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Prevalence and Molecular Diversity of *Legionella pneumophila* in Domestic Hot Water Systems of Private Apartments

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Authors' contributions

This work was carried out in collaboration between all authors. Author KMK designed the study and managed the literature searches. Authors AMMH and JAO did the laboratory work, wrote the protocol and wrote the first draft of the manuscript. All authors managed the analysis of the study, read, corrected and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To determine the prevalence of *Legionella* spp. in domestic hot water systems and evaluate the molecular diversity among these *Legionella* spp. Isolates.
Place and Duration of Study: Sample collection area was the city of Aqaba, Jordan, between May and December 2012. Sample analysis was done in Ben-Hayyan international laboratories, Aqaba city, and the molecular microbiology laboratories, Taibah University, Saudi Arabia.
Methodology: Two hundred (200) water samples were collected randomly from hot water tanks of private apartments, and were tested for the occurrence of *Legionella* spp. using direct membrane filtration method followed by species identification using Gram stain, the API 20NE biochemical system and the *Legionella* species latex agglutination test. Genotype characterizations of the *Legionella* isolates was carried out using DNA extraction followed by RAPD-PCR amplification with OP-A3 primer and analysis of the resulting patterns.
Results: Of the 200 samples, 17 (8.5%) were positive for the presence of *Legionella* spp. A

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total of 15 (88.2%) out the 17 positive samples were confirmed as *Legionella pneumophila*, 10 of them were of serogroup 1 and 5 isolates were of serogroup 2-14, the remaining two isolate were *Legionella* species other than *L. pneumophila*. RAPD-PCR analysis classified all 17 *Legionella* isolates into three groups. Serogroup 1 isolates were classified into group A, serogroup 2-14 isolates in group B and *Legionella* spp. isolates in group C. Group A was further sub-clustered into two subgroups, genotype A_1 containing isolates collected from hot water tanks of a temperature set at 25-30°C and A_2 containing isolates collected from hot water tanks of a temperature set at 55-80°C.

Conclusion: This study showed the colonization of the plumbing systems of private houses by *Legionella* spp. and demonstrated that the temperature of the water tanks maybe one of the most important factors that affect the genotypic behavior of *Legionella pneumophila*.

Keywords: Legionella pneumophila; Aqaba city; water heating system; RAPD-PCR; genotyping.

1. INTRODUCTION

Legionella spp. are gram negative, rod shaped, aerobic, flagellated, non spore former pathogenic bacteria that belongs to the gamma Proteobacteria [1]. *Legionella* is responsible for uncommon type of human respiratory disease called Legionnaires' disease or legionellosis which can be fatal if not treated [2-3].

By 2005, 48 species of *Legionella* with about 64 serogroups have been identified. Most notably is *Legionella pneumophila* which displays 15 serogroups. The majority of human cases (more than 80% worldwide) are due to the serogroup1 (Lp1) [4-5].

L. pneumophila usually enters the lung after inhalation of contaminated aerosol droplets of water [6]. Once the bacteria transfer to the alveoli, it undergoes phagocytosis by alveolar macrophages. The inhibition of phagosomal fusion gives the bacteria the opportunity for intracellular multiplication until it ruptures the cell and transfer to other macrophages [7].

Tolerance to elevated temperature is one of the most critical factors that affect the presence and multiplication of *L. pneumophila* in hot water systems [8-9]. Although *L. pneumophila* optimal temperature range of reproduction is between 30°C and 35°C, still it can survive in water at temperatures ranging from 0 to 70°C [10-12].

Another factor that affects the survival of *L. pneumophila* is its ability to grow and proliferate intracellularly inside biofilms of other organisms such as fresh water protozoa which is formed at the solid–liquid interfaces or at the liquid–air interface [13]. Also factors such as, the presence of sediment materials, stagnation, roughness of surfaces, flow rate, materials from which surface is made and disinfectants' concentration affect the accumulation of *L. pneumophila* in the pipelines, and sometimes support its colonization of the water systems in a way that makes it hardly eradicated [14].

DNA-based typing methods are becoming increasingly useful for performing epidemiological surveys of *Legionella*, and the success of these epidemiological surveillance studies is related to the typing procedures applied [15-21]. Randomly amplified polymorphic DNA (RAPD) analysis among other genetic typing procedures has shown to be simple, rapid,

easy to interpret, low cost and flexible tool to trace *Legionella* isolates epidemiologically, with great discriminatory power to distinguish between its strains [15,16,18].

Clear understanding of the factors affecting the colonization of water pipes and domestic water systems by *Legionella* spp. will help in determining the control and the prevention strategies for legionellosis. In Jordan very few studies have been carried out on the prevalence of *Legionella* spp. in domestic water. This study gives an idea about the prevalence of *Legionella* spp. in domestic hot water systems in Aqaba city which consequently help in controlling these pathogens. We also evaluated the molecular diversity among some of the isolated *Legionella* strains using random amplified polymorphic DNA (RAPD)-PCR typing.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Random collection of 200 water samples was carried out from the same number of private apartments located within ten different residential areas in the city of Aqaba. Collection of samples was taken from various hot water outlets over a period of eight months starting from May and till the end of December 2012. All buildings were supplied by the same water network, and being treated with chlorine dioxide. Hot water samples were drawn from the bathroom outlets (showers or taps) and placed in pre-sterilized 250ml borosilicate sterile glass bottles for bacteriological analysis, after a flow time of 3–5 min to eliminate any residual water present inside the tap or flexible shower pipes. In order to neutralize the residual free chlorine, 10% sodium thiosulphate was added to all collected samples. After collection samples were transferred to the laboratory on dry ice and were analyzed within 12 h of collection.

2.2 Bacteriological Identification

Isolation of *Legionella* species was conducted according to Leoni *et al*. using direct membrane filtration method with slight modification [14]. Briefly, 100 ml of the collected water sample was placed in a 50ºC water bath for 30 min then concentrated through a Sartorius nitrocellulose membrane filters with nominal pore sizes of 0.45µm using Sartorius manifold filtration unit (polyamide filter, Millipore, Bedford, MA, USA). After filtration, 25ml of acid buffer (pH 2.2 – 2.4) was added directly to the membrane and incubated for 15 minutes at room temperature, followed by washing away the acid buffer using 20 ml Page's saline, this heat and acid treatment steps are very useful in the decontamination of other micro-organisms. The filter membranes were then placed on duplicate plates of CYE agar (charcoal yeast extract base agar, Oxoid) with BCYE growth supplement (ACES buffer/potassium hydroxyde, L-cysteine, ferric pyrophosphate, alpha-ketoglutarate) and MWY selective supplement (glycine, vancomycin, polymyxin B, anisomycin) and incubated at 35ºC, in 2.5% CO_2 for 7–14 days [20]. The plates were examined at periodic intervals starting from day three of incubation, for the typical ground-glass appearances of the *Legionella* species colony using low magnification plate microscope (Leica Microsystems, Wetzlar, Germany).

The selected colonies from the previous step were subjected to species identification using Gram stain, the API 20NE biochemical system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's protocol. The *Legionella* species latex agglutination

serotype test which consist of blue latex particles linked to specific rabbit antibodies of different serogroup antigens of *L. pneumophila* (Oxoid kit, code DR0800, Thermo Fisher Scientific, USA) allowed the separate identification of *Legionella pneumophila* serogroup 1 and serogroups 2-14.

2.3 RAPD PCR Analysis

Nucleic acid extraction from *Legionella* isolates was performed using the NucliSENS easyMag automated extraction system (bioMérieux, France). Manufacturer's DNA extraction protocol was used with elution volume of 25 µl, and was kept at –20°C until use for RAPD-PCR amplification. Oligonucleotide primer was commercially synthesized by Operon (Operon, A Qiagen Company, Qiagen GmbH, Germany). OP-A3 (5'-AGTCAGCCAC-3') 10-mer primer was resuspended in TE buffer, stored at -20ºC, and 10 µM (10 pm/µl) working solution was prepared to be used in RAPD-PCR typing. The reaction mixture (25 µl) contained 10 mM Tris–HCI pH 7.5, 50 mM KCI, 1.5 mM MgCl2, 0.5 mM spermidine, 0.1 mM dNTPs, 15 pmol of the RAPD primer, 20 ng genomic DNA, and 0.8 U of Taq DNA polymerase. Amplification was carried out in a heated-lid automated DNA thermal cycler (Perkin-Elmer Applied Biosystems Inc., USA) for 40 cycles, each consisting of a denaturing step of 1 min at 94ºC, followed by annealing step of 1 min at 36ºC and an extension step of 2 min at 72ºC. The last cycle was followed by 5 min of long extension at 72ºC. The amplification products were separated by gel electrophoresis in 2.2% agarose (Sigma Aldrich Chemical Co., St. Louis, USA) in 45 mM Tris–borate, 1 mM EDTA buffer (pH = 8.0), containing ethidium bromide at 0.5 µg/ ml at a constant voltage of 5 V/cm. The gels were photographed under UV transillumination using gel documentation system (GelDoc 2000, Bio-Rad, UK).

2.4 Analysis of RAPD PCR Data

The RAPD-PCR profiles are defined only by the pattern of presence and position of bands on the gel. Gel images were then analyzed for genetic similarity among isolates using the Gelcompar II software (Applied Maths, Kortjik, Belgium). The similarities between DNA fingerprints were calculated with the band-matching Dice coefficient. A pairwise similarity (or distance) matrix was developed and cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic averages (UPGMA) method with a high Cophenetic correlation of 0.5, suggesting that the dendrogram preserves the pair wise distances between the original un-modeled data points. Highly related strains (groups) were defined as those having at least 90% homology on the basis of banding patterns.

2.5 Statistical Analysis

All statistical analyses were performed using IBM SPSS statistics (V. 19.0, IBM Corp., USA). Data were expressed using cross-tabulation as both number and percentage for the categorized data.

3. RESULTS AND DISCUSSION

Two hundred hot water samples taken from ten different residential areas within Aqaba city were tested for the presence of *Legionella*. Seventeen out of the 200 samples (8.5%) gave positive results displaying an auxotrophy for cysteine, a known characteristic of *Legionella*

spp. This percentage is found to be in agreement with other studies (ranging from 6.4–32%) regarding the prevalence of *Legionella* spp. in a single household [22-23].

Oxoid latex agglutination tests allowed us to show that 15 out of the 17 *Legionella* isolates belonged to the species *pneumophila* (88.2%), while the remaining two isolates (11.8%) were found to be other *Legionella* species. From the fifteen *L. pneumophila* isolates ten were confirmed as *L. pneumophila* serogroup 1 and five were *L. pneumophila* serogroup 2-14. Similar results regarding the prevalence of *L. pneumophila* in domestic hot water systems were also recorded in other studies [11-12, 14]. The areas along which sampling was performed and the number and percentages of positive samples in each residential area are summarized in table 1.

In this study 10 out of the 17 positive samples were collected in summer (from May to September). During this period of the year the water heating systems in Aqaba city are usually turned off, and thus the water temperature inside the heater storage tank ranges between 25 to 30°C. Still this temperature was found to support the survival and the growth of *Legionella* isolates. The remaining seven positive samples were collected in winter (mainly November and December), a period during which water heating systems are usually turned on with variable heat set points. Six out of the seven positive samples were collected from water heating systems set between 55°C to 60°C to prevent scalding. This finding along with other reports [9,14,22] suggests that the temperature of the water heating systems is the most important factor in occurrence of *Legionella* in the hot water tanks.

In contrast with the results reported by Krojgaard et al. [23], regarding the isolation of *Legionella* from newly built block of flats in Denmark, water samples collected from all three newly established residential areas within Aqaba city tested negative for the presence of *Legionella*. This may be due to the fact that these areas have a new water piping network and new heaters compared to the other ones covered in this study.

All 17 isolates were selected for RAPD-PCR analysis, taking into consideration the species and temperature at which the domestic hot water system was operating. OP-A3 RAPD primer was employed in this study for it has been successful in sub-grouping *Legionella* isolates [24]. RAPD-PCR amplification of the selected *Legionella* isolates' DNA showed a number of major bands (ranging from 200 bp to over 3 kb). Data interpretation criteria were based on differences in formation and/or position of the bands, The RAPD banding patterns generated by primer OP-A3 for the selected *Legionella* isolates are shown in Figure 1.

Similar to previous studies [15-16,18,24] the DNA-typing methods used in the present study exhibited the ability to group the selected *Legionella* isolates, and were also able to discriminate differences in highly related isolates both visually and with the aid of computer-assisted procedures.

Fingerprinting generated by OP-A3 was further evaluated using a well-proven computer-assisted data analysis system, to define related strains on the basis of RAPD-PCR banding patterns. In agreement with the dendrogram of genetic similarity all selected isolates were classified into three major groups (A, B and C). Group A included all *L. pneumophila* serogroup 1 isolates. Group B included *L. pneumophila* serogroup 2-14 isolates and group C contained the remaining two *Legionella* spp. isolates, as it is shown in Fig. 2.

Table 1. Distribution of the positive *Legionella* isolates recovered in this study in accordance to their frequency of occurrence among the different residential areas[a]

Residential Area	No. of collected water Samples	No. of positive samples	positive samples (%)	Isolate number	Species and serotype	Mean Count CFU/100ml	Heater[b] temperature set Range (ºC)
Alamyia	20	1	5	10	*L. pneumophila* 2-14	80	25-30
Al-Mahdood	20	1	5	5	*L. pneumophila* 1	360	25-30
Al-Balad	35	3	8.5	8	*Legionella* spp.	70	25-30
				9	*Legionella* spp.	250	25-30
				11	*L. pneumophila* 1	100	25-30
Al-Sadisa	15	0	0	–	–	–	55-60
Al-Thalitha	20	2	10	4	*L. pneumophila* 2-14	330	25-30
				13	*L. pneumophila* 2-14	370	80-90
Al-Sharqyia	20	5	25	1	*L. pneumophila* 1	20	25-30
				6	*L. pneumophila* 1	400	25-30
				14	*L. pneumophila* 1	40	55-60
				15	*L. pneumophila* 1	30	55-60
				16	*L. pneumophila* 1	200	55-60
Al-Gharbyia	20	4	20	2	*L. pneumophila* 1	1×10^4	25-30
				3	*L. pneumophila* 2-14	230	55-60
				7	*L. pneumophila* 1	60	25-30
				17	*L. pneumophila* 1	22	55-60
Al-Khamisa	15	0	0	–	–	–	25-30
Al-Thamina	15	0	0	–	–	–	25-30
Al-Ashira	20	1	5	12	*L. pneumophila* 2-14	440	55-60

[a]200 samples collected from the same number of private apartments

[b]in summer (May to September) water heating systems are turned off, and the water temperature inside the heater storage tank ranges between 25 to 30°C. in winter (November and December), water heating systems are turned on with heat set points usually between 55°C to 60°C.

Group A was further sub-divided into two sub groups (A1 and A2), the first contained *L. pneumophila* serogroup 1 isolates collected from water heaters set at a temperature ranging between 25-30ºC, the second sub group contained *L. pneumophila* serogroup 1 isolates collected from water heaters set at temperature ranging between 55-60ºC, suggesting that, a correlation between genotypes and the temperature at which the water heating systems were operating may be found and that the temperature of the water tanks maybe one of the most important factors that affects the genotypic behavior of *L. pneumophila*

Although differences in the RFLP patterns were noted among the *L. pneumophila* serogroup 2-14 isolates collected from water heaters set at a temperature ranging between 25-30ºC and isolates collected from water heaters set at temperature ranging between 55-60ºC, however, these variations were not taken into account when evaluated using Gelcompar II computer-assisted data analysis system and the patterns of all *L. pneumophila* serogroup 2-14 isolates were interpreted as being similar and thus were kept in the same group. This may be due the fact that the total number of isolates belonging to *L. pneumophila* serogroup 2-14 was too small to be analyzed properly, which necessitates trying to put more effort in properly identifying the *L. pneumophila* 2-14 serogroups and observe their genetic diversity using various DNA typing methods in future work. The characteristics along with molecular profiles of the selected *Legionella* isolates are all summarized in table 2.

In our study only one positive isolate (with a genotype pattern similar to those collected from heating water tank with temperature set at 50 – 60°C) was collected from water heater set at maximum point ranging between 80 – 90°C, and to confirm the ability of the isolate to survive this elevated temperature (80 – 90°C) it was further inoculated on duplicate slants of CYE broth with BCYE growth supplement and MWY selective supplement (as previously explained in the materials and methods section) and incubated at different temperatures ranging from 40–80°C for 24hr, after which the isolate was transferred to duplicate plates of CYE agar and incubated at 35°C for 3–7 days to observe colony formation. NO growth was detected on plates inoculated with isolates grown for 24 hr over 60°C. These results as previously reported suggests that, hot water systems set at maximum heating point may be responsible for the relatively low number of *Legionella* isolates colonizing them, and that the isolation of *Legionella* from these hot water systems maybe due to the fact that sometimes the actual temperature in the bottom of the tanks is much lower than the set one [10,16,25].

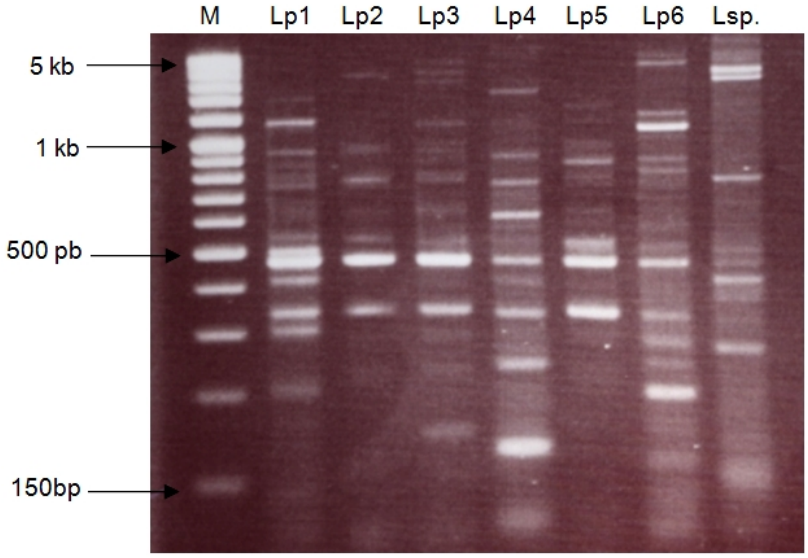

Fig. 1. Random amplified polymorphic DNA fingerprinting of some representative *Legionella* isolates obtained using primer OP-A3. Lanes M: DNA molecular size marker (100bp plus DNA ladder). Lanes (Lp1, 2, 3 and 5) are *L. pneumophila* 1 isolates No. 1, 14, 15, and 7 respectively. Lanes (Lp 4 and 6) are *L. pneumophila* 2-14 isolates No. 10 and 12 respectively. Lane Lsp refers to the *Legionella* spp. isolate No. 9.

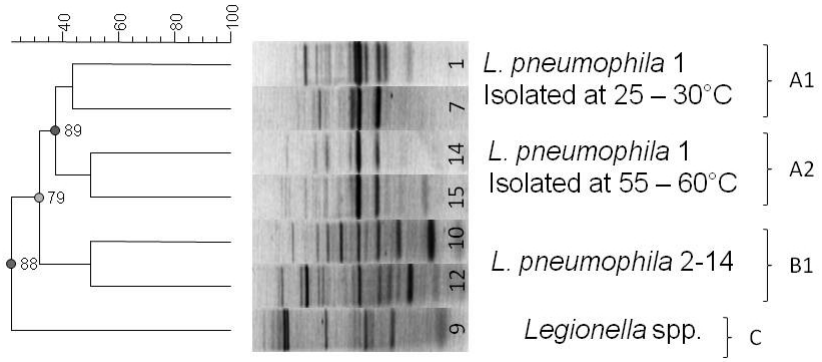

Fig. 2. Dendrogram representing the clusters of similarities and gel band patterns of Random Amplified Polymorphic DNA of some representative *Legionella* isolates typed with OP-A3 primer. The scale at the top represents percent similarity. The isolate numbers and genotypes are listed to the right of the figure and correspond to those in table 2

Table 2. The Random amplified polymorphic DNA types generated from the selected *Legionella* isolates using OP-A3 primer

Isolate number	Species and serotype	Heater temperature set Range (ºC)	RAPD Type
1	*L. pneumophila* 1	25 – 30	A_1
2			
5			
6			
7			
11			
14	*L. pneumophila* 1	55 – 60	A_2
15			
16			
17			
4	*L. pneumophila* 2-14	25 – 30	B
10		25 – 30	
3		55 – 60	
12		55 – 60	
13		80 – 90	
8	*Legionella* spp.	25 – 30	C
9	*Legionella* spp.		

4. CONCLUSION

The current study showed the colonization of the plumbing systems of private apartments located within different residential areas in the city of Aqaba by *Legionella* spp. with a maximum count of 1×10^4 CFU/100 ml. This study also provided data on the relation between RAPD genotypes profiles and the tolerance of the *L. pneumophila* isolates recovered in this study to elevated temperature. The results of the genotyping suggested that there is a high degree of heterogeneity among *L. pneumophila* serogroup 1 isolates collected from water heaters set at temperature ranging between 25-60ºC. We can also suppose that this high tolerance of *L. pneumophila* serogroup 1 isolates to elevated temperature enhance their capacity towards colonizing these water heating system and may result in increasing the genetic diversity among the isolates. Such findings may be useful as baseline information on the colonization of the water heating systems of private apartments located within the city of Aqaba by *L. pneumophila*.

In this manuscript it is also important to point out that, one of the limitations to this study was the small number of the total *L. pneumophila* isolates, but despite the limited number of tested isolates, the results observed in this study should be viewed with concern and necessitates continuous monitoring of *Legionella* through similar surveillance studies in future work. These studies should be conducted to include other cities and other water systems in the whole middle eastern area in order to give us a clear image about the prevalence of *Legionella* and further try to develop an international legislation and prevention measures to aid in reducing the *Legionella* related pneumonia cases, and also to help in the control of *Legionella*, which consequently help in preventing travel-associated legionellosis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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