INVESTIGATION OF LEGIONELLA SPECIES IN TEHRAN'S HOSPITAL WATER SUPPLIES

MOHAMMAD RAFIEE[1], JAHANGIRI-RAD MAHSA [2]

ABSTRACT. – Investigation of Legionella species in Tehran's hospital water supplies. The present work was performed to investigate the presence of Legionella spp. and its common species in hospital water supplies. Considering the drawback of culture method, polymerase chain reaction (PCR) assays were developed to detect the gene 16S rRNA regardless of the bacterial serotype. Four well-established DNA extraction protocols (freeze & thaw and phenol-chloroform as two manual protocols and two commercial kits) were examined and critiqued to release DNA from bacterial cells. A total of 45 samples were collected from seven distinct hospitals' sites during a period of 10 months. The PCR assay was exploited to amplify a 654-bp fragment of the 16S rRNA gene. Legionella were detected in 13 samples (28.9%) by all of the methods applied for DNA extraction. Considerable differences were noted in the yield of extracted nucleic acids. Legionella were not detected in any of the samples when DNA extraction by freeze & thaw was used. Omitting this method and comparing manual protocol with commercial kits, Kappa coefficient was calculated as 0.619 with $p < 0.05$. Although no meaningful differences were found between the kits, DNA extraction with Bioneer kit displayed a higher sensitivity than classical Qiagen. Showerheads and cold-water taps were the most and least contaminated sources with 55.5 and 9 percent positive samples, respectively. Moreover two positive samples were identified for species by DNA sequencing and submitted to the Gene Bank database with accession Nos. FJ480932 and FJ480933.

Keywords: Legionella, Hospital water supplies, DNA extraction, PCR, 16S rRNA

1. INTRODUCTION

Legionella are gram-negative, aerobic, and sporeless bacteria which some of their species like *Legionella pneumophila* are implicated in severe pulmonary nosocomial infections (Legionnaire's Disease) and Pontiac fever, especially in immuno-compromised patients, as well as in the elderly (Morio et al.2008, Leont et al, 2001). Indeed twenty one species of *Legionella* are pathogens for humans, especially in patients with the chronic pulmonary disease (Sambrook and Russell, 2001). Inhalation or micro aspiration of *Legionella* from contaminated environmental sources such as hot water systems and cooling towers water is the most frequently route of transmission.

[1] Department of Environmental Health Engineering, School of Public Health, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[2] Young Researchers and Elites Club, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Transmission has also been reported via nebulizer and showers in contaminated water as used (Fields et al. 2002, Doust et al. 2008). Although, its occurrence as a seasonal pattern is very common, its negative impact on health and hygiene requires a specific treatment cycle that is quite often a combination of management and sanitary stages. Hospitals are common habitats for the bacterium, where the bacterial niches are amply found. Hospitals provide the most likely places for susceptible people to contract the diseases. Outbreaks of Legionellosis have been reported from hospital patients in many countries with an incidence range of 0 to 47% (Morio et al.2008, Yu et al. 2008). Furthermore, there has been a steady increase in the incidence of sporadic cases reported. Consequently, national *Legionella* surveillance programs (Joly et al. 2008, Pryor et al. 2004, Bartie et al. 2003). In Iran, however, hospital-acquired Legionnaire's Disease has rarely been reported and environmental surveillance for *Legionella* in hospital water systems to provide useful data for risk assessment and prevention of hospital-acquired Legionnaire's Disease has never been systematically performed. DNA-based techniques are innovative tools for routine quality control assessment in environmental water samples and are thought to be valid alternatives for cultural.

The *polymerase chain reaction (PCR)* is considered the most adaptable and prevalent DNA-based assay technique, which is highly specific and sensitive alternative method to standard culture isolation, especially when rapid results are needed. In the context of this view, this study was conducted to investigate the presence of bacteria belonging to the *Legionella* genus in water supplies of some hospitals in Tehran, the capital city of Iran. The impact of water quality on *Legionella* existence was also determined. In spite of the large amount of the data available for the various indicator species in water samples, to our knowledge, this is the first attempt to gather information to monitor the presence of this bacterium in Iranian hospital water systems and there is no systematic study to assess the efficiency of chlorine disinfection. For more confirmation, randomly two of isolates determined as *Legionella* spp. were sequenced as well.

2. DNA extraction and PCR assay

One liter of samples was filtered through 0.2µm mixed cellulose ester membrane filters (Schleicher & Schuell) in a stainless-steel filter holder with a water aspirator. Each membrane was aseptically scraped, cut into smaller pieces and placed into sterile, screw-capped containers with 10 ml of the original sample. The samples then were sonicated for 5 min (Bandelin Sonorex) at 35 KHz, and shaken for 15 min to dislodge bacterial cells from the membranes. The eluate was transferred into a 15 ml conical centrifuge tube and centrifuged (2000g, 20min) to remove cell debris. Total DNA was extracted from concentrated water samples using freeze & thaw and/or phenol & chloroform methods. DNA extraction using freeze & thaw method was conducted by placing of 1mL of each concentrated water sample within 1.5mL microtubes and alternating application of freezing the samples in liquid nitrogen and their incubation in water bath in the temperature of 100 °C for three times. The

suspension was then centrifuged again (18000g, 10min) and an aliquot of 20 µL from the bottom of the tubes was transferred to new microtubes.

Extracted DNA was stored at -20°C until PCR. Amplification reactions were preformed according to what described earlier by Hsu. The PCR primers LEG 225 (5'-AAGATTAGCCTGCGTCCGAT-3') and LEG 858 (5'-GTCAACTTATCGCGTTTGCT-3') were used to amplify a 650 bp fragment of the 16S rRNA gene of *Legionella* species. Each 25 µl of reaction contained 20 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 20 pmol of each primer, and 1u of Tag Polymerase (Roche Biotech) in the PCR buffer. The cycling conditions were 94ºC for 5 min, followed by 30 cycles at 95ºC (30 sec), 64ºC and 74ºC for 20 sec each, and 1 cycle of 72ºC for 5 min in Thermocycler (Techne USA). PCR products were loaded onto a 2% agarose gel containing ethidium bromide. To confirm the obtained results, DNA sequencing was prepared by the 16S rRNA. The PCR products of two *Legionella* isolates were sequenced at MWG (mwg_biotech.com, Germany).

3. Comparison among DNA extraction methods

Several studies have compared DNA extraction methods and reported that their abilities to recover bacterial DNA were different, indicating that no single DNA extraction method is optimal for all bacteria (Whitehouse and Hottel. Queipo-Ortuño et al. 2008). In this study, four different procedures (two commercial extraction kits and two classic manual protocols) were evaluated and compared to obtain DNA from hospital water samples. DNA was extracted from water samples using these four methods and followed by PCR amplification of bacterial 16S rRNA gene. Overall, *Legionella* were detected in 13 samples (28.9%) by all four methods used for DNA extraction. The comparison of these methods regarding their relative ability to extract DNA and detect *Legionella* from samples bore significant differences according to the results obtained (Table 1). As shown in this table, *Legionella* were not detected in any of the samples when DNA extraction by freeze & thaw method was performed. Likewise, samples revealed low levels DNA of *Legionella* (4/45 corresponding to 8.9%) by Qiagen kit. DNA extraction by phenol-chloroform and Bioneer kit, however, revealed the most positive samples for *Legionella*, i.e., 8 out of 45 (17.7%) and 12 out of 45 samples (26.6%) showed contamination with *Legionella*, respectively. Better performance of Bioneer kit may be driven from the fact that columned method was applied for DNA extraction of cells (DNA adsorbed to membrane column), though DNA was precipitated by alcohol in the case of Qiagen kit. Four samples were detected positive by Qiagen DNA extraction kit, which were positive with Bioneer DNA extraction kit too and three of which were also positive with phenol-chloroform. One sample was positive only by phenol-chloroform and four only using Bioneer kit. The 32 resting samples were negative by all three methods.

Table 1. Legionella prevalence by the source

Sampling source	*Legionella* positivity (No. of positive/total No.)				
	DNA extraction protocol				Total*
	Manual		Kit		
	Freeze & Thaw	Phenol-chloroform	Qiagen	Bioneer	
Cold water tap	0/11	1/11	0/11	1/11	1/11 (9.1%)
Hot water tap	0/8	0/8	0/8	2/8	2/8 (25.0%)
Showerhead	0/9	4/9	1/9	4/9	5/9 (55.5%)
Cooling tower	0/13	2/13	2/13	4/13	4/13 (30.8%)
Hot water tank	0/4	1/4	1/4	1/4	1/4 (25%)
Total*	0/45 (0%)	8/45 (17.7%)	4/45 (8.9%)	12/45 (26.6%)	13/45 (28.9%)

*Considering overlap.

4. Legionella prevalence and species identification

Identification of *Legionella* specie was based on the presence of an amplified product of 654 bp. Analysis of PCR results revealed a great diversity with regard to the sources from which samples were taken. Figure 1 also demonstrates the results of *Legionella* monitoring in the seven hospitals by the source. In general, showerheads were the most contaminated source with 58.3 percent positive samples. Similarly, the samples from cooling waters, hot water taps, and hot water tanks yielded fairly comparable results (30.8%, 25%, and 25%, respectively). However, this was not the case with the cold-water tap samples and only 9% were positive for *Legionella (Table 2)*. For medical units with a high risk of legionellosis five positive samples in all were detected [Pediatrics stem cell transplantation (0), Bone marrow transplantation (0), Cancer Dep. (1), Maternity (1), Cardiac surgery (0), ENT (2), and Infant (1) wards. Two positive samples were identified for species by DNA sequencing. DNA for sequencing was prepared by the 16S rRNA. The PCR products of two *Legionella* isolates were sequenced at MWG (Germany), DNA sequence was used to search the Gene Bank database, and the database entry with the highest percentage similarity was taken to identify the species. Nucleotide sequences data have been submitted to the Gene Bank database with accession Nos. FJ480932 and FJ480933.

5. CONCLUSIONS

Considering the important role of DNA extraction methods with regard to performance in downstream molecular applications and the usual low amount of bacteria in environmental water samples such as hospital water, the use of optimized methods in detection of *Legionella* by PCR assays is critical. Molecular techniques based on PCR assay offer a rapid, practical,cost-effective and sensitive alternative for detection of *Legionella*. Although the concentration of *Legionellae* in the sampled hospital water systems was not determined, given the high positive rate of *Legionella* colonization, hospital-acquired legionellosis might be under diagnosed in Tehran. It calls for urgent control measures to minimize the transmission rate of *Legionella* from the source to the host and to prevent an outbreak.

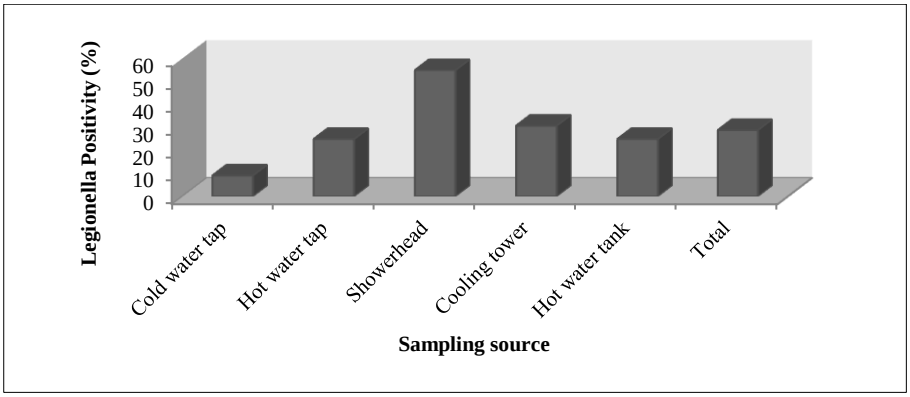

Fig. 1. ***Legionella prevalence by the source***

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