International Journal of Environmental Health Research

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/cije20

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Version of record first published: 12 Dec 2011

To cite this article: K. Fragou, P. Kokkinos, C. Gogos, Y. Alamanos & A. Vantarakis (2011): Prevalence of Legionella spp. in water systems of hospitals and hotels in South Western Greece, International Journal of Environmental Health Research, DOI:10.1080/09603123.2011.643229

To link to this article: http://dx.doi.org/10.1080/09603123.2011.643229

*International Journal of Environmental Health Research*
2011, 1–15, i*First* article

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# Prevalence of *Legionella* spp. in water systems of hospitals and hotels in South Western Greece

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(*Received 28 July 2011; final version received 28 September 2011*)

The aim of the present study was to determine the prevalence of *Legionella* spp. in water systems of hospitals and hotels located in South Western Greece, to study the molecular epidemiology of the isolated strains and their possible association with bacterial contamination (total count and *Pseudomonas aeruginosa*), the water pH, and temperature. A prevalence survey for *Legionella* spp. by culturing techniques in water distribution systems of eight hospitals and nine hotels occurred in South Western Greece. Water sampling and microbiological analysis were carried out following the ISO methods. *Legionella pneumophila* was detected in 33% and 36% of the distribution systems of hospitals and hotels, respectively. Our survey results suggest a frequent prevalence of elevated concentrations of *Legionella* spp. in water systems of hospitals and hotels. Our investigation has confirmed the need to regularly monitor the microbiological condition of water systems in hospitals and hotels.

**Keywords:** *Legionella* species; hospitals; hotels; water distribution systems; microbiological surveillance



## 1. Introduction

*Legionelleae* are Gram-negative bacteria, which are able to reproduce at temperatures between 25°C and 43°C and survive in temperatures of up to 55–60°C (Leoni et al. 2001). Human infection occurs through inhalation of contaminated aerosols with *Legionella* species (Edelstein 1998). *Legionella* are the etiological agents of both Legionnaires' disease and Pontiac fever (Palmer et al. 1995; Nazarian et al. 2008). Until today, at least 50 species of *Legionella* have been characterized, and more than 20 species are associated with human diseases (Fields et al. 2002; Bartram et al. 2007; Declerck et al. 2007; Diederen 2008). *Legionella pneumophila* is the most common pathogenic species, responsible for the majority of Legionellosis, worldwide (Declerck et al. 2007).

*Legionella* is an opportunistic pathogen with widespread distribution in the environment; existing as part of the natural microbial flora of many aquatic ecosystems such as surface and ground waters (Riffard et al. 2001). The most common sources of infection are cooling towers, evaporative condensers, hot and cold water systems, spa pools, and a variety of other artificial sources (Bartram et al.

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ISSN 0960-3123 print/ISSN 1369-1619 online

http://dx.doi.org/10.1080/09603123.2011.643229
http://www.tandfonline.com

2007). Very low concentrations of *Legionella* in natural habitats can increase markedly in man-made hot water systems where water temperatures are below 55°C. As there have been no reported cases of inter-human transmission, it can be presumed that the environment is the only source of the infection. A dose–response relationship for the development of *Legionella* infections has not, however, been established yet (Bartram et al. 2007). Bacteria are ubiquitous in natural and artificial aquatic environments, such as rivers, ponds and lakes, swimming pools, artificial water supplies, and water distribution systems (Huang et al. 2004; Hsu et al. 2006; Yu et al. 2008).

Many studies have demonstrated that the major sources for Legionnaires' disease are the potable water systems of large buildings including hospitals and hotels (Lu et al. 1993; Yu and Stout 2000; Codony et al. 2002; Moritz et al. 2010). Hot potable water (30–55°C) that is dispersed by shower-heads, faucets, etc. (Marrie et al. 1992) is the most common source of nosocomial (Mathys et al. 1999) and community-acquired Legionellosis (Pedro-Botet and Sabria 2005) worldwide. Reports have identified the water distribution systems and the cooling towers as the major reservoirs of *Legionella* in hospitals (Yu 1998). Parameters such as water temperature, flow, stagnation, low disinfectant residuals, pipe materials, degree of pipe corrosion, high water shear stress, and flushing are well known factors favoring bacterial growth and associated with the source of the *Legionella* outbreaks (Exner et al. 2005; Yoder et al. 2008). Furthermore, microbial biofilms and protozoa are known to play a major role in the growth and proliferation of *L. pneumophila* (Murga et al. 2001; Borella et al. 2005). Few studies have investigated the role of the heterotrophic plate count (HPC) when monitoring the prevalence of *Legionella* in hot water distribution systems (Kusnetsov et al. 2003; Edagawa et al. 2008; Moritz et al. 2010; Volker et al. 2010). Bacteria exhibit the ability to survive in an incredibly wide range of temperatures. Consequently, as for nosocomial cases, *Legionella* growth appears to be favored by warm temperatures, noting that maximum *L. pneumophila* growth has been reported between 30°C and 46°C (Mathys et al. 2008). Aquatic bacteria such as *Pseudomonas* species are presumed to provide nutrients for the growth of *Legionella* bacteria (Kramer and Ford 1994).

*L. pneumophila* serogroup 1 (Lp1) causes the majority of cases reported in Europe (Ricketts and Joseph 2007) and in the US (CDC 2007). Legionnaires' disease is a significant cause of community- and hospital-acquired pneumonia, and the case fatality rate (CFR) can be as high as 30%, particularly for hospital-acquired infections or in cases of immunosuppression (Blasi 2004; Dominguez et al. 2009).

After the detection of sporadic, nosocomial, or community outbreaks, several typing methods and appropriate identifications are significant for epidemiologic investigations. The European Working Group for Legionella Infections (EWGLI) developed a standard sequence-based method, for the typing of *L. pneumophila* serogroup 1 (Gaia et al. 2005; Ratzow et al. 2007). Sequence-based typing (SBT) may be useful in identifying the source of infection, demonstrating the link between clinical and environmental isolates (Galli et al. 2008).

It is admitted that hospital-acquired Legionnaires' disease is underdiagnosed in Greek hospitals. The same situation occurs in many other countries (Sabria and Yu 2002). In Greece, *L. pneumophila* was isolated and identified from hotel's water distribution systems associated with 30 cases of Legionellosis, for the first

time in 1989 (Alexiou et al. 1989). In a recent study, *L. pneumophila* serogroup 1 was isolated in Greek potting soils (Velonakis et al. 2009). In Greece, there is no specific legislation for *Legionella*, and prevention of Legionnaires' disease is covered by general Public Health legislation. A remarkable study, which was conducted during an extended inspection program for the Athens 2004 Olympic Games, determined the contribution of standardized scored inspections in the assessment of the presence of *Legionella* bacteria in man-made water systems (Hadjichristodoulou et al. 2006).

A total of 818 cases of Legionnaires' disease with onset of illness in 2009 were reported to the European Surveillance Scheme for Travel-Associated Legionnaires' Disease (Joseph et al. 2010). Clusters and sporadic outbreaks occur each year all over the world, associated with hotel's water systems contaminated by *Legionella*. A few studies have reported colonization by *Legionella* bacteria in hotel water systems in Greece and none in Patras. Patras is called the gateway to the West, as commercial center, a large port and a hub for trade and communication with Italy and other European countries.

Hospital-acquired Legionnaires' disease has rarely been reported in Greece, and environmental cultures for *Legionella* detection in hospital and hotel water distribution systems in South Western Greece have never been systematically performed. Environmental surveillance for *Legionella* detection in hospital and hotel water supplies can provide useful data for risk assessment and the prevention of hospital-acquired Legionnaires' disease.

In the present study, we conducted an environmental survey in eight hospitals at South Western Greece and nine hotels located in Patras. The objectives of the study are as follows: (1) to investigate the frequency of *Legionella* colonization in hospital water systems and hotel water supplies, (2) to assess the prevalence of *Legionella* spp. and serogroups involved, and (3) to evaluate the relationship between *Legionella* contamination in hot and cold water and microbiological and physicochemical water parameters (total count at 22°C and 37°C, temperature, pH), which are suspected to be related to the presence and/or growth of the *Legionella* bacteria.

## 2. Materials and methods

### *2.1. Sample collection*

During this study (July 2008–December 2009), 116 water samples were collected from hot and cold water distribution systems of hospitals and hotels located in South Western Greece (Figure 1). The number of collected samples in each sampling site was determined by considering the size of both hospitals and hotels. Sampling was carried out by suitably trained personnel according to British Standard BS 7592 (British Standard 1992).

Sampling points included distal sites throughout the entire building, multiple floors, and wings in areas of greatest concern, such as multiple units housing patients and clinics at high risk for Legionnaires' disease (gynecological, pathology, cardiological, surgery, respiratory, and pediatric clinic). Hotels with more than 50 rooms were included in this study. An adequate environmental survey should include a sufficient number of samples collected from a variety of locations. So, the sampling points represented the entire distribution network of the water system and likely points of use for patients and residents of hotels.

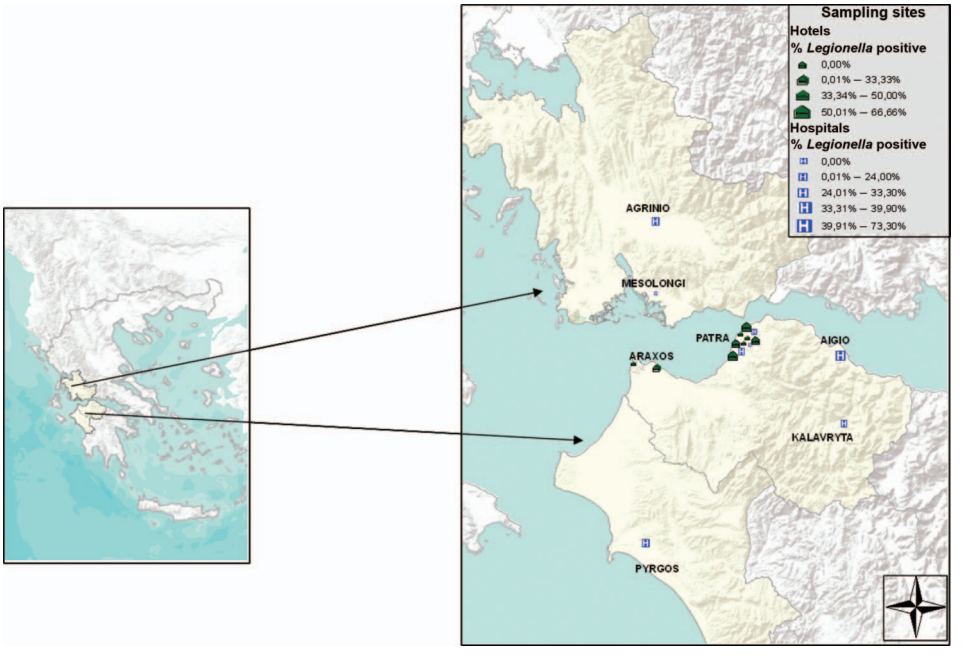


Figure 1. Map of all sites sampled and spatial distribution of positive samples for *Legionella pneumophila* (%).

The mean age of the buildings was 34 years, but the water systems of some of them had been partially renovated over the years.

As it concerns with the number of samples obtained, the Pittsburgh guidelines were followed, which propose sampling based on the size of the hospital (10 sites in a hospital with fewer than 500 beds; in larger facilities, two additional sites for every 100 beds) (Approaches to prevention and control of Legionella infection in Allegheny County health care facilities 1997).

### 2.2. *Study hospitals and hotels*

The size of hospitals ranged from under 100 beds ($N = 4$) to over 300 ($N = 1$). The size of hotels ranged from under 50 rooms ($N = 2$) to over 100 rooms ($N = 2$). All hospitals and hotels were supplied with chlorinated domestic drinking water.

### 2.3. *Hospitals*

From eight hospital units, 91 water samples were collected (Figure 1), each one consisting of a single building with one to five floors. Water samples were collected from tap (43 samples, volume of 1 L), shower head (44 samples, volume of 1 L), and cooling towers (four samples from the inlet, outlet, and recirculation). The local water network supplied water to all the hospitals, which was treated with chlorine dioxide. Hot water samples (44 samples) were collected from the flexible shower heads of different clinics and stored in sterile glass bottles (1 L), after a flow rate of approximately 1–2 min to eliminate any cold water present inside the flexible shower. In order to neutralize residual free chlorine, 10% of sodium thiosulphate

was used as neutralizing agent and added in the used sterile bottles (1 ml $L^{-1}$). Water samples were transported at ambient temperature and protected from sunlight. Samples were returned to the laboratory promptly after collection and examined within 24 h.

### 2.4. Hotels

From nine hotels located in the city of Patras, 25 water samples (1 L) were collected (Figure 1). Of the water samples derived from hotels' rooms, 9 water samples were taken from tap (cold water) and 9 samples from the flexible shower heads (hot water). Water samples (7) were collected from the cooling towers of hotels. The water was treated with chlorine dioxide. Thus, a neutralizing agent (10% sodium thiosulphate) was added in the used sterile bottles (1 L).

Water samples were transported at ambient temperature and protected from sunlight. Samples were returned to the laboratory promptly after collection and examined within 24 h.



### 2.5. Microbiological analysis

Mixed cellulose ester (0.22 $\mu$m pore sized, 47 mm diameter Millipore MA, US) were used to concentrate each water sample of 1 L for the detection of *Legionella* spp. The membranes were then placed aseptically in screw capped containers with 10 mL of sterile distilled water. Samples were vortexed to dislodge bacterial cells from the membranes. Heat treatment (incubation for 30 $\pm$ 1 min in a water bath at 50°C) and acid treatment (incubation for 5 $\pm$ 0.5 min with acid buffer at pH 2.2 $\pm$ 0.2) were used to reduce the number of the non-*Legionella* bacteria in the concentrated samples according to ISO 11731 (1998).

Plates containing buffered charcoal yeast extract-a (BCYE-a) agar (Oxoid, Hampshire, England) and glycine, vancomycin, polymyxin B, cycloheximide (GVPC) BCYE- a agar (Oxoid, Hampshire, England) were inoculated with a 0.1 mL concentrated sample. The plates were incubated at 36°C $\pm$ 1°C in a humidified atmosphere of 2.5% $CO_2$ for at least 10 days. Colonies suspected of being *Legionella* were sub-cultured on BCYE (+) agar (with L-cysteine) and BCYE (−) agar (without L-cysteine). Colonies growing only on BCYE (+) with cysteine were tested for *L. pneumophila* and *Legionella* species using the agglutination test (*Legionella* latex test, Duopath Glisa rapid test, Merck Darmstadt, Germany). Colony counts were enumerated as colony forming units per liter of water (CFU/L).

In parallel, all samples were tested for total count (22°C and 37°C) and *Pseudomonas aeruginosa* presence according to ISO 6222 (1999) and ISO 16266 (2006), respectively.

### 2.6. Physicochemical analyses

Water temperature and residual free chlorine (DPD method, colorimeter Hach Lagne, Pocket Colorimeter II, Düsseldorf, Germany) were determined at the time of sample collection. Water conductivity and pH (pH – meter Consort C830, Basic Multi-Parameter Analyzer, Turnhout, Belgium) were measured at the laboratory.

### *2.7. Nucleic acid extraction and PCR amplification*

Nucleic acids from pure *Legionella* cultures were automatically extracted using the QIAcube and DNeasy Blood and Tissue Kit (Qiagen, UK) according to the manufacturer's instructions.

*Legionella* DNA was amplified using two primer pairs for mip-74F [5′-GCT GCA ACC GAT GCC AC-3′], mip-595R [5′-CAT ATG CAA GAC CTG-3′], and flaA genes, using the primer pair flaA-619F [5′-TTTCTCTGGCGCAAGCTTCC-3′], flaA-846R [5′-GCTGCTTTGGCATAGGCAG-3′]. The reaction mixture consisted of 10 pmoles of each primer, 200 $\mu$M dNTPs, 2.5 mM $MgCl_2$, 5 U/$\mu$l Taq DNA polymerase (Qiagen, US); and 20–100 ng template DNA were determined by semi quantitative method.

The reaction mixture was subjected to enzyme activation for 5 min at 95°C followed by 35 cycles of amplification. Each amplification cycle consisted of denaturation for 30 s at 95°C, primer annealing for 30 s at 55°C, and elongation for 40 s at 72°C. A final step of 10 min at 72°C was performed to ensure complete extension (EWGLI 2009).

### *2.8. Sequence analysis – phylogenetic tree analysis*

Positive PCR products for *mip* and *flaA* genes were purified with the QIAquick PCR purification kit (Qiagen, US) in the QIAcube automated system according to manufacturer's recommendations and were confirmed by sequencing. Nucleotide sequencing reaction of the positive samples was performed in both directions by the Sequencing Unit of the Department of Immunology and Histocompatibility (School of Medicine, University of Thessaly, Greece). The obtained nucleotide sequences were analyzed by BLAST program at the NIH website (NCBI, National Centre for Technology Control, NIH, US) and were compared with each other and with other published sequences. Multiple alignments were performed with the Clustal X program. The neighbor-joining method has been applied for the phylogenetic tree analysis, the reliability of which was assessed by bootstrap resampling (1000 pseudoreplicates), using MEGA 4.0.2 program (Saitu and Nei 1987; Thompson et al. 1997; Kumar et al. 2001).

### *2.9. Statistical analyses*

Statistical calculations were made with Graph Pad Prism 5 for correlation coefficients. A significant correlation among samples was defined by a $P$ value of less than 0.05.

## 3. Results

### *3.1. Prevalence in hospitals*

Water distribution systems of hospitals (33%) were colonized by *L. pneumophila*. Two hospitals were found free of *Legionella* species in their water supplies (Table 1). In hospital A, all the samples of hot water collected from the clinics included in the study were found positive for *L. pneumophila*. Water samples derived from hospital B (73%) were positive for *L. pneumophila*, and the most commonly isolated species were *L. pneumophila* strain Corby. *L. pneumophila* was detected in cold and hot water samples from clinics of hospital F ($>10^3$ CFU $L^{-1}$).

*L. pneumophila* was also detected in hot and cold water samples from the ground floor clinics of hospital C. In hospital D, its concentration was found to be below $10^2$ CFU $L^{-1}$. Total count at 37°C and 22°C and the isolation of *P. aeruginosa* (cooling towers) was frequently found at higher concentration, than in other facilities (clinics), but the colonization was limited to single outlet rather than involving the whole system (Table 2). No correlation between *Legionella* presence in hospital potable water and hospital size was found in this study ($P > 0.05$).

### 3.2. Prevalence in hotels

Five water supplies of hotels were found to be colonized by *L. pneumophila* (Table 1). In hotel B of Patras (three positives out of six water samples), *Legionella* was detected in the cooling tower and in the second floor, in cold and hot water samples.

In hotel D, *L. pneumophila* was detected in the cooling tower at $10^2$ CFU $L^{-1}$. Hot water samples from two hotels were positive for *L. pneumophila* at low levels (243–400 CFU $L^{-1}$). Total count at 22°C and 37°C ranged between 0 CFU $ml^{-1}$ and $10^3$ CFU $ml^{-1}$, with the highest concentration found in cooling towers. Overall, *P. aeruginosa* was only detected in six samples at low concentration and only three



Table 1. Distribution of samples tested positive for *Legionella* species.

| Facilities | | Positive taps | | Positive showers | | Positive cooling towers | | Positive cold water points | | Positive hot water points | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *No. | (%) | *No. | (%) | *No. | (%) | *No. | (%) | *No. | (%) |
| Healthcare | Public hospitals | 8 | 21 | 21 | 48 | 1 | 25 | 9 | 19.6 | 22 | 49 |
| Community | Hotels | 1 | 11 | 5 | 56 | 3 | 43 | 4 | 28.6 | 5 | 45.5 |
| Total | | **9** | **19** | **26** | **49** | **4** | **36** | **13** | **22** | **27** | **48** |

Note: *Number.

Table 2. Distribution of samples tested positive for *Legionella* species (count level $<10^2$, $10^2$–$10^3$, $10^3$–$10^4$ CFU $L^{-1}$) and *P. aeruginosa*.

| | Facilities | | | |
|---|---|---|---|---|
| | *Healthcare* Public hospitals | | *Community* Hotels | |
| **Positive samples** | | | | |
| | *No. | (%) | *No. | (%) |
| *P. aeruginosa* | 31 | 34 | 6 | 24 |
| *Legionella* species and *P. aeruginosa* | 11 | 12 | 3 | 12 |
| *L. pneumophila* | 30 | 33 | 9 | 36 |
| *L. pneumophila* (CFU $L^{-1}$) | | | | |
| $<10^2$ | 6 | 6.5 | 4 | 16 |
| $10^2$–$10^3$ | 7 | 7.7 | 4 | 16 |
| $10^3$–$10^4$ | 17 | 18.7 | 1 | 4 |

Note: *Number.

samples (12%) were positive for both microorganisms (Table 2). A correlation between *Legionella* concentration from water distribution systems of hotels and hotel size was found in this study ($P < 0.05$). Hotels with a large number of rooms had a high colonization of *L. pneumophila*.

Considering the overall results obtained from water distribution systems of hotels and hospitals, the prevalence of *Legionella* species does not correlate with the presence of total count, either at 22°C or 37°C ($P > 0.05$). As shown in Figure 2, the prevalence of *Legionella* in water samples was frequent at temperatures ranging between 20°C and 50°C (Figure 2). Overall, concentrations of *Legionella* species lower than $10^2$ CFU $L^{-1}$ were detected in 8.6% of total water samples, and 15.5% of total samples were found with high concentrations ($10^3$–$10^4$ CFU $L^{-1}$). As shown by chlorine measurements of the collected samples, hospitals and hotels water distribution systems were free of chlorine. No significant correlation between *Legionella* concentration from water distribution systems of hotels and hospitals and pH was found in this study ($P > 0.05$). Our data for hospitals and hotels indicate that there was no correlation between the growth of *P. aeruginosa* and *Legionella* concentration ($P > 0.05$).

### *3.3. Sequence based-typing results*

The phylogenetic tree analysis based on *mip* gene sequences of 17 Greek strains of the present study and 23 reference strains retrieved from GenBank database is shown in Figure 3. For the analysis, *L. adelaidensis* was used as an outgroup. As expected, the Australian strain U91606 is in a distinct clade to all other *Legionella* strains. *Legionella bozemanii, Legionella anisa, Legionella parisiensis, Legionella gormanii, Legionella lonbeachae, Legionella jordanis, and Legionella israelensis* form a distinct group, which is differentiated from the Greek strains and the reference strains of *L. pneumophila*. Greek strains showed a high homology to *L. pneumophila* strains

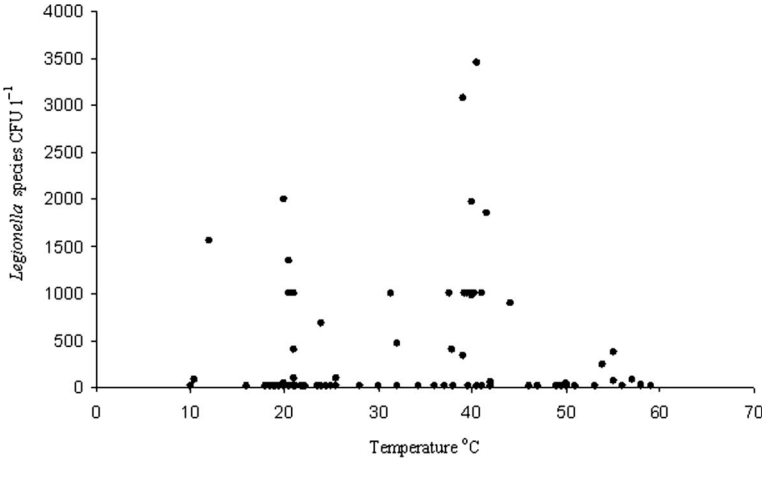


Figure 2. Scatter plot depicting the relationship between *Legionella* species and temperature of water samples. Each dot indicates the number of *Legionella* species (CFU $l^{-1}$) and the temperature (°C) at the same sampling point. Significantly decreased concentrations of *Legionella* bacteria above the temperature of 60°C is noticed.

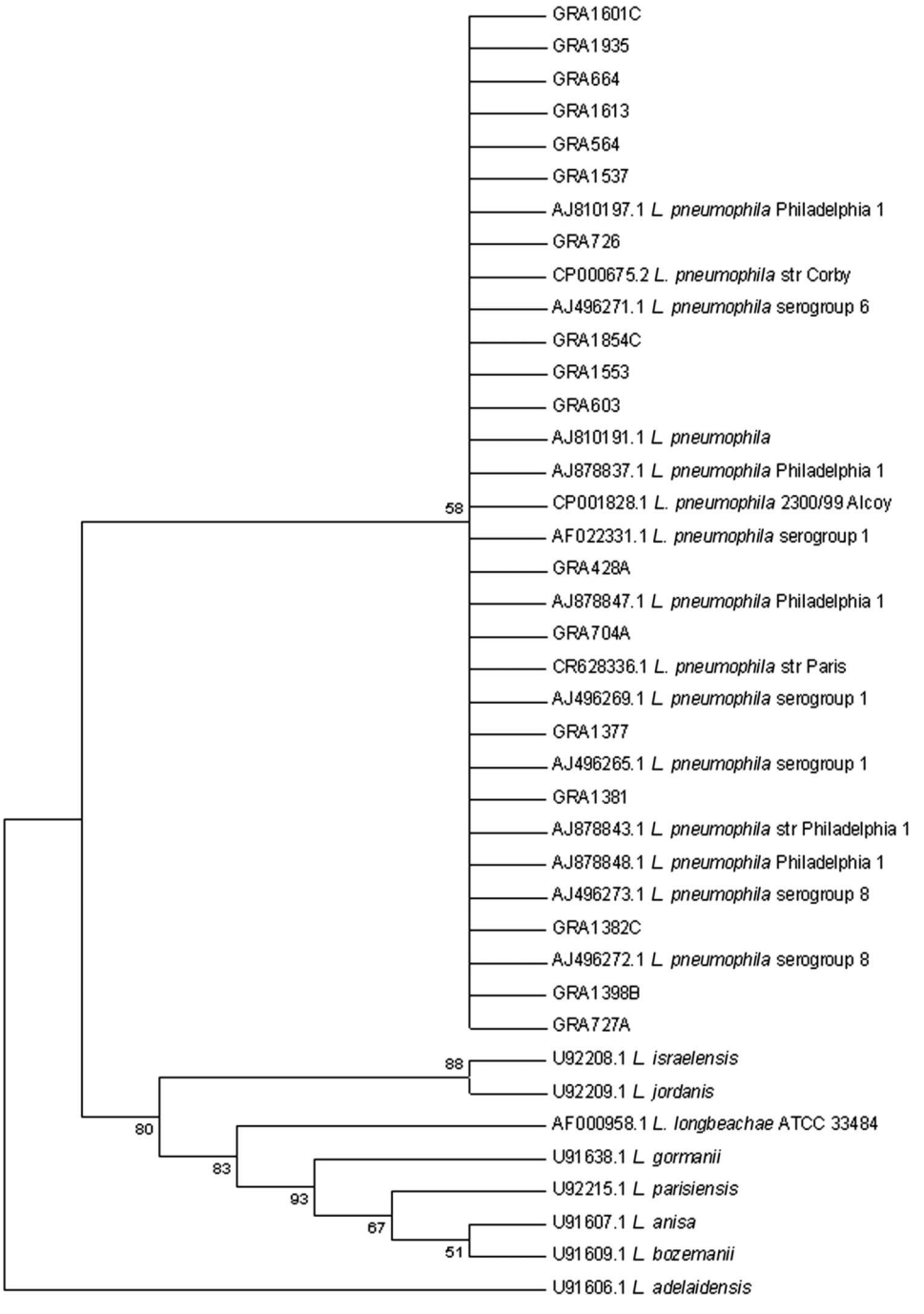


Figure 3. Phylogenetic tree analysis of *mip* gene sequences, from 17 Greek and 23 reference strains, depicting the relationship between the environmental *L. pneumophila* strains of the present study compared to strains retrieved from GenBank database. Numbers under branches are bootstrap percentage values, calculated from 1000 bootstrap replicates. Greek strains are abbreviated as GR followed by the sample registration number. GenBank accession numbers of the reference sequences along with the corresponding strain name are included in the phylogram. *Legionella adelaidensis* was used as an out group. Strains with reference numbers CP001828 and CR628336 were derived from Spain and France, respectively.

isolated during a study of genotypic characterization of *Legionella* species isolated in Italy.

Figure 4 shows the phylogenetic tree analysis of *flaA* gene sequences, from nine Greek and 11 reference strains. Similarly to the analysis based on *mip* gene sequences, a high similarity was found with Italian strains.

## 4. Discussion

The present study indicates a widespread environmental colonization of water systems of large buildings, such as hospitals and hotels in the study area, by *Legionella* species, especially at hot water distribution networks. *L. pneumophila* was found in 33% and 36% of the water samples collected from health care (hospitals) and community buildings (hotels), respectively. Of the total 39 isolates, *L. pneumophila* was the most frequently detected species. The molecular analysis of genomic DNA from environmental isolates confirmed the similarity of the isolates, which were typified as *L. pneumophila*, subgroup Philadelphia. Although surveys of *Legionella* colonization in hospitals have been conducted in many countries with positive rates ranging from 12% to 85% (Liu et al. 1993; Sabria et al. 2004), this is

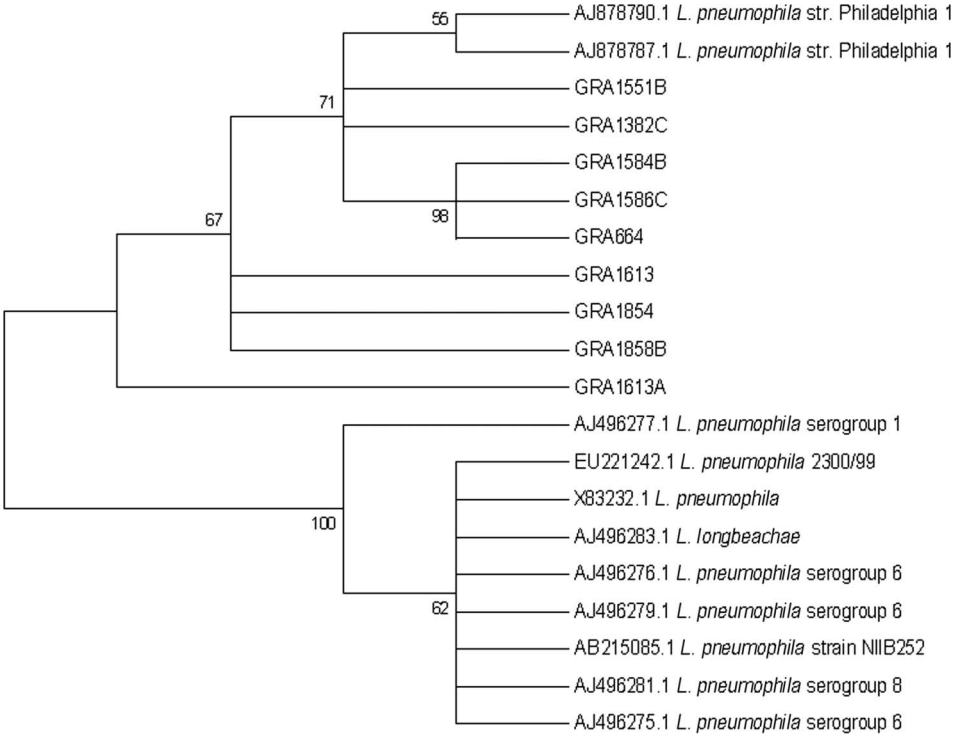


Figure 4. Phylogenetic tree analysis of *fla*A gene sequences, from 9 Greek and 11 reference strains. Numbers under branches are bootstrap percentage values, calculated from 1000 bootstrap replicates. Greek strains are abbreviated as GR followed by the sample registration number. GenBank accession numbers of the reference sequences along with the corresponding strain name are included in the phylogram. (AJ878790 isolate 1, AJ878787 environmental control, AJ496277 isolate Trento 49, AJ496276 isolate Trento 36, AJ496279 isolate Pavia 37, AJ496281 isolate Trento 36, AJ496283 isolate Pordenone 1, AJ496275 Chicago isolate.)

the first sort-scale hospital survey in South Western Greece. In US national surveillance study of 20 hospitals, the water distribution systems of 14 hospitals were colonized with *Legionella* in the same survey, 43% of the hospitals had environmental positive rates for *Legionella* (Stout et al. 2007). Also in a study performed in 12 hospitals (with positive samples $>30\%$, for *Legionella*) the prospective clinical surveillance in 92% of the hospitals found cases of nosocomial Legionellosis (Sabria et al. 2004).

Similar studies in hospitals and hotels referred a percentage of positive samples ranging from 30% to 87% in hospitals (Vickers et al. 1987; Alary and Joly 1992; Sabria et al. 2001; Legnani et al. 2002) and from 27% to 43% in hotels (Leoni et al. 2005). In studies of Italian hotels, *L. pneumophila* was found in 62.2% (Borella et al. 2005) and in 60.9% (Leoni et al. 2005) of the samples examined.

Furthermore, data showed that positive samples may be up to 100% in Italian hospitals (Leoni et al. 2005), 62.5% in Taiwan (Yu et al. 2008), and 61.5% in Greece (Mavridou et al. 2008). *L. pneunophila* serogroup1 was the most frequently detected isolate, with 80% of the positives found in hospitals in Taiwan, and 72.5% of the isolated strains in Greece. *Legionella* have been isolated from hotel water distribution systems throughout the world. Several data suggest that *Legionella* infections in hotels are not randomly distributed, and that certain hotels tend to transmit *Legionella* persistently. Another study concerning hotels in five European countries (Austria, Spain, Germany, Italy, and the UK) found an average colonization rate of 55%, ranging from 33% in the UK to 66% in Spain (Starlinger and Tiefenbrunner 1996).

Although previous data support that *L. pneumophila* serogroup 1 is the most common isolate found in humans, recent studies show that an increasing number of cases is attributed to other non-*pneumophila* species and different serogroups. In particular, a large European study has shown that 33.9% of hospital-acquired infections were caused by *Legionella* non-*pneumophila* 1 (Napoli et al. 2009), and the environmental contamination by *Legionella* non-*pneumophila* 1 should not be under-evaluated.

It has to be noticed that in our study, the numbers of the studied hotels and hospitals and the corresponding sampling points was lower, compared to the aforementioned studies. This results in a lower percentage of *Legionella* positive water distribution systems in our study. Furthermore, the fact that water samples were collected during the first two weeks of July during a hot summer period suggests that exposure to *Legionella* could be more intense due to high atmospheric temperatures. These extreme ambient conditions of high temperatures cause an intensive use of the cooling systems in hospitals and hotels, which may facilitate the contamination and infection by the bacteria. Furthermore, the stagnation of the water in the storage tanks enhances the biofilm formation and this can explain the higher levels of bacteria in centralized systems. In some hotels, and mainly in hospitals, stagnation and distribution is facilitated by the intermittent use of hot water and also by the complexity of the systems, sometimes resulting in the closure of pipelines and the creation of dead-ends.

A critical issue is the importance of cooling towers as sources of Legionnaires' disease and the need for a mandatory registration of all cooling towers. After the isolation of *Legionella* from a cooling tower near a hospital dealing with cases of Legionnaires' disease (Dondero et al. 1980), cooling towers were thought to be the main source of nosocomial Legionellosis. Because Greece has thus far not

experienced large outbreaks linked to cooling towers, registration of such systems has not been mandatory. However, the presence of such a register could facilitate the source identification and would reduce the time required to conduct an environmental investigation.

It is important to underline that simple measurement of CFU does not give the real estimate of the infection risk. The concentration of *Legionella* species in a hospital's water system is not necessarily constant over time (Trerotoli et al. 2003).

Overall, a few water samples contaminated by *Legionella* showed higher pH, and this suggests that the pH affects the solubilization of metals such as copper that is often present in the waterlines, which has been demonstrated to represent an inhibiting factor on the development of *L. pneumophila* in many studies (Kusnetsov et al. 2003).

Furthermore, *P. aeruginosa* was rarely found and detected at low concentrations, maybe affecting single sample points, rather than the whole water supply system. Considering from the obtained results, a significant correlation was not found between *L. pneumophila* and total count at 37°C and 22°C.

Regulations regarding the control of *Legionella* species in all water distribution systems should be established in Greece. Control measures should be intensively applied during summer period, especially in hotels operating seasonally. Furthermore, the prevention should be coupled with the provision of proper information on the importance of routine maintenance of cooling towers and water systems in order to avoid preventable diseases. Our results suggest that a multi-professional approach must be applied for the control and management of *Legionella* species in water systems. Risk assessments and integrated water safety plans, involving surveillance programs and training, should be carried out.



## 5. Conclusions

Our results suggest that a microbiological and environmental surveillance should be more frequent to control the environmental spread of *Legionella* species at South Western Greece. Cases of hospital-acquired Legionnnaires' disease and travel-associated outbreaks may have occurred in South Western Greece, but only few have been detected. Future studies in Greece should directly evaluate levels of water distribution system contamination in relation to Legionnaires' disease cases and outbreaks. We recommend that the Greek public agencies consider mandating the environmental surveillance of *Legionella* in all hospitals and hotels, as is now implemented in many European countries (Anonymous 2005).

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