagnostic PCR assays of *L. pneumophila* have specific target regions within the 16S rRNA genes or the *MIP* gene. Our *mip* gene appears that this region cover all serogroups of *L. pneumophila* as determined by blasting of Genetic databases. Using *mip* gene in real time PCR assay, we found 12 positive results among 262 respiratory specimens. It was more sensitive than culture since the latest method yielded colonies from only 4 specimens. Moreover, real time PCR could detect the

organism while its number was low in the specimens, i.e., it enumerated 820 and 241 copies/ml from patients B2 and D3, respectively (Table 1). In patient B1, despite counting high copy number by real time PCR, the culture was negative due to consumption of antibiotics.

This technique proved to be specific for *L. pneumophila* as it gave negative results with DNA templates from other organisms. Our designed real time PCR assay showed high sensitivity (4/4, 100%), specificity (250/258, 96.90%), technical specificity (9/9, 100%) and could detect 200 copies of target (the first standard dilution). Based on the results obtained by real time PCR assay, we estimate the prevalence of *L. pneumophila* in patients at ICUs at Tehran Hospitals to be 4.58%. During 1995–2005 more than 32,000 cases of Legionnaires' disease and over 600 outbreaks has been reported to the European Working Group for Legionella Infections (EWGLI) [24]. The overall proportion of cases related to hospital infections halved during the 3 years in the Europe from 12.8% in 2000 to 6.0% in 2002. At the same time, 73% of the total nosocomial cases belonged to France, Italy and Spain [25]. Information concerning the legionellosis in the Middle East region is limited to the studies that focused on CAP. In one study conducted in Saudi Arabia, none of 500 patients with CAP were found to be infected with *L. pneumophila* [26]. The second report from that country stated incidence of 2.6% of legionellosis among CAP patients [27].

Differences in rates of infections have been attributed to factors such as practitioner awareness, sensitivity of the surveillance system, impact of legislation for controlling the development of the bacteria in the aquatic environment, and environmental factors [26–29]. For example, in Hospital E, none of the specimens gave positive result using both methods. This may be due to good hygienic condition of environment in this hospital since it uses good air filtration system in the cooling towers. Water is likely the source of infection in the hospitals where our patients were positive for *L. pneumophila* since the sampled water from these hospitals were positive by both techniques in this study (data is not shown).

With the current estimated frequency of HAP with *Legionella* at Tehran hospitals (4.58%), control measures should be taken to reduce the burden of infection. The risk of infection with this organism among the patients should not be ignored. Improvement in isolation of this organism and its identification is helpful in diagnosis. That is important for the laboratories that lack the molecular facilities such as real time PCR. While the organism was identified by culture or real time PCR, patients can be treated successfully by azithromycin. In a life threatening disease such as legionellosis, patient can be treated much earlier if the involved organism is detected by real time PCR.

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