Surveillance of *Legionella* species in hospital water systems: the significance of detection method for environmental surveillance data

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ABSTRACT

Monitoring of hospital water systems to prevent and control nosocomial legionellosis is important from a public health perspective. This study was conducted to survey the prevalence of *Legionella* contamination of hospital waters. A total of 44 water samples from the hot-water system of 11 hospitals were tested for *Legionella* by a culture method and a nested polymerase chain reaction (PCR) assay with *Legionella*-specific primers to identify the more sensitive method. Some physicochemical parameters and heterotrophic plate counts of water samples for possible association with *Legionella* contamination were also determined. The contamination rate of hospitals in our study varied between 64% (eight of 11)–100% based on culture method and nested PCR, respectively. Of the 44 water samples examined, 23% were positive for *Legionella* spp. by the culture method, while the nested PCR assay using the primers LEG448-JRP revealed 66% of the water samples being positive. Given the importance of monitoring hospital water systems for the presence of *Legionella* spp., the present PCR assay proved highly applicable for practical and sensitive surveillance of *Legionella* in such water systems. In addition, rapid monitoring of *Legionella* contamination could eliminate the potential exposure of high-risk patients through effective control measures.

Key words | culture, hospitals, *Legionella*, PCR, water

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INTRODUCTION

Legionella species, ubiquitous Gram-negative bacteria, are found in various natural and man-made aquatic environments (Pepper & Gerba 2004; Chin *et al.* 2005). A total of 54 species of *Legionella* have been characterized, 25 of which have been associated with legionellosis (Buse *et al.* 2012), an infection that may present as Legionnaires' disease with acute pneumonia or Pontiac fever, a self-limiting influenza-like illness (USEPA 1999; Lucas *et al.* 2011). However, *L. pneumophila* is the most common pathogenic species (Fields *et al.* 2002; Devos *et al.* 2005; Lee *et al.* 2010) and the causative agent of the majority of community and nosocomially acquired legionellosis (USEPA 1999; Lucas *et al.* 2011). Nosocomial legionellosis accounts for about 25–45% cases of reported Legionnaires' disease (Buse *et al.* 2012) and its incidence appears to be on the rise (Lin *et al.* 2011). Presence of *Legionella* in hospital drinking water is recognized as the major source of nosocomial legionellosis (Kusnetsov *et al.* 1993; USEPA 1999; Lin *et al.* 2011). *Legionella* spp. can aerosolize during water activities and be inhaled by individuals. Human inhalation of contaminated aerosols leads to *Legionella* infections (USEPA 1999), especially in highly susceptible individuals such as immunosuppressed or immunocompromised patients (Sehulster & Chinn 2003). The rate of mortality due to *Legionella* infections may exceed 30% for the patients (Breiman & Butler 1998). Because of the ongoing increase in immunocompromised patients (Anaissie *et al.* 2009), hospitals are now more often facing the problem of legionellosis. Therefore,

doi: 10.2166/wh.2013.064

the first approach is to prevent and control nosocomial legionellosis by monitoring of hospital potable water systems (Sehulster & Chinn 2003). Given the importance of surveillance of *Legionella* in hospital drinking water systems, rapid and sensitive detection methods are essential. The culture technique is the most commonly used method for surveillance of *Legionella* in hospital water systems (Devos *et al.* 2005; Ditommaso *et al.* 2011). However, due to the fastidious nature of the organism, successful culture requires a highly selective medium. Furthermore, *Legionella* are slow growing and require a prolonged incubation period (Templeton *et al.* 2003). Other issues with culture-based detection include the presence of *Legionella* in a viable but non-culturable (VBNC) state, overgrowth by other bacteria especially in environmental samples, and the method is labour-intensive (Devos *et al.* 2005; Buse *et al.* 2012). Polymerase chain reaction (PCR)-based methods could improve the speed, specificity and sensitivity of *Legionella* detection in environmental samples (Waterer *et al.* 2001).

This study was conducted to survey the prevalence of *Legionella* contamination of hospital water systems using culture and a nested PCR assay with *Legionella*-specific primers in order to identify the more sensitive method. Some physicochemical parameters and heterotrophic plate counts (HPC) of water samples for possible association with *Legionella* contamination of water system were also determined.

MATERIALS AND METHODS

A total of 44 water samples were collected from 11 hospitals of Isfahan University of Medical Sciences, Iran. Water samples were taken from four different points of the hot water system, including tap water outlets and showers at each hospital. For the microbiological analyses, two 500 mL water samples were collected in sterilized glass bottles. An additional water sample (100 mL) was taken for physicochemical analyses.

Culture method

Water samples were concentrated by membrane filtration (0.22 μm, 47 mm diameter, Millipore). Membrane filters were washed in 10 mL of the sterile phosphate buffer solution, shaken for 15–30 min and then placed in an ultrasonic bath for 5 min for optimum clean-up of the filters. Samples were subjected to both acid and heat treatment as described by Leoni & Legnani (2001). From all the concentrated and treated samples, aliquots of 200 μL were plated in duplicate on BCYE Agar (Buffered Charcoal Yeast Extract medium (Fluka) with added ferric pyrophosphate (Sigma-Aldrich), cysteine (Sigma-Aldrich)) and CCVC agar (BCYE agar with added CCVC selective supplement (Sigma-Aldrich)). The plates were incubated at 37 °C in a 90% humidified environment with 2.5% CO_2 and examined for 4–10 days for the presence of *Legionella* spp. Suspected colonies were subcultured on BCYE agar with and without cysteine for verification. Isolates which grew on BCYE with cysteine, but not on the other media, were subsequently confirmed and identified with cultural and biochemical testing as described by Leoni & Legnani (2001). Finally, HPC were determined using R2A agar (Merck) and incubated at 35 °C for 48–72 h.

PCR detection

Water samples were concentrated by membrane filtration and centrifugation. To extract the DNA, pellets were resuspended in distilled water and frozen in liquid nitrogen and heated in boiling water three times. The DNA was further extracted and purified using Promega DNA Extraction Kit (Promega Wizard® Genomic DNA Purification Kit, Madison, USA) according to manufacturer's instructions. In the first PCR step, a ~1,420 bp fragment of the 16S rRNA gene region of bacteria was amplified using the bacterial primer set Eubac27F and 1492R (Lane 1991) to check the nucleic acid extraction as well as the presence of inhibitors. For the detection of *Legionella* species, a nested PCR technique was applied in order to increase the sensitivity. In the second PCR step, the LEG448-JRP primer set was used for optimum sensitivity (Yamamoto *et al.* 1993; Jonas *et al.* 1995). LEG448 is similar to JFP (Jonas *et al.* 1995) with the exception of one more base pair (G) at the 5′-end.

PCR amplification was conducted in a final volume of 25 μL consisting of 2.5 μL of 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTPs, 2 units of Taq DNA polymerase, and 1 μL of DNA. All PCR assays contained a

positive (DNA of *L. pneumophila*, NCTC 12821, FEPTU, HPA Center for Infections, London, UK) and a negative (molecular grade water without DNA) control. PCR was performed with an initial denaturation step for 5 min at 95 °C followed by 30 cycles of 94 °C for 45 s, 55 °C for 1 min and 72 °C for 90 sec, and a final extension at 72 °C for 5 min. PCR products were analyzed by agarose gel electrophoresis using 1.5% gels containing ethidium bromide together with a DNA molecular weight marker. Gels were viewed on a UV transilluminator (UV Tech, France), and DNA fragment sizes were compared with the 50 and 100 bp ladder DNA.

DNA sequencing for identification of *Legionella* species

A number of PCR products were analyzed for DNA sequencing and DNA sequences analysis was undertaken by BLAST algorithms and databases from the National Center for Biotechnology (www.ncbi.nlm.nih.gov).

PCR sensitivity evaluation

To evaluate the sensitivity of the nested PCR test, a suspension of *L. pneumophila* with a concentration equivalent to 10^8 cells per mL, according to the McFarland turbidimetric standards, was made. Serial dilutions were prepared from the suspension, and DNA subsequently extracted and tested by nested PCR.

Physicochemical analyses

Water temperature, pH (Corning pH Meter), and residual free chlorine (METERRC) were determined at the time of sample collection. Concentrations of iron, manganese and zinc were measured by flame atomic absorption spectrophotometer (Perkin-Elmer 2380). Turbidity was also measured (Eutech Instruments Turbidimeter TN-100).

Statistical analysis

All statistical calculations were made with SPSS software. Results were analyzed by correlation analysis, *t*-test, one-way analysis of variance, and chi-square test. A *P*-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Colonization of the water supply in a hospital has been shown to correlate with the incidence of nosocomial Legionnaires' disease (Kool *et al.* 1999) and the presence of *Legionella* in hospital water systems poses a serious health risk (Wellinghausen *et al.* 2001; Lin *et al.* 2011). Several surveys have been performed to assess the contamination rate of hospital water with *Legionella* (Leoni *et al.* 2005; Lee *et al.* 2010; Napoli *et al.* 2010; Tesauro *et al.* 2010). Based on the surveys 12–75% of hospitals were contaminated with *Legionella* (Hall *et al.* 2003). In the study, a total of 44 hot water samples were collected from 11 hospitals in Isfahan and examined for the presence of *Legionella* spp. by culture and nested PCR. Of the 11 hospitals surveyed, *Legionella* species were detected in eight (64%) of the hospitals by the culture method. We found that no more than two of the four water samples (50%) from each hospital were positive by culture (Table 1). Results of the nested PCR, however, revealed that at least one sample per hospital was positive by PCR, and so *Legionella* spp. were detected in all (100%) hospitals. The culture method also revealed 23% (10 of 44) of the water samples being positive for *Legionella* spp. (Table 1). Cell concentrations of *Legionella* spp. ranged from 35 to 350 CFU/L, with a mean of 135 CFU/L. In contrast to some studies (Leoni & Legnani

Table 1 | Results of culture and nested PCR for detection of *Legionella* spp. in hospital water samples

	Number of positive samples (%)	
Hospital No.	**Cultured method**	**Nested PCR**
Hospital 1	1 (25)	2 (50)
Hospital 2	1 (25)	3 (75)
Hospital 3	2 (50)	3 (75)
Hospital 4	1 (25)	3 (75)
Hospital 5	0 (0)	1(25)
Hospital 6	2 (50)	3 (75)
Hospital 7	1 (25)	2 (50)
Hospital 8	2 (50)	3 (75)
Hospital 9	0 (0)	4 (100)
Hospital 10	0 (0)	2 (50)
Hospital 11	0 (0)	3 (75)
Total	10 (23)	29 (66)

2001; Bartie *et al.* 2003), the selective medium, CCVC, could not increase the recovery of *Legionella* spp., and no statistically significant difference was seen between the detection of *Legionella* by the two types of media.

By nested PCR, the LEG 448-JRP primer set gave a 387 bp fragment in 66% (29 of 44) of the samples (Table 1). However, all samples that were found positive with the culture method were also positive with the PCR assay with LEG448-JRP primer set (Table 1) and PCR inhibition was seen in none of the 44 samples tested. Figure 1 shows agarose gel electrophoresis of PCR products amplified by the LEG448-JRP primer set.

These results suggest that the PCR assay is more sensitive than the culture method. Several studies have found higher sensitivity of PCR based methods than culture methods (Devos *et al.* 2005; Edagawa *et al.* 2008). Wellinghausen *et al.* (2001) reported the rates of detection of *Legionella* to be 98.7% by PCR compared to 70.1% by culture. Since in some cases bacteria were not detected with the culture method but with the PCR assay, it may be that they are in a VBNC state that is still virulent and thus able to cause human infection (Buse *et al.* 2012). Moreover, some *Legionella* species are sensitive to selective agents in the medium (Bopp *et al.* 1981; Miyamoto *et al.* 1997; Devos *et al.* 2005). Overgrowth by other bacteria, especially in environmental samples, could also hamper identification of *Legionella* spp. by culture. However, the PCR assay would detect dead cells and so there may be a risk of overestimation of positive samples; but the difference is probably not related to such detection; indeed, *Legionella* spp. colonize hospital water systems as biofilm microorganisms. Biofilm formation plays a key role in the persistence of *Legionella* in the water systems and protects the bacteria from adverse environmental conditions, including water disinfection (USEPA 1999). Therefore, if disinfection of hospital water system was not performed, it is unlikely that water samples contain dead cells.

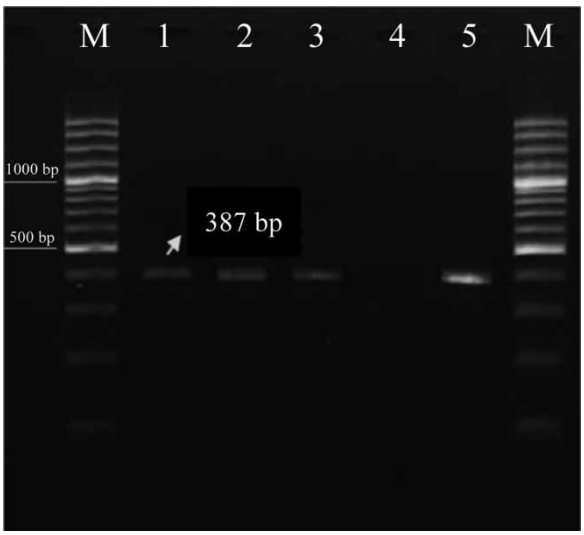

Figure 1 | Agarose gel electrophoresis of PCR products amplified by the LEG448-JRP primers.

Choosing the target gene for the PCR assay is an important factor for the efficiency and specificity of the detection (Miyamoto *et al.* 1997). In the present study, the sensitivity of the nested PCR was determined using extracted DNA from serially diluted *L. pneumophilla* samples. The lowest detection limit was 1CFU/tube by the nested PCR with the LEG 448-JRP primers. We used the 16 s rRNA multi copy gene as the PCR target in the first PCR and nested PCR to further increase sensitivity. However, the nested PCR assay is a qualitative method that only recognizes the presence or absence of *Legionella* and quantitative techniques such as real time PCR should be taken into use in order to further understand the prevalence of *L. pneumophila* (Devos *et al.* 2005). Nonetheless, there is a controversy about the importance of quantitative assays of *Legionella* (Leoni *et al.* 2005) and according to the guidelines of Centers for Disease Control and Prevention (CDC) there is no acceptable level of *Legionella* contamination (Sehulster & Chinn 2003). This stresses the importance of a rapid and sensitive detection method for *Legionella* in water samples to eliminate the potential exposure of at risk patients.

For confirmation, 25% of the PCR products were sequenced and the sequences deposited in the GenBank database (Table 2). Comparison of the 387 bp 16S rRNA gene sequence with sequences available in GenBank indicated that all sequences belonged to the genus of *Legionella*. This proves that no false-positive results were obtained. *L. pneumophila* was the most frequently detected species (82% of the samples) (Table 2). Biochemical testing of *Legionella* colonies revealed that most of them were *L. pneumophila*. Other investigators have also reported *L. pneumophila* as the most common isolate in aquatic samples (Wellinghausen *et al.* 2001; Devos *et al.* 2005; Leoni *et al.* 2005). Sequencing of the PCR products in the

Table 2 | Sequence based identification of *Legionella* spp. by BLAST searches

No.	Strain[a]	Accession No. in GenBank
1	*L. pneumophila*	JX962714
2	*Legionella* sp.	JX962717
3	*L. pneumophila*	JX962713
4	*L. pneumophila*	JX962710
5	*L. pneumophila*	JX962707
6	*Legionella* sp.	JX962711
7	*L. pneumophila*	JX962712
8	*L. pneumophila*	JX962708
9	*L. pneumophila*	JX962706
10	*L. pneumophila*	JX962715
11	*L. pneumophila*	JX962709

[a]Most closely related organism.

study of Devos *et al.* (2005) showed that 76% of the *Legionella* contaminated aquatic samples were positive for *L. pneumophila*. Whereas this species accounts for the great majority of *Legionella* infections, our result has confirmed the need for regulatory monitoring and disinfection of hospital hot drinking water systems.

Several factors may contribute to the survival and amplification of *Legionella*. In the present study, the possible association between the presence of *Legionella* and the microbial and physicochemical quality of the water samples was also evaluated. Mean concentration of heterotrophic bacteria was 41 CFU/mL. Kusnetsov *et al.* (1993) found that the total number of bacteria was much lower in *Legionella*-positive samples than in *Legionella*-negative samples in cooling water systems. In contrast, the results of Edagawa *et al.* (2008) showed a significant association between *Legionella* contamination and HPC in hot water systems. However, our study showed no relationship between the HPC and the presence of *Legionella*. This was also demonstrated by Leoni & Legnani (2001).

Optimum temperatures for reproduction of *Legionella* range from 32 to 45 °C (USEPA 1999). In our study nearly all samples were in the optimum range and statistical analysis showed no significant correlation between the temperature and presence of *Legionella* spp. The results of the physicochemical analyses of hot water samples are presented in Table 3. Moreover, no correlation was seen between the presence of *Legionella* and physicochemical parameters; only a statistically significant difference was seen in relation to the contamination by *Legionella* detected by culture and the levels of manganese ($P = 0.01$). The results of Leoni *et al.* (2005) showed lower content of magnesium in water samples contaminated by *Legionella*. However, there are conflicting reports about factors affecting *Legionella* growth and survival, and our results confirm the reports which found *Legionella* in a wide range of aquatic environments.

CONCLUSION

Active surveillance of hospital water systems by a sensitive, rapid and practical method is an important tool for effective prevention of *Legionella* infection. The nested PCR assay described here provides sensitive surveillance of hospital water systems. In addition, rapid monitoring of *Legionella* contamination could eliminate the potential exposure of high-risk patients through effective control measures.

ACKNOWLEDGEMENT

This research was conducted with funding from the vice chancellery for research at the Isfahan University of Medical Sciences (Grant No. 387346) as a MSc dissertation.

Table 3 | Physicochemical characteristics of the water samples

	Temperature (°C)	pH	Turbidity (NTU)	Residual chlorine (mg/L)	Fe (mg/L)	Mn (mg/L)	Zn (mg/L)
Minimum	29.00	5.20	0.52	0.00	0.11	0.04	0.05
Maximum	48.00	7.60	1.80	0.21	1.14	1.10	2.63
Mean	38.26		1.00	0.06	0.61	0.34	0.29
Standard deviation	4.76		0.32	0.05	0.28	0.24	0.42

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First received 30 January 2013; accepted in revised form 25 July 2013. Available online 12 September 2013