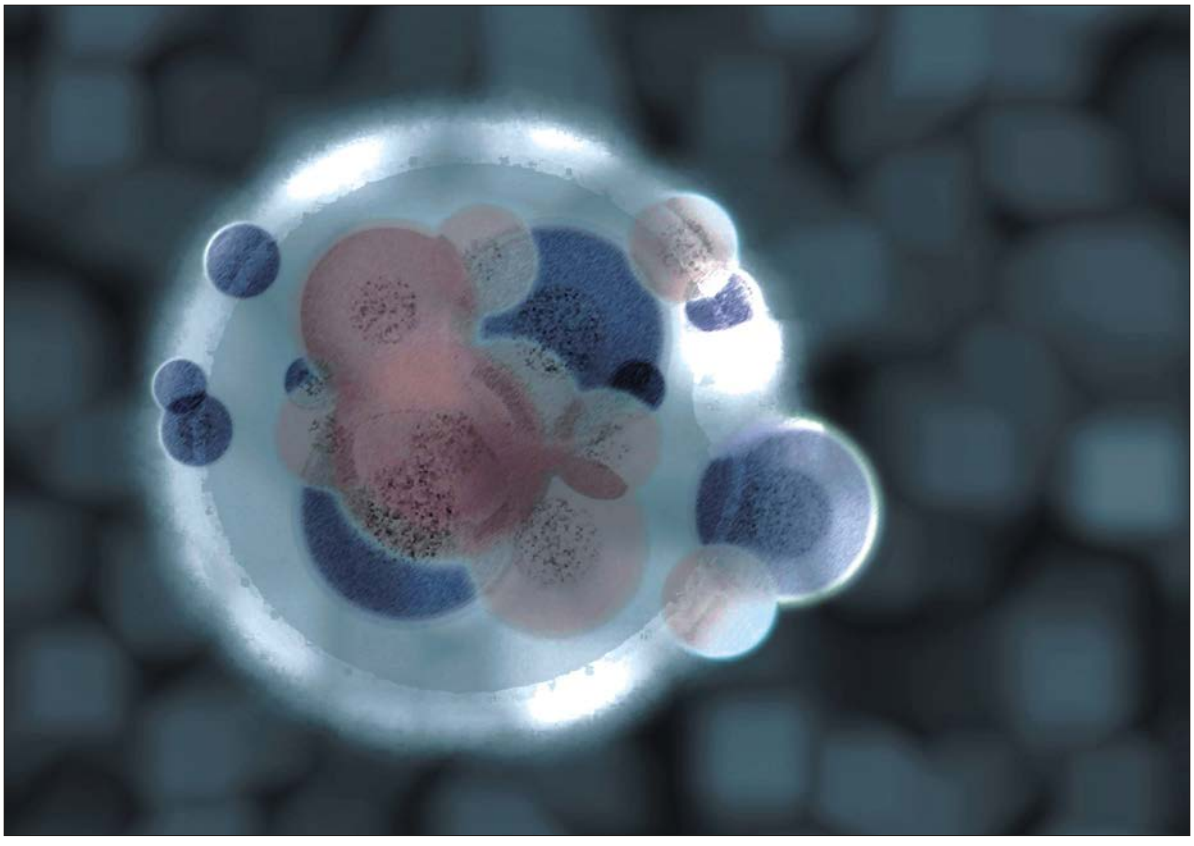

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Detection of *Legionella* in Hospital Water Supply using *Mip* Based Primers

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Abstract: The aim of this study was to evaluate the risk of legionellosis within the hospital water supplies. *Legionella* species are ubiquitous in various water systems. It is not surprising that hospital waters may be colonized with legionnaire bacterium. Cold and hot water samples from different hospitals of Tehran were screened, using standard culture method and PCR technique. A total of 132 cold and hot water samples were collected during the summer of 2006. All samples were concentrated and cultured on standard BCYE agar after pretreatment. The *mip* gene based primer system were then used to detect mip protein of *L. pneumophila* within each hospital water sample. The results were obtained from culturing and PCR compared to each other. The water samples that analyzed in this study were collected from hospitals and medical institution located in different parts of the city. *Legionella* were isolated from the total of 30 water samples (22.7%). The *mip* based primer sets by amplicon size of 630 bp detected *Legionella* genome from approximately (37%) of the culture positive samples that indicating they could be considered as *L. pneumophila*. The results showed that the risk of nosocomial legionellosis could not be excluded from the city hospitals due to significant contamination of the hospital water supplies. We concluded that hospital water screening for *Legionella pneumophila* contamination will help the diagnosis the threat of nosocomial outbreak of legionnaires' disease and also the evaluation of decontamination process of water supplies within high risk areas.

Key words: *Legionella* spp., hospitals water supplies, *mip*, PCR

INTRODUCTION

Legionella pneumophila is recognized as an important cause of atypical pneumonia. Legionnaire's disease is known to cause hospital-acquired pneumonia and may occur sporadically or as outbreak (Yu, 2000; Garcia-Nunez *et al*., 2008). *Legionella* spp. is ubiquitous in many water systems; it is not surprising that hospital water may be colonized with *Legionella pneumophila* and other species. Water systems of large buildings such as hospitals are often contaminated with *Legionella* and therefore, represent a potential danger to patients (Ozerol *et al*., 2006; Singh and Coogan, 2005; Kooler *et al*., 1999; Patterson *et al*., 1997). However, there is some controversy about the relationship between the presence of *Legionella* in hospital water systems and nosocomial legionellosis. The incidence of hospital-acquired pneumonia due to *Legionella* has been reported as ranging from 0-47% (Veronesi *et al*., 2007).

The presence of *Legionella* in hospital facilities, particularly in hot water distribution systems and cooling towers (Rivera *et al*., 2007) is an increasing problem. The most frequently described route of transmission is by inhalation of contaminated aerosols (Kool *et al*., 1999). Transmission has also been reported via nebulizer (Woo *et al*., 1992) and showers (Breiman *et al*., 1990; Fujimura *et al*., 2006) in contaminated water as used.

L. pneumophila is the most pathogenic *Legionella* species. It is also the cause of the frequent contaminations in environmental water sources. For risk evaluation of nosocomial legionellosis, surveillance of hospital water systems is needed. The degree of *Legionella* contamination in hospital water supplies has shown to be correlated with the incidence of nosocomial legionnaire's disease (Kool *et al*., 1999).

Culture method for the isolation of *Legionella* is gold standard, but it has limitations such as fastidious growth requirements of organisms, long incubation periods and the presence of viable and non-culturable *Legionella*. Recently, applying PCR technique, overcome the limitation of culture method. The *mip* gene PCR has shown to be specific for detection of all serogroups of

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L. pneumophila (Bej *et al*., 1991) PCR have been reported as a complementary method for screening of environmental samples (Ferreira, 2004).

The aim of this research was to screen the hospital water samples for *Legionella* contamination. A total of 132 water samples in various hospitals in Tehran were inspected. The culture positive samples results confirmed by *mip* PCR in colony and water samples. The results showed that routine surveillance for a hospital water distribution system is fundamental for public health and must include, as a priority, monitoring of *L. pneumophila.*

MATERIALS AND METHODS

Sample collection: A total of 132 cold and hot water samples were collected from the hospitals located in different areas of Tehran city, during the summer of 2006. The samples were obtained from tap water, shower head, nebulizers of hospital wards, cooling tower and chiller. Each sample was prepared in four liter sterile container according to the standard methods described previously (Hosseini Doust and Seal, 1998). Each water sample was concentrated aseptically by membrane filtration, using a three-piece PVE manifold (Millipore SA) and cellulose type HA membranes with a pore size of 0.2 μm (Millipore Corporation). The filter was then removed from the apparatus, cut to pieces and placed in 100 mL sterile plastic container along with 50 mL of the original filtrate and shacked vigorously for 30 min, in order to release bearing microorganisms.

Culture of water samples: All the samples were then kept at room temperature until use for further examination. A portion of 10 mL of the concentrated sample was removed aseptically from each container and subjected to heat treat (50°C) for 30 min (Palmer *et al*., 1993). An aliquot of 100 μL from each water sample was inoculated on to either Buffered Charcoal Yeast Extract (BCYE) agar containing α-ketoglutarate, glycine, vancomycin, polymyxin and cyclohexamide (Oxoid). The BCYE medium was supplemented with ferric pyrophosphate and L-cystein (Palmer *et al*., 1993).

The inoculated agar plates were sealed in plastic bags and incubated aerobically for up to 14 days at 37°C with a daily check of growth after three days. The colonies containing gram-negative bacteria grown after 4-7 days were isolated and examined for ability to grow on media with and without L-cystein. If the isolates grew up with typical colony morphology on BCYE agar at the presence of cysteine only, but not on BCYE without cysteine or sheep blood agar they were regarded as *Legionella.*

DNA extraction and PCR: In order to achieve PCR experiment, an aliquot of 1 mL from each water sample was subjected to 3 × freezing-boiling within 1.5 mL ependroff tubes in liquid nitrogen (5 min each) boiled for 10 min. The cell debris were then removed by centrifugation at 2000 for 2 min and the supernatants were centrifuged again for 10 min at 18000 x g and an aliquot of 20 μL from the bottom of the tubes was subjected to PCR experiment. Negative and positive controls under the same condition were included in all the PCR experiments. *Legionella mip* gene were amplified by gradient thermocycler.

For PCR amplification of the *mip* gene, a 630 bp fragment was amplified. Two 20-base oligonucleotides (Lpm-1, Lpm-2) bracketing a 600 base pair DNA fragment were synthesized as primers Lpm-1 (5-GGT GAC TGC GGC TGT (Jaulhac *et al*., 1998) TAT GG) was located at nucleotides 853-872 from the coding strand. Lpm-2 (5- GGC CAA TAG GTC CGC CAA CG) was located at nucleotides 1465-1484 complementary to coding strand.

PCR amplification was performed in a 0.5 mL reaction tubes in a total volume of 50 μL amplification mix, with 15 μL of sample DNA, 3 μL of $MgCl_2$ (final concentration, 4 mM), 50 pmol of each of primer and 1.0 units of taq DNA- polymerase and PCR-grade sterile water to a final volume of 50 μL. The PCR was performed in a gradient thermal cycler by the following protocol:

Initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 94°C for 1.5 min, annealing at 62°C for 2 min, extension at 72°C for 1.5 min and final extention at 72°C for 5 min (Fig. 1).

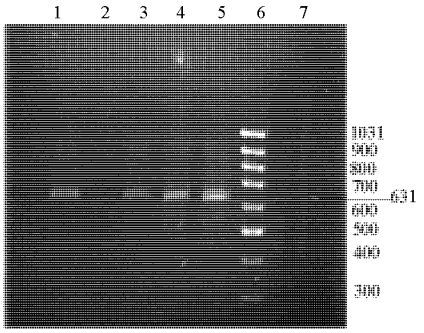

Fig. 1: Agarose gel electrophoresis of amplified DNA (35 cycle) from *Legionella* DNA extracted of hospital water by using mip primers. Lane 1 Marker, Lane 2, 3, 4: *Legionella* DNA extracted from water, Lane 5 water without *Legionella*, Lane 6: *Legionella pneumophila* as control positive, Lane 7: *Staph aureus*, Lane 8: *E. coli*, Lane 9: *P. aerojinosa*

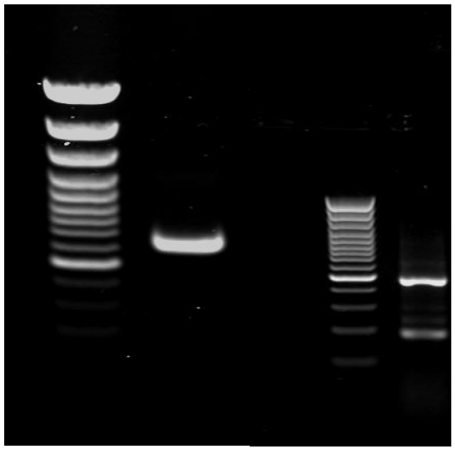

Fig. 2: RFLP analysis of mip amplification products. 630 bp amplification products were treated with kpn 1 restriction enzyme, two No. 400 and 200 bp fragments was yielded and showed the reaction specificity

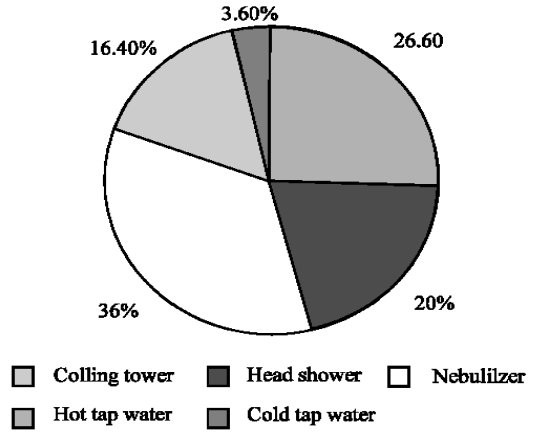

Fig. 3: *Legionella* spp. isolated from water of hospital water supplies by Conventional culture and PCR using *mip* primers

For quantification of the *mip* gene PCR product, a standard curve from one ng to one fg of *L. pneumophila* serogroups one DNA per PCR was also generated and detected by *mip* gene PCR.

Ten microliters of each sample, (containing amplified DNA) was electrophoreses through a 1.5% Agarose gel at 80 V in 0.5x TEB buffer (44.5 mM Tris HCl, 44.5 mM boric acid, 1.25 mM disodium EDTA). After staining with ethidium bromide (5 μg mL^{-1}), the gel was photographed under UV light.

RFLP analysis: PCR products (10 μL) were digested with Kpn1 (Fermentas) for 8 h at 37°C according to manufacturer's and the restriction fragments were analyzed by gel electrophoresis at 120 V in 1x Tris-acetate-EDTA buffer in 2.5% agarose gel and stained with Ethidium bromide. The fragments were visualized by UV light and the sizes of the restriction products determined (Fig. 2).

RESULTS AND DISCUSSION

The ecology of *Legionella* in hospitals water storage and distribution systems has been well documented. Little is known regarding the quality of water supplied to high-risk units. In the present study, various water distribution systems of the hospitals were screened for the presence of *Legionella* spp. by culture and PCR. The total of 132 samples from tap water, shower head, nebulizer and campus water systems (hot water, cold water and cooling tower) in the ICU, respiratory, CCU, renal transplant dialysis and oncology wards were cultured for *Legionella.*

Legionella spp. was isolated from the 30 (22.7%) water samples. *Legionella* containing the *mip* gene was isolated from 10 samples (7.57%) of hospital water supplies (Fig. 3).

Mip gene of *L. pneumophila* was detected from 2 (9.55%), 1 (6.6%), 1(7.4%) and 3 (12%) from ICU, CCU, renal transplant ward and nebulizer water samples, respectively (Table 1). Of the total of 132 water samples, 4 (3.03%) samples were determined to be positive only by PCR.

Previous investigators have shown that hospital water supplies may be contaminated by one of the multiple strains of *Legionella* spp. and that these strains can persist in water systems for long periods (Lawrence *et al*., 1999; Palmer *et al*., 1993). According to recent international surveys, 12-75% of all hospital water systems are contaminated with *Legionella* (Tkatch *et al*., 1998). The degree of *Legionella* contamination in hospital water supplies have been shown to correlate with the incidence of nosocomial legionnaires' disease (Ozerol *et al*., 2006).

Colonization of water systems by *Legionella* spp. is ubiquitous in hospitals throughout the world. Patients undergoing bone marrow or solid organ transplants and other immune-suppressed patients are at the highest risk for infection.

In this study the highest levels of contamination were found in the ICU and nebulizers water, respectively (Table 1). *Legionella* spp. was isolated from 8 (26.6%) cooling tower water samples, 11 (20%) head showers of which 9 (16.4%) isolates were from hot water and 2 (3.6%) from cold water samples (Fig. 4).

Table 1: Comparison of culture results with *mip*+ and *mip*- PCR results

	Culture		PCR	
Hospital wars	No.	%	*mip*+	*mip*-
ICU	8	26.9	2	6
CCU	4	13.4	1	3
RT & dialysis	2	6.6	1	1
Pulmonary dis.	2	6.6	1	1
Infection dis.	2	6.6	1	1
Pediatrics	1	3.4	1	0
Oncology	2	6.6	1	1
Nebulizers	9	36.0	3	6
Total	30	100.0	11	19

ICU: Intensive Care Unit; CCU: Critical Care Unit; RT and Dialysis; Renal transplantation and dialysis, Dis; disease

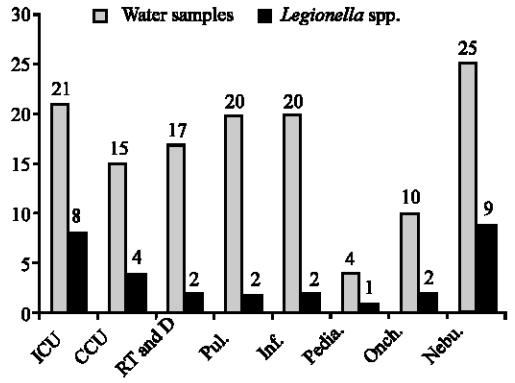

Fig. 4: Distributions of *Legionella* positive samples between collected water samples. ICU, Intensive Care Unit, CCU, Critical Care Unit, RT and Dialysis; Renal transplantation and dialysis, Pul., Pulmonary disease, Inf. infection disease, Pedia, Pediatrics, Onch., Oncology, Nebu., Nebulizer

Cooling towers have been implicated for nosocomial legionellosis. Recent studies, documenting the genetic identity of clinical and environmental isolates have proven that local outbreaks of legionnaire's disease may indeed originate from contaminated cooling towers (Keller *et al*., 1996). *Legionella* residents within biofilm are a particular problem in cooling tower systems. In addition to cooling towers, hot water system, respiratory therapy equipments, showers and faucets, whirlpool spas (Jernigan *et al*., 1996) have been linked to the increased occurrence of nosocomial legionnaire's disease. Hospital hot water systems are frequently colonized with *Legionella* species. In hospital wards, where immune-suppressed patients are treated, hot water systems should ideally be free of *Legionella* contamination. The control of hot water system colonization in hospitals is crucial to prevent clinical disease in patients and health care workers at risk. Several reports have shown a clear association between the presence of *Legionella* in hot water systems and the occurrence of legionellosis. These bacteria are present at the highest concentrations in biofilms within hot water systems (Stout *et al*., 1985). In the present study we isolated the *Legionella* from 16.4% of the hospitals hot water system and 36% of the nebulizers.

In this study, a *L. pneumophila*-specific PCR assay, based on the *mip* gene of *L. pneumophila* was developed. In total of 30 positive samples with culture, only 10 (33.33%) samples were positive for *mip* gene. The most likely explanation for this finding is that the *mip* target is specific for *L. pneumophila* (Cianciotto *et al*., 1989). Another possible explanation is that there are two species of *Legionella* in the sample, *L. pneumophila* and another *Legionella* species.

We found that four samples were positive with PCR but not with culture. The reason is not clear, but the occurrence of dead or non-culturable *Legionella* cells in water systems is a possible explanation.

CONCLUSION

The results suggest that both culture and PCR have advantages and limitations. PCR is sensitive and rapid. Non- viable and non-culturable *Legionella* are also detected by PCR. Culture method for isolation of *Legionella* is gold standard, but it has limitations such as fastidious growth requirements of organisms, long incubation periods and presence of viable and nonculturable *Legionella*, but the results of culture can be stored and used for exact identification and epidemiological studies. A clear advantage of PCR is its ability to detect *Legionella* in water samples contaminated by other fast growing bacteria. PCR and culture complement each other. PCR also promises to be rapid and sensitive technique to detect *Legionella* in both clinical and environmental specimens.

It is concluded that rapid detection and elimination of *L. pneumophila* in hospital water systems is necessary, in order to prevent further contaminations, routine environmental culture of the hospital water supply for *Legionella* has proven to be an important strategy in prevention. Hospital-acquired legionniares' disease can be prevented by disinfection of hospital water systems.

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