

Research Paper

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

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 houses, 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 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 *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 A₁ containing isolates collected from hot water tanks of a temperature set at 25-30°C and A₂ 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 but 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 aerosols droplets of water [6]. Once the bacteria transferred to the alveoli, it undergo phagocytosis by alveolar macrophage, 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 [15-20]. 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. MATERIAL AND METHODS

2.1 Collection of samples

Water samples (about 200 samples) were collected over a period of eight months starting from May and till the ended of December 2012, from various hot water outlets of private houses located within different areas in Aqaba city. 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 50°C 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 minute 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 35°C, in 2.5% CO₂ 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 (bio-Me'rieux, Marcy l'Etoile, France) and the *Legionella* species latex agglutination test (Oxoid kit, code DR0800, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. This serotype test allows 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 -20 °C, 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–HCl pH 7.5, 50 mM KCl, 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 94°C, followed by annealing step of 1 min at 36°C and an extension step of 2 min at 72°C. The last cycle was followed by 5 min of long extension at 72°C. 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 PCR profiles are defined 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 nine different residential areas within Aqaba city were tested for the presence of *Legionella*, only 17 out of the 200 samples (8.5%) gave positive results displaying an auxotrophy for cysteine, a known characteristic of *Legionellae* 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 [21-22].

Oxid latex agglutination tests which consist of blue latex particles sensitized with specific rabbit antibodies of different serogroup antigens of *L. pneumophila*, 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 *Legionella pneumophila* isolates ten were confirmed as *Legionella pneumophila* serogroup 1 and five were *Legionella pneumophila* serogroup 2-14. Similar results regarding the prevalence of *Legionella 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, 21] suggests that the temperature of the water heating systems is the most important factor in occurrence of *Legionella* in the hot water tanks.

In our study only one positive sample was collected from water heater set at a maximum temperature ranging between 80 – 90°C suggesting that hot water systems set at maximum heating point may be responsible for the relatively low number of isolates colonizing them. Another explanation to the isolation of *Legionella* from hot water systems set at the maximum heating point is that sometimes the actual temperature in the bottom of the tanks may be much lower than the set one [10, 16, 22].

In contradiction 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.

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

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

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

201
202 Group A was further sub-divided into two sub groups (A1 and A2), the first contained *L.*
203 *pneumophila* serogroup 1 isolates collected from water heaters set at a temperature ranging
204 between 25-30°C, the second sub group contained *L. pneumophila* serogroup 1 isolates
205 collected from water heaters set at temperature ranging between 55-60°C, suggesting that, a
206 correlation between genotypes and the temperature at which the water heating systems
207 were operating may be found and that the temperature of the water tanks maybe one of the
208 most important factors that affects the genotypic behavior of *Legionella pneumophila*

209
210 Slight differences in the RFLP patterns were noted among the *L. pneumophila* serogroup 2-
211 14 isolates collected from water heaters set at a temperature ranging between 25-30°C and
212 isolates collected from water heaters set at temperature ranging between 55-60°C, however,
213 these small variations were not taken into account and the patterns of all *L. pneumophila*
214 serogroup 2-14 isolates were interpreted as being similar and were thus kept in the same
215 group. The characteristics along with molecular profiles of the selected *Legionella* isolates
216 are all summarized in table 2.

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226 **Table 1. Distribution of the positive *Legionella* isolates recovered in this study in accordance to their frequency of occurrence**
227 **among the different residential areas.**
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Residential Area	No. of collected water Samples	No. of positive samples	positive samples (%)	Isolate number	species And serotype	Count CFU/100ml	Heater temperature set Range (°C)
Alamyia	20	1	5	10	<i>L. pneumophila</i> 2-14	80	25-30
Al-Mahdood	20	1	5	5	<i>L. pneumophila</i> 1	360	25-30
Al-Balad	35	3	8.5	8	<i>Legionella</i> spp.	70	25-30
				9	<i>Legionella</i> spp.	250	25-30
				11	<i>L. pneumophila</i> 1	100	25-30
Al-Sadisa	15	0	0	—	—	—	55-60
Al-Thalitha	20	2	10	4	<i>L. pneumophila</i> 2-14	330	25-30
				13	<i>L. pneumophila</i> 2-14	370	80-90
Al-Sharqyia	20	5	25	1	<i>L. pneumophila</i> 1	20	25-30
				6	<i>L. pneumophila</i> 1	400	25-30
				14	<i>L. pneumophila</i> 1	40	55-60
				15	<i>L. pneumophila</i> 1	30	55-60
				16	<i>L. pneumophila</i> 1	200	55-60
Al-Gharbyia	20	4	20	2	<i>L. pneumophila</i> 1	1×10 ⁴	25-30
				3	<i>L. pneumophila</i> 2-14	230	55-60
				7	<i>L. pneumophila</i> 1	60	25-30
				17	<i>L. pneumophila</i> 1	22	55-60
Al-Khamisa	15	0	0	—	—	—	25-30
Al-Thamina	15	0	0	—	—	—	25-30
Al-Ashira	20	1	5	12	<i>L. pneumophila</i> 2-14	440	55-60

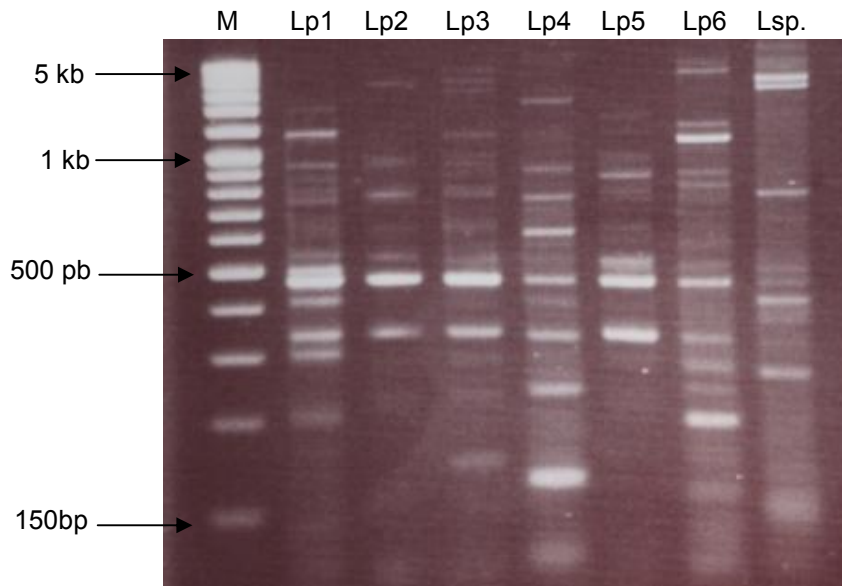


Fig.1. Random amplified polymorphic DNA fingerprinting of some representative *Legionella* isolates obtained using primer OP-A3. Lanes M: DNA molecular size marker (100bp plus DNA ladder). Lanes (Lp1, 2, 3 and 5) are *L. pneumophila* 1 isolates No. 1, 14, 15, and 7 respectively. Lanes (Lp 4 and 6) are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

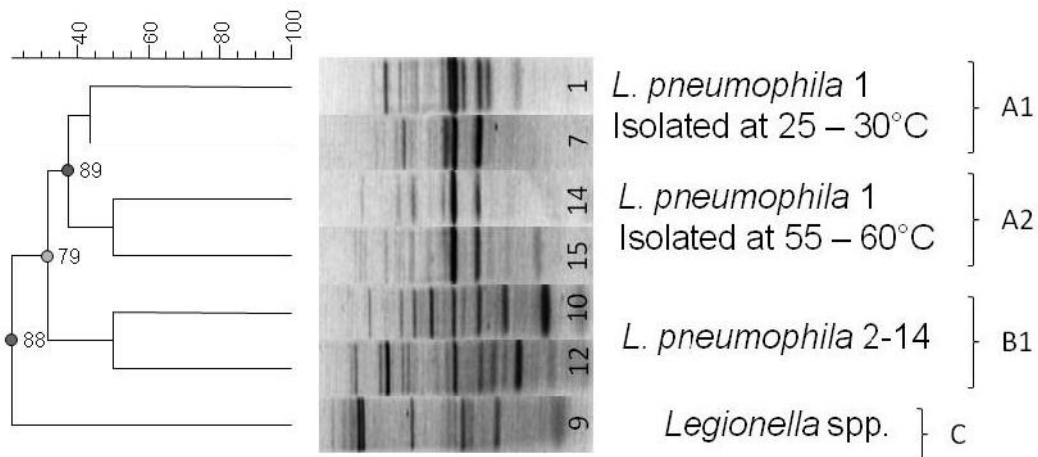


Fig.2. Dendrogram and RAPD-PCR gel band patterns of some representative *Legionella* isolates typed with primer OP-A3. The scale at the top represents percent similarity. The isolate numbers and genotypes are listed to the right of the figure and correspond to those in table 2.

248 **Table 2. RAPD PCR types generated from the selected *Legionella* isolates and OP-A3**
 249 **RAPD primer**
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Isolate number	species And serotype	Heater temperature set Range (°C)	RAPD Type
1 2 5 6 7 11	<i>L. pneumophila</i> 1	25 – 30	A ₁
14 15 16 17	<i>L. pneumophila</i> 1	55 – 60	A ₂
4 10 3 12 13	<i>L. pneumophila</i> 2-14	25 – 30 25 – 30 55 – 60 55 – 60 80 – 90	B
8 9	<i>Legionella</i> spp. <i>Legionella</i> spp.	25 – 30	C

251 252 **4. CONCLUSION**

253
 254 The current study showed the colonization of the plumbing systems of private houses
 255 located within different residential areas in the city of Aqaba by *Legionella* spp. with a
 256 maximum count of 1×10^4 CFU/100 ml. This study also provided data on the relation between
 257 RAPD genotypes profiles and the tolerance of the *L. pneumophila* isolates recovered in this
 258 study to elevated temperature. The results of the genotyping suggested high degree of
 259 heterogeneity among *L. pneumophila* serogroup 1 isolates collected from water heaters set
 260 at temperature ranging between 25-60°C. We can also suppose that this high tolerance of *L.*
 261 *pneumophila* serogroup 1 isolates to elevated temperature within the water heating system
 262 is in relation with the genetic diveristy among the isolates and may result in enhancing their
 263 capacity towards colonizing these water systems. Such findings may be useful as baseline
 264 information on the colonization of the water heating systems of private houses located within
 265 different residential areas in the city of Aqaba by *L. pneumophila*.

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 267 Further similar studies should be conducted to include other cities and other water systems
 268 in the whole middle eastern area in order to give us a clear image about the prevalence of
 269 *Legionella* and further try to develop an international legislation and prevention measures to
 270 aid in reducing the *Legionella* related pneumonia cases, and also to help in the eradication
 271 of *Legionella*, which consequently help in preventing travel-associated legionellosis.

272 273 274 **COMPETING INTERESTS**

275
 276 Authors have declared that no competing interests exist.

277 **AUTHORS' CONTRIBUTIONS**

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279 All three authors contributed equally to this work.

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