Investigation of Virulence Genes and Biofilm Formation Among Legionella Pneumophila Isolated from Hospital Water Sources

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Abstract

Background: *Legionella pneumophila* as a ubiquitous bacterium is inherently resistant to chlorine in tap water. It can easily enter water piping systems and get transmitted to immunocompromised populations and cause severe pneumonia. Owing to the fact that its presence in water sources doesn't necessarily lead to onset of disease; therefore, several factors such as inhaled bacteria dose, virulence factors and diversity of serogroups can be considered as contributing factors. The main aim of current project was to investigate the contamination rate of hospital water systems with *Legionella* by culture and evaluate presence of major virulence factor genes as well as the ability to form biofilms among the *Legionella* isolates.

Results: Twelve (12%) of the 100 water samples produced positive results in culture method. Additional confirmation was performed by PCR method with specific primers for *Legionella* genus (16SrRNA) and *pneumophila* species (*mip*). Fifty (5%) samples of 12 with positive culture have a colony forming unit higher than 1000cfu/100 ml. *Legionella* were isolated with a rate of 8%, 3% and 1% from shower heads, oxygen humidifier bottle and water bath, respectively. PCR assay for the virulence genes showed that all 12 (100%) isolates were positive for *mip* genes, 9 (75%) were positive for *dot* gene, 8 (66.66%) were positive for *hsp*, 6(50%) were positive for *lvh* and 4(33.33%) for *rtx.* Two of the isolates displayed higher ability to form biofilm in reference to the standard strain.

Conclusion: Although the presence of *Legionella pneumophila* in hospital environment does not necessarily confer a threat to public health; continuous monitoring of water sources should be conducted in order to avoid elevated concentration of this bacterium and visible biofilm formation.

Background

Legionella pneumophila is a facultative intracellular pathogen, which is the etiological agent of *legionellosis*, legionnar's disease [1–4]. *Legionella pneumophila* as a ubiquitous bacterium is resistant to chlorine in treated water and can easily enter piping systems and transmitted to human through contaminated artificial and natural water systems including cooling systems, air conditioning, shower baths and drinking water and cause mild to severe pneumonia with high mortality among immunocompromised populations [5–7]. The bacterium enters respiratory tract by infected droplets and swallowed by macrophages. It prevents the fusion of lysosomes to contaminated phagosomes [8, 9]. The bacterium is an opportunistic pathogen that can cause disease in compromised immune system individuals and a common cause of hospital acquired pneumonia [10]. Environmental surveillance in hospitals as performed by culturing of water supplies is useful for risk assessment and prevention of disease [11]. Owing to the fact that presence of *Legionella* in water source doesn't always leads to disease; therefore, several factors such as inhaled bacteria dose, virulence factors and diversity of serogroups can effect on this process [12, 13]. Virulence factors of *legionella* are LPS, flagella, T2SS secretion system and some other membrane proteins [14]. Many of these are found in the *legionella pneumophila* wall compositions, which include OMVs, peptidoglycans associated with lipoprotein (PLA),

phospholipase A, external membrane proteins, HSP60, FeoB, and mip [15]. *lvh* is a locus derived from proteins of the type 4 secretion system that is involved in invasion and bacterial virulence [16]. The rtxa protein in *Legionella pneumophila* causes the bacterium bind to host cells and invades them. This bacterial superficial protein also plays a role in the binding and entry of bacteria to the *Acanthamoeba castellanii* protozoan and its survival in nature. In rtxa mutants, the ability to bind and entery of the bacterium into epithelial cells and monocytes is reduced [17]. The *L. pneumophila* major outer membrane protein (MOMP) is involved in the attachment to host cells [18]. The heat shock protein (Hsp60) is also important for attachment to and invasion of a HeLa cell model [19]. Mip, the macrophage infectivity potentiator, is a membrane-associated homodimeric protein that is mainly found on the bacterial surface [20]. One mechanism that has been proposed for establishment of the unique *L. pneumophila* replication vacuole involves the Dot/Icm secretion system [21].

The main aims of current project was to investigate the contamination frequency of hospital water systems with *Legionella* by cultural methods, evaluate the water condition for the presence of *legionella* and detection of major virulence factor genes and ability for biofilm formation among *Legionella* isolates for risk assessment and prevention.

Results

Collection and measurement of temperature, pH and residual chlorine of water samples

During one year period, from December 2018 to December 2019, a total of 100 water samples were collected from nine hospitals in Tehran, Iran. Samples were from oxygen humidifier bottle (43%), shower head (46%) and water bath (11%). All samples had residual chlorine between 0.3 to 0.6 mg/ml (Fig. 1). The pH of the water samples were between 5.6 to 6 (Fig. 1). Temperature of the samples was between 25–30 C in 43%, 31–36 C in 9% and 37–41 C in 48% of the cases (Fig. 1).

Concentration And Treatment Of Water Samples

For this step, we examined two different concentrations (centrifugation and filtration) and then used two different treatments methods for each concentration (heat and acid). Water samples were concentrated with centrifuge at 3000 rpm for 10 minutes at 4º C. The deposits were resuspended in 5 ml of original water sample as concentrates. In concentration by filtration, at first all water samples were passed through 0.45 µm pore size nitrocellulose membranes. Then the membranes were aseptically removed, put into sterilized 50 ml tube and resuspended in 10 ml of the original water samples. Each concentrated water samples was shaken for 30 min to get out bacterial cells from the membrane. To exclusion of non-*legionella* bacteria, the concentrated water samples were diluted (1:10) in Kcl-Hcl solution (pH: 2.2), mixed and incubated at room temperature for 4 minutes. In an alternative treatment for concentrated water, heating at 50ºC for 30 min was used.

Culture of water samples on GVPC (Glcine, Vancomycin, Polymyxin and Cycloheximide)

A 0.1 ml volume of concentrated and treated water samples were spread onto the surface of BCYE supplemented with glycin, vancomycin Hcl (1 µg/ml), polymyxin B (79.2 IU/ml), and cycloheximide (80 µg/ml) (GVPC agar). Plates were incubated in candle jar (3–5% Co2) at 37ºC in a humidified atmosphere.

Identification of Legionella pneumophila **colony**

For verification of suspected colonies with the typical ground glass appearance on GVPC, these colonies were inoculated on BCYE with or without L-cysteine and onto non selective media such as blood agar. Identification of *Legionella pneumophila* were performed with gram stain and biochemical tests. Strains unable to grow on media without L-cycteine and blood agar were further analyzed by PCR with specific primers for *Legionella* genus (*16srRNA* gene) and *pneumophila* specie (*mip* gene).

DNA extraction from Legionella pneumophila **colonies**

Freshly grown *legionella* colonies on GVPC medium were suspended in distilled water and 200 µl of the suspension was used for DNA extraction and purification according to a commercial kit manufacture instruction. 50 µl of lysis solution was added into each microcentrifuge tube containing *legionella* suspension for breakage of the cell membrane. The tubes were vortexed and then incubated at 95 °C for 10 min in a hot plate and then were left to equilibrate at room temperature for 5 minutes. The tubes were then vortexed and centrifuged at 6,000 rpm for 2 minutes and the eluted DNA was transferred to an eppendorf tube. Quality of the extracted DNA was assessed by optical density at 260 nm and electrophoresis on agarose gel. Extracted DNA from *legionella* colonies was stored at − 20 °C for a maximum of 2 days.

Biochemical Analysis, Culture And Pcr Methods On Water Samples

None of the 12 suspected colonies grown on GVPC with a negative reaction in gram staining when cultured on blood agar and BCYE without L-cystein showed any growth. Identification of these suspected colonies were performed by biochemical test such as positive oxidate test and weak reaction in catalase test. Additional molecular confirmation was performed by PCR method with specific primers for *Legionella* genus (16srRNA) and *pneumophila* species (*mip*). All of the isolates were positive as *Legionella pneumophila*. Using culture and PCR methods for isolation and detection of *Legionella* in water samples shown that 12 and 42% of the hospital water samples were colonized by *Legionella*, respectively. All of the samples that were positive in culture methods (12%) were also positive by PCR. *Legionella* was isolated with a rate of 8%, 3% and 1% from shower heads; oxygen humidifier bottle and water bath respectively (Fig. 2). Fifty (5%) samples from 12 with positive culture have a colony forming unit higher than 1000 cfu/100 ml and the rest had values. A correlation was found between *Legionella*

culture positivity rate and temperature of water samples in analysis by chi-square and likehood test (p value = 0.000, r = 0.493). No significant correlation was found between residual chlorine of water samples and the presence of *Legionella* with chi-square and Fisher test (p value = 0.313). A correlation was detected between the presence of *Legionella* and pH (p = 0.000; r = 0.546). Since sample collection continued for a period of one year, the isolation rate in summer and spring was 10 cases of 12 (83.33%) as compared to winter and autumn which was 2 cases (16.66%).

Detection of virulence factors in the Legionella pneumophila **isolates**

To estimate whether *Legionella* isolates are pathogenic for human the presence of virulence genes including *mip, dot, hsp, rtx and lvh* were detected among the isolates. Findings showed that 12 (100%) isolates were positive for *mip* genes, 9 (75%) were positive for *dot* gene, 8 (66.66%) were positive for *hsp*, 6(50%) were positive for *lvh* and 4(33.33%) for *rtx* (Fig. 3, Fig. 4*)*. Twelve cases showed eight virulence patterns that were reported in Table 2 and Fig. 5. All of the isolates had at least two of these virulence factors.

Biofilm Formation Assay Among Isolates

The ability of *Legionella pneumophila* for biofilm formation were estimated and results revealed that two isolates in first day have a higher ability to form biofilm in reference to the standard strain and this ability increased to eight and ten isolates compare to standard strain in third and ninth days (Table 3).

Table 2
Different patterns of virulence genes among isolated *Legionella* strains.

Strains	*Lvh*	*Rtx a*	*Hsp60*	*Dot*	*Mip*	*16srRNA*
Lp1	+	+	+	+	+	+
Lp2	-	-	+	-	+	+
Lp3	+	+	+	-	+	+
Lp4	+	-	+	+	+	+
Lp5	-	-	+	+	+	+
Lp6	-	-	+	+	+	+
Lp7	-	-	-	+	+	+
Lp8	+	+	-	+	+	+
Lp9	+	-	-	+	+	+
Lp10	-	-	-	+	+	+
Lp11	-	-	+	+	+	+
Lp12	+	+	+	-	+	+

Table 3
Results of OD mean for biofilm formation among isolated *Legionella* strains

Legionella strains	First day	Third day	Ninth day
Negative control	0.009 ± 0.001	0.009 ± 0.001	0.009 ± 0.001
Positive control	0.123 ± 0.004	0.644 ± 0.028	1.297 ± 0.070
LP1	0.139 ± 0.001	0.982 ± 0.081	2.008 ± 0.020
LP2	0.101 ± 0.001	0.975 ± 0.223	1.767 ± 0.042
LP3	0.145 ± 0.001	0.905 ± 0.014	1.463 ± 0.026
LP4	0.099 ± 0.001	0.935 ± 0.002	1.841 ± 0.131
LP5	0.93 ± 0.001	0.851 ± 0.093	1.397 ± 0.035
LP6	0.105 ± 0.001	0.804 ± 0.042	1.785 ± 0.0151
LP7	0.098 ± 0.001	0.608 ± 0.013	1.917 ± 0.084
LP8	0.103 ± 0.004	0.500 ± 0.023	1.536 ± 0.082
LP9	0.105 ± 0.001	0.646 ± 0.046	1.188 ± 0.082
LP10	0.103 ± 0.001	0.804 ± 0.042	1.767 ± 0.042
LP11	0.096 ± 0.001	0.608 ± 0.013	1.841 ± 0.131
LP12	0.093 ± 0.001	0.851 ± 0.093	1.297 ± 0.035

Discussion

Water supplies in hospitals are the major infectious sources for *Legionella*. In many nosocomial outbreaks of *Legionella*, water supplies were the most frequent culprit [22]. Elderly individual, immunocompromised as well as patients having undergone surgery are more at risk and nosocomial Legionnar's disease showed a higher fatality than community ones [1]. For these individuals, aerosols generated from water supplies containing *Legionella pneumophila* can be a source of *Legionella* infection. Previous studies described that the isolation of *Legionella* SPP from shower heads, oxygen humidifier bottles and water baths are highly prevalent [5]. In the present study using culture method indicated that [12] 12% of the water sources including shower heads, water baths and oxygen humidifier bottle of nine hospitals were colonized with *Legionella* SPP. We could isolate *Legionella* with a rate of 8%, 3% and 1% from shower heads, oxygen humidifier bottle and water bath, respectively. The result of colony count of these 12 samples showed only 5 samples had a critical concentration of *Legionella* (more than1000cfu/100 ml) and rest had a moderate risk of *Legionella* concentration (lower than 1000 cfu/100 ml). Factors such as the present of viable but non culturable bacteria, loss of viability of bacteria after collection and low concentration of *Legionella* in the samples may influence on

unsuccessful isolation of *Legionella* in water samples [23]. The PCR method with *16SrRNA* gene target of *Legionella* showed a rapid and sensitive test. Twelve samples in a total of 100 gave positive results for *Legionella* using the culture methods; whereas, 42 samples were positive when PCR was used. All samples that were found with the culture method were also positive with the PCR assay. Using primer specific for *mip* gene confirmed that all the isolates in culture (100%) were *Legionella pneumophila*. There was a significant difference between PCR and culture results for detection of *Legionella*. Several studies described PCR to have a higher rate of detection than culture methods [24–26]. Molecular methods were suggested to be fundamental when an outbreak occurs. Molecular methods have several advantages such as rapidity, precision and are useful for analysis of samples usually contaminated with microorganisms other than *Legionella*. However, isolated DNA may not come from live microbial cells [27, 28]. The physicochemical composition of water samples can affect the colonization and proliferation of *Legionella* in hospital water supply. The residual chlorine plays a very important role in clearance of the bacterium. The residual chlorine in our *Legionella* positive samples were between 0.3 or 0.6 mg/L. Several previous researches reported that there was no detection of *Legionella* SPP when residual chlorine was over 0.4 mg/L [29, 30]. Concerning the physicochemical analysis of water samples in this study, we found *Legionella pneumophila* isolated from chlorinated water samples. Prior studies showed that *Legionella* has reduced viability at higher pH [31]. In this study, there was a significant association between *Legionella* isolation and pH of the water samples. The mean temperature of the samples positive for *Legionella* was lower (25–30ºC) compared to the mean temperature for those negative for *Legionella pneumophila* (31–41ºC). This result was consistent with a research in Ohio which showed that water temperature had an association with *Legionella* colonization [27]. However, is not consistent with a research in Italy that showed presence of *Legionella* was not affected by water temperature [32]. Sample collection in this study was carried for a full one year. All positive samples were collected in autumn, spring and summer with a high rate in summer (10%) and autumn (2%). *Legionella* not detected during winter. The main reason was high temperature and humidity during the summer months which is optimum for *Legionella* growth. Additionally, free living amoebae were also abundant mainly during autumn [33]. The existence of virulence genes in isolates from water sources is important to determine whether the isolates can have pathogenic potentials. When the virulence genes were studied, every isolate appeared to have different virulence pattern. The isolates Lp1, Lp3, Lp4 and Lp8 most the effective bacteria in 12 of experimented followed by the Lp12. Many studies explained the relationship between putative virulence genes and pathogenesis [1]. In this study, some isolates had least virulence genes. Hung et al reported *lvh* and *rtx* genes as pathogenesis markers that can be used for determining the infection potential of an isolate [12]. In our study (33.333%) of the isolates showed a positive result for both of these genes. Previous studies revealed that *rtx* to be involved in the attachment and entry to *Legionella pneumophila* into acanthamobea, human epithelial and monocytes cells and pore formation in host membrane [34]. Hsp60 of *L. pneumophila* through a mechanism that involves surface interaction modulated macrophage function [1]. *Legionella* attach on surface and secret polysaccharide products for biofilm formation. The potential colonization of *Legionella* isolates could be analysed by the ability to produce biofilm on a polyethylene surface. Biofilm is an ideal microenvironment for proliferation, survival, dissemination and the likely the pathogenesis of *L. pneumophila*. Because *Legionella* in biofilm can

become highly resistant to biocides, pH, excess temperature and chemical factors, outbreaks have been attributed to biofilm. In the present study, (71.35%) of the isolates had a strong potential for biofilm formation. This ability appeared in the first day of the assay and increased on third and ninth days.

Conclusion

Findings of this project showed that although the presence of *Legionella pneumophila* does not necessarily equate a threat to public health but continuous monitoring and checking of the water sources should be performed in order to avoid high concentration of this bacterium and visible biofilm formation.

Materials And Methods

Collection and measurement of temperature, pH and chlorine of water samples

In this project, during a one year period between September 2018 and September 2019, a total of 100 water samples were taken from different hospital water supplies in Tehran, Iran (Table 1).

Five hundred millimeter or fifty millimeters of water samples were collected in sterile bottles or small polypropylene containers respectively. Water temperature, chlorine and pH were measured at time of sample collection and then 0.1 M sodium thiosulphate was added to neutralize residual free chlorine in the samples. All samples were transported to the Microbiology laboratory of Iran University without delay.

Pcr Methods For Determination Of Virulence Genes

The virulence factor genes (*rtx, dot, hsp, mip and lvh*) were assessed by amplification with specific primers (Table 2) for all legionella isolates. PCR reactions were prepared in 25 µl consisting of 2.5 µl of 2X master mix, 0.2 Mm of each primers and 2 µl of DNA template. Initial denaturation of *legionella* chromosome DNA was carried out at 95ºC for 5 min then 35 cycles of PCR amplification followed with 95ºC for 30 s, 58ºC for 1 min and 70ºC for 90 s with a final extension at 70ºC for 5 min. PCR products were analyzed on 1.5% agarose gel containing 0.5 µg/ml safe stain. Gels were visualized in a gel document device.

Determination of biofilm formation in Legionella pneumophila **isolates**

The *legionella* biofilm formation was performed based on Hinder et al protocol [35]. The *legionella* isolates freshly grown on BCYE agar and suspended to an OD of 0.3 at 600 nm. 200 µl of the *legionella* suspension were added to 96 wells microplates. After attachment of *Legionella* to the polysterene wells, 300 µl BYE broth was added for extended biofilm. The well contents were collected and washed gently three times. The wells were filled with 1% crystal violet at 15 min. After washing steps, the dye which had been absorbed by biofilm was revealed using ethanol: acetone (80:20) incubation for 15 min. Solutions

were subjected to another microplate and quantified by spectrophotometer at 595 nm. The results were interpreted basis on Stepanovic et al. [36] Table 1.

Table 1
Primer sequences for amplification of virulence factors of *Legionella pneumoniae*

gene	Primer sequences 5′ -> 3′	Product size	reference
16 s rRNA	AGGGTTGATAGGTTAAGAGC	386	[37]
16 s rRNA	CCAACAGCTAGTTGACATCG	386	[37]
*lvh*1/*lvh*B3	ATTGGGAGCTTCTGGCAATA	186	[38]
*lvh*2/*lvh*B3	GCTGGGGTGACCTTTGAATA	186	[38]
rtx1/*rtxA*	GCTGCAACCACCTCTTTGAT	181	[38]
rtx2/*rtxA*	CAGGGGCTGGTTATGTTGAT	181	[38]
*dot*1/*dot*A	CAAATCCGGCATTCAAAATC	174	[38]
*dot*2/*dot*A	CTATTGTCGCCTTGGGTGTT	174	[38]
*hsp*1/*hsp*60	GCGAATCGTTGTTACCAAAGAAAAC	401	[39]
*hsp*2/*hsp*60	CAATTTGACGCATTGGAGATTCAATAG	401	[39]
*mip*1/*mip*	GGTGACTGCGGCTGTTATGG	632	[39]
*mip*2/*mip*	GGCCAATAGGTCCGCCAACG	632	[39]

Statistical analysis

All data collected from each assay and analysis on hospital water samples were imported into an Exel file (Microsoft office, Microsoft Excel). Statistical analysis was performed using IBM SPP statistics software (v.22). Quantitative variables are expressed either mean values with standard deviation. Chi-square, Fisher's exact and likehood ratio tests were used to identify any association between each parameter and presence of Legionella in water by culture. A finding with a p value of less than 0.05 was considered to be statistically significant.

Abbreviations

PLA
peptidoglycans associated with lipoprotein
GVPC
Glcine, Vancomycin, Polymyxin and Cycloheximide
MOMP

major outer membrane protein
Hsp60
heat shock protein

Declarations

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Authors' contribution

M conceptualized the study. SH, NA provided resources. SH, FM performed the experiments. NA , FM performed statistical analysis. SM, NM and SH wrote the original draft. SM, SH, NA, FM revised and edited the manuscript. All authors read and approved the final

Ethics approval and consent to participate

The study was approved by the Research Ethics Boards at Iran University of Medical Sciences.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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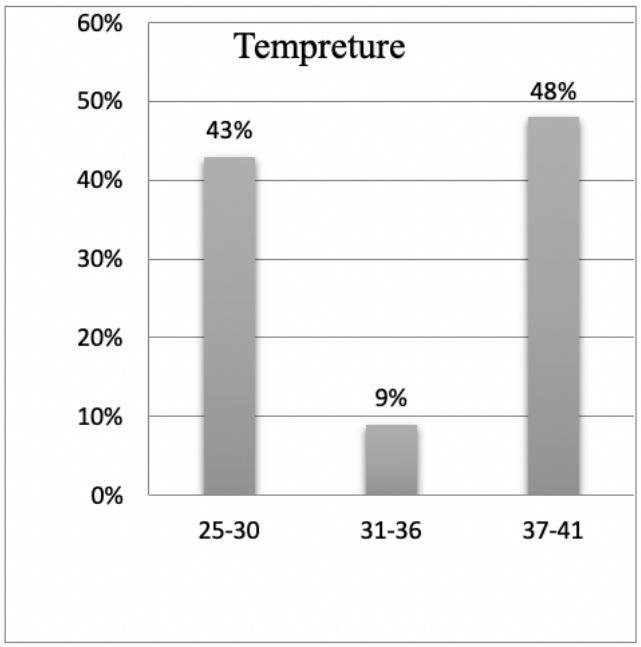
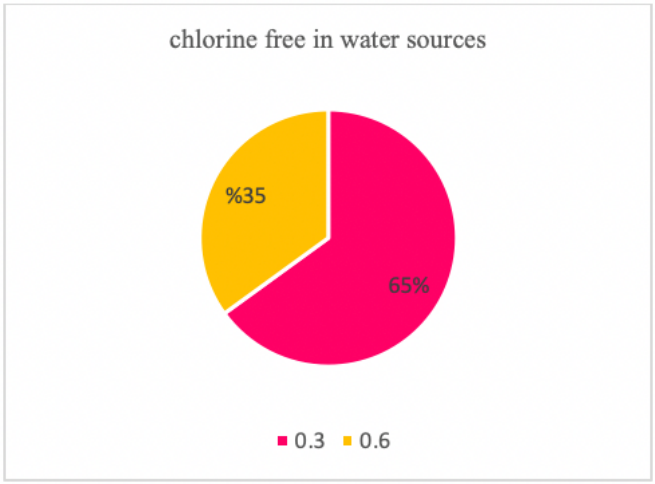
Figures





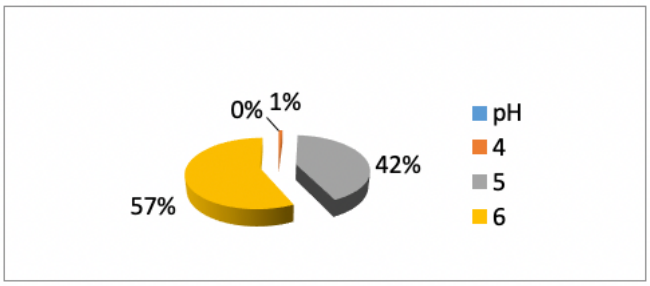


Figure 1

Results of physicochemical assay (pH, Tempreture and residual chlorine) on water samples.

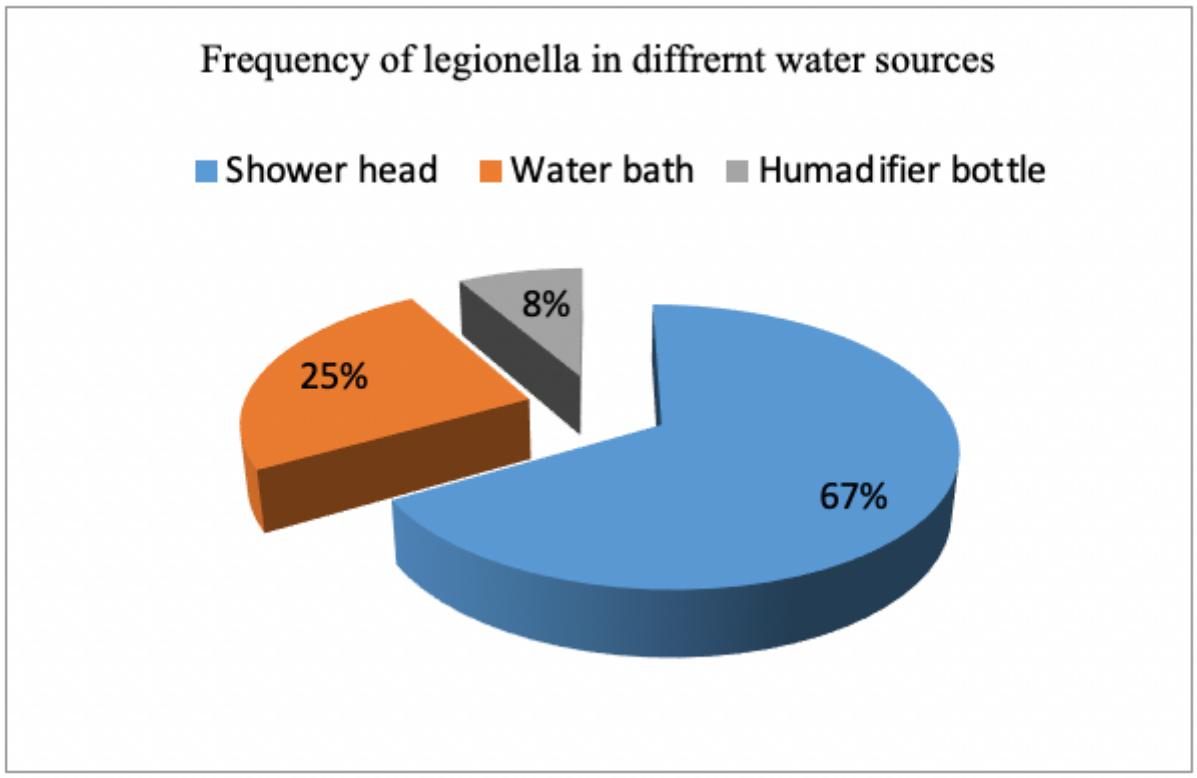


Figure 2

Frequency of Legionella in different water samples.

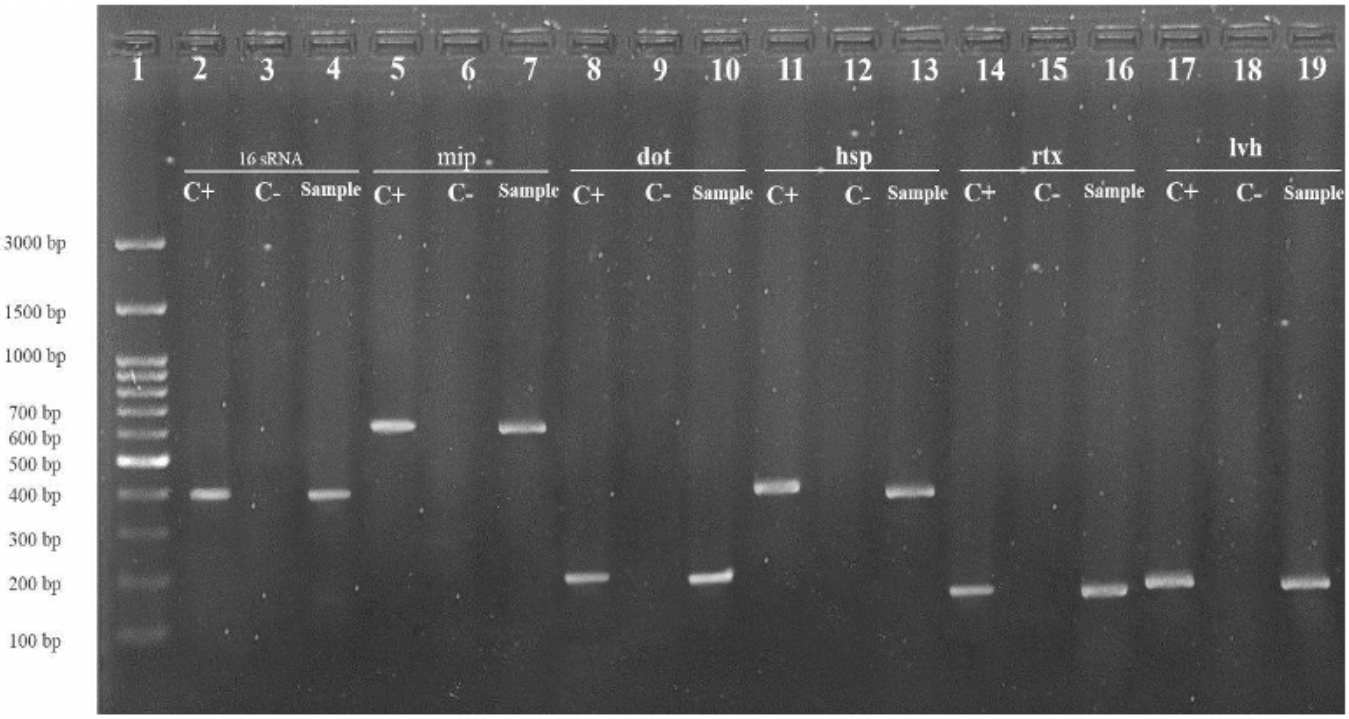


Figure 3

Electrophoresis of PCR product of virulence genes (mip, dot,hsp,rtx, lvh).

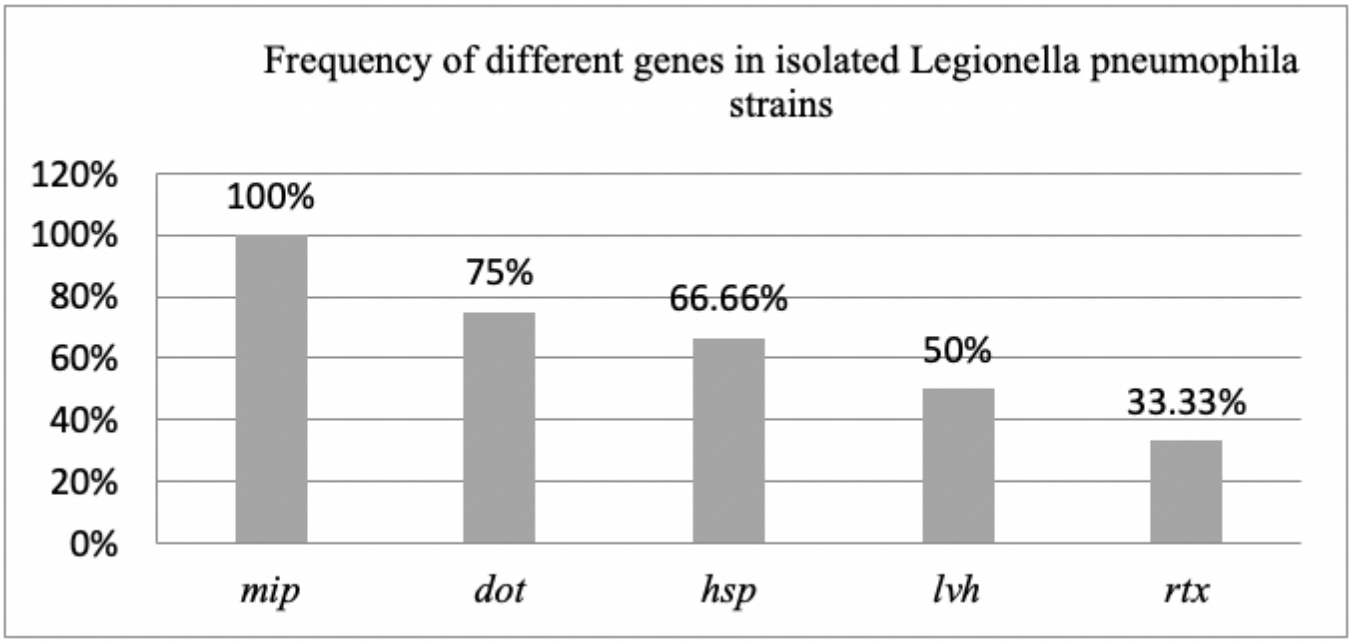


Figure 4

Frequency of different genes in isolated Legionella pneumophila isolates

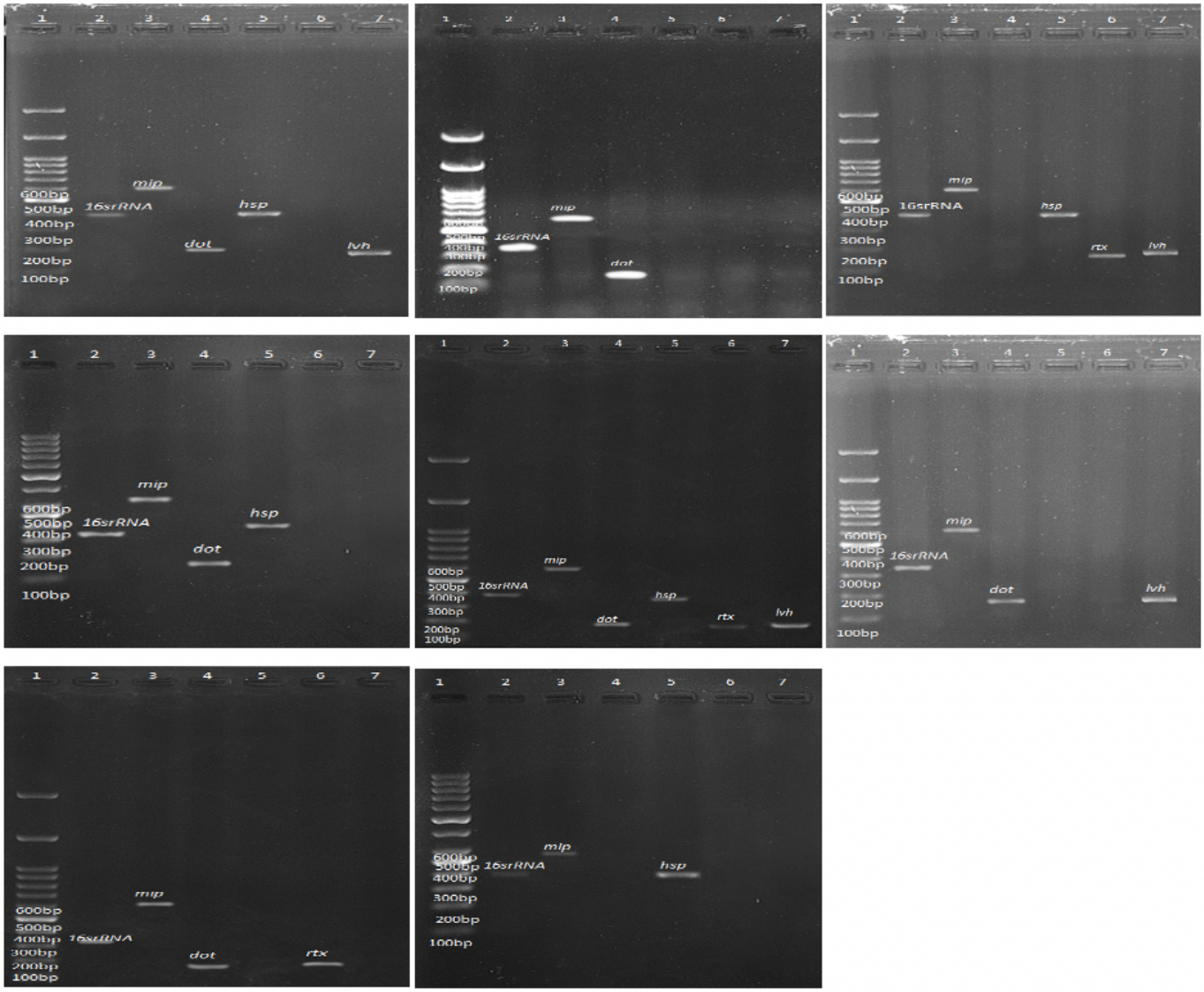


Figure 5

Electrophoresis of PCR products of different genes in isolated Legionella strains (16srRNA, mip, rtx, lvh, hsp and dot) on gele agarose.