Genotyping of *Legionella pneumophila* serogroup 1 strains isolated in Northern Sicily, Italy

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SUMMARY

During a three-year period, from April 2002 to May 2005, one hundred-forty-seven samples, taken from technical systems of water distribution at point of use, were repeatedly collected at six different sites in Northern Sicily and assayed for the presence of *Legionella pneumophila* serogroup 1 and serogroups 2 to 14. At the first samplings, the water distribution systems of all the sites were heavily contaminated, and disinfection treatments by the superheat and flush method were therefore performed. Treatments were always successful against *L. pneumophila* sg.1, but only in a few cases against all other serogroups. Eighty-six strains of *L. pneumophila* sg.1, isolated from 26 of these samples, were characterized by amplified fragment length polymorphism (AFLP) analysis and sequence-based typing (SBT) procedure. Perfectly overlapping results were obtained by both the procedures and four genotypes were identified, accounting for all the isolates. The easy transferability of the SBT data through a web-based database made it possible to identify the presence in Northern Sicily of the two SBT types most commonly circulating in Europe.

KEY WORDS: Legionella pneumophila, Surveillance, Water distribution system, Molecular typing, Amplified fragment length polymorphism (AFLP), Sequence-based typing (SBT)

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INTRODUCTION

Legionellae are normal inhabitants of the fresh water environment, pathogenic for humans and responsible, particularly *Legionella pneumophila* serogroup (sg.) 1 (Joseph, 2002), for infections (legionellosis) invariably acquired from environmental sources and clinically characterized by features ranging from mild respiratory illness to acute life-threatening pneumonia. Legionellosis may occur in dramatic outbreaks associated with hotels, hospitals and large building complexes, but may be most often sporadic, nosocomial, and community-acquired (Rodgers, 1998). Inhalation of aerosolized contaminated water produced by sources such as air-conditioning evaporative condensers, humidifiers, nebulizers, potable and hot water supplies, and domestic and hospital showerheads causes acquisition of the disease, whose progression, severity and outcome are critically conditioned by host and microbial-related factors (Cianciotto, 2001; Edelstein and Cianciotto, 2001) as also - and possibly mainly - (Tercelj-Zorman *et al.*, 2004) by the microbial exposure dose.

When a new case occurs, epidemiological investigations are usually performed to locate the source and extent of infection, adopt proper preventive measures, and identify any legal responsibilities. Comparison is required between clinical and environmental isolates and typing procedures must be used that are able to differentiate

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isolates below the serogroup level. As serotyping is insufficient for epidemiological ascertainment (Fry *et al.*, 1999), genotyping must also be performed.
Among the different genotyping procedures, those allowing a true "epidemiological typing", i.e. the allocation of the isolates to previously defined types, if possible by means of on-line identification libraries (Le Flèche *et al.*, 2002), are certainly advantageous when a long-term follow-up in hotels, hospitals, or other public or private institutions is required. This is also the cases when, as frequently occurs in legionellosis, travellers are involved and comparisons may be required among results obtained by laboratories in different parts of the world so that typing data can be exchanged rather than strains across countries and borders.
With respect to *Legionella pneumophila* sg.1, one of these procedures, amplified fragment length polymorphism (AFLP) analysis, has been adopted as an international standard by members of the European Working Group for Legionella Infections (EWGLI) (Fry *et al.*, 2000), and assessed for reliability and discriminatory power in some recent interlaboratory studies (Fry *et al.*, 2000; Fry *et al.*, 2002). Another procedure, a sequence-based typing (SBT), has been also proposed (Gaia *et al.*, 2003) and improved (Gaia *et al.*, 2005) with the selection as genetic markers of six bacterial genes, all likely to be under selective pressure, and with the definition of a consensus sequence-based epidemiological typing scheme. Online databases, established for both AFLP and SBT, are accessible at the EWGLI web site (http://www.ewgli.org) and open, in particular the SBT database, for submission of novel allele types and profiles. The SBT method, when compared with other typing procedures (Amemura-Maekawa *et al.*, 2005; Scaturro *et al.*, 2005), showed excellent reproducibility, genetic stability and the advantage of requiring less subjectivity in interpretation of the results. SBT has therefore been proposed as a new gold standard for the typing of *L. pneumophila* (Gaia *et al.*, 2005), at least, as the proposing authors claim, until a larger data set is established and reviewed.
With the aim of contributing to this evaluation, but also of obtaining information on the types circulating in our geographical area, we used SBT and AFLP analysis to characterize 86 recent isolates of *L. pneumophila* sg.1 derived from 26 samples taken from technical systems of water distribution at point of use. Samples were collected in Northern Sicily during a three-year period from April 2002 to May 2005 and results are here shown and discussed.

MATERIALS AND METHODS

Sample collection and treatment
A total of 147 samples of fresh tap-water (51), hot tap-water (48), swabs or concretions from faucets or showerheads (48) were collected and assayed for the presence of *Legionella pneumophila* sg.1. Tap-water was allowed to flow for 3-5 min to allow elimination of any contaminant present inside the tap; one-litre volumes of both fresh and hot water samples were then aseptically collected in sterile glass bottles, directly from outlets, and 1 ml L^{-1} of a 10% solution of sodium thiosulphate was added to each bottle to neutralize any residual free chlorine. Swabs were plunged in 2-4 ml of water from the same distribution system where samples were collected. Faucet filters and showerheads were closed in sterile envelopes. All samples were put in a chilled cold box (at 4°C) and transferred to the laboratory where analyses were started as soon as possible on the same day as collection.
In the laboratory, tap-water samples were concentrated by filtering the whole 1 litre samples through sterile 0.22 µm filter membranes (Millipore, Bedford, MA, USA) usinig a vacuum pump. After filtration, membranes were aseptically removed, placed in sterile, screw-capped containers with 10 ml of the original filtrates, and vigorously vortex-mixed for 5 min. Swabs were swirled in their collection water; concretions from faucet filters and showerheads were mechanically removed and dissolved in 10 ml of sterile distilled water. In both these cases, samples were centrifuged at 1,500 G for 10 min and the final pellets were resuspended in 2 ml of sterile distilled water.

Bacterial strains
According to the Italian national guidelines for prevention and control of legionellosis (Anonymous, 2000), one half of each sample was placed in a 50°C water bath for 30 min to reduce

contamination by thermosensitive micro-organisms. Aliquots of 0.1 ml of the samples (with and without heat pre-treatment) were spread on plates of MWY agar (Legionella MWY selective medium, Oxoid LTD, Basingstoke, Hampshire, England) and incubated at 37°C, in 2.5% CO_2, for 7-14 days. All colonies with morphology similar to that of *Legionellaceae* (gray, glistening, convex, and circular with a uniform edge) were subcultured on MWY (Oxoid) and on blood and McConkey agar (Becton Dickinson, GmbH, Heidelberg, Germany) plates. The colonies grown on MWY agar, but not on blood and McConkey agar, were Gram-stained and identified on the basis of their biochemical (oxidase, catalase, hydrolysis of hippurate, β-lactamase) and serological features (using a commercially available latex agglutination test kit for *Legionella pneumophila* serogroups 1 to 14, Oxoid). When present, up to ten colonies of *Legionella pneumophila* sg.1 from each sample were collected for typing. Colony counts were performed considering a dilution factor of 1:100 for the 10 ml tap-water samples and of 1:20 for all the other 2 ml samples.

AFLP analysis

Amplified Fragment Length Polymorphism (AFLP) analysis was carried out according to Fry *et al*. (2000) and the Standard EWGLI AFLP Protocol version 1.2 (available at http://www.ewgli.org), with minor modifications.

Briefly, nucleic acids were extracted from cultures of *Legionella pneumophila* sg.1 by using guanidinium thiocyanate and diatoms, as described by Boom *et al.* (1990). Extracted and purified genomic DNA was eluted with 100 µl of sterile distilled water, spectrophotometrically quantitated by measuring the absorbance at 260 nm, and assessed for integrity by agarose gel electrophoresis.

Restriction-ligation reaction was performed with the Pst I and T4 DNA-ligase enzymes (Roche Diagnostics, Mannheim, Germany) and with approximately 1.5 µg of genomic DNA, at 37°C for 3 h, and the tagged DNA fragments were purified by standard phenol-chloroform-isoamylalcohol extraction and selective precipitation, at -20°C for 16 h, by adding sodium acetate (to a final molarity of 3 M) and 2.5 volumes of ice-cold 98% ethanol. After centrifugation at 18,000 G for 20 min, and washing twice with 70% ethanol, the dried pellet was dissolved in 100 µl of sterile distilled water. Before PCR reaction, the dissolved pellet was diluted 1:100 with sterile distilled water and 5 µl of this dilution was used as template DNA in PCR reaction.

The amplification reaction was performed with 75 ng of the selective primer AFLP-*Pst*I-G: 5'-GACTGCGTACATGCAGG-3', in a reaction mixture of 25 µl, using the PCR kit puRe *Taq* Ready-To-Go PCR beads (Amersham Biosciences, Little Chalfont Beckinghamshire, United Kingdom) and the PCR cycling conditions described in the EWGLI Protocol. In a few assays, the AmpliTaq Gold polymerase (Applied Biosystems, Roche, Branchburg, New Jersey, USA) was also used as an alternative. In these cases, amplification was carried out in a reaction volume of 50 µl, with an initial denaturation step of 10 min at 95°C to allow the activation of the enzyme; each reaction mix contained 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM of each deoxynucleotide (Amersham Biosciences), and 2.5 U of the enzyme. The amplified products were electrophoresed in a 1.5% agarose gel at 7 V/cm for 2 h in Tris-borate-EDTA buffer, stained with ethidium bromide (0.5 1 µg/ml), and scanned in a Geldoc instrument (Bio-Rad), or digitally recorded under UV transillumination. Each gel contained at least two ladders (GeneRuler DNA ladder mix; MBI, Fermentas, United Kingdom) at each end, and a reagent blank containing all the reaction components of the reaction mixture with the exception of the template DNA (substituted with sterile distilled water).

The criteria for interpreting the AFLP patterns were those established by the members of the EWGLI:

1) only AFLP fragments in the range between 3500 and 300 bp were considered;
2) the band position tolerance was 2%;
3) if there was no difference or a one-band difference between the samples, they were scored as indistinguishable types, whereas, if two or more bands differed between the samples, they were regarded as unique types.

To try a designation of the identified AFLP types, according to the EWGLI recommendations (Fry *et al*., 2002), the molecular weights of the respective AFLP fragments, as defined by gel scanning, were compared with those in the EWGLI AFLP database.

SBT analysis

Sequence-Based Typing (SBT) analysis was essentially performed according to the EWGLI SBT protocol version 2.0 (available at http://www.ewgli.org). Chromosomal DNA was extracted from the 86 isolates of *Legionella pneumophila* sg.1 by heating emulsified colonies in 0.5 ml sterile water and at 100°C for 10 min. After centrifugation at 18,000 G for 20 min, supernatants were collected and 5 µl lysates were used for PCR amplification reactions. Partial amplification (245 to 648 bp) of the six genes (*flaA*, *mompS*, *proA*, *asd*, *mip*, *pilE*), as suggested by Gaia *et al*. (2005), was performed with the oligonucleotide primers designed by the Authors. The same primers were used for amplification and sequencing of all targets, including *mompS*. Amplification was performed in a reaction volume of 100 µl, each mix containing 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM of each deoxynucleotide (Amersham Biosciences), 10 pmol of each primer (Invitrogen, Life Technologies), and 2.5 U of AmpliTaq Gold polymerase (Applied Biosystems). After an initial enzyme-activation step of 10 min at 95°C, the reaction mixture was subjected to 35 cycles of amplification in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). Each cycle consisted of 30 s at 94°C for denaturation, 30 s at 50°C (for *flaA*, *mompS* and *proA*) or 55°C (for *asd*, *mip* and *pilE*) for annealing, and 30 s at 72°C for elongation; the last cycle was followed by a final extension at 72°C for 5 min. Amplified products were subjected to gel electrophoresis in a 2% agarose gel and subsequently purified for sequencing by using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences). Sequences of purified amplicons were directly determined, with forward and reverse primers, using the ABI PRISM BigDye Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the instructions supplied by the manufacturer, and a model 310 ABI DNA sequencer (Applied Biosystems) for the analysis of products.

Sequence analyses were performed using the BioEdit version 7.0.0. For all analyses, the data obtained with the forward and reverse sequencing primers were combined and aligned manually to produce a consensus sequence. Consensus sequences trimmed to the correct length were then compared with those in the EWGLI SBT database to assign the allele numbers, and for each isolate the combination of alleles at each of the six loci was defined as the Sequence-Based Type or allelic profile.

RESULTS

In the three-year period, from April 2002 to May 2005, 147 environmental samples were collected at six different sites in North Sicily, and analyzed for the presence of *Legionella pneumophila*. The sites were two Units at Palermo City Hospital (A), a hotel (B), a private house (E), and a dental surgery (F) in the city of Palermo, and two hotels by the sea, respectively east (C) and west (D) of Palermo. Sites were sampled repeatedly (three to seven times) during the study, and the presence of *Legionella pneumophila*, both of serogroup 1 and of serogroups 2-14, was observed, at all the sites, in 62 out of the 147 samples (42.2%) (Table 1). *Legionella pneumophila* sg.1 was found in 26 of these samples (17.7 %), either alone (8 samples) or with other serogroups (18 samples). Fourteen of the 26 samples positive for *L. pneumophila* sg.1 were derived from the fresh water distribution systems and seven from the hot water distribution systems, while five were biofilms, collected by swabs, or concretions obtained from faucets or showerheads; with reference to the three typologies of samples (fresh water, hot water, bioflms or concretions), percentages of *L. pneumophila* sg.1 isolations were 27.5, 14.6, and 10.4, respectively.

At site C, *L. pneumophila* sg.1 was occasionally isolated, at the seventh and last sampling, only from fresh tap water, in low number and along with other serogroups. At this site, *Legionella pneumophila* sg. 2-14 was isolated throughout the observation period, with the single exception of the sixth sampling time; its initial concentration, at the first and second sampling, and both in fresh and hot tap water, was $>10^5$ cfu l^{-1} (data not shown).

At all other sites, *L. pneumophila* sg.1 was found just at the first sampling, either alone or with other serogroups, in more than 30% of the collected samples (33.3%, 100%, 33.3%, 66.7%, 50% at sites A, B, D, E, and F, respectively). In many cases, it

TABLE 1 - *Environmental samples assayed for the presence of Legionella pneumophila sg.1 genotypes.*

Site of origin (room)*	*Sampling*		*Collected samples*		*Samples positive for L.pneumophila*				*Typed strains of L. pneumophila sg.1 (n.)*
					serogroup 1		*other serogroups*		
	n.	*time*	*n.*	*(type)** from (room)*	*n.*	*(type)(room)*	*n.*	*(type)(room)*	
A	1°	Apr. 03	9	(x, y, z) (a, b, c)	3	(x, y, z) (a)	1	(y) (a)	21
[RDW]	2°	May. 03	9	(x,y,z)(a,b,c)	0		2	(x,y)(a)	0
(a,b,c)	3°	Jan. 04	9	(x,y,z)(a,b,c)	0		2	(x,y)(a)	0
	4°	Feb. 05	9	(x,y,z)(a,b,c)	2	(x)(a,b)	3	(x,y)(b)-(x)(c)	11
A	1°	Apr. 03	3	(x,y,z)(a)	1	(x)(a)	0		4
[ICU]	2°	May 03	3	(x,y,z)(a)	0		0		0
(a)	3°	Jan. 04	3	(x,y,z)(a)	0		0		0
	4°	Feb. 05	3	(x,y,z)(a)	0		1	(x)(a)	0
B	1°	Jul. 03	9	(x,y,z)(a,b,c)	9	(x,y,z)(a,b,c)	9	(x,y,z)(a,b,c)	25
(a,b,c)	2°	Jul. 03	9	(x,y,z)(a,b,c)	1	(y)(c)	3	(y)(a,b,c)	1
	3°	Aug.03	9	(x,y,z)(a,b,c)	0		0		0
C	1°	Aug. 02	6	(x,y,z)(a,b)	0		4	(x,y)(a,b)	0
(a,b)	2°	Oct. 02	6	(x,y,z)(a,b)	0		4	(x,y)(a,b)	0
	3°	Jan. 03	6	(x,y,z)(a,b)	0		4	(x,y)(a,b)	0
	4°	Apr. 03	6	(x,y,z)(a,b)	0		4	(x,y)(a,b)	0
	5°	Jun. 03	6	(x,y,z)(a,b)	0		4	(x,y)(a,b)	0
	6°	Oct. 03	6	(x,y,z)(a,b)	0		0		0
	7°	Apr. 04	6	(x,y,z)(a,b)	2	(x)(a,b)	4	(x,y)(a,b)	4
D	1°	Aug. 04	3	(x,y,z)(a)	1	(y)(a)	1	(y)(a)	5
(a)	2°	Sep. 04	3	(x,y,z)(a)	0		0		0
	3°	May 05	3	(x,y,z)(a)	0		0		0
E	1°	Aug. 03	3	(x,y,z)(a)	2	(x,y)(a)	0		3
(a)	2°	Oct. 03	3	(x,y,z)(a)	1	(x)(a)	1	(x)(a)	1
	3°	Jan. 04	3	(x,y,z)(a)	0		1	(x)(a)	0
F	1°	Jul. 03	4	(x,x1,y,z)(a)	2	(x,z)(a)	2	(x1,z)(a)	3
(a)	2°	Oct. 03	4	(x,x1,y,z)(a)	2	(x,x1)(a)	2	(x1,z)(a)	8
	3°	Jan. 05	4	(x,x1,y,z)(a)	0		2	(x1,z)(a)	0

*A = Palermo City Hospital [RDU, Respiratory Disease Unit; ICU, Intensive Care Unit]; B = Hotel in Palermo City; C = Hotel by the sea (east of Palermo); D = Hotel by the sea (west of Palermo); E = Private house in Palermo City; F = Dentist's surgery in Palermo City. **x,x1 = fresh tap-water; y = hot tap-water; z = swab sample or concretions from faucet or showerhead.

was present in the fresh tap water as well as in the hot tap water and in biofilms and concretions, and in many cases its concentration, in the water samples, was in the range of 10^3-10^5 cfu l^{-1} (data not shown).

At all the sites, disinfection treatments of the entire water distribution systems were carried out by the superheat and flush method (Stout and Yu, 2004). Although they had to be repeated at sites B, C, E, and F, the treatments were always successful against *L. pneumophila* sg.1 but, at least in some cases, not against all other serogoups.

At site A, after two repeated negative samplings, *L. pneumophila* sg.1 was again found, together with other serogroups, in two out of four samples of fresh tap water, 22 months after the first isolation and the disinfection treatment.

Among all the colonies of *Legionella pneumophila* assayed for serogrouping, 86 colonies, in the cultures derived from the 26 positive samples, were recognized as belonging to serogroup 1.

The 86 strains of *L. pneumophila* sg.1 obtained from these colonies were all typable by AFLP analysis. As shown in Figure 1, by this typing procedure, four AFLP-distinguishable patterns could

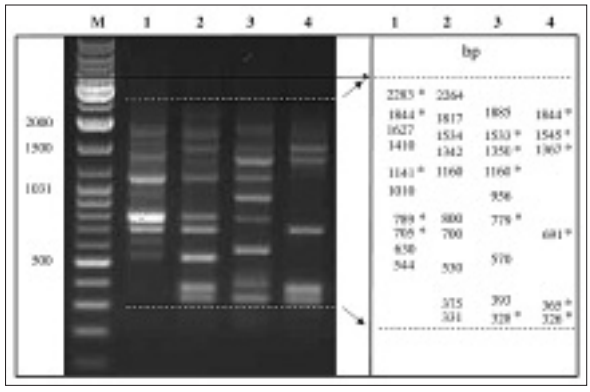

FIGURE 1 - *Lanes 1-4 show genomic patterns of Legionella pneumophila sg.1 isolates, representative of AFLP genotypes PA1-4, respectively; lane M shows a DNA ladder mix. The MWs of all the considered fragments are also shown, with an asterisk near MWs that, in genotypes PA1, PA3 and PA4, were in a likeness range of 2% with respect to those in genotype PA2.*

be identified and referred to four different AFLP genotypes, which were arbitrarily designated PA1, PA2, PA3, and PA4. When compared with each other, PA2 exhibited five bands in common with PA1 and PA3 and six bands in common with PA4 (their MWs are marked with an asterisk in Fig.1), PA1 exhibited two bands in common with PA3 and PA4, PA3 exhibited three bands in common with PA4. When compared with genotypes in the EWGLI database (Table 3), the highest similarities were observed with the EWGLI AFLP types 012 Rome (TS), 028 Rome (TS), 002B Lugano, and 020 Rome (TS), with likeness scores ranging from 70.6 to 90.9. In all cases, when assays were repeated, patterns remained stable also after three cycles of freezing and thawing or five passages of the strains in the laboratory. Only when the indication of the Standard EWGLI AFLP Protocol version 1.2 to use pure *Taq* Ready-to-go PCR beads, instead of the AmpliTaq Gold polymerase, was not respected, did the higher molecular weight fragment disappear from the patterns of genotypes PA1 and PA2 (data not shown).

The distribution of the AFLP genotypes of the 86 environmental isolates of *L. pneumophila* sg.1, according to their sites and samples of origin, is detailed in Table 2. Genotype PA2 was found in 19 out of the 26 positive samples (73.1%) and it was the only genotype isolated at sites A, C, E, and F (55 strains, from 15 samples); at site A, the 11 strains isolated 22 months after the initial disinfection treatment also belonged to this genotype. Genotype PA4 was isolated only at site D (five strains, from the only positive sample, at this site). Genotype PA1 (13 strains, from six samples) and genotype PA3 (eight strains, from four samples) were isolated, with genotype PA2 (five strains, from four samples), at site B; at this site, the presence of strains belonging to more than one genotype was observed in three out of the ten positive samples.

All the 86 isolates of *L. pneumophila* sg.1 were also typable by SBT analysis; PCR products of the expected sizes and nucleotide sequences were yielded from all the six gene fragments investigated and an allelic profile of the six genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA*) was obtained for each isolate. Four different allelic profiles were identified and four SBT groups were defined, each of them perfectly overlapping one of the four, previously described AFLP genotypes (Table 3); all the strains belonging to the prevalent PA2 genotype exhibited the profile "1, 4, 3, 1, 1, 1", whereas the other strains, with genotypes PA1, PA3, and PA4, were characterized by the profiles "11, 14, 16, 16, 15, 13", "2, 10, 3, 1, 9, 4" and "4, 7, 11, 3, 11, 12", respectively. All these profiles were already present in the EWGLI SBT database.

The analysis of the sequences, including, for each gene, the number of the allelic types, the number of polymorphic sites, the percentage of nucleotide substitutions, and the number of synonymous and non nonsynonymous polymorphisms, are shown in Table 4. Three or four allelic variants were observed in each locus. The minimal and maximal numbers of polymorphic sites were found in *mip* and *pilE* genes, respectively; the highest percentage of nucleotide substitutions in the polymorphic sites was found in the *pilE* gene (11.4%). There were no preferential regions of polymorphic sites on the gene fragments, except for the *mip* gene, on which polymorphic sites started at nucleotide number 112. Synonymous polymorphisms were prevalent in genes *flaA*, *pilE,* and *proA*, while nonsynonymous polymorphisms were prevalent in genes *asd*, *mip,* and *mompS*.

When compared with the more frequently isolated strains characterized by allelic profile 1, 4, 3, 1, 1, 1, arbitrarily defined as genotype II, (Figure 2), genotypes with allelic profiles 11, 14, 16, 16, 15, 13 (I), 2, 10, 3, 1, 9, 4 (III) and 4, 7, 11, 3, 1, 4 (IV) exhibited percentages of cumulative se-

TABLE 2 - *Distribution of AFLP genotypes of Legionella pneumophila sg.1 isolates from different environmental sites.*

*Origin of the isolates**				*N° of isolates of AFLP genotype*			
Site	*Room*	*Sampling n°*	*Sample type*	*PA1***	*PA2***	*PA3***	*PA4***
A [RDU]	a	1°	x		10		
			y		1		
			z		10		
	a	4°	x		10		
	b	4°	x		1		
A [ICU]	a	1°	x		4		
B	a	1°	x	5	1		
			y			4	
			z	1	2	1	
	b	1°	x	2			
			y			1	
			z	1	1		
	c	1°	x	1			
			y			2	
			z	3			
	c	2°	y		1		
C	a	7°	x		2		
	b	7°	x		2		
D	a	1°	y				5
E	a	1°	x		1		
			y		2		
	a	2°	x		1		
F	a	1°	x1		1		
			z		2		
	a	2°	x		7		
			x1		1		
Total				13	60	8	5

*As in Table 1. **Local arbitrary designation.

TABLE 3 - *AFLP genotypes and SBT allelic profiles of Legionella pneumophila sg.1 isolates.*

AFPL genotype			*SBT allelic profile*	*N° of isolates*
Local designation	*Most similar EWGLI AFLP type*	*Score of similiraty*	*(flaA, pilE, asd, mip, mompS, proA)*	
PA1	012 Rome (TS)	77.8	11,14,16,16,15,13	13
PA2	028 Rome (TS)	90.9	1,4,3,1,1,1	60
PA3	002B Lugano	80.0	2,10,3,1,9,4	8
PA4	020 Rome (TS)	70.6	4,7,11,3,11,12	5

TABLE 4 - *Sequence variations in the six SBT gene fragments of all the 86 tested isolates of Legionella pneumophila sg.1, evaluated by comparison with the EWGLI reference alleles.*

Gene	*N° of alleles*	*N° of polymorphic nucleotide sites*	*% of nucleotide substitutions*	*N° of synonymous polymorphisms*	*N° of nonsynonymous polymorphisms*
flaA	4	16	8.8	16	0
pilE	4	38	11.04	29	9
asd	4	32	6.8	4	28
mip	3	9	2.2	0	9
mompS	4	30	8.5	5	25
proA	4	22	5.4	21	1

quence variation of 5.7%, 0.9%, and 2.0%, respectively; synonymous and nonsynonymous polymorphisms were similarly distributed in genotypes I and IV, while synonymous polymorphisms were prevalent among the few substitutions of genotype III (Table 5). Nucleotide substitutions were 125 (5.8%) and 107 (5.0) in genotype I, with respect to genotype III and IV, and 52 (2.4) in genotype III, with respect to genotype IV.

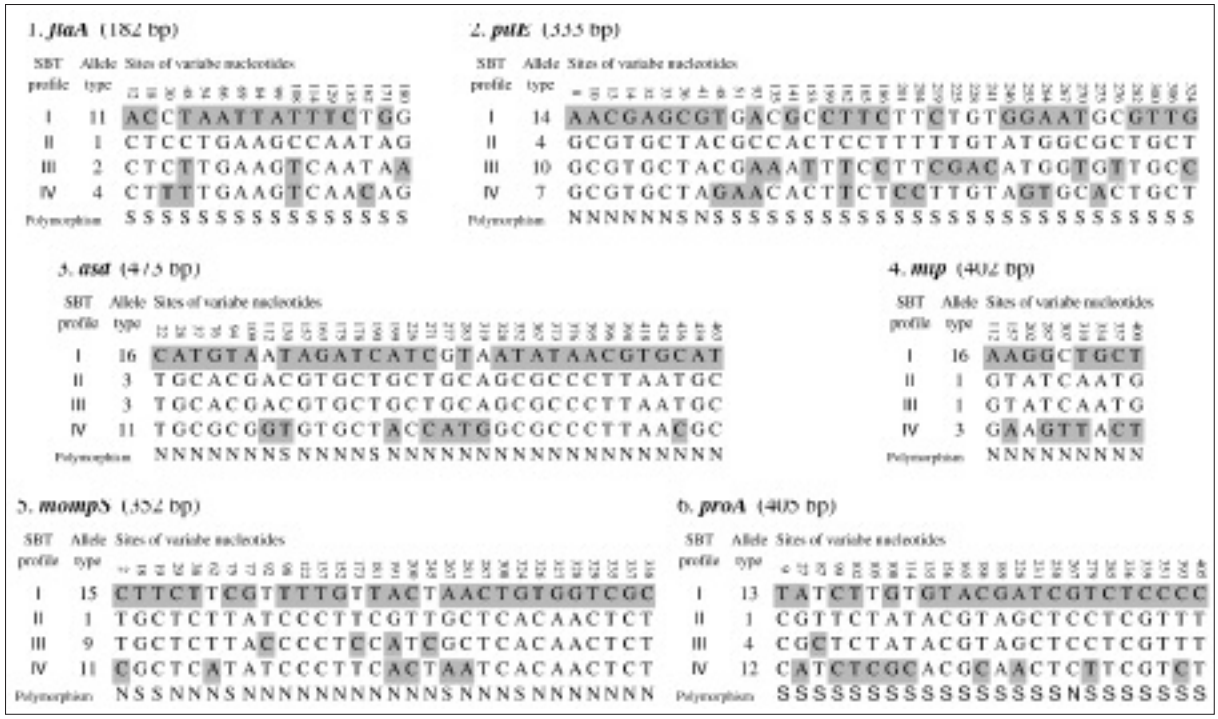

FIGURE 2 - *Polymorphic sites in the gene fragments, flaA, pilE, asd, mip, mompS, and proA, of the SBT genotypes of Legionella pneumophila sg.1 characterized by allelic profiles 11,14,16,16,15,13 (genotype I), 1,4,3,1,1,1 (II), 2,10,3,1,9,4 (III) and 4,7,11,3,11,12 (IV). Numbers in parenthesis indicate the sizes of the sequenced gene targets; numbers over nucleotides identify their position from the first base of determined region; shaded letters show variability with respect to nucleotides in the genes of the more frequently isolated genotype II; letters below each polymorphic site indicate synonymous (S) and nonsynonymous (N) polymorphisms.*

TABLE 5 - *Cumulative sequence variation in the SBT allelic profiles of genotypes I, III and IV of Legionella pneumophila sg.1, compared with the more frequently isolated genotype II.*

SBT allelic profile (genotype)*	*N° of polymorphic nucleotide sites*	*% of nucleotide substitutions*	*N° of synonymous polymorphisms*	*N° of nonsynonymous polymorphisms*
11,14,16,16,15,13 (I)	122	5.07	58	64
1,4,3,1,1,1(II)	0	0	0	0
2,10,3,1,9,4 (III)	20	0.9	16	4
4,7,11,3,11,12 (IV)	43	2.00	25	18

*Arbitrary designation.

DISCUSSION

All six sites assayed for the presence of *Legionella pneumophila* sg.1 had been occasionally frequented by individuals who were recognized as being infected (e.g. a boy with pneumonia, at site A) or who, subsequently, and in some other place, developed diseases diagnosed as legionellosis. Although in these cases no relationships were established between clinical and environmental bacterial strains, *L. pneumophila* sg.1 was isolated from five out of the six investigated sites, and *L. pneumophila* sg. 2-14 from all of them. The analyses were carried out in application of the Italian guidelines for the prevention and control of legionellosis (Anonymous, 205), and the percentages of colonization were observed to be higher than the indeed high percentages of colonization recently described in Italy, in many hotels in five different towns (Borella *et al*., 2005), as well as in some hospitals and private apartments (Leoni *et al.*, 2005) and in some dental units (Zanetti *et al.,* 2000) in the city of Bologna.

Disinfection treatments, by the superheat and flush method, were performed when risk indexes for legionellosis were observed, namely when *L .pneumophila* was isolated from more than 30% of the samples (Allegheny County Health Department, 1997; Best *et al*., 1983) or when its presence exceeded, in many samples, the concentration of 10^3-10^5 cfu l^{-1} (EWGLI, 2005). Although *L. pneumophila* sg.1 is described (Borella *et al*., 2005) as being more resistant than *L. pneumophila* sg.2-14 to both high temperatures and hyperchlorination (employed in the superheat and flush method for an initial 12-14 hour disinfection of the water tanks), treatments were successful against *L. pneumophila* sg.1, but not always against all other serogroups. An explanation for this apparent paradox could be the existence, at all the investigated sites, in addition to some ecological niches favorable to *L. pneumophila* proliferation, also of factors such as a high water hardness or a long-term low free chlorine concentration, which are indicated as potentially responsible (Borella *et al*., 2004; Borella *et al*., 2005) for the persistent colonization by *L. pneumophila* sg.2-14. Another explanation could be referred to the likely presence, in the water distribution systems, of free-living amoebae and to their ability to resuscitate *L. pneumophila* (Garcia *et al*., 2007). In this case the hypothesis of a lesser efficacy of this event with respect to *L.pneumophila* sg.1 should be advanced and explored.

Even when a truly effective disinfection treatment is carried out, the elimination of *Legionella* has usually proved temporary (Rodgers, 1998). In the course of our investigations, *L. pneumophila* sg.1 was reisolated only once (at site A) and only 22 weeks after treatment. Another occasional appearance of *L. pneumophila* sg.1 was observed at site C, originally uncontaminated by this serogroup of the micro-organism, after six negative samplings performed during a period of about two years. In both its reisolation at site A and its occasional appearance at site C, *L. pneumophila* sg.1 was present, in small number, only in some samples of fresh tap water. These observations could be indicative of a relatively low cir-

culation of *L. pneumophila* sg. 1 in Northern Sicily. They are however consistent with the general epidemiology of *L. pneumophila*, which is characterized by the presence of the micro-organism, in small numbers, in many natural aquatic habitats, owing to its capacity to survive water treatment processes, its passage, always in small numbers (Stout *et al., 1985*; Witherell *et al.,* 1988), in man-made habitats, and its proliferation, especially in the water distribution systems of such habitats, when favorable water temperatures (25° to 42°C), physical protection (biofilm), and nutrients (Steinert *et al.,* 2002), or free-living amoebae (Greub and Rauolt, 2004) are present.
The origin of the 86 typed strains of *L.pneumophila* from only six sites, and the isolation of many of them only from the very first samplings taken at five out of these sites, might help to explain why only four genotypes of *L. pneumophila* sg. 1 were detected and why a complete relationship was observed between the two performed typing analyses. One genotype (PA4) was isolated only from a hotel located by the sea, about twenty kilometers west of Palermo. Another genotype (PA2) appeared to be widely prevalent, in that it was present in four of the five initially contaminated sites, all in the city of Palermo, either alone, in three of them, or with the last two genotypes (PA1 and PA3), in the fourth. Strains of *L. pneumophila* sg.1 occasionally found in the fresh tap water at site C, a hotel east of Palermo, and at site A, after disinfection, also belonged to this prevalent genotype.
The observation that all the strains belonging to one and the same AFLP genotype also showed one and the same SBT allelic profile confirms the validity and robustness of both the typing procedures. However, whereas an SBT allelic profile consists of six completely identified nucleotide sequences, a range of variability is admitted in the definition of the AFLP genotypes, and some difficulty has been recognized in comparing AFLP banding patterns, even when they were obtained by means of well-standardized methods, and in allowing, by AFLP, simple and significant interlaboratory evaluations (Fry *et al.,* 1999; Gaia *et al.,* 2005). We employed the last 1.2 version of the EWGLI protocol to perform AFLP analysis, and we photographed and digitally recorded gels to obtain image capture; nevertheless, when the images were submitted to the EWGLI website to be compared with the EWGLI AFLP database, only strains belonging to genotype PA2 exhibited a likeness score higher than 90 with a well-characterized existing isolate. On the contrary, all the allelic variants of the six genes included in the SBT profile and all the four SBT genotypes described in this paper proved to be already present in the EWGLI SBT database.
The SBT analysis showed that *L. pneumophila* sg.1 strains prevailing in our samples belonged to SB type 1, 4, 3, 1, 1, 1, the most commonly isolated in Europe (Fry *et al.,* 2005) and present also in Japan (Amemura-Maekawa *et al.,* 2005) and Canada (Wong *et al.*, 2006). As some other isolates (from our site D) belonged to SB type 4, 7, 11, 3, 11, 12, the second most common in Europe (Fry *et al.,* 2005), it is possible to consider Northern Sicily a well-integrated part of a general European, if not universal, ecosystem where *L. pneumophila* sg.1 appears to be not only ubiquitous and characterized by common epidemiological behaviors but also easily spreading, at least with respect to some genotypes.
Some authors (Amemura-Maekawa *et al.,* 2005) consider SBT less effective than other typing procedures in discriminating between strains with genomic differences; others (Scaturro *et al.,* 2005) propose SBT as the new gold standard for the typing of *L. pneumophila*, when high discrimination has to be guaranteed. In our experience, both AFLP and SBT exhibited good discrimination capacity, with differences, among the genotypes less frequently identified, involving four to eight bands, in the first analysis, and 52-125 nucleotides, in the second, mainly within *pilE*, *flaA*, and *mompS* genes. However, when strains with the more frequently observed allelic profile 1, 4, 3, 1, 1, 1 were compared with strains of all the other three genotypes, differences were in the range of 4-5 bands, with AFLP, and of 20-122 nucleotide substitutions, with SBT. At least on the basis of these observations, SBT, even if still performed in respect to the EWGLI protocol version 2.0 and not extended by the recently proposed use of *neuA* as a seventh allele (Ratzow *et al.,* 2007), appears to be characterized not only by an excellent reproducibility and a capacity to allow easy transferability of data through web-based databases, but also, at least with respect to AFLP, by an equal or perhaps higher discriminatory power.

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