

Detection of *Legionella* Contamination in Tabriz Hospitals by PCR Assay

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ABSTRACT

Purpose: The present study was designed to evaluate the occurrence of *Legionella* contamination in the tap water of Tabriz hospitals, Azerbaijan, Iran. **Methods:** One hundred and forty water samples from diverse water supply systems of 17 hospitals were collected and analyzed for the presence of *Legionella* spp. by PCR assay. **Results:** In this study, 10 of 140 (7.1%) samples were positive for *Legionella* which *L. pneumophila* was detected in 4 (2.85%) water samples. **Conclusion:** In conclusion, hospital potable systems are the primary reservoirs for Legionnaires' disease. This study concludes that *Legionella* spp. are present in aquatic hospitals environment of Tabriz. Due to the serious risk of infections, it is better to make efforts to eliminate *Legionella* spp. in water supplies.

Introduction

Legionella spp. are gram-negative, fastidious and ubiquitous bacteria responsible for mild upper respiratory tract infections or pneumonia following inhalation of contaminated water droplets from a variety of water sources.¹ Probably 3 to 8% of all community-acquired pneumonias are caused by *Legionella* spp, and 85% of those pneumonias are caused by *Legionella pneumophila*.² Non-*L. pneumophila* species have also been reported to be infectious.³ The case-fatality rate for patients with legionellosis is 5 to 30%, with the elderly and immunocompromised patients being at greater risk.¹ *Legionella* infections can be the cause of extrapulmonary inflammatory diseases too.² Although hospital cases are not infrequent, most cases occur sporadically.¹ Numerous reports have shown an obvious association between the presence of legionellae in hot water supplies and the occurrence of legionellosis.¹⁻³ Therefore, for risk assessment of nosocomial *Legionella* infections, examination of hospital water systems is required.⁴ *Legionella* are detected by various methods. Culture is considered the gold standard for the laboratory detection of *Legionella* infections. It has a sensitivity of 50 to 90%; however, colonies appear after 3 to 4 days.² *Legionellae* are slow-growing bacteria, and successful culture needs selective media.⁵ Nucleic acid amplification tests have been shown to be helpful for the detection of *Legionella*.⁶ Recently, novel methods have been developed for finding of *Legionella* in water samples

by PCR methods to overcome the limitations of culture. PCR techniques have the advantages of quickness, detection of nonculturable legionellae, and easier handling of large samples.⁴

This study presents the results of an environmental investigation on *Legionella* spp. that was carried out on the water distribution systems of 17 healthcare facilities in Tabriz, Iran.

Materials and Methods

Sample collection

A total of 140 tap water samples were collected from diverse water supply systems of 17 hospitals. The size of hospitals ranged from under 200 beds to 500 beds. All hospitals were supplied with chlorinated domestic drinking water. All samples were transported to the laboratory of microbiology department and kept at 4 to 8°C until analysis, and water samples were concentrated by filtration through 0.45µm pore size nitrocellulose membranes (HTTP, Millipore, Ireland). The membranes were aseptically removed, put into sterilized 50mL tubes and resuspended in 10mL of the original water samples. Each concentrated water sample was shaken for 30 min to get out bacterial cells from the membranes.⁷ For acidic treatment, the chloride acid was used as the acidic buffer with a pH of 2.2 over a period of 15 minutes.⁸ The overgrowth of faster growing bacteria and fungi was decreased with the heat treatment of samples.⁷

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Microbiological analysis

Aliquots of 100µL of prepared samples were inoculated on plates of buffered charcoal yeast extract agar (BCYE) with supplements (Difco Laboratories, Detroit, Mich., USA), and the plates were incubated at 37 °C in a humidified atmosphere with 2.5% CO₂ for 6-14 days. Colonies with the typical ground glass form of *Legionella* were Gram stained and subcultured on selective BCYE [containing polymyxin B (80U/ml), vancomycin (0.5µg/ml) anisomycin (80µg/ml), cefamandole (4µg/ml)] and non-selective media, such as sheep-blood agar and MacConky agar. Colonies that grew on selective BCYE agar but not on non-selective media were considered supposed *Legionella*. The identification of *Legionella* spp. were done by biochemical tests.^{7,9}

DNA extraction and PCR assay

DNA extraction was performed as described previously.¹⁰ The PCR test was carried out in a 25µl of reaction mixture with the follow compositions: DNA extract (5µl), 0.1 units *Taq* DNA polymerase (Fermentas), 1.5mM magnesium chloride, 0.5mM of deoxy-nucleotriphosphate, 2.5 mM buffer, and 0.5µM of each primer (Table 1). For amplification, an initial denaturation for 4min at 95 °C was followed by 30 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s. In this study, DNA of *Legionella pneumophila* ATCC 33152 was used as positive control and DNA of a laboratory isolate strain of *E. coli* as negative control. The PCR products were analyzed by agarose gel electrophoresis using 1.5% agarose gel containing ethidium bromide, and gels were viewed on a UV transilluminator.

Table 1. Primers for PCR amplification of *Legionella* spp.

Target	Sequence	Product size (bp)	Ref
16S rRNA	GCTAATCTTAAAGCGCC	212 bp	11,12,13
	CCTGGCTCAGATTGAACG		
Mip	GCATTGGTGCCGATTTGG	168 bp	9,12
	GCTTTGCCATCAAATCTTTCTGAA		

Results

In this research, various water supplies of hospitals were investigated for the presence of *Legionella* spp. by culture and PCR methods.

Overall, 140 samples from various areas of hospitals were cultured, and *Legionella* spp. were isolated from 8(5.7%) of samples. Table 2 shows the number of *Legionella* spp. and the source of samples and the detecting methods. Two strains isolated from the water samples were identified as *L. pneumophila* by culture.

In order to detect *Legionella* spp., at first 16Sr RNA gene was amplified using PCR test (Figure 1). Of the total of 140 samples, 10 (7.1%) were PCR positive for 16Sr RNA. *L. pneumophila* was detected in 4 (2.85 %) samples of hospital water systems by Mip gene PCR. In this study, the culture method showed excellent correlation with PCR results (*P*v 0.001).

Table 2. The detection of *Legionella* spp. according to the source and the methods.

Source	Culture	PCR
Entrance water system	1	1
Heating system	1	3
Bathroom	3	3
Kitchen	3	3
Patient room	0	0
Ventilator	0	0
Total	8	10

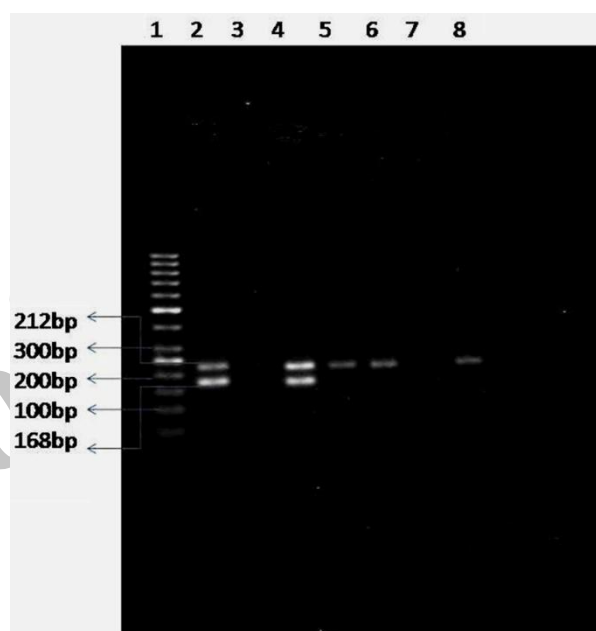


Figure 1. Amplified products of *Legionella* spp. by PCR, lane 1: 50 bp DNA ladder, lane 2: *Legionella pneumophila* positive control, lane 3: negative control, lane 4: *Legionella pneumophila* sample, lane 5, 6 and 8: *Legionella* spp. samples, lane 7: negative sample.

Discussion

The levels of *Legionella* contamination in hospital water system have been reported to correlate with the occurrence of nosocomial *Legionella* diseases.¹⁴ For risk evaluation of nosocomial *Legionella* infections; surveillance of hospital water systems is needed. A sampling program may be designed to perform an overall evaluation of the level of contamination of the hospital, or to detect the presence of *Legionella* during epidemiologic studies of clinical cases. In the absence of ascertained cases of legionellosis, the value of routine environmental sampling aimed at primary prevention is controversial.¹⁵ Although surveys of *Legionella* colonization in hospitals have been conducted in the UK, Canada, USA, and Spain¹⁶, but in Azerbaijan region, the health care facilities don't obtain routine environmental cultures. The results of this study showed that *Legionellae* was detected in Tabriz hospitals. To the best of our knowledge, this is the first

report of *Legionella* spp. isolation in this area. Many techniques have been reported to detect *Legionella*, including culture,^{7,13,17} Direct Florescence,^{7,17,18} specific PCR,^{7,17,19,20} and real-time PCR.^{4,6,11,13} Isolation of *Legionellae* spp. from water samples by culture technique is generally preferred. Culture is the gold standard and very important for antibiotic susceptibility tests, but it has some limitations such as the growth requirements of the organisms, long incubation periods, overgrowth of other bacteria, and nonculturable *Legionellae* in some environmental samples.⁴ PCR method has some advantages and some limitations. Non viable and non cultivable *Legionella* are detected by PCR. Our genus-specific 16S rRNA PCR test was detected all *Legionella* species. The 16S rRNA gene is exceptionally suited as a target gene since it exists in several copies per genome and thus allows a high sensitivity of the PCR. Another method using *mip* or 16S rRNA gene sequencing was also considered promising because it offer sequence-based identification for *L. pneumophila* and non- *L. pneumophila* species, but it is complex and time-consuming.³ We found 2 samples were positive for PCR but culture negative; it may be due to dead or non-cultivable *Legionella* spp. Dusserre et al. showed that *L. pneumophila* DNA detected by PCR in water samples treated with chlorine. Indeed, culture became negative after the addition of 0.5ppm chlorine for 24 h, whereas the PCR was preserved.²¹ In this study, 2 and 4 isolated strains were identified as *L. pneumophila* by culture and *mip* gene, respectively. There are nearly 40 specious *Legionella* spp. and *mip* target is specific for *L. pneumophila*.

In a previous study have shown that hospital water system may be contaminated by *Legionella* spp., and these strains can persist for long periods in water.¹⁴ According to an international study, 12-75% of all hospital samples are contaminated with *Legionella* in hospitals.²² Samples of hot water and biofilms were collected from 41 hospitals in Italy's Piemonte region between June 1999 and March 2008, and *Legionella* spp. were isolated from 32% of them.¹⁵ In some studies conducted in Iran, prevalence of *Legionella* isolation from hospitals was 36.6% and 22.7% (8, 14). In a USA national surveillance study of 20 hospitals in 13 states, 6 hospitals were colonized with *Legionella*.²³ In another study from Spain, the rate of *Legionella* contamination in 12 hospitals was reported 30%.²⁴ There was any information on the prevalence of the *Legionella* spp, and the value of this bacterium as a nosocomial pathogen is not clear in the Tabriz. Our study confirms that a large portion of water systems in hospitals are contaminated with the bacterium and this may play a key role as a risk factor in the safety of patients in hospitals.

Legionella species have been isolated from a wide variety of water types.⁷ In this research, the numbers of *Legionella* positive samples from the kitchens,

bathrooms, internal distribution system, and cooling tower were 3, 3, 3, and 1, respectively.⁸ Moreover, several authors have described the isolation of *Legionella* spp from showers, cooling towers and boilers,^{4,7} which is in agreement with the findings of our study. The results of the present study show that temperature has a key role in the positive samples. In this study, 13.72% of hot water samples were *Legionella* positive by PCR. The control of hot water supplies in hospitals is crucial, and several reports have shown a clear association between the presence of *Legionella* in hot water systems and occurrence of legionellosis.^{14,23}

Conclusion

In conclusion, hospital potable water systems are the primary reservoirs for Legionnaires' disease. Therefore, surveillance of water supplies of hospital is important, and the infection can be prevented by disinfecting hospital water. This study shows that both culture and PCR methods could detect *Legionella* spp. in water supplies. However, PCR is more sensitive and faster than culture.

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Conflict of Interest

There is no conflict of interest in this study.

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