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Original Article

The Efficacy of Residual Chlorine Content on the Control of *Legionella* Spp. In Hospital Water Systems

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Abstract

Background: Outbreaks of legionellosis may be a side effect of institution-water treatment. However, the long-term outcomes and the predictive factors of *Legionella* prevalence in such systems have still not been fully studied. This study was therefore conducted to investigate the prevalence of *Legionella* spp. and to evaluate the role of bacteriological water quality parameters on its prevalence and removal in hospital water systems.

Methods: A total of 45 samples were collected from distinct sites at seven hospitals in Tehran, Iran. The prevalence of this bacterium was assayed through a sensitive and specific technique for DNA detection using PCR. Multivariable stepwise regression analysis was used to explore the independent effects of the baseline factors on the incidence of *Legionella*. Two positive samples were also identified for species by DNA sequencing.

Results: *Legionella* were detected in 31.1% of samples. Showerheads and cold-water taps were the most and the least contaminated sources with 55.3 and 9 percent positive samples, respectively. Total mean of residual chlorine was 0.38 mg/L, with the peak value of 1.7 mg/L. *Legionella* detection was proportional to the residual chlorine content of water and the results indicated that residual chlorine content is a critical factor in the incidence and proliferation of *Legionella* (r=-0.33). The prevalence of *Legionella* also coincided with the prevalence of HPC and amoeba cysts.

Conclusion: The high positive rate of *Legionella* colonization shows that hospital-acquired legionellosis might be under diagnosed in studied hospitals. Further, *Legionella* colonization is independent of the type of water, system characteristics and of preventive maintenance measures.

Keywords: *Legionella*, Hospital water supplies, Residual chlorine, Cooling tower, PCR

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 immunocompromised patients, as well as in the elderly (1, 2). Indeed twenty one species of *Legionella* are pathogens for humans, especially in patients with the chronic pulmonary disease (3). 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 trans-

mission. Transmission has also been reported via nebulizer and showers in contaminated water as used (4, 5). Although, *Legionella* 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% (1, 5-7). Furthermore, there has been a steady increase in the incidence of sporadic cases reported. Consequently, national *Legionella* surveillance programs have been established for regular monitoring of environmental samples in these countries (6, 8-9). 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 culture methods. The 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. This method is especially favorable when the samples contain abundant and diverse microbiota and when fastidious and slow-growing bacteria like *Legionella* are to be detected. Despite the fact that culture method for isolation of *Legionella* is approved by International standard Organization (ISO) and many other national standards for water quality determination; over the past few years, molecular techniques based on 16S rRNA gene beside other genetic markers have been developed to analyze bacterial communities in environmental samples (10, 11).

It is believed that the presence of *Legionella* is related to the physicochemical characteristics of water. The correlation between the occurrences of *Legionella* and water quality parameters is important to identify the main factors promoting the growth of *Legionella* in hospital water systems and to optimize the facility maintenance and operation. However, it appeared that results concerning the physicochemical characteristics of water and *Legionella* occurrence were often inconsistent or even contradictory and strong disparities in relationship have also been reported. 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.

Materials and Methods

Sample collection and preparation

Forty five samples were collected from distinct sites at seven hospitals underlying Tehran University of Medical Sciences. This cross-sectional study was conducted from June 2011 to January 2012. Hospital water facilities sampled included tap cold and hot water, shower, cold water tank, hot water tank, and cooling tower water. Samples were collected in 1-litre sterile bottles directly from the outlet. Before sampling, a sterile swab was inserted into faucet outlets to dislodge the sediment. At the moment of sampling the electric conductivity (EC), temperature, pH, and free chlorine (DPD method) of each sample were recorded. Concentrations of hardness, calcium, magnesium (calculation method), iron and manganese (atomic absorption method) were determined in laboratory. Furthermore, samples were analyzed for microbiological quality using the standard plate

method to determine the total heterotrophic counts per milliliter at 37 °C (HPC) and the presence of amoeba cyst in water. HPC values were determined with the pour-plate method using R2A medium. Volumes of 1 ml were spread on plates and colonies were counted after 10 days of incubation at 37 °C. Analysis of the presence of amoeba cysts in the water was based on morphological characteristics of cysts. After filtration of samples through a 0.22 µm mixed cellulose ester membrane filters (Schleicher & Schuell), the filter was separated and directly placed on the non-nutrient agar medium prepared with Amoeba Page Saline. Plates were then kept at room temperature for two weeks and were monitored daily for the growth of amoeba during this period. Reversed contrast phase microscope with a 10x objective was used to investigate the presence of amoeba. All of the experiments were measured according to standard procedures reported in Standard methods (12).

DNA extraction, PCR assay, gel electrophoresis and DNA sequencing

One liter of samples was filtered through 0.22 µm mixed cellulose ester membrane filters in a stainless-steel filter holder with a water aspirator. Each membrane was aseptically scraped, cut into smaller pieces and placed into sterile containers with 10 ml of the original sample. The samples were then sonicated for 5 min (Bandelin Sonorex), 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 (2000 g, 20 min) 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 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 (18000 g, 10 min) 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 (10). 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 94 ºC for 5 min, followed by 30 cycles at 95ºC (30 sec), 64 ºC and 74 ºC for 20 sec each, and 1 cycle of 72 ºC 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).

Statistical analysis

Statistical analysis was worked out using SPSS software. Quantitative variables were expressed as mean ± standard deviation when the data was normally distributed, while variables were expressed as a median (range) when the data was not in a normal distribution. Quantitative variables were compared by Student's test. The X^2 test was used where applicable to compare categorical variables. Univariable logistic regression analysis and multivariable logistic stepwise regression analysis were used to explore the independent effect of the baseline factors on the incidence of *Legionella*. Differences were considered significant at a *P*-value of 0.05 or less.

Results

Identification of *Legionella* specie by PCR in samples 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. Table 1 demonstrates the results of *Legionella* monitoring in seven hospitals by the source. Quality characteristics of

hospital water sources on leginella detection are shown in table 2.

Table 1: *Legionella* prevalence by the source

Sampling source	*Legionella* positivity, % (No. of positive/total No.)
Cold water tap	9.1 (1/11)
Hot water tap	25.0 (2/8)
Showerhead	55.5 (5/9)
Cooling tower	30.8 (4/13)
Hot water tank	25 (1/4)
Total*	28.9 (13/45)

* Considering overlap

According to the table the Cooling towers water showed considerably higher mean HPC values than other water environments. As shown in this table, HPC values in the sampled waters from cooling towers have been reported as TNTC (too numerous to count), and the median values for other samples were 72.1 CFU/ml. This finding is expected and is in accordance with the nature of such systems. Cooling towers were therefore excluded in the further analysis of the HPC data and a moderate positive correlation (r=0.37) was found between HPC values and *Legionella* presence.

Amoeba was detected in 21 out of 45 (i.e., 46.7%) investigated hospital water samples. Excluding cooling tower samples, all polluted with amoeba, there were two hospitals with no positive sample taken from water sources.

Discussion

Legionella prevalence and species identification

In general, showerheads were the most contaminated source with 55.3 percent positive samples. Similarly, the samples from cooling waters, hot water taps and cold-water tanks yielded fairly comparable results (37.5%, 25%, and 20% respectively). However, this was not the case with the cold-water tap samples and only 9% were positive for *Legionella* and no *Legionella* was detected in hot water tank samples.

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 (mwg_biotech.com, 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 No. FJ480932 for L. *pneumophila* and FJ48093. Detection of *Legionella* in aquatic environments has been demonstrated in other researches (10, 13, 14). Although PCR inhibitor may interfere with the results obtained by PCR, it has shown higher sensitivity than culture as demonstrated by Lye et al., and Morio et al. (1, 15). This may be derived from the relatively low concentrations of *Legionella* and supported by the fact that *Legionella* bacteria are commonly present in aquatic environments in the viable but non-culturable status which cannot be detected by culture (16, 17).

In our study, although no statistical difference among *Legionella* positive rates in various sources was found ($P>0.05$), the positive rate itself showed the severity of contamination. Therefore, even though the results obtained by PCR are not a valid determinant of *Legionella* viability in the environmental samples, it should be seriously considered as a potential public health threat. Owing to the lack of epidemiological and ecological studies, no *Legionella* outbreaks have been reported in environmental water samples in Tehran or other cities of Iran till now. However, considering patients' complaint about their acquired pulmonary diseases at hospitals, the results of this study showed that sporadic or even a fairly high incidence of *Legionella* might have occurred but neglectfully distinguished as other pulmonary diseases.

Table 2: Quality characteristics of hospital water sources (routine monitoring data of a 1-year period)

sample	Water quality characteristics												*Legionella* positivity
	Physicochemical										Microbiological		
	T (°C)	pH	EC (µS/cm)	Alk[a]	Hard[a]	Ca[a]	Mg[a]	Fe[a]	Mn[a]	Cl_2[a]	TPC	Amoeba positivity	
Cold water tap	20.0	7.7	393	86	156	51.2	6.83	0.098	0.008	1.7	0	No	No
	24.5	7.6	383	86	172	50.4	11.22	0.056	0.015	0	2	No	No
	17.0	7.2	365	90	176	52	11.2	0.083	0.007	0.2	1	No	No
	25.0	7.9	374	88	192	59.2	10.7	0.044	0.001	0.4	TNTC[b]	Yes	No
	23.0	7.8	382	88	208	59.2	14.64	0.071	0.008	1.5	4	No	No
	19.0	7.6	383	96	188	64	6.8	0.060	0.013	0	120	No	No
	21.0	7.3	380	88	146	56	1.46	0.011	0.024	1.2	20	No	No
	19.0	7.5	697	96	204	60.8	12.7	0.059	0.003	0.4	0	No	Yes
Hot water tap	41.0	7.6	408	84	156	52	6.34	0.202	0.016	0.2	0	No	No
	49.0	7.6	390	86	158	51.2	7.32	0.410	0.003	0	4	No	No
	49.0	7.3	410	82	144	48	5.8	0.150	0.016	0.7	1	No	No
	27.0	8.2	393	84	168	59.2	4.88	0.375	0.011	1	218	Yes	No
	34.0	7.9	378	88	170	52.8	9.3	0.040	0.018	0.4	21	No	No
	40.0	7.8	384	84	166	56	6.34	0.020	0.006	0.1	122	Yes	Yes
	35.0	7.4	378	120	152	51.2	5.85	0.208	0.022	0	27	Yes	Yes
	40.0	7.5	674	124	188	60.8	8.78	0.344	0.016	0.3	5	No	No
Showerhead	26.0	7.5	400	84	175	52.8	10.5	0.053	0.010	0	8	Yes	No
	45.0	7.4	375	86	156	50.4	7.32	0.034	0.010	0	6	No	No
	35.0	7.5	395	80	152	55.2	3.42	0.140	0.012	0.2	3	No	No
	25.0	8.0	380	84	160	52	7.32	0.150	0.005	0	TNTC	Yes	Yes
	31.0	8.0	386	88	160	54.4	5.85	0.020	0.014	1.4	TNTC	No	No
	27.0	7.8	375	88	160	56	4.88	0.203	0.009	0.5	TNTC	Yes	Yes
	33.0	7.6	380	84	188	52	9.27	0.025	0.017	0	70	No	Yes
	36.0	7.5	678	120	190	59.2	10.25	0.02	0.007	0.7			No
Cooling tower	17.0	7.8	18700	238	5940	60	1060	0.109	0.015	0	TNTC	Yes	No
	18.0	7.9	5900	164	2120	390	280	0.313	0.015	0	TNTC	Yes	Yes
	25.0	7.8	1400	60	504	152	30.25	0.055	0.008	0	TNTC	Yes	No
	17.0	8.2	6570	206	1700	256	258	0.410	0.031	0	TNTC	Yes	Yes
	19.0	8.2	7440	170	1640	184	288	0.530	0.028	0	TNTC	Yes	Yes
	17.0	7.9	-	190	2750	340	463.6	0.875	0.088	0	TNTC	Yes	No
	19.0	8.2	-	192	1100	184	156	0.749	0.070	0	TNTC	Yes	No
	20.0	8.1	19970	228	4400	520	756.6	0.169	0.012	0	TNTC	Yes	Yes
	19.0	8.2	10630	208	2200	320	341.6	0.840	0.105	0	TNTC	Yes	Yes
	22.0	9.4	3210	516	134	19.2	21	0.719	0.095	0	TNTC	Yes	Yes
	21.0	11	48440	480	7680	473.6	1585	0.20	0.063	0	TNTC	Yes	No
	21.0	10	17000	324	8960	488.4	1890	0.175	0.048	0	TNTC	Yes	Yes
Cold water tank	18.0	7.3	371	104	162	51	8.4	0.044	0.015	1.2	0	No	No
	19.0	7.5	400	85	135	47	4.3	0.039	0.033	0.9	0	No	No
	21.0	7.3	424	94	175	53	10.5	0.106	0.008	1.5	0	No	No
	22.0	7.4	398	91	172	50.5	11.2	0.091	0.020	1.1	3	No	No
	19.0	7.5	656	124	248	64	21.47	0.072	0.015	0.4	6	No	Yes
Hot water tank	40.0	8.6	340	88	192	76.8	13.2	0.144	0.052	1	18	Yes	No
	48.0	7.9	398	-	-	-	-	-	-	0	100	No	No
	35.0	7.6	385	92	216	54.4	19.5	1.20	0.165	0	TNTC	Yes	No
	40.0	7.7	447	84	56	11.2	6.83	0.54	0.079	0	48	No	No

a: as mg/L- b: TNTC: Too numerous to count; >300 CFU

Relationship between the occurrence of Legionella and water quality

Previous studies have shown that the incidence of pulmonary diseases at hospitals associated with *Legionella* differs markedly with physicochemical properties of water.

In the present study of seven hospitals in Tehran, total mean of residual chlorine was 0.38 mg/L, with the peak value of 1.7 mg/L. *Legionella* detection was proportional to the residual chlorine content of water and the results indicated that residual chlorine content is a critical factor in incidence and proliferation of *Legionella* (r=-0.33). However, even a concentration of 0.4 mg/l of residual chlorine showed to have no effect on disinfecting the bacterium. This study was generally conducted during the warm seasons of the year, when the municipal drinking water was super chlorinated due to water shortage and subsequent loss of water pressure in the distribution system at some hours of the day. Although *Legionella* is susceptible to disinfection, and it is believed to reduce the potential risk of contracting infections (18)**,** the characteristics of cooling tower facilities make it impossible to maintain adequate disinfectant residuals. Although many guidelines recommend conscientious maintenance and though such recommendations seem reasonable, to the best of our knowledge, there is not any documented data on the minimization of *Legionella* colonization in cooling towers by maintenance measures and also on the usefulness of control measures in preventing outbreaks of Legionnaires' disease from cooling towers. Routine maintenance measures have been recommended for general quality of hospital water, not on *Legionella* colonization. On the other hand, some species of *Legionella* have shown an ability to survive in high free chlorine concentration, the condition that inhibits other bacterial groups (19). Many microorganisms of aquatic origin, such as *Pseudomonas*, produce bacteriocins that may actually be inhibitory to *Legionella spp.* Thus, chlorine levels that eliminate these microorganisms could increase the population of indigenous *Legionella.* Furthermore, *Legionella* spp. are more resistant to chlorine than other bacteria because they can attain a viable but nonculturable state, be protected by amoebae, and/or survive in pipe biofilms (19). It has therefore been deliberated that the scientific method has been less successful in the prevention of legionellosis contracted from cooling towers than from drinking water systems (7). Floating and attached biofilms are common in such aquatic environments. The presence of *Legionella* and some of its amoeba hosts has been proved in these biofilms from both anthropogenic and natural aquatic systems (20), which explain why the sampled cooling towers in this study generally demonstrated higher positive results for *Legionella* than other sources. Many studies have documented that *Legionella* need an amoeba host for replication in the biofilms and also amoebae are able to resist different treatments and survive under stressful environmental conditions, such as high chlorine levels. Therefore, they probably act as reservoirs for *Legionella*, allowing quick recolonization of the system once the treatments are interrupted (20-22). To assist with confirmation, it has been recently shown that *Legionella* may be necrotrophic, allowing bacterial growth despite water treatment (23). Another hypothesis that may validate this finding is that cooling towers are generally operated at temperatures close to the optimal growth temperature of *Legionella* (35°C), which may lead to high bacterial concentrations (4). It should be also mentioned that cooling towers are more liable to colonization owing to their aged-old and low hydraulic circuit.

Besides old and partially somewhat damaged water piping in most of the hospitals under study, survival and extension of amoeba in the nature and especially in water support its high presence in hospital water samples. Nevertheless, based on table 2, the chlorine concentration in most of the samples (excluding cooling towers) was greater than 0.2 mg/L, confirming that water quality standards, both cold and hot tap water samples, especially in old hospitals had amoeba contamination. This can be rooted from the fact that amoeba have high resistance against water chlorination, as the minimum concentration of 1.5 mg/L of free residual chlorine has been reported to be effective against Acanthamoeba cyst (a well-known *Legionella* amoeba host) (24). A variety of microorganisms such as *Legionella* spp., which nest in the form of endosymbiont in the amoeba as amoeba-

associated bacteria, can survive after chlorination and applying other disinfectants (25). A fairly strong and significant correlation (r=0.46) was found between the presence of amoeba and *Legionella* in the present study ($P<0.01$).

Conclusion

Molecular techniques based on PCR assay offer a rapid, practical, cost-effective and sensitive alternative for detection of *Legionella*. Although the concentration of *Legionella* 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. In light of these data, it can be concluded that *Legionella* colonization in environmental waters such as hospital water systems is a deep-rooted phenomenon. Moreover, *Legionella* are able to persist and increase with time, independent of the type of water, system characteristics and of preventive maintenance measures.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

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