## Original Article

# Identification of Legionella in the Hot Water Supply of a General Hospital in Isfahan

H. Movahedian Attar PhD*, M.R. Shahmansouri PhD*, A.A. Neshat MSc**, M. Fazeli PhD***

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***Background:*** Legionella is a gram negative, aerobic, and sporeless bacterium which is readily found in ventilation systems, cooling towers, hot water distribution systems, bathrooms, swimming pools, and fountains. Legionella is implicated in the legionnaires' and Pontiac fever diseases. Hospitals are common habitats for the bacterium, where the bacterial growths are amply found and that provide the most likely places for susceptible people to contract the diseases. Given the importance of hospitals in this regard, this survey was carried out in a General Hospital in Isfahan.

***Methods:*** For the purposes of this study, a total of 30 samples were taken according to standard methods from hot water distribution system at various points in the kitchen, the bathrooms, the internal distribution system, and the cooling towers (8, 8, 8, and 6 samples, respectively). After that temperature, pH, and residual chlorine of samples were determined, the samples were transferred to lab where they were inoculated on a base medium of BCYE-a and two selective media of GPVA and CCVC. The plates were then incubated at a temperature of 37 ˚C and a humidity of 90%. The colonies were then identified and counted.

***Results:*** The tests showed that 11 from the total 30 samples were contaminated with Legionella, accounting for 36.6% of the samples. The numbers of Legionella positive samples from the kitchens, bathrooms, internal distribution system, and cooling tower were 4, 3, 3, and 1, respectively. From the total 30 heat and acid pretreated samples inoculated on base and selective media, 36.6% were Legionella positive while from the untreated samples, 6% grew on the base medium and 23% on selective media. Total mean of residual chlorine was 0.25 mg/l, pH= 7.6 , and average temperature was 31.1 ˚C. The results of biochemical tests Blood agar, Catalz, Urea agar, Gelatin agar, Motility, and gram staining were –, +, –, +, +, and –, respectively. In addition to these tests, Legionella colonies were grown by Direct Fluorescent Antibody, confirming their presence.

***Conclusion:*** The results indicate that temperature is a critical factor in Legionella sp.'s proliferation. An average 0.25 mg/l of residual chlorine showed to have no effect on disinfecting the bacterium. Heat pretreatment proved to be more effective than the acidic one in removing nuisance factors and, finally, bacterial growth was higher on the selective media than on the base medium.

***Keywords***: Legionella, water supply, hospital hot water.

Legionella bacterium was identified as the causal agent of legionnaires' disease in 1976 and its role in various outbreaks of the disease was determined[1]. Legionella is a gram negative, aerobic, sporeless, and rodlike bacterium with 42 different species[2]. It has been found to cause the two diseases of Legionnaires' and Pontiac fever[3]. Although everybody may be susceptible to these diseases, middle-aged people and the elderly, especially smokers and those with chronic pulmonary diseases and immunity system deficiencies such as AIDS, cancer, diabetes, or patients with kidney failures receiving dialysis are especially susceptible[3, 4]. The bacterium is found both in natural aquatic bodies and in water distribution systems as in hotels, hospitals, clubs, public buildings, and factories. Contaminated water

*Assistant Proffessor and Associate Proffessor, Department of Environmental health, School of Public Health, Isfahan University of Medical Sciences, Isfahan, Iran.
** Department of Environmental health, School of Public Health, Isfahan University of Medical Sciences, Isfahan, Iran.
***Associate Proffessor, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

distribution system in hospitals is one of the most known sources of Legionnaires' disease[5]. The bacterium is more resistant than other organisms to common standard disinfecting methods. It may, therefore, be found even in disinfected waters with residual chlorine content[6]. WHO proposes a number of methods such as heat shock, discharge of hot water remaining for long periods in pipes, and observance of the procedures for constructing new distribution systems[4].

A great many outbreaks of this disease have been reported in different parts of the world, provoking a large number of studies on Legionella aimed at reducing the health risks associated with this bacterium. Annually 8,000 to 18,000 cases of the Legionnaire's disease are reported in USA, of which 10 to 20% are the cases reported during epidemics with a death toll of about 20 to 40 percent[7]. Hospitals in different countries around the globe have been surveyed for Legionella contamination. The results of the surveys in UK revealed that the water distribution systems in 70% of the 40 studied hospitals contained Legionella growths. This is while in one study 60% of the studied hospitals in western Pennsylvania (15 hospitals), 23% in Scotland (39 hospitals), 68% in Quebec, Canada (84 hospitals), and in another study 12% of the hospitals in UK (out of 17 hospitals) were found to be Legionella positive[8].

Like most other pathogens, Legionella has also imposed rather high expenses on public health systems in most countries. Pneumonia is the sixth cause of deaths in the United States, including an annual expense of around $23 billion. This is while Legionella pneumophila alone accounts for 4.1 to 20.1% of pneumonia cases[9]. From these, 20 to 30% of cases have been related to nosocomial agents[10]. Not many studies have been conducted in Iran on bacterial identification. In one study in 1994, 289 samples which included 187 environmental samples were used to separate 7 different strains of Legionella pneumophilla[11]. In another study in 1997, no Legionella strains were removed from 200 clinical samples[12].

No study has been conducted in Isfahan to remove and identify Legionella from water distribution systems in hospitals. Given the fact that hot water is supplied in hospitals, the possibility for Legionella growths in places like bathrooms, kitchens, cooling towers, and the distribution system and the consequent infections cannot be ignored. The present study is an attempt to determine the frequency of Legionella in the water consumed in a general hospital in Isfahan.

## Materials and Methods

This study was conducted over the period from January to December 2003. The culture media used consisted of BCYE-a (Buffered Charcoal Yeast Extract-alpha) as the base medium, and GPVA (Glycine, Polymyxin B, Vancomycin, Anisomycin) and CCVC(Cephalotin, Colistine, Vancomycin, Cyclohexamide) as selective media. In containers prepared according to standard methods, thirty 3 liter samples of water were taken from four different parts of the hospital[13]. The points and number of sampling included 8 samples of hot water from the kitchen, 8 samples from the internal distribution system, 8 samples from the bathroom, and 6 samples from the circulating water in the cooling tower. After that temperature, pH, and residual chlorine of the samples were measured, they were transferred to the lab to be concentrated on nitrocellulose filters with a mesh pore of 0.45μm. The filter was then removed from the unit and placed aseptically in 10 ml of buffered phosphate solution, previously prepared and sterilized. The solution was kept in steam bath for 10 minutes. In order to remove interfering nuisances in the solution, 15 samples were subjected heat pretreatment while the other 15 were subjected to acidic treatment. The chloride acid and potassium chloride were used as the acidic buffer with a pH of 2.2 for acidic treatment over a period of 15 minutes[13]. The heat treatment was accomplished by keeping the buffer phosphate solution containing the sample at a temperature of 50° C for 30 minutes[14] .The distribution of samples on culture media consisted in three untreated samples of 0.1 ml each inoculated on the base medium and three untreated samples of 0.1 ml each inoculated on the selective media. The same inoculation was repeated after acidic or heat treatment of samples. One of the three plates from each medium was cultured using the steric method while one was cultured linearly. Upon culturing, the samples were incubated at a temperature of 37° C and a humidity of 90%[13]. The colonies took three to four days to form when the conducting confirming tests on the samples became possible. These tests included cultures on blood agar, urea agar, Catalz, gelatin melting, motility, and gram staining. In one case,

however, the DFA (Direct Fluorescent Staining) test was used to confirm the colonies formed. All the tests were conducted according to standard methods for the examination of water and wastewater tests[14]. The analysis of the results was performed according to Freidman and Coxon non-parametric statistical analysis methods.

## Results

From the 30 samples taken from the under question hospital, 11 were Lagionella positive upon culture which account for 36.6% of the total samples. The greatest number of positive samples belonged to the group taken from the kitchen sampling point, 50% of which samples were positive on the average. The lowest number of positive samples belonged to the cooling tower sampling point which account for 16% of the total number of samples. From the 15 samples receiving heat treatment, 23% were found to be Legionella positive. From the samples subjected to acidic treatment, 13% were positive. From the total number of samples subjected to both types of treatment, only 7 samples were Legionella positive on the selective media and only 2 samples on the base medium. These account for 23% and 6% of the total samples, respectively. Figure1 shows the colonies formed on the base medium (BCYE-a). Tables 1 and 2 present the overall results from the tests performed on the samples taken from different sampling points in the hospital.

**Table 1**. Overall results from the tests to determine Legionella frequencies

| Sampling point | Mean Temperature (˚C) | Mean pH | Total mean residual Chlorine(mg/l) | No. of samples | No. of positive samples | Percent |
|---|---|---|---|---|---|---|
| Kitchen hot water tap | 43.1 | 7.5 | 0.26 | 8 | 4 | 50 |
| Bathroom hot water tap | 38.6 | 7.6 | 0.25 | 8 | 3 | 37.5 |
| Internal distribution system | 34.8 | 7.6 | 0.23 | 8 | 3 | 37.5 |
| Cooling tower discharge | 8 | 7.6 | 0.26 | 6 | 1 | 16.6 |
| **Total** | - | 7.6 | 0.25 | 30 | 11 | 36.6 |

**Table 2**. Average No. of bacteria observed in positive cases of the samples taken from different points (CFU/L)

| Sampling point | Non-pretreated Samples | | *Pretreated samples* | |
|---|---|---|---|---|
| | Selective media | Base medium | Selective media | Base medium |
| Kitchen hot water tap | 644 | 166 | 1775 | 1375 |
| Bathroom hot water tap | - | - | 922 | 466 |
| Internal distribution tap | 388 | 366 | 1588 | 1366 |
| Cooling tower discharge | 166 | - | 466 | 300 |

## Discussion

The results from the present study show that temperature has a role to play in the positive samples such that lower temperature of the water from the cooling tower resulted in lower number of positive cases (Table 1). The statistical analysis also confirms this point by indicating a significant difference among the temperatures of the samples taken from the kitchen, bathroom, internal distribution

system and those taken from the cooling tower ($P<0.05$) while no significant difference is observed among the other samples. According to Table 1, the mean total residual chlorine was 0.25 mg/l; as despite this quantity of residual chlorine, Legionella bacteria were observed in the samples, it may be rightly concluded that the bacterium is resistant to this level of chlorine concentration. This finding agrees well with the results obtained by Berg et al[6]. No significant differences were observed among the different samples taken in this study with respect to their pH levels ($P>0.05$). According to Wadowsky et al, the acceptable pH level for Legionella varies between 5.5 and 9.2 [6]. As the pH levels in the samples were in this range, we can say that pH was not a limiting factor. Statistical analysis shows a significant difference between the results obtained from the two sets of treated and untreated samples ($P<0.05$) and, therefore, pretreatment yields more Legionella positive samples and greater numbers of colonies. As also mentioned earlier, the percentage of positive samples on selective media outnumbered those on the base medium. This also agrees well with the results from the work by J. Verran et al (1994) on water samples in England[14]. Diagram 1 represents the percentages of positive cases in the modes of pretreated and untreated and the two selective and base culture media.

From the two pretreatment methods employed in this study, the heat pretreatment method was more effective in removing nuisance elements than the acidic pretreatment method. This finding matches the results from J. Varren et al[14]. Diagram 2 is a comparison of the positive cases from the two heat and acidic pretreatment methods.

## Conclusion

The results obtained from the present study reveals that temperature was more effective on Legionella growth in the samples and that the lower temperature of the water in the cooling tower resulted in reduced number of positive cases observed in the tests. Total residual chlorine at a concentration of 0.25 mg/l was not capable of disinfecting Legionella, meaning that the bacterium remains resistant at such low chlorine concentrations. Pretreatment was shown to be effective in producing better conditions for Legionella growths in the samples. The study also showed that Legionella grows better on the selective media than on the base medium and, consequently, the number of colonies grown on the former was lower.

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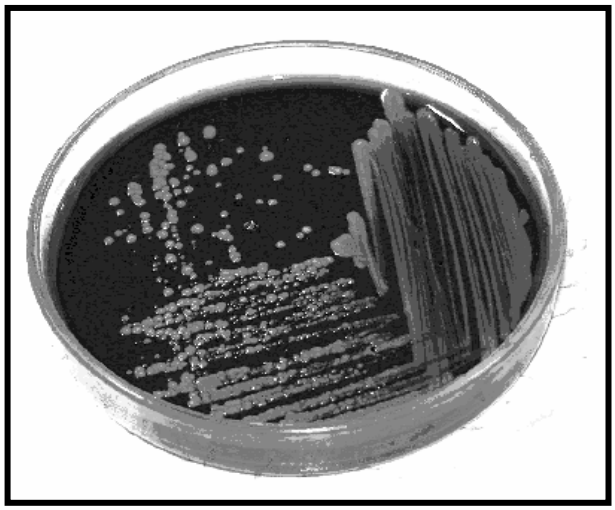

**Figure 1**. Legionella bacteria grown on BCYE-a medium

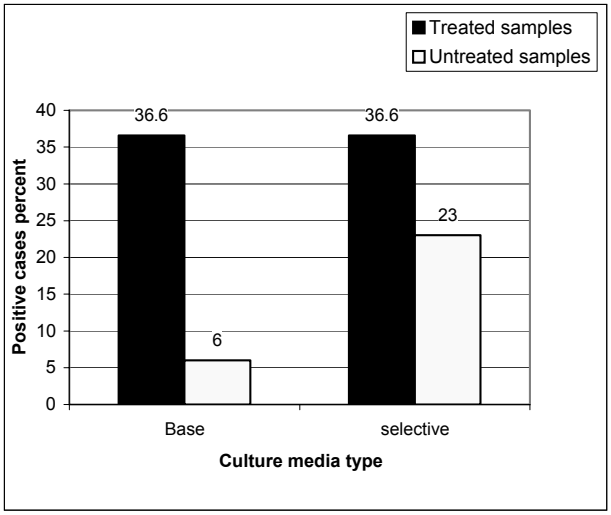


**Diagram 1.**Percentage of positive cases observed in the pretreated and untreated samples on the selective and base media.

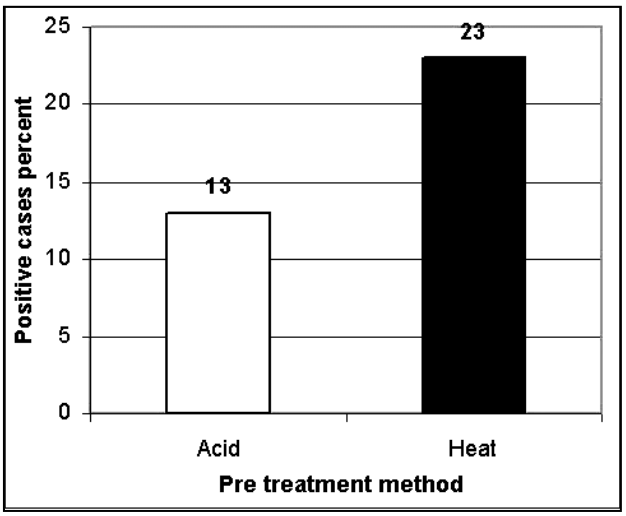


**Diagram 2.** Percentage of positive cases observed in the heat and acidic pretreated samples.

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