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 litter of water samples from 77 water-coolant systems 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 *Legionella* 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 systems.
system of different hospitals, nursing home and university
gent hostel in Kerman, Iran.

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 MilliQ Lab 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
Cinnagen 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 Techrne 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 1X 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 \textit{L. pneumophila} and pose serious concern. So, we recommend avoid using such type of coolant system in the hospitals and nursing homes.

![Fig. 1](image1.png)

\textbf{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](image2.png)

\textbf{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 \textit{Legionella} (P = 0.843).

<table>
<thead>
<tr>
<th>S. G.</th>
<th>F.</th>
<th>\textit{L. spp}</th>
<th>P.</th>
<th>\textit{L. p.}</th>
<th>P.</th>
</tr>
</thead>
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<td>2.6%</td>
</tr>
<tr>
<td>1006-1010</td>
<td>21</td>
<td>10</td>
<td>13%</td>
<td>3</td>
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</tr>
<tr>
<td>1011-1015</td>
<td>9</td>
<td>6</td>
<td>7.8%</td>
<td>3</td>
<td>3.9%</td>
</tr>
<tr>
<td>1016-1020</td>
<td>14</td>
<td>5</td>
<td>6.5%</td>
<td>3</td>
<td>3.9%</td>
</tr>
<tr>
<td>1021-1025</td>
<td>10</td>
<td>3</td>
<td>3.9%</td>
<td>2</td>
<td>2.6%</td>
</tr>
<tr>
<td>&gt; 1026</td>
<td>4</td>
<td>1</td>
<td>1.3%</td>
<td>1</td>
<td>1.3%</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>30</td>
<td>39%</td>
<td>14</td>
<td>18.2%</td>
</tr>
</tbody>
</table>

\textbf{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 sewage system, so all infective agents are not.
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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REFERENCES