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UNIVERSITÀ DEGLI STUDI DI TORINO

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Does better identification of the *Legionella pneumophila* serogroup 1 strains by Sequence-Based Typing (SBT) allow for the implementation of more effective contamination control strategies and more targeted intervention measures?

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Abstract

The purpose of this study was to investigate the distribution of *L. pneumophila* sg 1 sequence types within the water supplies of 56 healthcare facilities (HCFs) in the Piemonte region.

Using Sequence-Based Typing (SBT) we typed 22 environmental strains of *L. pneumophila* sg 1 obtained during routine testing from 2004 to 2009.

Thirty-three percent of the STs obtained from our analysis were unique to the EWGLI database.

Of the 22 analysed environmental strains, 11 STs were found in man-made water systems that were associated with human disease. Four STs, which in our study belonged to strains isolated in hospital with reported clinical cases, were already known in the literature. The presence of these STs was confirmed by reports of clinical and nosocomial cases (single cases or clusters), especially for ST 1, ST 23, and ST 42, the most frequently and widely distributed STs worldwide

This is the first report in Italy where environmental strains of *L. pneumophila* isolated from a large geographical area have been identified using SBT. This study confirms that the circulation of some

STs is correlated with the development of cluster epidemics in the community or even isolated cases of nosocomial origin. Better identification of the strains which cause the majority of disease would allow for more targeted intervention measures.

Keywords

Legionella Pneumophila, Molecular Typing, Risk Management

Abbreviations

DLV	Di-Locus Variants
EWGLI	European Working Group for Legionella Infections
HCFs	Healthcare Facilities
MAb	Monoclonal Antibodies
SBT	Sequence-Based Typing
SLV	Single-Locus Variants
ST	Sequence Type

1. Introduction

The genus *Legionella* contains 58 species [1], all of which are ubiquitous environmental bacteria.

Of the 20 species associated with human disease, a single species, *Legionella pneumophila*, is responsible for 90% of reported cases of legionellosis in the United States [2]. In Europe, approximately 70% of *Legionella* infections are caused by *L. pneumophila* serogroup 1 [3].

Among the *L. pneumophila* sg 1 strains, some monoclonal subgroups (Knoxville, Philadelphia, Benidorm, and France/Allentown) display the virulence-associated epitope recognised by MAb 3/1; these strains are associated with hospital outbreaks, travel-associated infections or community-acquired infections [4; 5]. There are some published data [4; 6; 7; 8; 9] on the serogroup and monoclonal subgroup distribution of *L. pneumophila* in man-made water systems and whether they are associated with human disease. It is acknowledged, however, that other techniques may be more useful in studying individual outbreaks, because identification of microvariation is required in order to distinguish between circulating strains within a geographical area.

In addition to phenotypic typing method, a number of molecular methods have been successfully employed locally for epidemiological purposes. These include ribotyping, amplified fragment length polymorphism (AFLP) analysis, pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) analysis, restriction endonuclease analysis (REA), and arbitrarily primed PCR, Multilocus sequence typing (MLST) and Sequence-Based Typing" (SBT).

A Sequence-Based Typing (SBT) approach has been developed and evaluated for implementation in the investigation of outbreaks of legionellosis caused by *Legionella pneumophila* [Gaia 2003 Gaia et al. (2005) Ratzow et al. (2007)].

The use of six-gene (*flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA*) SBT scheme yielded a discriminatory power (D value) of 0.932 (95% CI, 0.913 to 0.951) for the panel of 79 unrelated strains, but with the addition of the *neuA* gene this increased to 0.963 (95% CI, 0.952 to 0.974), which is above the value recommended for a good epidemiological typing system (Struelens.) The seven-gene SBT scheme of *L. pneumophila* produces robust,

epidemiologically concordant, and highly discriminatory data that can be easily exchanged between laboratories. (Ratzow 2007 gaia 2005)

The discriminative capacity of the method is not well determined to date for *Legionella pneumophila* serogroups other than serogroup 1. Fenduky found a limited genotypic variability of serogroup 6 isolates that decreased the usefulness of genotyping methods as SBT. Further knowledge regarding the epidemiological typing of serogroup 6 isolates from different countries by the SBT method is essential.

Furthermore, it has been shown that some genotypes of *L. pneumophila* serogroup 1 may be widespread in large geographical areas (Bonura) therefore, genotyping results should be used in conjunction with clinical and epidemiological data.

In our previous study (submitted to journal) we identified the environmental strains of *L. pneumophila* sg 1 obtained from the routine testing of healthcare facilities (HCFs) in the Piemonte region between 2004 and 2009 using the monoclonal antibody MAb 3/1 of the Dresden Panel. We collected strains from 56 HCFs and found *L. pneumophila* sg 1 in 22 HCF water supplies. The results of MAb were compared to the reports of hospital-acquired legionellosis from 2004 to 2009, and, among the strains of *L. pneumophila* sg 1, the monoclonal subgroup with the virulence-associated epitope recognised by MAb 3/1 was isolated in samples from the hospitals that reported 85% of legionellosis.

In our previous study, we demonstrated a statistically significant association between the occurrence of legionellosis cases and the colonisation of water supplies by MAb 3/1-positive *Legionella* strains in HCFs in the Piemonte region.

In the present study we used Sequence-Based Typing (SBT) to further analyse these environmental strains of *L. pneumophila* sg 1. The purpose was to investigate the distribution of *L. pneumophila* sg 1 sequence types within the water supplies of HCFs in the Piemonte region.

2. Materials and Methods

2.1. DNA sequence analysis

Genotyping was conducted via the standard sequence-based typing (SBT) method (Version 4.2) of the European Working Group for *Legionella* Infections (EWGLI) using 7 genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) [10; 11]. Nucleotide analyses used the SBT database available on the EWGLI website [12], and the sequences were compared with those in the SBT database, which are also available on the website [13].

2.2. Allele identification and Sequence Typing (ST)

The SBT database was used to assign the specific allele number corresponding to the gene locus. For each isolate, the combination of seven alleles at each of the loci was defined as the allelic profile or SBT by using the predetermined order: *fla*, *pil E*, *asd*, *mip*, *momp*, *proA* and *neu A*.

Putative novel variants were submitted to the site ‘Sequence Quality Tool’ for verification and assignment of new allelic numbers. New combinations of allelic numbers were also submitted to the curators via the EWGLI website for assignment of new ST numbers. In this study, genes that were not amplified are indicated as allele ‘0.’

Diversity was estimated by calculating Hunter and Gaston’s modification of Simpson’s index of diversity, as previously described [14].

3. Results

SBT analysis was used to assign *L. pneumophila* sg 1 isolates to 18 different STs, with only one ST found per structure.

The strains were isolated in samples from 22 different HCFs in the Piemonte region: three each from the north and the south and sixteen from the centre (Figure 1).

Of the 22 analysed environmental strains, 11 STs were found in man-made water systems that were associated with human disease, and MAb 3/1 positive isolates accounted for 36% of all strains (8/22).

All 22 strains but one were typable by SBT. The gene *asd* in isolate number 11 was not amplified (indicated by an allele number of “0”) and was not given a ST number (Table 1). The 21 remaining environmental isolates were divided into a total of 18 distinct allelic SB types and showed high profile diversity (IOD=0.981).

Identical SBT was found among strains belonging to different MAb 3/1 subgroups. In our study, SBT 59 was found in 3 strains isolated from 3 HCFs: the strains from hospitals N°8 and N°9 were MAb 3/1 negative, and the strain from hospital N°12 was MAb 3/1 positive.

ST 363 occurred only twice among our strains. Both isolates obtained from two hospitals were MAb 3/1 negative.

ST 313 and ST 363 are single-locus variants (SLV) of ST 59; ST 964 is a di-locus variant (DLV) of ST 59; ST 1169 is a DLV of 59 and ST 476 is a SLV of ST 1.

The data were uploaded to the EWGLI SBT database to allow for comparison between countries. Six STs (1, 751, 23, 42, 59, and 12), which in our study belonged to strains isolated in hospitals with reported clinical cases, were already known to be associated with community-acquired legionellosis and nosocomial clinical cases elsewhere in Europe [15; 16; 17; 18; 19] (Table 2). The involvement of these strains in human disease was confirmed by reports of clinical cases (either single cases or clusters), especially for ST 1, ST 23 and ST 42, which are the most frequently and widely distributed STs worldwide [20; 21 [qin t](#)].

Of the five STs isolated from HCFs without reported cases of legionellosis (363, 313, 476, 104, and 68), only ST 68 has been associated with clinical cases in 6 countries worldwide (Table3).

Seven STs (1170, 1169, 1107, 1162, 964, 955, and 1231) were new to the EWGLI database. The allelic profiles of ST 1170, ST 1169, and ST 1107 were isolated from environmental samples that

came from hospitals with legionellosis cases, and their virulence was demonstrated by their positivity versus MAb 3/1. ST 1162, ST 964, ST 955, and ST 1231 were isolated from environmental samples from hospitals without reported cases and were negative versus MAb 3/1.

4. Discussion

We collected 22 environmental and unrelated isolates of *Legionella pneumophila* sg 1 from man-made water systems as part of routine sampling.

Thirty-three percent of the STs obtained from our analysis were unique to the EWGLI database.

Four STs, which in our study belonged to strains isolated in hospital with reported clinical cases, were already known in the literature. The presence of these STs was confirmed by reports of clinical and nosocomial cases (single cases or clusters), especially for ST 1, ST 23, and ST 42, the most frequently and widely distributed STs worldwide [20; 21 **qin t**].

ST 1 appears to be the most prevalent ST in the world and is the most commonly reported ST in the EWGLI database. It has been associated with nosocomial outbreaks and community-acquired legionellosis.

ST 23 has been associated with a cluster of travel-associated legionellosis in Italy [16] and in Greece [17] ST 42 has been associated with community-acquired legionellosis in England [7] and in Belgium [19], and ST 59 has been associated with community-acquired legionellosis in Sweden [18] and in the UK [7]

This study confirms that the circulation of strains ST 1, ST 23, ST 42, and ST 59 is correlated with the development of cluster epidemics in the community or even isolated cases of nosocomial origin. Our laboratory is involved in the environmental monitoring of many HCFs in the Piemonte (area 25,399 km², population 4,446,230); thus, we collected environmental samples from the water systems of 56 HCFs that were representative of the wider *L. pneumophila* population in our region.

This is the first report in Italy where environmental strains of *L. pneumophila* isolated from a large geographical area have been identified using SBT and MAb subtyping. Moreover, the study contributed to the EWGLI-SBT database and to the knowledge of European *L. pneumophila* diversity.

5. Conclusion

SBT is a powerful tool for typing strains of *L. pneumophila* and allows for an easy comparison of the data from different countries. The European EWGLI database, which currently contains 1698 (accessed 03-03-2014) STs, can be used in both epidemiological investigations and in the assessment of the risk associated with environmental reservoirs. Many of the guidelines and official recommendations for preventing the transmission of Legionnaires' disease recommend action based only on certain concentrations of legionellae or on the number of sites that are positive for the bacteria [22 ;23]. None of the guidelines discriminate based on the large number of *Legionella* species or serogroups, even though it is clear that they differ in their capacity to cause disease [2].

The STs most commonly found in sporadic and outbreak-associated Lp1 populations may have an increased ability to cause disease and thus may require special attention when detected. [Kozak-Muiznieks NA](#)¹

Better identification of the strains which cause the majority of disease would allow for more targeted intervention measures.

Hospitals colonised by more virulent strains should be advised to increase their clinical and epidemiological monitoring efforts and to implement more effective contamination control strategies.

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Table 1. 18 SBT profiles of 22 *Legionella pneumophila* environmental isolates.

Health Care Facilities	Allelic profile	ST	Reactivity vs MAb 3/1
1	1,4,3,1,1,1,1	1	+
2	8,10,3,15,9,14,6	751	+
3	6,10,15,3,9,4,13	1170	+
4	2,3,9,10,2,1,6	23	+
5	7,6,17,3,9,11,6	1169	+
6	8,10,3,15,18,1,8	1107	+
7	4,7,11,3,11,12,9	42	+
8	7,6,17,3,13,11,11	59	-
9	7,6,17,3,13,11,11	59	-
10	11,14,16,1,15,13,6	12	-
11	3,13,0,3,9,9,11	n.d.	-
12	7,6,17,3,13,11,11	59	+
13	7,6,3,3,13,11,11	363	-
14	7,6,17,6,13,11,11	313	-
15	2,6,1,6,9,4,11	1162	-
16	1,4,3,13,1,1,1,	476	-
17	7,6,3,3,13,11,11	363	-
18	3,13,1,28,14,9,3	68	-
19	4,6,17,6,13,11,11	964	-
20	3,10,1,1,14,9,1	104	-
21	2,10,17,3,9,4,11	955	-
22	3,1,22,26,6,10,12	1231	-

New profiles are in bold

Table 2. Occurrence of sequence type among health care facilities with reported cases of legionellosis and comparison with submissions from other countries (source: EWGLI).

ST	No. of worldwide strains isolated from environmental sources	No. of worldwide strains isolated from patients	No. of countries that reported legionellosis
1	515	417	23
751	1	4	2
23	46	457	21
42	36	141	25
59	43	43	9
12	2	2	1
1170, 1169, 1107	1	0	0

New profiles are in bold

Table 3. Occurrence of sequence type among health care facilities without reported cases of legionellosis and comparison with submissions from other countries (source: EWGLI).

ST	No. of worldwide strains isolated from environmental sources	No. of worldwide strains isolated from patients	No. of countries that reported legionellosis
363	4	1	1
313	2	1	1
476	1	2	2
104	8	5	3
68	26	16	8
1162, 964,955 1231	1	0	1

New profiles are in bold

Figure 1: Geographic distribution of Sequence Type and occurrence of legionellosis in Piemonte region.

