

Detection of *Legionella pneumophila* in Cooling Water Systems of Hospitals and Nursing Homes of Kerman City, Iran by Semi-Nested PCR

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Abstract—*Legionella pneumophila* is involved in more than 95% cases of severe atypical pneumonia. Infection is mainly by inhalation the indoor aerosols through the water-coolant systems. Because some *Legionella* strains may be viable but not culturable, therefore, *Taq* polymerase, DNA amplification and semi-nested-PCR were carried out to detect *Legionella*-specific 16S-rDNA sequence. For this purpose, 1.5 liter of water samples from 77 water-coolant system were collected from four different hospitals, two nursing homes and one student hostel in Kerman city of Iran, each in a brand new plastic bottle during summer season of 2006 (from April to August). The samples were filtered in the sterile condition through the Millipore Membrane Filter. DNA was extracted from membrane and used for PCR to detect *Legionella* spp. The PCR product was then subjected to semi-nested PCR for detection of *L. pneumophila*.

Out of 77 water samples that were tested by PCR, 30 (39%) were positive for most species of *Legionella*. However, *L. pneumophila* was detected from 14 (18.2%) water samples by semi-nested PCR. From the above results it can be concluded that water coolant systems of different hospitals and nursing homes in Kerman city of Iran are highly contaminated with *L. pneumophila* spp. and pose serious concern. So, we recommend avoiding such type of coolant system in the hospitals and nursing homes.

Keywords—*Legionella pneumophila*, water-coolant system, semi-nested -PCR.

I. INTRODUCTION

DIFFERENT researchers showed that there are strong relations between Legionnaires' disease and water-coolant systems. In general, the children under two years old, the elderly and immuno-compromised people are seriously prone to acquire *Legionella* infection [1]. Since infection to these

bacteria is not contagious, and inhaling the contaminated aerosol is the only way of infection to these bacteria, having the clean air is important parameter to prevent the pulmonary infection. Similarly, these bacteria hide inside the amoeba they can not be destroyed by chlorinated water, or any other bactericidal agent [2]. *Legionella* spp are facultative intracellular parasite of some free-living amoeba such as *Balamuthia mandrillaris*, *Acanthamoeba*, and *Hartmannella*. They can provide a niche for them. Furthermore, Infection of amoebas by *L. pneumophila* enhances the bacterial infectivity for mammalian cells and lung tissues. *L. pneumophila* can replicate with in the amoeba about four to five log cycles from 24 to 72 h. after infection [3, 4].

L. pneumophila alone is the causative agent of high percentage of hospital acquired atypical pneumonia; about 5% of sporadic *Legionella* diseases are nosocomial legionellosis, while 3.8% of which are fatal *L. pneumophila* [5, 6]. Because some *Legionella* strains may be viable but not culturable, PCR of 16S rDNA gene is exceptionally well suited in identification of *L. pneumophila* in water cooling system [7, 8, 9]. Based on these data we decided to amplify the 16S-rDNA gene of the samples to verify the presence of *Legionella* spp. including *L. pneumophila* [10, 11].

This bacterium is facultative intracellular, flagellated, gram negative rod, spore less, aerobic, sized 0.3 to 0.9 μm , more resistant than a lot of other organisms to common standard disinfectant [10]. So far 42 different species of *Legionella* had been discovered, among them *Legionella pneumophila* is involved in more than 95% of cases of severe atypical pneumonia [12]. In the lung, *L. pneumophila* multiplies intracellularly in the monocytes [7]. Iranian researchers rarely employed 16S rDNA PCR to detect *Legionella* spp. Some researchers during the summer of 1997 collected 108 samples from cooling system of three different hospitals in Tehran; in one of them they found *Legionella* [13]. 187 samples collected from six different hospitals in Tehran and tested by *Legionella* Research Center in Lion of France in which the samples of five hospitals had *Legionella* [14]. In another research in Iran, the researchers had six positive cases by PCR on 32 samples which were negative by culture, they clarify that the sensitivity and specificity of PCR is triple than those of culture [15].

There were no reports in the prevalence of *L. pneumophila*, therefore this study deals with PCR amplification and semi-nested PCR detection of *L. pneumophila* in water-coolant

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II. MATERIALS AND METHODS

A. Water sample collection

1.5 liter of water from 77 water-coolant system was collected each in brand new plastic bottles (purchased from Mahboub Plastic Co. of Kerman) during summer season of 2006, from four different hospitals (three of which belong to Kerman Medical University), two nursing home, and one gent hostel. The samples were transferred to the laboratory in less than one hour and were kept in the refrigerator (4°C) until the time of filtration. Each sample was filtered separately in sterile condition through the Millipore Membrane Filter (Mixed Cellulose Ester). The membrane filter (0.2µm mesh, 47mm diameter) were purchased from Schleicher & Schuell Company of Germany.

B. DNA extraction

Membranes were bend-broken to small pieces while they were inside the plastic gloves. Each smashed filter was transferred to a brand new glass test tube then 1.5 ml sterile NaCl 0.5% was added. The tubes were vortexed vigorously for at least three minutes, and then the emulsified liquid part were transferred to a 1.5ml eppendorf tube and centrifuged at 10,000 rpm for at least five minutes. The supernatant was discarded, and DNA was extracted by DNP™ Kit from Cinnagene Inc Ltd, Iran. Briefly, the pellets were resuspended in 100µl of protease buffer then placed in 95°C hot block for 10 minutes. 100µl of sample was mixed with 400µl of lyses solution and vortexed for 15-20 seconds or until completely homogenized. 300µl of precipitation solution was added, mixed by vortex for 3-5 seconds and kept in -20°C for 20 minutes. Then centrifuge at 12000 rpm for 10 minutes. The supernatant were discarded, to prevent cross contamination the tubes were decanted each on a brand new tissue paper. One ml of wash buffer was added to pellet, mixed gently by vortex for 3-5 second and centrifuged at 12000 rpm for 5 minutes. The pellets were suspended in 50µl of solvent buffer, gently shake, and placed in a 65°C hot block for 5 minutes. Undissolved materials were precipitated by centrifugation at 12000 rpm for 30 seconds. The supernatant which contain the DNA were transferred to new tubes and stored at -20°C until the time of PCR which was not more than 24 hours, 10µl of DNA solution was used for PCR.

C. PCR

To perform PCR, 10µl of template DNA was used for a total volume of 25µl, 1X- PCR buffer (50 mMol/l KCl, 10 mMol/l Tris-HCl, pH 8.3, 1.5 mMol/l MgCl₂, 0.001% gelatin, and 1.25 mMol/l dNTPs), 2.5U Taq polymerase, and 50 pmol/l of each primer. Forward primer was an 18 mers known as *Leg 120 v* (5'-CGC GTA GGA ATA TGC CTT-3'), the reverse primer had also 18 mers called *Leg 1036-r* (5'-GCA GCA CCT GTA TCA GTG-3') [7]. The tubes were then transferred to Techne Thermal Cycler (model TC-512). After an initial denaturing step of 5 minutes at 93°C, 35 cycles of 45

sec. at 93°C, 45 sec. at 50°C, 90 sec. at 72°C, and 10 min. as final extension at 72°C were performed. The PCR product was electrophoresed in a 1% agarose gel containing 0.5µg/ml ethidium bromide resulting in a 930-bp band.

D. Semi-nested PCR

A. Semi-nested PCR was carried out with 2.5µl of the first PCR products in 25µl 1-X PCR buffer (see above) and 50 pmol/l of each primer. Forward primer was an 18 mers known as *LPNE-614-v2* (5'-GGG CTT AAC CTG GGC AGG-3'); the reverse primer was *Leg-1036-r* (as mentioned before) which was used in the first round of PCR. Amplification was performed with an initial denaturing step of 5 min. at 80°C and 30 cycles of 30 sec. at 94°C, 30 sec. at 65°C, 90 sec at 72°C, and the final extension was 3 min. at 72°C resulting in a 440bp product. *L. pneumophila* serogroup No.1 (ATCC 33152) was used as positive control. De-ionized distilled water was used as negative control for both primary and nested PCR. Gene-Ruler 100bp DNA ladder from Fermentas Life Sciences Company (Germany) was used to measure the size of PCR products.

III. RESULTS AND DISCUSSION

Since *Legionella* spp are facultative intracellular bacteria, some free-living amoeba such as *Balamuthia mandrillaris*, *Acanthamoeba*, and *Hartmannella* can provide a niche for them. Infection of amoebas by *L. pneumophila* enhances the bacterial infectivity for mammalian cells and lung tissues. *L. pneumophila* can replicate with in the amoeba about four to five log cycles from 24 to 72 h. after infection.

Primarily, we tried to amplify 16S-rDNA gene using PCR technique in order to detect most *Legionella* from water coolant samples. For PCR experiments, 16S rDNA primers were designed according to the available DNA sequence by the blust X system. Out of 77 water samples that had been tested in this study, 30 (39%) were positive by PCR test for different *Legionella* species. The amplified DNA product of the first round of the PCR on 16S-rDNA gene had a 930-bp band corresponding to relevant gene as shown in the figure 1. To identify the *L. pneumophila* from other *Legionella* species, semi-nested PCR was carried out on DNA product of the first round of PCR, only 14 (18.2%) positive samples which had a 440 bp band were considered as *L. pneumophila* as shown in figure 2. The extra bands which are in the figure 2 were due to amplification of nonspecific DNA that was existed in DNA samples. However, the sharp band of 440 bp indicates the presence of *L. pneumophila* in the semi-nested PCR product. Since we carried out semi-nested PCR of positive *Legionella* samples in the water coolant system, therefore there was no need of sequencing for detection of species.

Although different researchers collect their water samples from different sources and employed different methods for diagnosis of either *Legionella* spp or *L. pneumophila* alone, but our results is in the same result-range of them [16].

Since, there was no any information exist on the prevalence of the *L. pneumophila* in the Kerman City Iran, and significance of this bacterium as a nosocomial pathogen,

therefore, this study confirmed that a large portion of water coolant systems in hospitals and nursing home have been contaminated with this bacterium and play an pivotal role in the safety of patients in hospitals and elderly living in the nursing home in Kerman City.

From the above results it can be concluded that water coolant systems of different hospitals and nursing homes in Kerman city of Iran are highly contaminated with *L. pneumophila* and pose serious concern. So, we recommend avoid using such type of coolant system in the hospitals and nursing homes.

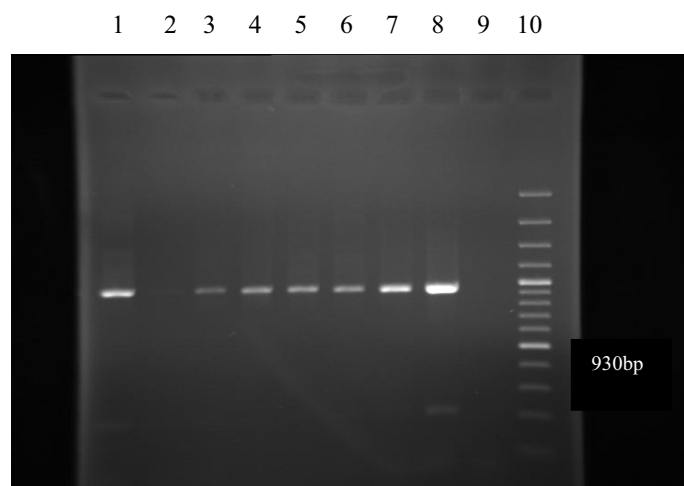


Fig. 1: Results of gel electrophoresis obtained from water samples of first stage PCR. Lanes 1, 2, 3, 4, 5, 6, and 7 are water samples. Lane 8 is positive control (930 bp). Lane 9 is negative control and lane 10 is DNA size standard. The ladder is DNA fragments in 100 base pairs each. The ladder contains two reference bold-bands (1000 and 500 bp) for easy orientation.

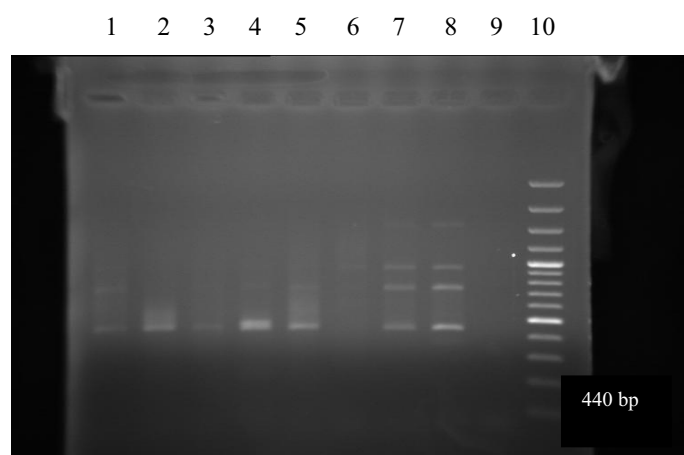


Fig. 2: Results of gel electrophoresis obtained from semi-nested PCR. Slot 1, 2, 3, 4, 5, 6, and 7 are the results of semi-nested PCR for samples. Lane 8 is positive control (440-bp). Lane 9 is negative control and lane 10 is DNA size standard. The ladder is DNA fragments in 100 base pairs each.

Based on the information that we gain during this study, we strongly recommend to avoid using water coolers for home, hospitals, and especially for elderly in the nursing home

because their immune system are very weak. At the same time because this organism is an intra cellular bacteria treatment of this infection is very difficult. So preventing from infection to these bacteria is very important.

We also measured the specific gravity of samples with refractometer. The specific gravity of samples was from 1001 up to 1048. We classified the sample's gravity for each five degrees as it is shown in Table - 1. The data of table one were analyzed by the computer software of SPSS-6.0 through the T-test program but statistically there were not any relation between the concentration of salt in the cooling-water-system and the frequency of isolated *Legionella* ($P = 0.843$).

TABLE I
THE PREVALENCE OF *LEGIONELLA* SPP. AND *LEGIONELLA PNEUMOPHILA* REGARDING TO THE SPECIFIC GRAVITY OF THE SAMPLES

S. G.	F.	<i>L. spp</i>	P.	<i>L. p.</i>	P.
1001-1005	19	5	6.5%	2	2.6%
1006-1010	21	10	13%	3	3.9%
1011-1015	9	6	7.8%	3	3.9%
1016-1020	14	5	6.5%	3	3.9%
1021-1025	10	3	3.9%	2	2.6%
> 1026	4	1	1.3%	1	1.3%
Total	77	30	39%	14	18.2%

S. G. = Specific Gravity

F. = Frequency

L. spp = *Legionella spp*

P. = Percen

L. p. = *Legionella pneumophilla*

P. = Percent

IV. CONCLUSION

Since infection to these bacteria is not contagious, and inhaling the contaminated aerosol is the only way of infection to these bacteria, having the clean air is the first important parameter to prevent the pulmonary infection. Because these bacteria hide inside the amoeba they can not be destroyed by chlorine in the water, or any other bactericidal agent. Cleanliness of cooler seems to be useless, because the bacteria or amoebas containing the bacteria can dislocate from any place such as pounds, sewage, swamp, or any place to another by wind and contaminate the water of coolers, by then continually multiply inside the water-tray of the cooler, then spray into the indoor area as aerosol and cause pulmonary infection. The best is to avoid using water cooler and replace them with any other cooling system. Regarding the other air-conditioner although they have drains but because drains in them are piped out to swage system, so all infective agents are

continually subtracts from the indoor air and washes away by the drains water. So employing non-water cooler seems to be much safer compare to water coolers.

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