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Prevalence and molecular distribution of *Legionella pneumophila* in cold water taps across Alborz province, Iran

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ABSTRACT

Background: The aim of this study is to explore the distribution of *Legionella pneumophila* (serogroups 1–6) in cold water taps across Alborz province, Karaj, Iran. In addition, some other non-fastidious gram-negative bacteria not belonging to the *Enterobacteriaceae* family (GNB-NE) were followed.
Subject: A total of 162 water samples were collected from 6 countries and tested by culture and genus-specific polymerase chain reaction (PCR). The *L. pneumophila* isolates were subsequently analyzed by monoclonal antibodies (MAbs) for serogroup identification, molecularly by PCR, and sequencing for genetic diversity. The patterns of resistance to antibiotics were determined by disk diffusion.
Results: Fifteen (9.25%) samples were detected as positive for *L. pneumophila* by PCR and 12 (7.4%) samples yielded growth in culture. All isolates of *L. pneumophila* were belonged to serogroup 1. Fifteen GNB-NE species were isolated from the samples, including nine isolates of *Pseudomonas aeruginosa* and six isolates of *Acinetobacter* spp. Antibiotic resistance was detected in 3 isolates of *P. aeruginosa*.
Conclusions: Despite the prevalence of *L. pneumophila*, few clinical cases have been reported, which may be due to underdiagnosis of the disease caused by it. Therefore, the prompt attention should be paid to do clinical diagnosis of legionellosis by the effective diagnostic tests from patients with compatible clinical symptoms.

1. Introduction

Legionella pneumophila (L. pneumophila) bacteria was discovered following a pneumonia outbreak at the 1976 American Legion Convention in Philadelphia.[1] It was isolated from the infected lung tissue and caused the epidemic Legionnaires' disease.[2] It is known that pneumonia comes to pass in approximately 95% of the *L. pneumophila* infections.[3] It rarely causes Pontiac fever, which is an influenza-like infection in humans.[4]

Until now, 53 species of the *Legionella* genus has been known, of which seven of them are divided into serogroups.[5,6] The bacterial strains in these serogroups are genetically homologous (based on DNA hybridization experiments); however, they are discernible through their specific reactivity to the antibodies.[1] Since now, the relationship between the infection by eighteen species of *L. pneumophila* and patients with pneumonia has been found.[5]

The incidence of legionellosis has been reported worldwide[7]; however, the accurate occurrence is usually undetermined due to the improper surveillance of the infected cases. The early detection of legionellosis relies on the increase of the physician's awareness about disease and development the requirement equipment to diagnose it.[7,8] The most preferable place for *L. pneumophila* is the aquatic environment.[8] According to the published reports, *L. pneumophila* can grow in biofilms, since it has adequate interplay with other organisms. It causes the increase of the survival duration and proliferation in water.[9–11] Therefore, they are resistant to the standard water disinfection methods and can pollute the drinkable water supplies.[9,11] Moreover, studies revealed the widespread incidence of *L. pneumophila* in the freshwater resources and marine waters.[12,13]

Although some phenotypic characteristics including gram stain, cell

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membrane fatty acid and ubiquinone content, morphology, and growth on the specific media are usually employed to diagnosis the *L. pneumophila* bacteria, more specific diagnostic techniques comprising DNA analysis and antigenic analysis should be utilized to discern individual species.[2,5,14]

For the first time, this study aimed to investigate the prevalence and genetic diversity of *L. pneumophila* in the cold water taps of the Alborz province. In addition, some other non-fastidious gram-negative bacteria not belonging to the *Enterobacteriaceae* family (GNB-NE) were traced and their antibiotic resistance were investigated.

2. Methods

2.1. Sample collection

Alborz Province is situated in North-West of Tehran. It has six counties, Karaj, Savojbolagh, Taleqan, Eshtehard, Fardis, and Nazarabad. A total of 162 samples were collected between 2015 and 2016, from 24 sampling sites along 6 counties. 1000 ml of water taps were filled into sterile non-toxic 1000 mL plastic Bottles and then the samples were transferred to the microbiology lab in less than 4 h.

2.2. Sample preparation and DNA extraction

The filtered samples were used for DNA extraction. The samples were filtered using cellulose nitrate membrane filters with a pore size of 0.45 μm (Sartorius AG, Goettingen, Germany). Membranes were broken into small pieces inside the 30 ml sterile plastic container with 25 ml original filtered water. The container was shaken at 37 °C for 24 h in order to release the bacteria from filter to the filtered water. Then, 10 ml subsample was taken from each filtered samples and treated by heating at 50 °C for 30 min to inactivate the organisms except *L. pneumophila* spp.[15] After treatment, 100 μl of each water sample was inoculated on blood agar and BCYE (Becton, Dickinson and Company, MD, USA) medium supplemented with glycine, vancomycin, cycloheximide, and polymyxin B (GVPC).The plates were incubated under microaerophillic conditions at 35 °C (90% humidity, 3% of CO2) for 7 days.[15]

The gram negative rods or coccobacilli were recognized as *L. pneumophila* if they grow on BCYE agar and showed positivity in catalase and oxidase tests.[16] Direct fluorescent antibody kit (Prolab, Texas, USA) was used to determine the serogroups.

To recover GNB-NE, 10 ml of each filtered samples were placed on the tryptic soya agar medium.[17]

2.3. Polymerase chain reaction (PCR) assay

DNA was extracted using the PrimePrep Genomic DNA isolation kit (GENET-BIO). For molecular identification of *L. pneumophila*, following primers of mip gene were used in PCR test. F: GGCATAGATGTTTATC CGG, R: GTGAAACCTGAAAACTTGCT. The mip gene of *L. pneumophila* encodes a 24 kDa protein that promotes the entry of bacteria into macrophages and amoeba.[18,19] The final optimized PCR reaction consisted of 1 μl of each primer (10 pmol), 0.5 μl dNTP (10 mM), 0.5 μl MgCl (100 mM), 0.2 μl (1 unit) Taq DNA polymerase (Metabion, Germany), 2.5 μl PCR buffer (10X), and 0.5 μl of DNA template (100 μg/ml) in total volume of 25 μl with double distilled water. The cycling program was adjusted as follows: 35 cycles of 1 min at 94 °C, 30 s at 49 °C, and 30 s at 72 °C, followed by a final extension of 6 min at 72 °C.

2.4. DNA sequencing and phylogenetic analysis

Firstly, the PCR products were purified using Sephadex. ABI (Applied Biosystems) BigDye 3.1 Chemistry was used for cycle sequencing. Double-stranded sequencing runs were performed on 3130 Genetic Analyzer (Applied Biosystems).The mip gene was used to identify the *L. pneumophila* genotypes. The sequencing outcomes were collected and aligned with each other using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The genotyping was carried out by phylogenetic analysis with reference sequences. The reference sequences were obtained from GenBank database (NCBI). Phylogenetic tree reconstruction was performed using MEGA software (version 6.06) (http://www.megasoftware.net), with the Maximum likelihood method (ML) in Kimura-two parameter substitution model with 1000 bootstrapping replicates.

2.5. Isolation and identification of GNB-NE

The tryptic soya agar (Merck KGaA, Darmstadt, Germany) and *Pseudomonas* selective agar base (Cetrimide Agar, Merck, Germany) were used for isolation of GNB-NE. The inoculated tryptic soya agar plates were incubated for 24 h at 37 °C. The grown colonies were counted and differentiated by biochemical test. The plates containing *Pseudomonas* selective agar base were incubated at 42 °C, and then positive oxidase test was performed for confirmation of the colonies.[15,17]

2.6. Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were performed using disk diffusion method on Mueller–Hinton agar. Antibiotics used in the disk diffusion method were chosen randomly from different antibiotic classes, which were including cephalothin (30 μg), cefepime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), meropenem (10 μg), imipenem (10 μg), piperacillin/tazobactam (10/100 μg), gentamicin (10 μg), colistin, gatifloxacin (5 μg), ampicillin (10 μg), and amikacin (30 μg). All antibiotic disks were purchased from Mast Company (UK).

2.7. Study selection & data extraction

In order to perform a comparative study, some related reports regarding to prevalence of *L. pneumophila* in Iran published between 2010 and 2018 were found in MEDLINE (PubMed) and the Institute for Scientific Information (ISI) database. Then, they were screened at three levels: title, abstract and main text levels. A total of 13 eligible papers were selected for further consideration. Afterwards, the following information was extracted from each study: the first author, year of survey, region, sample, sample size, confirmed cases, and prevalence.

3. Results

3.1. Isolation and identification of L. pneumophila strains

From 162 water samples, 15 (9.25%) were positive for *L. pneumophila* by PCR and 12 (7.4%) yielded growth in culture, which indicates a higher sensitivity of PCR in comparison with culture method. The number of CFUs for *L. pneumophila* in positive culture was 100 CFU/100 ml. We also isolated 6 isolates of *Legionella* other than *pneumophila* that grew on GVPC agar. The PCR were positive only for one sample for these isolates, because mip gene is solely present in the *L. pneumophila* genome. Nine isolates of *L. pneumophila* belonged to serogroup 1 as determined by direct fluorescent antibody. The remaining isolates were not identified as serogroup (Fig. 1).

3.2. Genotyping and phylogenetic analysis

To confirm genotyping by direct fluorescent antibody, a 402 bp PCR product of the macrophage infectivity potentiator protein (mip) was used to perform the phylogenetic analysis with the corresponding regions of 12 reference sequences of *L. pneumophila* serogroups 1–6, which are available in the GenBank database. The six samples that were not detected by serology tests in addition to one confirmed sample were

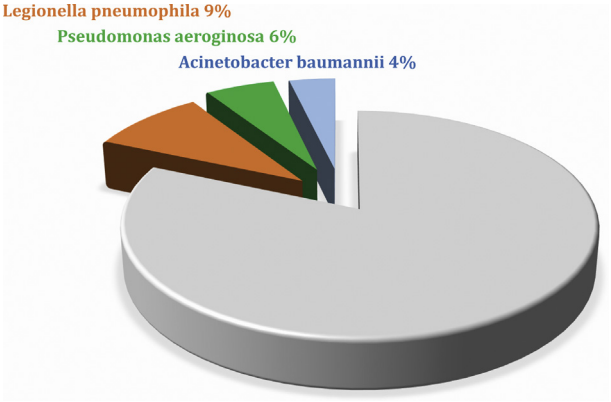

Fig. 1. Distribution of *L. pneumophila*, *P. aeruginosa*, and *acinetobacter baumannii* in samples of cold water tap in Alborz province, Iran.

sequenced to construct the phylogenetic tree. The nucleotide sequence analysis of seven isolates confirmed the circulating of *L. pneumophila* serogroup 1 in the Alborz province (Fig. 2). The *L. pneumophila* sequences demonstrated 98–99% homology with serogroup 1 sequences available in the GenBank database.

3.3. Isolation of (GNB-E) and GNB-NE in water samples

Gram-negative bacteria belonging to the *Enterobacteriaceae* family (GNB-E) were not found in the examined water samples. Fifteen GNB-NE species were isolated from the water samples, which included nine isolates of *P. aeruginosa* and six isolates of *Acinetobacter* spp. The number of CFUs detected for *P. aeruginosa* and *Acinetobacter* spp. were ranged from 50 to 300 CFU/100 ml and 20–100 CFU/100 ml, respectively. Three samples were simultaneously positive for *L. pneumophila* and *P. aeruginosa,* (Fig. 1).

3.4. Antibacterial susceptibility testing

In this study, only colistin antibiotic resistance was detected for 3 isolates of *P. aeruginosa* based on CLSI reference guide-lines for disk diffusion method.

3.5. Study selection & data extraction

Table 1 summarizes different reports on distribution of *L. pneumophila* in water of different regions and provinces of Iran (Table 1). The highest prevalence was reported by Asghari. F. B, which was found in the hospital water systems of Isfahan in 2013.[20] After that, the prevalence of 41.17% was reported by Tabatabaei. M. in the cold and warm water systems of South-West of Iran in 2016.[21]

4. Discussion

The pollution of water resources with *L. pneumophila* species can exacerbate the incidence of Legionnaires' disease. Therefore, the public water resources should be investigated in terms of the presence of *L. pneumophila*.

This report disclosed the presence of 15 and 12 *L. pneumophila* -positive samples using PCR and growth in culture techniques, respectively, in the cold water taps of Alborz province, Iran. However, only nine samples of 15 PCR-confirmed were detected by serology tests. The serogroup of nine isolates of *L. pneumophila* was determined as serogroup 1. In order to determine the serogroup of the other 6 PCR-confirmed, the phylogenetic tree was constructed and disclosed the same serogroup. In addition, the presence of six isolates of *L. pneumophila* was found.

Water is a proper medium for living *L. pneumophila*, which leads to

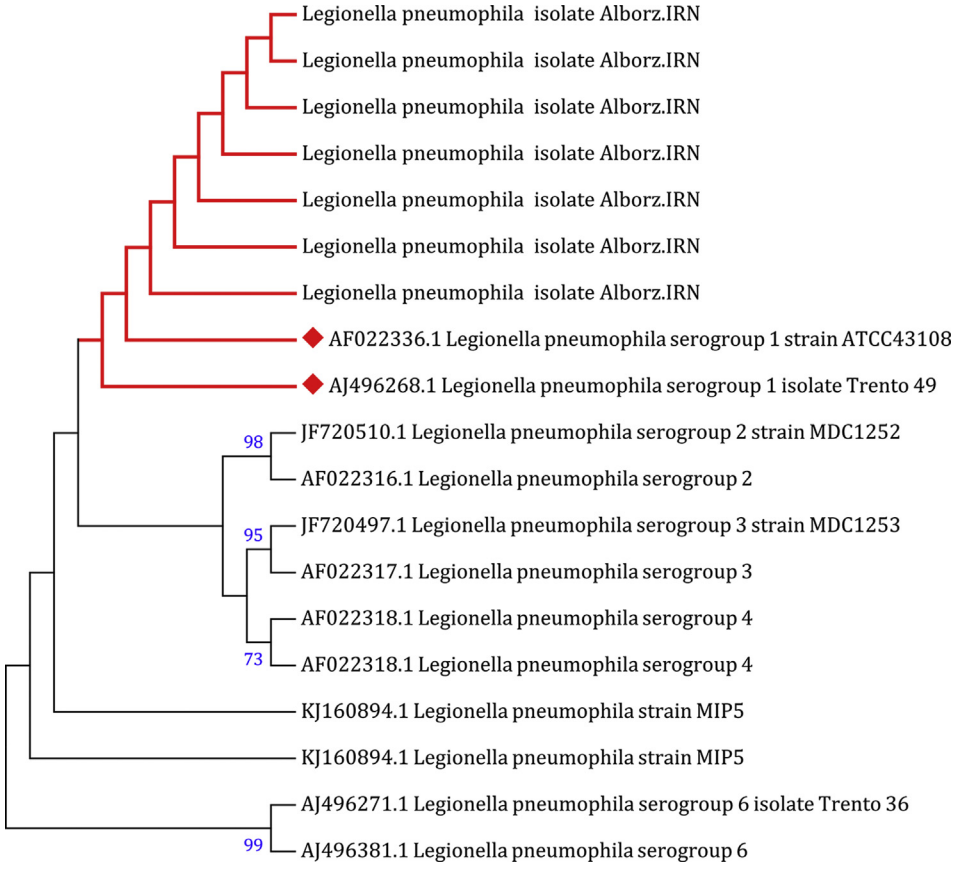

Fig. 2. The subtyping of *L. pneumophila* by mip gene sequences characterized in samples of cold water tap in Alborz province, Iran. Phylogenetic tree reconstruction was conducted using MEGA software (version 6.06), with the maximum likelihood method in the Kimura two parameter substitution model with 1000 bootstrap sampling. All obtained sequences made the same clade with the serogroup 1.

Table 1
Summary of the selected studies conducted on *L. pneumophila* distribution in water of different regions and provinces of Iran.

Row	Region	Sample	Sample size	Confirmed cases	Detection method	Prevalence	Ref
1	South-West of Iran	Cold and warm water systems	34	14	PCR	41.17%	21
2	Tehran	Water and dialysate samples	50	4	PCR	8%	22
3	Tehran	Hot and cold water systems	150	33	Biochemical tests	22%	23
4	Kerman and Bam	Cooling water systems	128	25	Real Time PCR	19.50%	24
5	Isfahan	Bio-aerosols generated from wastewater treatment plant	54	3	PCR	6%	25
6	Bandar-e-Abbas	Home air-conditioning system	66	14	Biochemical tests	22%	26
7	Tehran	Hospital Water Systems	45	14	PCR	31.10%	27
8	Tehran	Hospital water supplies	45	13	PCR	29%	28
9	Tabriz	Tap water of Tabriz hospitals	140	10	PCR	14%	29
10	Isfahan	Hospital water systems	44	29	PCR	66%	20
11	Tehran	ICU wards of Tehran hospitals	52	5	Real Time PCR	9.60%	30
12	Mashhad	Dental unit water line system	52	19	ELISA	36.1	31
13	Khuzestan	Fishponds, swimming pools and cooling towers	150	23	PCR	15.3	32

its survival period in the human water resources and subsequently infectious possibility of consumers.[33] The Legionnaires' disease caused by *L. pneumophila* infection has usually not been reported, because of inattention of clinicians to prescribe the laboratory tests and health authorities to trace the suspicious cases.[34] Therefore, the detection of *L. pneumophila* in public community resources can lead to make serious decisions to prevent the prevalence of disease caused by it.[35,36] The previously studies on the presence of *L. pneumophila* in Iran (Table 1) revealed high prevalence in different kind of water supply systems. Moreover, infection of hospitals water with *L. pneumophila* is more worrying, since the vulnerable groups are more exposed to the infection.[20] Also, the water and wastewater treatment system of Iran should be more considered in terms of eliminating *L. pneumophila* from urban water systems.

The gold standard for detection of *L. pneumophila* is culture, which confronts with some limitations and problems including time-consuming, low sensitivity, rather than molecular techniques, negative grow after initiation of antibiotic therapy, and need to proper preprocessing of samples. PCR technique can resolve the mentioned obstacles and increase the detection accuracy.[37,38] The selection of specific target regions of the *L. pneumophila* gene is important to reach precise results. To this end, we elected mip gene that covered all serogroups as specified by blasting of genetic databases. Using mip gene in PCR assay, 15 positive samples were detected among 162 water samples, while only 12 positive samples were found by culture. *L. pneumophila* serogroup 1 is the predominant cause of legionellosis in many, parts of the world, but in other areas, other serogroups are more important. For example, *Legionella longbeachae* is a major cause of legionellosis in Australia and New Zealand.[39] On the other hand, serogroup 1 is more important in pathogenesis and induction of clinical signs.[8] The genotyping results from this study confirmed the presence of serotype 1 in the water taps. This could have provide early warning to the relevant authorities.

Enterobacteriaceae is a large family of gram-negative, which usually housed in gastrointestinal tract of human and animals. Some members of this family have pathogenesis potential and are drug-resistant. In this study, the GNB-E was not detected in the water samples. However, nine isolates of *Pseudomonas aeruginosa* and six isolates of *Acinetobacter* spp. belonging to non-gram-negative *Enterobacteriaceae* were found. Moreover, the superinfection of *L. pneumophila* and *P. aeruginosa* were detected in three samples, which can lead to severe problems in the infected subjects. Although, only three isolates of *P. aeruginosa* showed colistin antibiotic resistance, the *Enterobacteriaceae* resistance must be considered in order to prohibit the following drug resistance among the infected cases.

5. Conclusion

The present study revealed that more attention should be paid to decontaminate of community water system from *L. pneumophila*, *P. aeruginosa*, and *Acinetobacter* spp. in Alborz Province, Iran. Moreover, the common serology technique cannot precisely detect the *L. pneumophila*; however, PCR seems to be a good replacement.

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Conflicts of interest

None declared.

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