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Isolation of *Legionella* species/serogroups from water cooling systems compared with potable water systems in Spanish healthcare facilities

J.-M. Rivera [a,b], L. Aguilar [c], J.J. Granizo [d], A. Vos-Arenilla [e], M.-J. Giménez [c], J.-M. Aguiar [f], J. Prieto [c,*]

[a] *Preventive Medicine Department, School of Medicine, Universidad Complutense, Madrid, Spain*
[b] *Bioseguridad Ambiental SL, Madrid, Spain*
[c] *Microbiology Department, School of Medicine, Universidad Complutense, Avda. Complutense s/n, 28040 Madrid, Spain*
[d] *Granadatos SL, Madrid, Spain*
[e] *Nursery Department, Nursery School, Universidad Complutense, Madrid, Spain*
[f] *Control Microbiológico SL, Madrid, Spain*

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Summary Surveillance of *Legionella* spp. in hospital water systems was performed in forty-four inpatient healthcare facilities in Spain during 2005–2006. A total of 2341 samples were collected: 470 from cooling systems (cooling towers) and 1871 from potable water systems. The latter included 211 from cold-water tanks and 260 from hot-water tanks, totalling 471 from central water reservoirs 136 from showers, 1172 from unfiltered taps and 92 from filtered taps, totalling 1400 from peripheral points. Temperature, chlorine levels and the presence of *Legionella* spp. were determined. In all, 373 (15.9%) samples yielded *Legionella* spp. Significantly higher isolation rates were obtained from cooling towers (23.8%) versus cold- and hot-water tanks (approximately 4.7%), due to the significantly higher number of samples positive for serogroup 1 (19.4 vs 0.9–3.5%). In potable water systems, no differences were found between central water tanks and showers, but significant differences in isolation rates between central water tanks and unfiltered taps were observed (4.7 vs 19.6%) due to differences in non-serogroup 1 *L. pneumophila*. Filters significantly

* Corresponding author. Address: Microbiology Department, School of Medicine, Universidad Complutense, Avda. Complutense s/n, 28040 Madrid, Spain. Tel.: +34 91 3941508; fax: +34 91 3941511.
E-mail address: jprieto@med.ucm.es

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decreased isolation rates of these serotypes (11 vs 0%). Some seasonal differences were noted, with higher isolation rates in summer for legionella serogroup 1 in cooling systems and for *L. pneumophila* serogroups 2—14 in potable water systems. In regression models, higher temperatures were associated with colonisation in cooling systems, while lower chlorine levels were associated with colonisation in potable water systems.

Introduction

Legionella is found in aqueous environments at temperatures ranging from 5 to 50°C, but for multiplication it requires temperatures between 25 and 42°C.[1] Under adverse environmental conditions such as changes in temperature or the presence of biocides, legionella may survive within free-living amoeba or extracellularly in a low metabolic state.[2] Intra- or extra-amoebic legionella organisms are biofilm-associated, and when environmental factors change either to shift the balance between protozoa and bacteria, or to disrupt the biofilm, release of planktonic legionella can result in human infection.[3—5] Nosocomial clusters of infection have been described in association with contaminated cooling towers and potable water supplies, but since 1985 virtually all hospital-acquired legionellosis has been linked to potable water.[6,7] Patients may be exposed by inhaling, aspirating or ingesting contaminated water.[6]

The percentage of distal sites positive for *Legionella pneumophila* is predictive of the risk of hospital-acquired legionellosis, but nosocomial infection may be underestimated, because not all nosocomial pneumonias are aetiologically assigned, and because the most widely used test (the legionella urinary antigen test) is specific for serogroup 1.[8,9] Among immunocompromised patients at least 20% of infections are caused by strains other than *L. pneumophila* serogroup 1.[10] These facts are important since the mortality of healthcare-associated legionellosis is roughly double that of community-acquired cases, reaching 40%.[11] Hospitals whose water supplies are not colonised by legionella do not have cases of nosocomial legionellosis.[8,12]

This study analyses differences in isolation of *Legionella* spp. and serogroups in samples from water-cooling systems compared with potable water systems in healthcare facilities in Spain.

Methods

Forty-four inpatient healthcare facilities from all over Spain were studied during 2005 and 2006. The facilities were those where Bioseguridad Ambiental SL was responsible for sampling and analysing water systems for legionella at least once a year, in compliance with Spanish regulations (R.D. 865/2003). A total of 2341 samples were collected: 470 from cooling systems (cooling towers) and 1871 from potable water systems (211 from cold-water tanks, 260 from hot-water tanks as central water reservoirs 136 from showers, 1172 from unfiltered taps, and 92 from filtered taps as peripheral points of water supply).

Sampling

One litre was collected from each central water reservoir (cooling towers for the cooling systems and cold-water tanks or hot-water tanks for potable water systems), scraping walls and collecting sediments if these were present. A volume of 100 ml was collected immediately after opening the valve from each of the 1400 peripheral sampling points (showers or taps); a sterile swab was then inserted into the faucet and introduced into a sterile vessel that afterwards was made up to 1 L using water.

Measuring temperature and chlorine levels

Immediately after collection, the water temperature was measured using a thermometer 'testo 106' (range: from −50 to +275°C) (Testo AG, Germany), and chlorine levels were measured with ISM Hanna HI 93734 (Hanna Instruments, USA).

Microbiological processing of water samples

Water samples were concentrated 100-fold immediately on arrival at the laboratory. Three 1 mL aliquots were used: one untreated, one heat-treated (50°C for 30 min), and one acid-treated (in 9 mL of HCl—KCl acid buffer at pH 2.2 for 5 min). Of each aliquot, 0.1 mL was plated onto GVPC (glycine, vancomycin, polymyxin B, cyclohexamide) selective agar medium (Oxoid Ltd, Basingstoke, Hampshire, UK). Plates were incubated at 36°C for 10

days and examined for growth every 48 h. Colonies morphologically consistent with *Legionella* spp. were plated onto buffered charcoal yeast extract (BCYE) agar (Oxoid) and blood agar (Oxoid), and incubated for 48 h. Colonies growing on BCYE agar but not on blood agar were definitively identified as *Legionella* spp. using a commercially available latex agglutination test (Oxoid, DR0800) that distinguishes *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2–14, and other *Legionella* spp. (including *L. longbeacheae*, *L. bozemannii*, *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei* and *L. anisa*).

Statistical analysis

Data were described by mean and standard deviation for quantitative variables and by percentages for qualitative variables. Comparison of percentages by Chi-squared test adjusted for multiple comparisons, odds ratio (OR) and 95% confidence interval (CI) were assessed using SPSS V14 (SPSS Inc., Chicago, IL, USA). Due to multiple comparisons, $P \leq 0.012$ was considered statistically significant.

Two multivariate regression models were used, one for cooling systems and one for potable water systems, in order to identify factors associated with legionella. The models were constructed using legionella colony counts ($\log_{10}$ cfu) as dependent variables, and temperature, monthly isolation rates and chlorine levels as independent variables. For potable water systems the sampling site was also included as an independent variable. *Legionella* spp. or serogroups were included as fixed effects in both models. Factorial models were designed, but in order to explore interaction between variables, exploratory models were also performed.

To investigate whether isolation rates varied with the time of year, the percentage of isolates per season (January–March, April–June, July–September and October–December) were determined. Positivity rates were then compared using the Chi-squared test with Yates' continuity correction or the Fisher's exact test when necessary. Due to multiple comparisons, $P \leq 0.012$ was considered statistically significant.

Results

Environmental conditions

Table I shows temperatures and chlorine levels at the different sampling sites. Higher levels of chlorine were measured in cooling systems and in central potable water reservoirs (water tanks) than at peripheral points (showers and taps). Temperature was higher in hot potable water systems (hot-water tanks, showers and taps) than in cold-water tanks or cooling systems.

Isolates per sample and types of isolates

Table II presents the positive samples and Table III the isolates, both broken down by sampling site. In all, 373 of 2341 (15.9%) samples yielded 384 isolates of *Legionella* spp.; 317 (82.5%) of these were *L. pneumophila* (169 were serogroup 1, and 148 were other serogroups) and 67 (17.5%) were other *Legionella* spp.

Cooling vs potable water systems

Significantly higher rates of positive cultures for legionella were obtained in cooling towers (23.8%) than in potable cold-water tanks (4.7%) or hot-water tanks (4.6%) ($P \leq 0.001$). The difference consisted mainly of *L. pneumophila* (21.9 vs 1.9–4.2%; $P \leq 0.001$), particularly serogroup 1 strains (19.4 vs 0.9–3.5%; $P \leq 0.001$) (Table II). Bacterial densities ($\log_{10}$ cfu/mL) in positive samples were significantly ($P \leq 0.001$) higher in cooling systems than in potable water systems (unfiltered taps), with mean legionella counts of 3.60 vs 3.17 $\log_{10}$.

Potable water systems

No significant differences were found in the frequency of isolation of *Legionella* spp. between cold-water tanks and hot-water tanks, nor between hot-water tanks and showers (Table II). However, unfiltered taps were significantly ($P \leq 0.001$) more likely than hot-water tanks to yield *Legionella* spp. (19.6 vs 4.6%). The differences consisted mainly of *L. pneumophila* (16.5 vs 4.2%; $P \leq 0.001$), particularly serogroups 2–14 (11 vs 0.8%; $P \leq 0.001$). Filtered taps were

Table I Temperature and chlorine levels (mean ± SD) of water collected at the different sampling sites

Sampling site	Temperature (°C)	Chlorine (ppm)
Cooling towers	22.6 ± 6.2	0.78 ± 1.22
Cold-water tanks	16.2 ± 5.4	0.69 ± 0.49
Hot-water tanks	56.4 ± 10.1	0.75 ± 0.45
Showers	46.0 ± 13.0	0.53 ± 0.40
Unfiltered taps	40.8 ± 14.3	0.54 ± 0.39
Filtered taps	45.6 ± 6.0	0.71 ± 0.46

Table II Number (percentage) of samples yielding *Legionella* spp., *L. pneumophila* or other *Legionella* spp. per sampling site

Sampling site	No. of samples	No. of (%) positive samples to				
		Legionella spp.	*L. pneumophila*			Legionella (other)
			All	Serogroup 1	Other serogroup	
Cooling towers	470	112 (23.8)[a]	103 (21.9)	91 (19.4)	12 (2.6)	18 (3.8)
Cold-water tanks	211	10 (4.7)	4 (1.9)	2 (0.9)	2 (0.9)	6 (2.8)
Hot-water tanks	260	12 (4.6)	11 (4.2)	9 (3.5)	2 (0.8)	1 (0.4)
Showers	136	7 (5.1)[b]	4 (2.9)	1 (0.7)	3 (2.2)	4 (2.9)
Unfiltered taps	1172	230 (19.6)[b]	193 (16.5)	64 (5.5)	129 (11.0)	38 (3.2)
Filtered taps	92	2 (2.2)	2 (2.2)	2 (2.2)	0 (0.0)	0 (0.0)
Total	2341	373 (15.9)[c]	317 (13.5)	169 (7.2)	148 (6.3)	67 (2.9)

[a] Nine samples yielding two species of Legionella.
[b] One sample yielding two species of Legionella.
[c] Eleven samples yielding two species of Legionella.

significantly ($P \leq 0.001$) less likely than unfiltered taps to yield *Legionella* spp. (2.2 vs 19.6%; OR: 0.09, 95% CI: 0.01—0.34), mainly due to the decrease in *L. pneumophila* (2.2 vs 16.5%; OR: 0.11, 95% CI: 0.01—0.43; $P \leq 0.001$), particularly *L. pneumophila* serogroups 2—14 (0 vs 11%; $P \leq 0.001$).

Seasonality

Figures 1 and 2 show the variation in isolation rates over the year in sampling sites in cooling towers and unfiltered taps. In cooling towers (Figure 1), significantly ($P \leq 0.001$) higher rates of isolation of *L. pneumophila* serogroup 1 were observed in the summer months, but there was no apparent seasonality for *L. pneumophila* serogroups 2—14 ($P > 0.4$) or for other *Legionella* spp. ($P > 0.1$). Conversely, in potable water systems (Figure 2), significantly higher isolation rates were found for *L. pneumophila* serogroups 2—14 in the summer versus each of the other seasons ($P \leq 0.001$), and for serogroup 1 versus autumn ($P \leq 0.001$), but not for other *Legionella* spp. ($P > 0.05$).

Multivariate analysis

Table IV shows regression data for the two models. In the cooling tower model, higher temperature was significantly ($P = 0.010$) associated with the isolation of legionella, but lower chlorine levels were not ($P = 0.302$). Seasonality did not have a significant effect in this model ($P = 0.444$). Interaction between variables did not improve the significance or explanation of the model (same r^2). In the potable water model, low chlorine levels and sampling from taps were significantly associated ($P = 0.004$) with detection of legionella. No significant association was observed with season ($P = 0.230$) or with temperature ($P = 0.280$). Interaction between variables did not improve the significance or explanation of the model (same r^2).

Discussion

Since the discovery in 1985 that hospital-acquired legionnaires' disease usually arises through the potable water supply, nearly all hospital outbreaks

Table III Number (percentage) of *Legionella* spp. and serogroups isolated in positive samples per sampling site

Sampling site	*Legionella* spp.			*L. pneumophila*		
	No. of isolates	*N* (%) species		No. of isolates	*N* (%) serogroup	
		L. pneumophila	Other spp.		1	Other
Cooling towers	121	103 (85.1)	18 (14.9)	103	91 (88.3)	12 (11.7)
Cold-water tanks	10	4 (40.0)	6 (60.0)	4	2 (50.0)	2 (50.0)
Hot-water tanks	12	11 (91.7)	1 (8.3)	11	9 (81.8)	2 (18.2)
Showers	8	4 (50.0)	4 (50.0)	4	1 (25.0)	3 (75.0)
Unfiltered taps	231	193 (83.5)	38 (16.5)	193	64 (33.2)	129 (66.8)
Filtered taps	2	2 (100)	0 (0.0)	2	2 (100)	0 (0.0)
Total	384	317 (82.5)	67 (17.5)	317	169 (53.3)	148 (46.7)

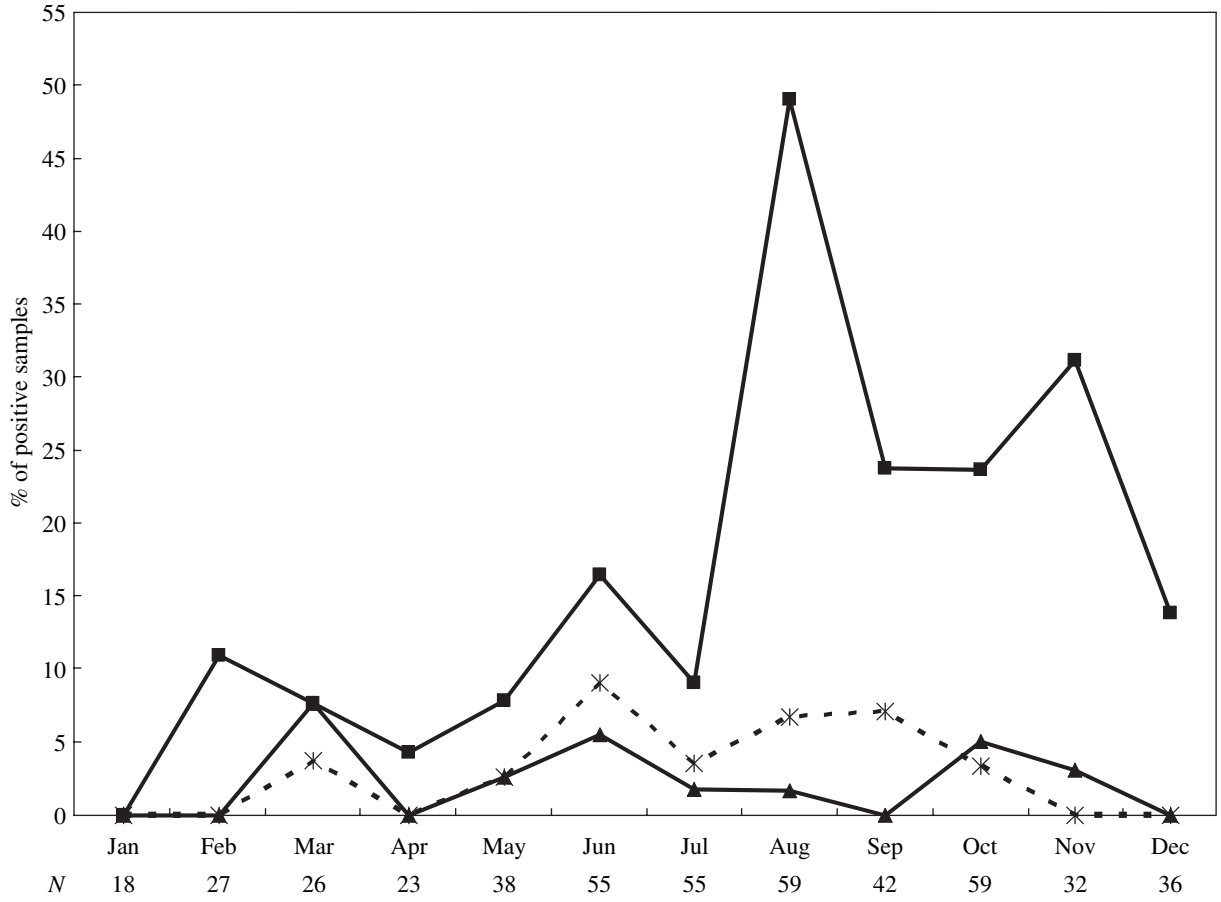

Figure 1 Percentage of positive samples in cooling systems, by month of the year. Black squares: *L. pneumophila* serogroup 1; black triangles: *L. pneumophila* serogroups 2–14; crosses/dashed line: other *Legionella* spp.). *N* = no. samples per month.

have been linked to potable water, and nosocomial cases linked to cooling water have all but disappeared.[7,13]

Hospital water supplies can be tested for primary prevention purposes in institutions without documented cases, or for secondary prevention during or following an outbreak.[10] Primary prevention remains controversial because some studies demonstrate a clear link between presence of legionella in water systems and nosocomial legionellosis, but others do not, the difference being explained in part by differences in populations studied, investigation of clinical cases, analysis of hospital water supplies and design of hospital water systems.[10] Other authors state that the percentage of distal sites that are positive for legionella correlates with the incidence of the disease, and conversely that, if there is not detectable legionella in the water supply, cases will not occur.[8] Previous studies have shown that the risk of nosocomial legionella is related more closely to the proportion of positive sampling sites than to the actual bacterial concentration present.[14,15] Since only a small proportion of exposed patients develop legionnaires' disease, the number of legionella-disseminating points (and subsequently the number of potentially exposed individuals) is a more important determinant of transmission than the infectious dose.[14] In this sense, highly susceptible patient populations (e.g. transplant patients) are at risk at lower levels of contamination, so policies for control of nosocomial disease should take into account the number of susceptible patients, the size of the hospital, and the disinfectant concentrations measured in patient rooms.[14]

The ecology of legionella seemed to differ in our study between cooling systems and potable water. In both cooling systems and potable water systems *L. pneumophila* was the main species isolated (85.1% and 83.5%, respectively; Table III). In cooling towers, serogroup 1 was the most frequent serogroup (88.3% of the 103 *L. pneumophila* isolates), and the isolation rate of this serogroup significantly increased during the summer. In potable water supplies, central reservoirs (cold- and hot-water tanks) and showers showed low rates of isolation of legionella, which supports the

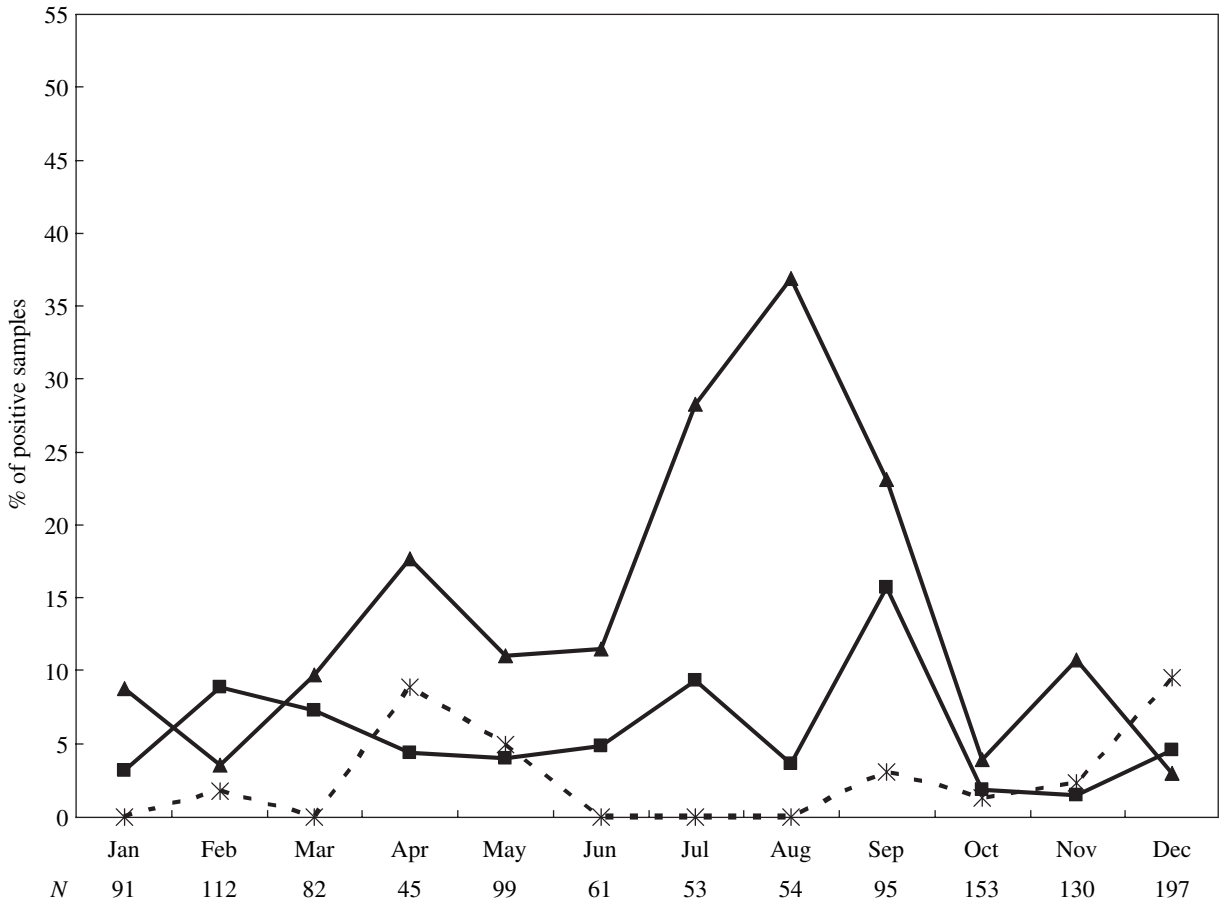

Figure 2 Percentage of positive samples in tap water, by month of the year. Black squares: *L. pneumophila* serogroup 1; black triangles: *L. pneumophila* serogroups 2–14; crosses/dashed line: other *Legionella* spp.). *N* = no. samples per month.

Table IV Multivariate analysis of the two models

Model	r^2	Variable	β coefficient	*P*-value
Cooling towers	0.960	Temperature	0.008	0.010
		Chlorine	0.001	0.302
		Monthly isolation rate	−0.004	0.444
		Species: *L. pneumophila*	Reference	–
		Species: Other spp.	0.233	0.281
		Serogroup: 1	−0.349	0.128
		Serogroup: 2–14	Reference	–
		Constant	3.248	0.001
Potable water	0.947	Temperature	0.001	0.280
		Chlorine	−0.052	0.004
		Monthly isolation rate	0.002	0.230
		Species: *L. pneumophila*	Reference	–
		Species: Other	−3.276	0.059
		Serogroup: 1	−0.679	0.071
		Serogroup: 2–14	Reference	–
		Sample: water tanks	Reference	–
		Sample: taps	0.537	0.004
		Sample: showers	−0.193	0.233
		Constant	2.275	0.001

previous assertion that showering carries a low risk of transmission.[7–16] However, in unfiltered taps we found high rates of *L. pneumophila*. This increase in isolation rates in taps may be due to the characteristics of the systems with segments prone to stagnation where legionella multiplies at high rates.[6] Two-thirds (66.8%) of isolates from unfiltered taps belonged to serogroups other than serogroup 1 (Table III). This is important with respect to nosocomial legionellosis because the most widely used rapid diagnostic test (the legionella urinary antigen test) is specific for serogroup 1. These non-01 serogroups showed seasonality, with significantly increased isolation rates in summer. As in other studies, the use of filters resulted in a highly significant decrease in isolation rates.[17,18] Considering that among immunocompromised patients the proportion of infection by *L. pneumophila* other of serogroup 1 can reach rates higher than 20%, the use of filters may be advocated in units housing haematopoietic stem-cell transplant or solid-organ transplant recipients.[10]

As in other studies, higher bacterial densities were found in cooling towers.[9] In multivariate analysis, higher temperature was predictive for legionella isolation, which is in accordance (considering Spanish weather) with the seasonality observed. In another Mediterranean country, summer seasonality was reported for legionella contamination of hotel water distribution systems.[19] In our study, potable water systems presented lower bacterial densities, and the highest isolation rate was found in unfiltered taps that presented mean chlorine levels (0.54 ppm) lower than those in central reservoirs (water tanks) (mean: 0.69–0.75 ppm). As in other studies, in our multivariate analysis low chlorine levels were predictive of legionella isolation in potable water systems.[15,20]

Although attempts to eradicate legionella in water supplies are often unsuccessful, and the Centers for Disease Control and Prevention argue that negative cultures may give a false sense of security, knowledge that water is colonised with legionella increases physicians' awareness and may improve the diagnosis of nosocomial infection.[12,17]

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