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Epidemiological Investigation of *Legionella pneumophila* Serogroup 2 to 14 Isolates from Water Samples by Amplified Fragment Length Polymorphism and Sequence-Based Typing and Detection of Virulence Traits

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ABSTRACT

The aim of this study is to explore the dispersion, clonality, and virulence of *Legionella pneumophila* serogroups 2 to 14 in the Greek environment. Eighty *L. pneumophila* serogroup 2 to 14 strains isolated from water distribution systems of hotels, hospitals, athletic venues, and ferries in Greece were tested by monoclonal antibodies (MAbs) for serogroup discrimination and molecularly by amplified fragment length polymorphism (AFLP) for genetic diversity. Fifty-six of 80 strains were also typed by the sequence-based typing (SBT) method. All strains were further analyzed for detection of two pathogenicity loci: *Legionella vir* homologue (*lvh*) and repeats in structural toxin (*rtxA*). Thirty-seven strains (46.2%) belonged to serogroup 6, 26 strains (32.5%) to serogroup 3, and 7 (8.8%) to other serogroups (4, 5, 8, and 10). Ten strains (12.5%) were nontypeable (NT) into the known serogroups. Thirty-nine different AFLP types were found among the 80 *L. pneumophila* serogroup 2 to 14 strains, and 24 different SBT types were found among the 56 strains tested. Among the 80 strains, the *lvh* locus was present in 75 (93.8%), the *rtxA* locus was found in 76 (95%), and both loci were found in 73 (91.3%) strains. This study showed that there is genetic variability of *L. pneumophila* serogroups 2 to 14 in the Greek environment as well as a high percentage of the pathogenicity loci. Introducing an effective diagnostic test for *L. pneumophila* serogroups 2 to 14 in urine and promoting the examination of respiratory specimens from patients hospitalized for pneumonia in Greek hospitals are essential.

IMPORTANCE

In this study, the dispersion, clonality, and virulence of environmental isolates of *Legionella pneumophila* serogroups 2 to 14 (Lp2–14) in Greece were investigated. Genetic variability of Lp2–14 in the Greek environment was identified together with the presence of the pathogenicity loci in a high percentage of the isolates. Despite the high prevalence of Lp2–14 in the Greek environment, no clinical cases were reported, which may be due to underdiagnosis of the disease. Almost all the legionellosis cases are diagnosed in Greece by using the urine antigen test, which is specific for Lp1. There is an urgent need to improve the clinical diagnosis of legionellosis by introducing an effective diagnostic test for Lp2–14 in urine and by promoting the PCR examination of respiratory specimens from patients with compatible clinical symptoms.

Legionella species are inhabitants of water and biofilms in both natural and industrial aquatic environments (1). *Legionella* spp. can cause Legionnaires' disease and the flu-like Pontiac fever in humans, through inhalation of *Legionella*-contaminated water aerosols (2, 3). In Europe, according to the World Health Organization (WHO), the incidence rate was 10 to 15 cases per million population, and the total case-fatality rate (CFR) was about 12% (4, 5). In the last surveillance report from the European Centre for Disease Prevention and Control (ECDC), for the year 2014, the notification rate of Legionnaires' disease in Europe for the same year was 13.5 cases per million inhabitants and the CFR was 8%. Specifically for Greece, the notification rate was 2.5 per million inhabitants and the CFR was 12% (6).

There are around 60 known *Legionella* species (http://www.bacterio.net/legionella.html), and among them, *Legionella pneumophila*, especially serogroup 1 (sg1), causes the majority of Legionnaires' disease cases (7, 8). Specifically, approximately 90% of Legionnaires' disease cases are caused by *L. pneumophila*. More than 75% of the total are due to *L. pneumophila* serogroup 1 (Lp1), and the remaining cases (ranging from 5% to 22%) are caused by the other serogroups, i.e., non-serogroup 1 of *L. pneumophila* (Lpnon-1) (9, 10). It should be noted that *L. pneumophila* presents a variety of 16 serogroups (11).

Different methods have been developed for molecular typing of the Lp1 strains, such as pulsed-field gel electrophoresis (PFGE),

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amplified fragment length polymorphism (AFLP) analysis, and sequence-based typing (SBT). These are useful tools during investigations of Legionnaires' disease cases, clusters, or outbreaks (12–14).

Lp1 has been studied for the presence of a variety of virulence genes, such as intracellular multiplication/defective in organelle trafficking (*icm*/*dot*) (15, 16), repeats in structural toxin (*rtxA*) (17), and *Legionella vir* homologue (*lvh*) (18). The *icm*/*dot* genes encode the type IV secretion system and are responsible for intracellular multiplication. The *lvh* locus derives proteins for a second type IV secretion system that contributes to conjugation and virulence (18). The role of *rtxA* is to help *Legionella* entry and adherence to the host cell (17).

One of the factors that can explain the high proportion of clinical cases of Legionnaires' disease reported to be due to Lp1 is the diagnostic method used in the hospital laboratories. In particular, Greek and other European hospitals use mainly the urine antigen test for lab diagnosis. The test detects only Lp1, implying that cases due to Lpnon-1 are probably underdiagnosed. Given that probably more than 10% of *Legionella* infections internationally (9, 10) are caused by serogroups other than Lp1, the investigation of the existence and the molecular epidemiology of such serogroups in the environment of a region with enhanced tourism and a prolonged warm vacation period are of obvious interest. Epidemiological data for Greece and Europe since 2011 show that the majority of cases were diagnosed with the urine antigen test at a percentage ranging from 82 to 96% and 77 to 82%, respectively (6, 19–21). Two recent studies in Greece performed environmental detection of *Legionella* in water distribution systems and molecular typing, but until now no other study in Greece had detected virulence genes (22, 23). The objective of this study was to investigate the genetic diversity of *L. pneumophila* sg2 to sg14 (Lp2–14) in the Greek environment and the existence of virulence genes among them.

MATERIALS AND METHODS

Isolates. Eighty-two strains of Lp2–14 serotyped by latex were randomly selected from 171 strains isolated from water samples collected during a 2-year period within the public health inspection program for the Athens Olympic Games in 2004. Confirmation of the serogroups was conducted by use of monoclonal antibodies (MAbs), and two of the strains proved to be serogroup 1 and were excluded from the study. The remaining 80 strains were used for typing by AFLP and detection of virulence genes. Due to financial restrictions, 56 of 80 strains were additionally typed by SBT.

The 80 strains included in the study have been isolated from the analysis of 1,870 water samples that were taken from 385 hotels (1,086 samples), 94 athletic venues (382 samples), 10 cruise ships (133 samples), and 21 ferries (269 samples). Parts of these results have been published elsewhere (24–26).

Water samples came from 13 different Olympic prefectures, which represent about 70% of the population of Greece and are distributed geographically in northern, central, and southern Greece. Four prefectures belong to Attica and nine to the rest of Greece.

Detection and enumeration of *Legionella* spp. in water samples. Water samples were analyzed with a method based on the ISO 11731:1998 standard (27). The final identification of the colony was made by the latex agglutination test (Oxoid). This test can discriminate Lp1 and Lp2–14 without specifying the exact serotype of the Lp2–14 strain, while it is capable of detecting a group of particular non-*pneumophila* species.

Serological typing. Further serogroup typing was conducted by using MAbs in an enzyme-linked immunosorbent assay (ELISA) and/or an indirect immunofluorescent method, to discriminate the strains of Lp2–14. Serogroup-specific MAbs of the Dresden panel were used when needed (9).

Molecular typing. The AFLP protocol was implemented as described previously (12, 13, 28). DNA extraction from *Legionella* strains has been conducted using the Nucleon BACC2 DNA extraction kit (Amersham Pharmacia Biotech). Bands within the range of 300 to 3,500 bp only were included for each pattern. The comparison of the AFLP profiles between different gels was performed by the software Gelcompare II (Applied Maths, Belgium). Cluster analysis was based on the Dice coefficient and the method of unweighted pair group method with averages (UPGMA). Strains with genetic homology of >90% were interpreted as homologues (29).

The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for Legionella Infections (ESGLI) SBT protocol for epidemiological typing of *Legionella pneumophila* (SBT protocol, version 5) was followed for the typing of the Lp2–14 strains (http://bioinformatics.phe.org.uk/legionella/legionella_sbt/php/sbt_homepage.php [protocols]). For a single strain, *neuAh* sequence-based typing (SBT protocol version 1) for epidemiological typing of *Legionella pneumophila* was applied (see URL above). The *Legionella* sequence quality tool (http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi) was used for the analysis of the primary sequence data. All data, including new sequence types (STs), were introduced in the ESGLI SBT database (http://bioinformatics.phe.org.uk/legionella/legionella_sbt/php/sbt_homepage.php [strain data submission]).

Detection of virulence traits. Two virulence gene loci (*lvh* and *rtxA*), previously proven to have a strong correlation with Legionnaires' disease in clinical cases by Lp1 (28, 30) were selected to be detected in Lp2–14 strains. Six pairs of virulence gene-based PCR primers have been used in this study. They were *lvh1-prpA* and *lvh2-prpA*, *lvh3-lvhB3* and *lvh4-lvhB4*, *lvh5-lvhB8* and *lvh6-lvhB9*, and *lvr1-lvrE* and *lvr2-lvrE* for the detection of the *lvh* region and *rtx1-rtxA* and *rtx2-rtxA* as well as *rtx3-rtxA* and *rtx4-rtxA* for the amplification of the *rtxA* region. The same primer sequences and PCR conditions were used as those described in the former studies (28, 30).

Statistical analysis. Fisher's exact test was used to explore any associations between categorical variables. A result was considered statistically significant when the *P* value was <0.05.

RESULTS

Detection and enumeration of *Legionella* spp. in water samples. Among 1,870 water samples, 172 (9.2%) were positive for Lp1, 171 (9.1%) for Lp2–14, and 45 (2.4%) for *Legionella* non-*pneumophila*. Analytical results for the total water samples are shown in Table 1 (24–26).

Serological typing. The 80 randomly selected study strains belonged to different serogroups. The major part consisted of 37 strains (46.2%) that were sg6, while 26 strains (32.5%) belonged to sg3. Seven strains (8.8%) belonged to various serogroups: 1 sg4 strain (1.3%), 1 sg5 strain (1.3%), 3 sg8 strains (3.7%), and 2 sg10 strains (2.5%). Ten strains (12.5%) were nontypeable (NT) into the known serogroups. A statistically significant difference ($P < 0.001$) in the distributions of serogroups between Attica and the rest of Greece was identified. In Attica, 23 of 37 strains (62.2%) were sg6, while in the rest of Greece, sg3 was the most frequent, since 22 of 43 strains (51.2%) belonged to sg3.

Molecular typing. (i) AFLP. Thirty-nine AFLP types were found among the 80 Lp2–14 strains. Among the 36 sg6 strains, 17 different AFLP types were found, and among the 27 sg3 strains, 14 different AFLP types were found. Nine NT strains belonged to 8 AFLP types.

As shown in Table S1 in the supplemental material, the most

TABLE 1 Site of environmental sample collection and *Legionella*-positive results

Facility type	No. of environmental samples	No (%) of positive environmental samples			
		Legionella spp.	*L. pneumophila* sg1	*L. pneumophila* sg2–14	*Legionella* non-*pneumophila*
Hotel	1,086	268 (24.7)	121 (11.1)	120 (11.0)	36 (3.3)
Cruise ship	133	0	0	0	0
Ferry	269	82 (30.5)	43 (16)	44 (16.3)	9 (3.3)
Athletic venue	382	15 (3.9)	8 (2.1)	7 (1.8)	0
Total	1,870	365 (19.5)	172 (9.2)	171 (9.1)	45 (2.4)

frequent AFLP type was AF8 ($n = 8$), followed by AF1 ($n = 7$) and AF6 and AF35 ($n = 4$ each). A statistically significant difference ($P = 0.001$) in the distributions of AFLP types between Attica and the rest of Greece was also found. In Attica, 18.9% of the strains belonged to AF5 and AF35 (7 of 37). In the rest of Greece, 18.6% of the strains belonged to one AFLP type, AF8 (8 of 43 strains).

(ii) SBT. Among the 28 sg6 strains, 11 different STs have been found. Among the 19 strains of sg3, 6 different STs have been found. Five NT strains belonged to 5 different STs. Overall, 10 new STs were found. They are ST1825, ST1929, ST1930, ST2082, ST2083, ST2084, ST2085, ST2086, ST2087, and ST2088. The detailed molecular typing results are presented in Table 2. Finally, a statistically significant difference ($P < 0.001$) in the distributions of STs between Attica and the rest of Greece was identified. The prevalent ST in Attica was ST68, with 7 of 25 strains (28%), while in other regions of Greece 8 of 31 strains (25.8%) belonged to ST728.

Comparison of SBT and AFLP. As shown in Table 2, for the 19 strains of sg3, 6 different STs and 10 different AFLP types were identified. For the 28 strains of sg6, 10 different STs and 18 different AFLP types were found. Comparing these results, it seems that strains of the same ST can belong to more than one AFLP type. For example, strains of ST68 presented the highest variability, belonging to 5 different AFLP types, while those belonging to ST87 belonged to 3 different AFLP types, as did strains of ST728.

Finally, the diversity of AFLP types of strains which belong in the same ST group is enhanced as the number of strains constituting the group increases. In cases of ST groups consisting of a low number of strains, a single AFLP type is observed for each ST.

Additionally, strains with identical AFLP types may belong to different serogroups. This is observed in the group of strains that belong to AFLP type 1, in which strains belonging to both sg6 and sg3 are included. In contrast, most of the strains with a common ST show stability to the kind of serogroup to which they belong. These data are summarized in Table S2 in the supplemental material.

Detection of pathogenicity genes. All strains were subjected to detection of pathogenicity gene loci *rtxA* and *lvh*. The *lvh* locus was present in 75 strains (93.8%), while the *rtxA* locus was found in 76 strains (95%). The presence of both loci was detected in 73 strains (91.3%). The simultaneous absence of the two genes has not been found in any strain.

The *lvh* and *rtxA* loci were detected in high proportions among the different serogroups. Results are shown in Table 3.

DISCUSSION

The results of the present study showed that a large variety of different Lp2–14 clones can be found in the environment. The most frequently identified serogroup proved to be sg6, followed by sg3. In a study that took place in France for the *Legionella* distribution of 259 clinical and 2,747 environmental strains, Lp1 comprised 95.4% of cases but only 28.2% of the environmental samples. Serogroup 6 and sg3, although making up a low percentage (2%) of the clinical cases, were the most common from the Lpnon-1 clinical cases. In environmental strains, the same two serogroups were also found in higher percentages, 10.8% (sg3) and 11.1% (sg6), making a sum of 21.9% (31). These specific serogroups are the most frequent and virulent Lpnon-1 serogroups among clinical cases (6, 9, 10). That means that there is a strong possibility of transmission of Legionnaires' disease caused by those serogroups in Greece in susceptible humans. Further, several case reports describe Lpnon-1 Legionnaires' disease in humans. They include serogroups 6, 3, 5, 8, 10, 11, and 12 (32–37). It is worth mentioning that coinfections in humans by two different serogroups have been reported as well (34, 37).

Our results are in concordance with data from a study in the Netherlands from 2002 to 2012, in which 24.7% (367/1,117) of environmental samples were positive for *Legionella*. Seventy-six of 367 samples (20.7%) were positive for Lpnon-1, and 26.4% (97/367) were positive for Lp1. Lp1 was found in 28 (19.7%) clinical cases, Lpnon-1 in 6 (4.2%), and *Legionella* spp. other than *L. pneumophila* in 22 (15.5%) (38). In England and Wales, from 2000 to 2008, Harrison et al. found 43.5% (120/276) Lp2–15 and 55.8% Lp1 (154/276) in environmental samples, while in clinical isolates only 2.4% (4/167) belonged to Lp2–14 (39). In contrast, in southeastern Italy from 2000 to 2009, Lp2–14 was found in community buildings at a prevalence of 40.8% (831/2038), versus 33% (673/2038) for Lp1. No specific data for serotyping exist for the clinical strains (40).

Anevlavis et al. reported in a study that from 1,390 community-acquired pneumonia (CAP) cases admitted in two hospitals of northern and southern Greece, only 435 (31.3%) had positive sputum cultures. The remaining 955 cases (68.7%) remained undiagnosed. These data show that a rather large percentage of CAP cases without a known etiologic agent exist in Greece. In combination with the facts that no rapid test for Lp2–14 is implemented and that the application of the sputum or BAL fluid culture for the isolation of *Legionella* spp. is rare, it is highly likely that Legionnaires' disease due to Lp2–14 is underdiagnosed (41).

In our study, 39 different AF types came from the 80 strains. The genetic clusters that were close to each other tended to share the same serogroup. For instance, the clusters AF8 to AF12 consisted of 15 strains; 12/15 strains belonged to sg3. In neighboring clusters, AF13 to AF18 (total, 10 strains), all strains belonged to sg6. In AF22 to AF28, 7/9 strains were nontypeable, and finally in AF31 to AF36, 12/13 strains belonged to sg6.

TABLE 2 Results of serological and molecular typing of all 56 *L. pneumophila* sg2–14 strains tested by SG, AFLP, and SBT typing methods, linked with environmental epidemiological data

Strain ID no.	Code of health inspection point[a]	Prefecture code[b]	AFLP (AF) type	ST	ST gene allele[c]							SG
					flaA	*pilA*	*asd*	*mip*	*mompS*	*proA*	*neuA*	
1	O-1	P-1	1	87	2	10	3	28	9	4	13	3
2	O-2	P-1	1	461	6	10	14	28	21	14	9	6
3	H-1	P-2	1	461	6	10	14	28	21	14	9	6
4	O-3	P-1	1	87	2	10	3	28	9	4	13	3
5	O-2	P-1	1	461	6	10	14	28	21	14	9	6
6	O-2	P-1	1	461	6	10	14	28	21	14	9	6
7	H-2	P-3	1	1825	6	10	15	3	21	4	15	6
8	H-3	P-4	2	1929	6	6	3	28	9	4	6	3
9	H-4	P-5	2	1929	6	6	3	28	9	4	6	3
10	H-4	P-5	2	1929	6	6	3	28	9	4	6	3
11	SP-1	P-2	3	1358	5	2	22	10	6	25	203	8
12	SP-1	P-2	3	1358	5	2	22	10	6	25	203	8
13	O-4	P-1	3	461	6	10	14	28	21	14	9	6
14	H-5	P-3	5	2083	2	10	15	28	21	4	9	6
15	H-5	P-3	5	2083	2	10	15	28	21	4	9	6
16	H-5	P-3	5	2083	2	10	15	28	21	4	9	6
17	H-6	P-6	6	2083	2	10	15	28	21	4	9	6
18	O-5	P-1	6	2084	6	10	19	2	19	4	9	6
19	H-7	P-6	6	2085	6	10	15	28	4	3	1	6
20	H-8	P-7	6	2083	2	10	15	28	21	4	9	6
21	H-9	P-8	7	993	10	22	7	28	16	18	13	3
22	O-3	P-1	8	728	2	10	3	28	9	4	3	3
23	O-3	P-1	8	728	2	10	3	28	9	4	3	3
24	H-10	P-1	8	728	2	10	3	28	9	4	3	3
25	H-10	P-1	8	728	2	10	3	28	9	4	3	3
26	H-10	P-1	8	728	2	10	3	28	9	4	3	3
27	H-11	P-9	8	728	2	10	3	28	9	4	3	3
28	H-12	P-10	8	421	2	10	3	3	9	4	3	6
29	H-13	P-9	8	2086	6	10	15	28	9	14	3	NT
30	H-14	P-9	9	1930	6	10	2	28	21	14	3	10
31	H-10	P-1	9	728	2	10	3	28	9	4	3	3
32	H-16	P-11	11	87	2	10	3	28	9	4	13	3
33	H-16	P-11	12	87	2	10	3	28	9	4	13	3
34	H-16	P-11	12	87	2	10	3	28	9	4	13	3
35	H-18	P-3	13	153	2	10	3	28	9	14	3	6
36	O-7	P-9	15	68	3	13	1	28	14	9	3	6
37	F-4	P-2	16	68	3	13	1	28	14	9	3	6
38	H-20	P-4	16	81	2	10	3	28	9	4	9	6
39	H-21	P-7	17	68	3	13	1	28	14	9	3	6
40	H-21	P-7	17	68	3	13	1	28	14	9	3	6
41	H-20	P-4	18	81	2	10	3	28	9	4	9	6
42	F-2	P-2	19	2082	2	10	15	28	21	4	207	NT
43	O-8	P-1	21	728	2	10	3	28	9	4	3	3
44	F-1	P-3	22	1575	4	3	18	10	5	1	6	NT
45	H-22	P-12	22	1226	7	10	17	28	13	11	3	4
46	H-5	P-3	24	2087	2	10	15	28	21	4	6	NT
47	H-5	P-3	28	2088	2	10	15	28	21	4	1	NT
48	H-22	P-12	29	361	7	10	17	28	13	11	13	3
49	H-25	P-9	30	297	7	10	17	28	13	4	13	3
50	H-27	P-12	32	649	3	13	1	3	14	9	3	6
51	F-2	P-2	34	1999	3	6	1	6	1	11	9	6
52	H-1	P-2	35	421	2	10	3	3	9	4	3	6
53	F-1	P-2	35	68	3	13	1	28	14	9	3	6
54	F-1	P-2	35	68	3	13	1	28	14	9	3	6
55	F-1	P-2	36	68	3	13	1	28	14	9	3	6
56	H-21	P-7	36	68	3	13	1	28	14	9	3	6

[a] O, house; H, hotel; F, ferry; SP, sports center; C, city.
[b] P-2, P-3, P-4, and P-7 are in Attica; P-1, P-5, P-6, P-8, P-9, P-10, P-11, and P12 are in the rest of Greece.
[c] Each number is the allele variant of the gene.

TABLE 3 Detection of virulence traits in *L. pneumophila* sg2–14 strains per type of serogroup

Serogroup	No. (%) of strains positive for *L. pneumophila* sg2–14		
	lvh locus	*rtxA* locus	*lvh* and *rtxA* loci
6	37/37 (100)	35/37 (94.6)	35/37 (94.6)
3	24/26 (92.3)	25/26 (96.1)	24/26 (92.3)
NT	9/10 (90)	9/10 (90)	9/10 (90)
8	2/3 (66.6)	3/3 (100)	2/3 (66.6)
5	1/1 (100)	1/1 (100)	1/1 (100)
10	2/2 (100)	2/2 (100)	2/2 (100)
4	0/1 (0)	1/1 (100)	0/1 (0)
Total	75/80 (93.8)	76/80 (95)	73/80 (91.3)

STs that were found in this study are encountered in the ESGLI database in clinical cases also. This means that inevitably they have caused pneumonia in humans. Among the 14 known STs, 11 are responsible for clinical cases. Additionally, 11 new STs have been found. The STs in clinical strains most often found in the ESGLI database from the STs of this study are ST68 and ST87. STs from our study that have not been found yet in the database of ESGLI as clinical strains are ST1575, ST1226, and ST361.

Results show that different clones of Lp2–14 are more prevalent in the Attica region than in the rest of Greece. ST68, which belongs to the more frequent STs of clinical cases in the ESGLI database, is encountered more often in Attica than in the rest of Greece. This differentiation is in agreement with the prevalence of serogroups in the two areas, since sg6 is prevalent in Attica whereas sg3 is prevalent in the rest of Greece. Both areas are urban, and there was not any specific predominance of a certain clone in a specific water site (hotel, ship, etc.). Thus, the most likely explanation for the differences in clone predominance is the geographical location.

In addition to those in this study, all strains have been examined for pathogenicity genes that have already been examined for Lp1. Specific genetic loci *lvh* and *rtxA* were chosen because they were suggested as appropriate indicators of *L. pneumophila* virulence after 19 major virulence genes were studied (42). A genetic element of the *lvh* locus in the genome of strain Philadelphia 1 (derived from the strain of the first outbreak in Philadelphia, PA) was detected in the genome of an Lp6 strain that has been sequenced in Canada (43). Different studies suggest that there is recombination and horizontal gene transfer through the plasmid pP36 of the *lvh* locus and other loci involved in type IV secretion systems (43, 44). *rtxA* encodes the *dot*/*icm*-regulated pore-forming toxin and plays a significant role in the entry of the bacteria into the host cell (17). Rtx toxin consists of two regions; the N-terminal region is responsible for adhesion, and the C-terminal region participates in adhesion and pore formation (42).

Our data showed that these genetic loci exist in Lpnon-1 strains at a very high percentage. This fact leads to the conclusion that Lpnon-1 has the pathogenicity mechanisms to adhere to, enter, and escape acidification of the vacuole in a host cell, a fact consistent with the virulence potential of these strains.

To our knowledge, no clinical case of Legionnaires' disease due to Lpnon-1 has ever been diagnosed in Greece. This can be attributed to the almost exclusive use of the immunochromatography (ICT) urine antigen test for the diagnosis of Legionnaires' disease in Greek hospitals. This test has excellent sensitivity for Lp1 (97.2% in concentrated urine samples) and specificity (100%) and is recommended for this diagnostic purpose (45). It is worth mentioning that the Binax NOW *Legionella* urinary antigen kit (ICT) had given a positive result for Lp3 and Lp6 when the detection limit was more than 100-fold higher than the detection limit for Lp1 (46).

The Biotest kit based on the enzyme immunoassay (EIA) method is the only diagnostic tool for rapid laboratory diagnosis of Legionnaires' disease caused by strains other than Lp1. Its sensitivity for Lpnon-1 is 86%, versus 94.6% for Lp1 (47).

The culture of clinical specimens such as sputum or bronchoalveolar lavage (BAL) fluid is extremely rare in Greek hospitals. This is also a common phenomenon in several other European countries (6). Surveillance data from the last report coming from ECDC show characteristically that for the year 2014, from the 27 cases reported from Greece, 26 (90%) were diagnosed by a urine test and 1 (4%) was diagnosed from the high titer of a single antibody, while none (0%) was confirmed by culture or PCR. These data can be considered representative for other European countries. From the total of 6,941 cases, 6,038 (87%) were diagnosed by the urine test, 577 (8%) by PCR, 247 (4%) by a high-titer single-antibody assay, 819 (12%) by culture, 67 (1%) by a 4-fold antibody rise, and only 2(<1%) by direct immunofluorescence (6). The majority of reported cases during the period 2010 to 2014 in Greece were diagnosed with the urinary antigen test (82% to 96%), while the cases diagnosed with culture were 4% to 10%, those with PCR 3% to 5%, and those with a single high titer 4% to 16%. The 4-fold titer rise and the direct immunofluorescence assays were never used (6, 19–21, 48). Respiratory secretion culture can be implemented mainly in Greek Public Health Laboratories. Even though it is so, clinical samples come in these laboratories usually after a positive urine test. Inevitably, an unspecified number of cases of Legionnaires' disease remain undiagnosed, a fact that makes the introduction of culture and PCR in the diagnostic protocol of possible Lp infection crucial.

One possible limitation of our study is the representativeness of the environmental samples and isolates of the Greek environment in both time and place. We acknowledge that during the planning phase representativeness was not perceived, but since the samples and isolates were from 13 prefectures covering more than 70% of the Greek population, we can assume that the isolates could be considered to be representative of the Greek environment.

Finally, based on these results, it would be useful to take into consideration the quick introduction of a proper diagnostic test for Lp2–14 in urine and even in upper respiratory specimens in the Greek hospitals, since we identified several dispersed clones of Lp2–14 existing in the Greek environment that could be possibly virulent for humans. A strong consideration of an alternative and more-cost-effective diagnostic approach would be to focus on the serogroups of each region in the clinical diagnostic setup in order to improve the diagnosis of the disease.

Further studies must be conducted to explore if the Lp1 strain has developed more pathogenicity mechanisms by natural selection than the Lpnon-1 strains. Exploring the virulence of Lp and mapping the clone dispersion of this bacterium in the environment could lead to more-advanced and -intensive measures for the control and prevention of legionellosis.

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