Prevalence of mip virulence gene and PCR-base sequence typing of Legionella pneumophila from cooling water systems of two cities in Iran

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ABSTRACT

Legionella pneumophila is the primary respiratory pathogen and mostly transmitted to human through water cooling systems and cause mild to severe pneumonia with high mortality rate especially in elderly both in hospitals and community. However, current Legionella risk assessments may be compromised by uncertainties in Legionella detection methods. Here, we investigated the presence of L. pneumophila mip gene in water samples collected from different hospitals cooling towers, nursing homes and building/hotels water coolants from two geographical locations of Iran (Kerman and Bam cities) during summer season of 2015 by both nested and real-time PCR methods. Analysis of the 128 water samples for presence of the mip gene by nested-PCR revealed, 18 (23%) positive cases in Kerman and 7 (14%) in Bam. However, when samples were tested by real-time PCR, we identified 4 more new cases of L. pneumophila in the hospitals as well as nursing homes water systems that were missed by nested-PCR. The highest rate of contamination was detected in water obtained from hospitals cooling towers in both the cities (p<0.05). Dendrogram analysis and clonal relationship by PCR-base sequence typing (SBT) of the L. pneumophila genomic DNAs in Kerman water samples showed close clonal similarities among the isolates, in contrast, isolates identified from Bam city demonstrated two fingerprint patterns. The clones from hospital water samples were more related to the L. pneumophila serogroup 1.

ARTICLE HISTORY

Received 19 January 2016
Revised 19 March 2016
Accepted 22 March 2016

KEYWORDS

Legionella pneumophila; mip gene; nested-PCR; real-time PCR

Legionella pneumophila is the primary human pathogen and is the causative agent of Legionnaires’ disease, also known as legionellosis. Potable water and water coolant containers are important source of both nosocomial and community acquired Legionella infections. Upon transmission to human, L. pneumophila infect and replicate within alveolar macrophages and spread to blood stream causing mortality rates approaching 30–40%. Outbreaks have been linked to a range of sources, including natural environments such as ground water as well as in technical water carrying systems like cooling towers, household coolers, spas, showerheads and drinking water. For these reasons some countries specifically regulate the surveillance and control of L. pneumophila in water regularly and assess its presence. In a survey conducted in Spain, the prevalence of L. pneumophila was found to be 66.6% of total water samples collected (449 confirmed cases of legionellosis). Furthermore, 42% of Italian hotels water cooling systems of different sizes were contaminated by L. pneumophila. Prevalence of L. pneumophila in water distribution systems in hospitals and public buildings of the Lublin region of eastern Poland was found to be 166 (74.77%) of hot water samples. In other study, more than 1100 cases of legionellosis in Japan, caused by contaminated artificial whirlpool spas or natural hot springs were presented in Infectious Agents Surveillance Report 2014.

Only a few factors have been detected and characterized that contribute to survival of the L. pneumophila in eukaryotic cells. The macrophage infectivity potentiator (mip) gene is described as a virulence factor necessary for optimal intracellular survival of this bacterium. The mip gene was first L pneumophila virulence-associated gene that required for efficient host cell infection. This gene encode a protein belongs to the class of FK 506-binding proteins catalyzing the slow cis/trans interconversion of polypeptide bonds in oligopeptides and well conserved in L. pneumophila.
Isolation of Legionella by culture method is considered the gold standard, but have several limitations namely long incubation period, the presence of viable but non-culturable (VBNC) cells and co-contamination with other microorganisms. Furthermore, the sensitivity of Legionella detection based on culture methods depends largely on the physiological state of the cells. Preliminary evidence indicates that PCR sensitivity and specificity are comparable to those of cell culture. Therefore, PCR-based techniques may best substitute for detection of this bacterium in water systems. Molecular methods described thus far targeted a number of sequences such as the 16S rRNA, the 23S–5S spacer regions and the mip gene of L. pneumophila. However, current Legionella risk assessments may be compromised by uncertainties in Legionella detection methods.

There is paucity of information regarding contamination rate of L. pneumophila in water system of different sources in Iran. Here, we investigated presence of L. pneumophila mip gene in a total of 128 water samples collected from different hospitals towers, nursing homes and building/hotels water coolants of 2 Iranian cities (Kerman and Bam) during summer season of 2015 (May to August) by both nested and real-time PCR. We also studied genetic relationship among the L. pneumophila genomic DNAs from these water samples by PCR-base sequencing method. Selection of the mip gene for screening purposes was based on its discriminatory power and frequent usage in other studies. Geographical locations and sampling sources are illustrated in Fig. 1. A total volume of one liter of each water sample was aseptically collected from the bottom or side of the vessels or reservoirs in 1.5 liter capacity polypropylene containers and placed in the sealed plastic bag in a temperature controlled box (the water systems were not treated with biocides). The samples were then transferred to the microbiology laboratory in less than 4 hour and kept in the refrigerator (4°C) for further analysis. Physico-chemical parameters of each water sample such as temperature, pH, turbidity, biological oxidation demand (BOD), chemical oxidation demand (COD) and total chloride content were examined according to standard method for the

Figure 1. The map and sources of water samples analyzed for detection of L. pneumophila in this study. Both the Kerman and Bam cities were located in south east of Iran. Number of hospitals, building (homes / hotels) and nursing houses that water samples were taken are included in this figure.
examination of water and wastewater treatment.\textsuperscript{19} 250 ml each water sample was then passed through 0.4 \mu m pore diameter membrane filter (Millipore, Bedford, USA), the filter coat was scraped by pipet tips and suspended in 5 ml of sterile TE-buffer (10 mM Tris-HCl, 1 mM EDTA) pH-8.0. One ml of each suspension was then transferred to sterile Eppendorf tubes (Eppendorf, Germany) and centrifuged at 7000 \times g for 10 min at 4°C, supernatant discarded and pellet was kept at refrigeration condition (4°C) for further analysis. DNA extraction was carried out with a commercially available kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer instructions. The quality of isolated DNA was measured by determination of absorbancy at the wave lengths $A_{260}$ nm and $A_{280}$ nm (ratio of these values between 1.7 and 1.9 indicates a high quality of the product).

The conventional PCR reaction was carried out with 5 \mu l of a 340-bp extracted DNA fragment of \textit{mip} gene (sequence was obtained from GenBank database; http://www.ncbi.nlm.nih.gov/GeneBank), 20 pmol forward (5’-AAAGGACATGCAAGACGCTAT-3’) and reverse (5’-ACGTTGCTGGCTTTAGGCTTT-3’) primers (GeneRay, Shanghai, China), 2U Taq DNA polymerase (Ampliqon, Denmark) with 3 \mu l deoxyxynucleoside triphosphates (dNTPs) in 10X reaction buffer containing 1.5 mmol l\(^{-1}\) MgCl\(_2\) in a total volume of 25 \mu l. Sterile distilled water was used as the negative control. DNA ladder was a ready to use plasmid double digest sized range 100-3000 bp obtained from SMOBIO Technology (Hsinchu, Taiwan). Specificity of the primers were checked by Primer Quest software tool (http://www.ncbi.nlm.nih.gov/GeneBank). Amplification was conducted in temperature gradient thermal cycler (Biometra-T300, Gottingen, Germany) with initial denaturation temperature at 95°C for 2 min, followed by 40 cycles of 94°C for 30 s, annealing 53°C for 30 s, extension 72°C for 30 s and a final extension at 72°C for 5 min. Nested-PCR was employed to amplify 124-bp \textit{mip} DNA fragment by using 2 specific primers (forward 5’-TTTGATGGCAAAGCGTACTG-3’ and reverse 5’-TTGCAAACCTTGAGCAATA-3’) as described previously.\textsuperscript{20} Here, the PCR condition was consisted of an initial denaturation at 95°C for 2 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min, respectively. Presence of the \textit{mip} gene in all PCR experiments was checked with positive control consist of \textit{L. pneumophila} serogroup-1 (GenBank accession number CR628336) kindly obtained from Department of bacteriology, Tarbiat Modarres University, Tehran, Iran.

The specificity of the nested-PCR was further confirmed by real-time PCR. Here, a specific set of primers were used for detection of \textit{L. pneumophila} \textit{mip} gene.\textsuperscript{16} The reaction mixtures were consisted of 2 X real-time PCR Master Mix Green with no Rox dye (Ampliqon, Denmark) along with 5 \mu l DNA template in 20 \mu l reaction mixture composed of 2.5 \mu l (0.5 \mu M) forward (5’-ACGGAAACGCAAATAGAAGA-3’) and reverse (5’-AACGCCCTGGCTTTTTTGT-3’) primers and 3.0 mM MgCl\(_2\). Five \mu l of nuclelease-free DDW was taken as a negative control for each run. The experimental LightCycler protocol consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing for 15 s at 55°C, extension for 30 s at 72°C and a cooling step (40°C for 2 min) with acquisition of data following extension step using Rotor-Gene 6000 (Corbett Research, Australia). PCR performance was confirmed to be reproducible at the threshold cycles (Ct) <35. For determination sensitivity and specificity of primers, a serial dilution in the range from 100 ng to 1 fg of purified \textit{L. pneumophila} serogroup -1 DNA was used. All samples were tested by 3-fold repetitions. Results were analyzed with the use of standard slope, provided by the producer (slope points were: 100,000, 10,000, 1,000, 100, 10, 1). Following amplification, melting curve was performed on the SYBER channel (at gain 70) using a ramping rate 0.5°C/10 s for 65–95°C. Melt (65–95°C) hold secs on the first step, hold 5 secs on next steps. One cycle melting curve was carried out and optimized using Rotor Gene software (QIAGEN, Hilden, Germany) for analysis of PCR amplification as described by manufacturer guidelines. \textit{L. pneumophila} serogroup -1 \textit{mip} gene was used to generate standard curve. The experimental points aligned in a straight line and correlation coefficients (R) was ascertained at 0.91658 (R\(^2\)=0.84) by following calibration equation was obtained Y = 0.34908x + 29.66623.\textsuperscript{8,16} The slope of 0.34908 corresponded to an amplification efficiency of 99.8%. Tm (temperature melting) value of the products was 87°C.

In order to confirm the in-silico findings, we performed sequencing of 340-bp PCR product of the \textit{mip} gene amplified from all positive cases. Sequencing was carried out by the Bioneer Company (Seoul, Korea) with Sanger dideoxy chain termination method using Applied Biosystems 3730/3730XL DNA Analyzers (Applied Biosystems, Foster City, CA, USA). Both strands of the amplicons were sequenced. For sequencing we used PFU polymerase instead of Taq DNA polymerase. Similarity searches for the Legionella \textit{mip} gene sequences verified using database provided by the European Working Group for Legionella Infections (http://www.ewgli.org/). Genetic relationship of \textit{L. pneumophila} genomic DNAs among Kerman and Bam isolates were performed directly by PCR- sequence based typing (SBT) method as described previously.\textsuperscript{22} Bands were arbitrarily chosen to range from 200 to 4000 bp. Strains that had fingerprinting patterns more than one band difference in terms of
Table 1. Distribution of *L. pneumophila mip* gene detected in water samples collected from different cooling water systems in Kerman and Bam cities by nested and real-time PCR.

| City  | Sources            | Nested- PCR | Real-time-PCR |
|-------|--------------------|-------------|---------------|
|       | No. of positive sample | Total No. of samples | Percent | No. of positive sample | Total No. of samples | Percent |
|       |                    |             |              |                     |                    |         |
| Kerman| Hospitals          | 11          | 47           | 23.4                | 13                  | 47       | 27.65   |
|       | Nursing home       | 2           | 8            | 25                  | 3                   | 8        | 37.5    |
|       | Building (home/hotels) | 5           | 23           | 21.7                | 5                   | 23       | 21.73   |
|       | Total              | 18          | 78           | 23                  | 21                  | 78       | 26.92   |
| Bam   | Hospital           | 2           | 21           | 9.52                | 3                   | 21       | 14.28   |
|       | Nursing home       | 1           | 12           | 8.3                 | 1                   | 12       | 8.3     |
|       | Building (home/hotels) | 4           | 17           | 23.5                | 4                   | 17       | 23.5    |
|       | Total              | 7           | 50           | 14                  | 8                   | 50       | 16      |

Note. Real-time PCR performance was confirmed to be reproducible at the threshold cycles (Ct) < 35.

Figure 2. Agarose gel electrophoresis amplification of *L. pneumophila* by A) conventional-PCR (340bp) and B) nested –PCR (124bp) of the *mip* gene detected in cooling water samples in this study. Lanes 7-9 and 12 are water samples from Bam. Lane 11 is positive control (340bp). Lanes 1 and 14 are ladder consist of 100 base pairs DNA fragments. NC = negative control.
size or intensity were considered distinct types. Banding patterns were analyzed by UPGMA (unweighted pair-group method with arithmetic averages) clustering method using Gel Compare II software version 4.0 (Applied Maths, Sint-Matens-latem, Belgium). Degree of homology was determined by Dice coefficient. Isolates that clustered >95% were considered related. All statistical analysis was performed using SPSS 17.0 (SPSS, Chicago, IL, USA). p-value greater than 0.05 was considered as statistically significant for 2-tailed test.

The average water temperature in Bam city (38 ± 0.3°C) was higher as compared to Kerman (33 ± 0.3°C). The pH of both water samples were alkaline (pH- 8.7). Turbidity was higher in Bam (NTU = 14.1), however, total hardness was higher in water samples collected from Kerman (THD = 699.0 mg l⁻¹) as compared to Bam city (THD = 563.1 mg l⁻¹). Average chloride ion concentration of water samples obtained from Kerman (826 mg l⁻¹) was higher than Bam (787 mg l⁻¹). Water conductivity was more or less same in both the cities

Figure 3. The real-time PCR detection of *L. pneumophila* *mip* gene isolated from water cooling systems investigated in this study. Panel: A) Real-time PCR of DNA extracted from water samples of Kerman and Bam. Panel: B) The standard curve with the CT plotted against the concentration of the starting quantity of template for each dilution

Figure 4. Dendrogram analysis of whole *L. pneumophila* genomic DNAs obtained from water samples of Bam and Kerman cities. Banding patterns were analyzed by UPGMA (unweighted pair-group method with arithmetic averages) clustering method using Gel Compare II software version 4.0 (Applied Maths, Sint-Matens-latem, Belgium). Degrees of homology were determined by Dice coefficient. Isolates that clustered >95% were considered related.
(4750 $\mu$S/cm$^{-1}$). The average BOD and COD of both waters were approximately similar (7 ± 0.2 mg l$^{-1}$). Analysis of the water samples for presence of the $mip$ gene by nested-PCR revealed, 18 (23%) positive cases in Kerman and 7 (14%) in Bam (Table 1, Fig. 2A, B). However, when samples were tested by real-time PCR, we identified 4 more new cases $L$. pneumophila in the hospitals as well as nursing homes water samples that were missed by nested-PCR. The highest rate of contamination was detected in water obtained from hospitals cooling towers in both the cities. For building (homes/hotels), the number was much lower ($p < 0.05$). The data obtained by real-time PCR are shown in Table 1 and Fig. 3 A, B. This results were further supported by sequencing of the $mip$ DNA extracted from each $L$. pneumophila isolates. A blast search of the GenBank database demonstrates a high specificity, with the only cross-reacting bacteria being $L$. worsleiensis (GenBank accession number LWU60164) with 89% homology. Pairwise alignments with $mip$ sequences in NCBI database searched by neighbor joining method showed 98% of homology with $L$. pneumophila accession number NC_002942.5. The dendrogram analysis and clonal relationship of $L$. pneumophila genomic DNA showed 2 patterns of fingerprints among isolates obtained from Bam water samples (Fig. 4). Indeed, DNAs from water samples 6 and 7 demonstrated different clonal patterns while, clone 5 was a singleton. In contrast, most members of the DNA isolated from Kerman hospital and nursing home water coolants especially isolates 9-13 were closely related (clones 1 and 18 were identical) as the predominant type, and distributed commonly throughout the majority of environmental facilities.

Water cooling systems are frequently used during summer season in 2 cities of Kerman and Bam situated near the central desert of Iran. No information exists on rate of contamination of different water systems in this region. For this reason we attempted to analyze water systems of 2 main cities of this region for the presence of $L$. pneumophila by molecular techniques. Evaluation of physico-chemical parameters of water samples revealed average change in COD, BOD, total dissolved solids, alkaline pH and chlorine had not influenced on the presence of $L$. pneumophila DNA and survival of organism in different cooling water systems ($P > 0.05$; the $\chi^2$ test). Legionella are presently identified by comparing their 16S rRNA or $mip$ gene sequences, with known sequences deposited in GenBank. To validate the nested -PCR, we performed real-time PCR analysis targeted the $mip$ gene, we found 4 more cases of $L$. pneumophila corresponds to approximately one-two genome more equivalent per reaction as compared to nested-PCR. Furthermore, real-time PCR offers a rapid amplification of several samples simultaneously and diminishes the likelihood of laboratory contamination. Indeed, real-time PCR can supplement gold standard culture based detection of Legionella in environmental samples. The yearly incidence of Legionnaires’ disease seem to be associated with climate changes, only 4% of cases were associated with a known outbreak or possible cluster. The dendrogram analysis of the $L$. pneumophila genomic DNAs investigated in this study revealed close proximity among the lineages in environmental water samples of Kerman city, while, 2 patterns of fingerprints were observed in dendrogram obtained from Bam city indicating clonal divergence in these strains. Our results showed the clones 5, 6 and 7 were isolated from hotels water samples showed close banding pattern and suggest the isolates were transferred from visiting individuals. $L$. pneumophila Serogroup -1 had entirely different banding pattern. This may suggest that our environmental isolates especially from building/hotel water coolants are not closely related to serogroup- 1. However, 3 isolates obtained from hospital towers water samples showed almost similar fingerprint with $L$. pneumophila Serogroup -1. Similar cases were observed from Kerman isolates. Georghiou et al., studied molecular fingerprinting of Legionella species from different water systems and revealed substantial variation among the fingerprints of different Legionella species and serogroups. More limited, but distinct, polymorphisms of the fingerprint were observed among epidemiologically unrelated isolates of $L$. pneumophila serogroup 1. It has also been found in several studies (8, 18, 23) that endemic clones of $L$. pneumophila causing apparently unrelated cases of legionellosis have the same molecular genotype. In a study carried out by sequence-based typing using 6 loci, flaA, pilE,asd, $mip$, mompS, and proA in Japan, indicated that all 10 isolates from cooling towers clustered into a unique type, which was distinct from strains of other environmental sources.

In conclusion, $L$. pneumophila represents a potential pathogen, especially for some risk groups such as elderly, ICU patients. This study highlights the need of continuous monitoring, and risk assessment of water supplies of large buildings and hospitals. One advantage of the molecular detection is that it enables rapid interventions to limit infections caused by $L$. pneumophila. Molecular typing showed closed lineage similarities between environmental isolates but hospital isolates yielded distinct clusters and similarity with serogroup-1. Further research must be carried out on serogroup-structure of MIP protein.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.
Acknowledgments

The physico-chemical parameters analyses of waters obtained in this study were performed by environmental quality center, Kerman, Iran. We also thank hospital staffs and owners of buildings / hotels for their cooperation for collection of water samples.

Funding

This study was funded by Bam University of Medical Sciences, Iran (grant number 11/94).

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