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Sequence-Based Typing for Legionella Pneumophila Isolated from Water Systems of Residential Facilities in Kuwait

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Abstract

A total number of 30 strains of *Legionella pneumophila* (sg1 = 6; sg3 = 20; sg4 = 1 sg7 = 1; sg10 = 2) were genotyped by the SBT method according to the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for Legionella Infections (ESGLI formerly EWGLI). A high proportion of isolates did not give a full 7 loci SBT profile due to amplification fail of the *flaA* loci, the *flaA* sequence of representative isolates were however revealed by whole genome sequencing. The *Legionella pneumophila* isolates were grouped into seven distinct Sequence Types, including one (ST1719) which were new to the SBT database of ESGLI and two (ST1223 and ST1718) which were recently also isolated from cooling towers in Kuwait but not have been reported from other countries.

SBT profile F, 14,16,25,7,13,24 (probably all ST336) (11 isolates of sg3) was the most prevalent genotype. The other prevalent STs identified were ST93 (nine isolates of sg3) and ST1 (five isolates of sg1). All of the ST1 sergroup1 isolates were of the Oxford/OLDA subgroup. This is the first study used to describe the use of SBT to characterize environmental *Legionella pneumophila* strains isolated from domestic water systems in Kuwait. This baseline data will form the basis for the development of a Legionella environmental surveillance program to be used for future epidemiological investigations.

Keywords: Legionaires' Disease; Legionella; Serogroups; SBT; ST Profile; Water Contamination; Kuwait

Introduction

Legionella pneumophila is a waterborne organism that has been increasingly recognized as the causative agent behind community-acquired and nosocomial pneumonia in humans. Potable water systems have often been implicated as the source in the outbreak of Legionnaires' disease [1,2]. Legionella spp. are Gram-negative bacteria that normally occupy natural aquatic environments where they can survive as intracellular parasites of protozoa. The majority of the community-acquired cases are caused by strains belonging to *Legionella pneumophila* serogroup 1 [3,4].

Molecular-based typing methods have often been used to characterize clinical and environmental *Legionella pneumophila* to determine the source of infection [5,6,7]. Sequence-based typing (SBT) is a highly discriminatory typing method for Legionella pnuemophila. Sequence-based typing using the seven gene locus (*flaA, pilE, asd, mip, mompS, proA, and neuA or neuAh*) will result in a distinctive allelic profiling of the isolates which are then differentiated into sequence types (ST) [5,8,9]. The ESGLI SBT scheme is presently recognized as the 'gold standard' tool for *Legionella pneumophila* typing [5,8,10].

Only two study reports the characterization of *Legionella* that has been isolated from potable water systems in Kuwait [11,12]. However, no DNA Sequence Based Typing (SBT) has ever been determined for legionellae that has been isolated

from Kuwait's potable water and compared with the ESGLI database. In this study, we investigated SBT as a molecular tool to characterize 30 *Legionella pneumophila* isolates from domestic water systems in Kuwait. The allelic profiles of these isolates were then compared to the EWGLI database. This data will provide a better understanding of the dominant genotype(s) present in Kuwait's environment.

Material and Methods

Legionella pneumophila

A total number of 30 environmental isolates of *L. pneumophila* were used in this study. These isolates have previously been isolated from domestic water system samples obtained from the faucets of wash basins and showerheads in bathrooms, taps from kitchens and cold and/or hot water tanks from seventeen different residential sites in Kuwait (Figure. 1)[12]. *L. pneumophila* was isolated on buffered charcoal yeast extract medium using standard methods AS/NZS 3896 [13].

Sero- and Subgrouping of *Legionella pneumophila*

The Oxoid Legionella Latex test was used to identify and differentiate between serogroup 1 and serogroups 2-14 (code DR0800; Oxoid; UK). The isolates were serogrouped and subgrouped if applicable using the Dresden panel of monoclonal antibodies as previously described [3,14].

Sequence Based Typing (SBT)

The genomic DNA was extracted from study isolates using the QIAamp DNA Mini kit (Qiagen). *L. pneumophila* isolates were genotyped using the seven gene protocol sequence-based typing (SBT) scheme developed by ESGLI as previously described [5,8]. Trace files with the obtained sequences were analyzed by using the Legionella SBT quality tool (http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi). New alleles and STs encountered for the first time in this study were submitted to the database (http://www.hpa.bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php).

Figure 1. Sample collection sites. Red stars: positive sites for legionella. Site 1: Residential complex; site 2: Governmental building.

Whole Genome Sequencing (WGS)

The genomic DNA was extracted from four representative isolates using the QIAamp DNA Mini kit (Qiagen) and was subjected to whole genome sequencing using the Illumina MiSeq platform with 250bp paired-end reads according to the manufacturer's instructions. The *flaA* loci were extracted from the de novo assemblies (CLC-bio vers. 8.0). Four isolates were selected among isolates that failed to give a *flaA* PCR product and showed one of the following four allelic profiles (1) F,14,16,16,15,13,2; (2) F,14,16,25,7,13,24; (3) F,14,16,65,7,13,217 or (4) F,14,16,19,15,13,215.

Cluster Analysis

A cluster analysis of the seven different Sequence Types found in this study was performed with BioNumerics v7.6 (Applied Maths), with the following settings Pearson correlation and the UPGMA method. The analysis was based on the sequences for the six loci *flaA, pilE, asd, mip, mompS and proA*. Sequences of the seventh loci *neuA* in the standard SBT scheme could not be revealed for all isolates (ST1223, ST1718, ST1719 and ST1300) and was not included in the analysis (for these isolates *neuA* homologue (*neuAh*) sequences could be revealed, and in this way the isolates could be allocated to a definite Sequence Type). The sequences for the type strains Philadelphia-1 (*Legionella pneumophila* pneumophila, serogroup 1, ST36) and Dallas-1E (*Legionella pneumophila* fraseri, serogroup 5, ST1300) were also included in the analysis.

Results

Sero- and Subgrouping of *Legionella pneumophila*

Two thirds of the *L. pneumophila* isolates belonged to sg3 (20 isolates) followed by sg1 (6 isolates). Sergroups 4, 7 and 10 were isolated at a lower frequency (1-2 isolates) as shown in Table 1. All of the six *L. pneumophila* sg1 isolates were of the Oxford/OLDA subgroup, and the sg4 isolate were of the Los Angeles subgroup as shown in Table 1.

Sequence Based Typing (SBT) & Whole Genome Sequencing (WGS)

Fourteen of the isolates failed to give a *flaA* PCR product using the standard SBT protocol. In order to reveal the sequence of the *flaA* genes and subsequently to allocate a full allelic profile and ST to the isolates, representatives for the different SBT profiles with *flaA* fails (n = 4) were subjected to WGS. The WGS of the four representatives revealed that all four strains were *flaA* allele type 11. The reason to the fail was found in the primer binding sites, which showed two and four mismatches in the forward and reverse primer binding sites respectively. Recently the same mismatches have been revealed in other *flaA* PCR negative isolates form Denmark, UK and Israel [15]. Eleven isolates had the allelic profile F,14,16,25,7,13,24 but only one of these isolates were subjected to WGS, revealing *flaA* allele type 11 and ST336. It is however very likely that all the other 10 isolates with this profile also had *flaA* allele type 11 and belonged to ST336. The SBT results of the 30 *L. pneumophila* isolates are shown in Table 1. The isolates were distinguished into seven different STs of which one ST, ST1719, were identified for the first time and were submitted to the ESGLI SBT database. ST1223 and ST1718 were recently reported as new STs isolated from cooling tower water in Kuwait [16]. With the exceptions for the sg1 ST154, the majority of *L. pneumophila* sg1 isolates belonged to ST1 (1, 4, 3, 1, 1, 1, 1) which accounted for 20% of the total isolates. Two STs was observed for the *L. penumophila* sg3 isolates, ST336 (probably 11 isolates) and ST93 (nine isolates).

Many of the isolates (n = 14; of sg 1, 3, 4 and 7) seem to be rather closely related as they all share the same three alleles for *pilE* (14), *asd* (16) and *proA* (13) and probably also share the same allele for *flaA* (11), but the latter was only documenter for four of the isolates. This profile is also recognized for the reference strains of serogroup 4 (Los Angeles-1; ST1334 (11, 14, 16, 25, 7,13, 206)), serogroup 5 (Dallas-1E; ST1300 (11, 14, 16, 18, 15, 13, 201)) and for the Lansing3 strain ST336 (11, 14,

Total Strain	Serogroup, mAb subtype	Seven gene SBT comprising flaA, pilE, asd, mip, mompS, proA, and neuA	ST	Source	Site No.
5	1, Oxford/OLDA	1,4,3,1,1,1,1	1	Bathroom, Kitchen	1 , 2
1	1, Oxford/OLDA	11,14,16,16,15,13,2	154	Bathroom	1
9	3	3,10,1,28,14,9,13	93	Bathroom, Tank,	1
1	3	11,14,16,25,7,13,24	336*	Kitchen	1
10	3	F,14,16,25,7,13,24	336#	Bathroom, Kitchen	1, 2
1	4, Los Angeles	11,14,16,65,7,13,217	1719*	Kitchen	1
1	7	11,14,16,19,15,13,215	1718*	Kitchen	1
2	10	1,4,3,5,1,1,213	1223*	Bathroom	1

Table 1. Sequence types among the 30 environmental strains of Legionella pneumophila isolated in Kuwait.

*Isolates only reported from Kuwait

flaA was not revealed for these 10 isolates but the SBT profile was identical to ST336 for six of the seven loci and was also serotyped as serogroup 3 as the ST336 isolate, so it is most likely that the seven isolates also belongs to ST336.

16, 25, 7, 13, 24)); which, all have been suggested as belonging to the subspecies *fraseri* of *L. pneumophila* [17]. A cluster analysis of the seven isolates showed that the seven STs cluster in two different groups (Figure 2), one together with the Philadelphia-1 strain (subspecies *pneumophila*) (ST1, ST93 and ST1223) and the others (ST154, ST336 and ST 1718) with the above mentioned common alleles cluster together with the Dallas 1E strain (subspecies *fraseri*). The clustering is independent of the serogroup distribution.

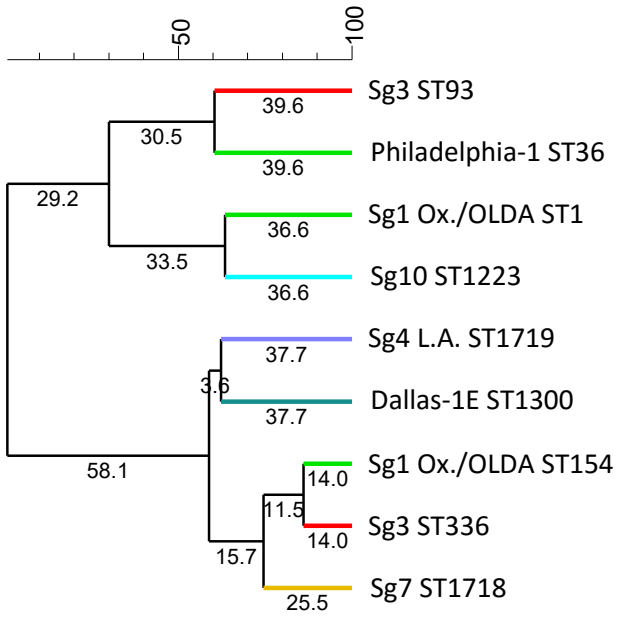

Figure 2. Cluster analysis showing the relative similarity for the seven different Sequence Types of *L. pneumophila* isolated from domestic water systems in Kuwait based on the sequences for the six loci *flaA*, pilE, asd, mip, mompS and proA. The sequences for the type strains Philadelphia-1 (*Legionella pneumophila* pneumophila, serogroup 1, ST36) and Dallas-1E (*Legionella pneumophila* fraseri, serogroup 5, ST1300) are also included in the analysis. Different colours represent different serogroups.

Discussion

This is the first study that provides a sequence-based typing for environmental *L. pneumophila* that is isolated from domestic water systems in Kuwait. This data also provides useful information for future epidemiological investigation of local and regional outbreaks of Legionnaires' disease. Our results are similar to those observed by Kozak et al. [18], who reported that 58% of the STs found were unique to the United States.

This study also reflects the distribution and profiles of *Legionella* genotypes circulating in Kuwait's environment. Serogroup 3 isolates (77.1%) dominated the total number of isolates from the domestic water systems [12]. Moran-Gilad et al. [19] also reported similarly that the most prevalent environmental stain in Israel was sg3 (52%), but the results are different from the other studies that reported that sg1 was the most frequently detected environmental isolate [20,21]. Furthermore, Doleans et al. [22] and Harrison et al. [23] have reported that sg3 isolation rate in France and the UK and Wales were only 14.3% and 12.0%, respectively. The sg3 ST93 and SBT profile F/11,14,16,25,7,13,24 (probably all ST336) were the most prevalent STs circulating in Kuwait's water systems accounting for 66,7% of all the isolates. ST93 has previously been implicated in a sporadic hospital case of Legionellosis [24]. In the ESGLI database ST93 is also recorded as a relatively common cause of community and nosocomial legionnaires´ disease and reported from several European countries and Japan. ST336 is on the other hand very rare, only one other strain of this ST has been submitted to the ESGLI database and this is a sg15 isolate (the Lansing-3 strain), so ST336 of serogroup 3 is for the moment unique for Kuwait. Little data is available on the virulence of sg3 isolates. Previous international survey studies have reported that *L. pneumophila* sg3 was responsible for only 1% of the outbreaks [4]. Serogroup 3 is however the second most common cause of legionnaires´ disease in Europe after sg1, but still only accounts for less than 5% of the culture confirmed cases [25]. The environmental predominance of sg3 with the allelic profile of ST93 and probably ST336 may suggest their ability to survive the harsh environmental conditions of Kuwait. Further studies will be needed to ascertain whether these predominant STs play a role in the transmission of community-acquired pneumonia.

Almost half of the isolates (n = 14) do share an allelic theme also seen in strains belonging to several members of the suggested subspecies *fraseri* of *L. pneumophila*. The reason to this high prevalence of strains with these common alleles for *flaA*, *pilE, asd* and *proA* in Kuwait is unknown. It is however interesting that both ST336 and ST1718 also are widely distributed in cooling tower water in Kuwait [16].

The ST1 (1,4,3,1,1,1,1), the most frequent profile reported in the world, was found in all but one of six sg1 isolates (see Table1). This ST has been commonly isolated from environmental samples and is associated with outbreaks of legionnaires' disease [26,27,28]. Notably, one sg1 subgroup Oxford/OLDA was ST154 which is a relatively rare ST. Furthermore, as all of the sg1 isolates belonged to the subgroup Oxford/OLDA they were negative for the MAb3/1 of the Dresden panel. MAb3/1 negative strains are common in the environment and Harrison et al. [23] reported that only 8.3% of the environmental *L. pneumophila* sg 1 isolates in the UK and Wales were MAb3/1 positive. In addition, MAb3/1 positive sg1 strains were implicated in 94% of legionellosis outbreaks in the United State [18] and can be considered as a virulence factor.

In conclusion, the data indicating that serogoroup 3 is predominant among environmental isolates of *L. pneumophila* from the domestic water systems in Kuwait, this observation might also be important for the selection of a diagnostic test for legionellosis in Kuwait. Urinary antigen test is the most popular diagnostic test worldwide, yet it can detect Lp serogroup 1 only, perhaps other diagnostic tests capable to detect sg3 (e.g.

culture and PCR) should be recommended for Kuwait.

In addition, this study provides essential data on the prevailing ST isolated from domestic water systems in Kuwait. This baseline data may also be used in risk assessment and risk management strategies for water decontamination of infected water systems. Furthermore, since a comprehensive ST for clinical isolates is not available in Kuwait, it is clear that most of the STs with the exception of ST1 and to some degree ST93 not have been reported as major causes of Legionaires' disease worldwide. The availability of clinical isolates may help in determining whether these isolates are responsible for future community acquired and nosocomial cases of legionnaires' disease in Kuwait or not.

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