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Legionella waterline colonization: detection of *Legionella* species in domestic, hotel and hospital hot water systems

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ABSTRACT

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Aims: An evaluation was made of the prevalence of *Legionella* species in hot water distribution systems in the city of Bologna (Italy) and their possible association with bacterial contamination (total counts and Pseudomonadaceae) and the chemical characteristics of the water (pH, Ca, Mg, Fe, Mn, Cu, Zn and Total Organic Carbon, TOC).

Methods and Results: A total of 137 hot water samples were analysed: 59 from the same number of private apartments, 46 from 11 hotels and 32 from five hospitals, all using the same water supply. *Legionella* species were detected in 40·0% of the distribution systems, *L. pneumophila* in 33·3%. The highest colonization was found in the hot water systems of hospitals (93·7% of samples positive for *L. pneumophila*, geometric mean: $2{\cdot}4 \times 10^3$ CFU l^{-1}), followed by the hotels (60·9%, geometric mean: 127·3 CFU l^{-1}) and the apartments with centralized heating (41·9%, geometric mean: 30·5 CFU l^{-1}). The apartments with independent heating systems showed a lower level of colonization (3·6% for *Legionella* species), with no evidence of *L. pneumophila*. Correlation analysis suggests that copper exerts an inhibiting action, while the TOC tends to favour the development of *L. pneumophila*. No statistically significant association was seen with Pseudomonadaceae, which were found at lower water temperatures than legionellae and in individual distribution points rather than in the whole network.

Conclusions: The water recirculation system used by centralized boilers enhances the spreading of legionellae throughout the whole network, both in terms of the number of colonized sites and in terms of CFU count.

Significance and Impact of the Study: Differences in *Legionella* colonization between types of buildings are not due to a variation in water supply but to other factors. Besides the importance of water recirculation, the study demonstrates the inhibiting action of copper and the favourable action of TOC on the development of *L. pneumophila*.

Keywords: hospital, hot water distribution system, hotel, *Legionella* species, private residence.

INTRODUCTION

On account of its tolerance to heat and low nutritional needs, *Legionella pneumophila* is widespread in many water systems and therefore poses a serious public health risk. The micro-organism passes from its natural reservoirs into the water distribution network, where the main source of contamination can be found. Pneumonia from *Legionella* is caught by inhaling particles of aerosol emitted by showers, taps, cooling towers, as well as by hydromassage tubs, spa waters, ornamental fountains etc. In hospitals micro-aspiration may be an important route of transmission for patients lacking normal respiratory reflexes (Yu 2000). In many outbreaks hot water systems are the most frequently involved source of infection (Meenhorst *et al.* 1985; Berthelot *et al.* 1998; Fields *et al.* 2002; Perola *et al.* 2002). Temperature is in fact one of the main factors associated with the presence of legionellae, which are able to reproduce between 20 and 45°C, and can remain viable at much higher temperatures, even up to 60°C.

Although *L. pneumophila* infections have till now been generally underestimated, largely because of lack of clinical

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awareness, outbreaks are often reported in community establishments and hospitals. Community-acquired cases of *Legionella* infection have, for example, been linked to exposure to water systems of hotels (Joseph *et al.* 1996; Benin *et al.* 2002; Fields *et al.* 2002) where a risk is created by the intermittent use of the water and the consequent stagnation, leading to the formation of biofilms in the piping. Furthermore, the presence of *L. pneumophila* in hospital water supplies is a well-known risk factor for nosocomial pneumonia (Colville *et al.* 1993; Berthelot *et al.* 1998; Perola *et al.* 2002); there is also evidence of a widespread diffusion of the bacteria in domestic hot water distribution systems (Stout *et al.* 1992; Marrie *et al.* 1994; Zacheus and Martikainen 1994).

The aims of the present study were to evaluate the frequency of colonization by *Legionella* species in different domestic, hospital and hotel hot waters and to examine the possible association with bacterial contamination and chemical parameters.

MATERIALS AND METHODS

Sample collection

An analysis was carried out of 137 hot water samples taken from various different hot water outlets in the city of Bologna (Italy): 59 samples from the same number of private apartments, 46 from 11 hotels, and 32 from five public hospitals. All buildings were supplied by the same water network which distributes water originating from both surface and deep sources, and which is treated with chlorine dioxide. At the time of sampling a questionnaire was used to obtain details of the water heating system: the type of distribution (centralized or independent, gas or electrically heated), the year of installation, the volume of the reservoir tank (if present), the distance of the boiler from the sampling point, the possible presence of a water-softening system. Hot water samples were drawn from the bathroom outlets (showers or taps) and placed in sterile glass bottles, after a flow time of 3–5 min to eliminate any cold water present inside the tap or flexible shower pipe. In order to neutralize the residual free chlorine, 10% sodium thiosulphate was added in sterile bottles for bacteriological analysis (1 ml l^{-1}), whereas acid-preserved glass bottles were used for chemical analyses.

Bacteriological analysis

Microbiological samples were analysed within 12 h of collection using the standard plate method to determine the total heterotrophic counts per millilitre at 36 and 22°C (plate count agar; Oxoid, Basingstoke, UK) and the standard MF technique to determine Pseudomonadaceae (CFC agar; Oxoid) (standard methods: APHA, AWWA, WEF 1998). The colonies grown on CFC agar were then subcultured and identified using the API 20NE biochemical system (bioMérieux, Marcy l'Etoile, France).

Isolation of *Legionella* species was performed by concentrating 2 l of water using 0·2 μm filter membranes (0·2 polyamide filter, Millipore, Bedford, MA, USA). Each membrane was resuspended in 10 ml of the original sample water, shaken for 15 min, spatulated and vortex mixed again for 3 min. A 5-ml portion of this suspension was placed in a 50°C water bath for 30 min for the decontamination of other micro-organisms. Aliquots of 0·1 ml of the original and concentrated samples (with and without heat pretreatment and 1 : 10 diluted or not) were spread on duplicate plates of CYE agar (charcoal yeast extract base agar, Oxoid) with BCYE growth supplement (ACES buffer/potassium hydroxyde, L-cysteine, ferric pyrophosphate, alpha-ketoglutarate) and MWY selective supplement (glycine, vancomycin, polymyxin B, anisomycin) and incubated at 35°C, in 2·5% CO_2, for 7–14 days (Leoni and Legnani 2001). All colonies on MWY with the typical ground glass appearance of *Legionella* species were subcultured onto BCYE medium and CYE medium, and incubated at 35°C for 2 days or more. The colonies grown on BCYE, but not grown on CYE, were re-isolated and identified on the basis of cultural (lack of growth on blood agar, fluorescence), biochemical (oxidase, catalase, hydrolysis of hippurate, β-lactamase) and serological features, using commercial antisera for *Legionella pneumophila* SG (serogroup) 1 to 14 (Polyclonal latex reagents, Biolife, Milan, Italy) and *Legionella* species (*Legionella* species latex test, Oxoid; agglutinating sera *Legionella bozemani* SG 1, *Legionella dumoffii*, *Legionella gormani* SG 1, *Legionella micdadei*, Biogenetics Diagnostics, Padova, Italy). The isolates with typical cultural and biochemical characteristics, but negative to serological analysis were tested by nested PCR for the identification of the 16S gene, specific of *Legionella* species.

Physical and chemical analyses

Water temperature, pH (direct reading pHmeter – Orion 701 A, Cambridge, MA, USA) and residual free chlorine (DPD method, colorimeter La Motte, Model DC 1100, Chestertown, MD, USA) were determined at the time of collection. Total concentrations of Ca, Mg, Fe, Mn, Cu and Zn were measured by Inductively Coupled Plasma emission spectrometry (ICP, Perkin-Elmer, Model Optima 3000 XL, Boston, MA, USA). The detection limits were 0·01 mg l^{-1} for Ca, 0·02 mg l^{-1} for Mg, 1 mg l^{-1} for Cu, 10 μg l^{-1} for Fe, 1 μg l^{-1} for Mn, 10 mg l^{-1} for Zn. Total Organic Carbon (TOC) was measured using the technique of infrared spectrometry with oxidation at high temperature (Schimadzu, Model TOC-Vcpn, Duisburg, Germany).

Statistical analysis

For the statistical analysis the bacteriological data were converted into $\log_{10}(x + 1)$ to normalize the non-normal distributions. The results were analysed by linear regression analysis, unpaired *t*-test, ANOVA to assess risk variables associated with microbiological contamination. Stat View II program for Macintosh computer was used (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Private apartments

Legionella species were detected in 30·5% of the domestic hot water systems, *L. pneumophila* in 22·0% and *L.* non-*pneumophila* in 8·5%. Table 1 shows the results of the microbiological parameters, divided according to the type of water heating system (centralized or independent). A higher level of *Legionella* colonization was found in the water of centralized systems (a total of 31), with statistically significant differences compared with the independent systems (unpaired t test, $P < 0{\cdot}001$). About 54·8% of centrally heated water samples contained *Legionella* species (range: 25–97 500 CFU l^{-1}), of which 41·9% were *L. pneumophila* and 12·9% other species. No system showed mixed contamination. Of the 13 positive for *L. pneumophila*, four were contaminated by SG 3 and SG 9, three by SG 6, two by SG 8; of the four systems positive for other *Legionella* species, one was contaminated by *L. micdadei*, one by *L. bozemanii* and two others by legionellae unidentifiable with the sera used in the investigation. Furthermore, the colonization of the centrally heated water does not appear to have been affected by the size of the system (number of apartments served), by the distance from the central boiler or by the presence or not of a softening device. Only 1 of the 28 independently heated systems (using a gas heater and a storage tank) was found to be contaminated by legionellae, of the *micdadei* species. As far as the other microbiological parameters are concerned (total bacteria counts at 22 and 36°C, total Pseudomonadaceae and *Pseudomonas aeruginosa*), no statistically significant differences were observed between the centralized and independent systems (unpaired *t*-test). Pseudomonadaceae were isolated from 57·1% of the independent systems (range: 14–8 200 CFU 100 ml^{-1}) and from 45·2% of the centralized systems (range: 4–5500 CFU 100 ml^{-1}); *P. aeruginosa* was only detected in two independent systems (7·1%) and in three centralized systems (9·7%). The other most frequently found species were *Stenotrophomonas maltophilia* (16·9% of samples), *Comamonas acidovorans* (10·2%) and *P. stutzeri* (6·8%).

Hotels

Forty-six water samples were collected from 11 hotels, ranging from two to six samples for each establishment depending on the size (from 24 to 174 rooms), the number of floors (from one to six) and the different distances from the central boiler (at least one sample per floor). Only in four hotels were all samples negative for *Legionella* species. The other seven (63·6%) showed wide colonization by *L. pneumophila* SG 1 (three hotels), SG 3 (three hotels) and mixed SG 3 and 6 (one hotel). Unlike the apartments, where only one species was isolated from each system, in three of the seven hotels positive for *L. pneumophila* there was also evidence of *L. micdadei* and *L. bozemanii*. Table 1 shows the microbiological characteristics of the hot water in the hotels. In the same hotel it was possible to find *L. pneumophila* in all the samples taken at different distances from the main boiler, at concentrations varying at the most by 1 log in the different sampling points, with no evidence of any relationship between the levels of contamination and the distance from the boiler, the size of the hotel (number of rooms) and the presence of a softening device. Pseudomonadaceae were found in 90·9% of the hotels, but nearly always at low levels and limited to a single sampling point rather than affecting the whole system. A notable variety in levels and number of species was observed within a given hotel. The most commonly isolated species were *C. acidovorans* (15·2% of samples), *S. maltophilia* (15·2%), *Burkholderia cepacia* (6·5%) and *P. stutzeri* (6·5%).

Hospitals

A total of 32 hot water samples were collected from five hospital units, each consisting of a single building with three to six floors. Sampling was carried out on each floor of every building, at different distances from the central boiler. All five were found to be contaminated by *L. pneumophila* SG 3 with 93·7% of positive samples, two of which also by *L. anisa* and one by *L. bozemanii*, although at low levels and not consistently in samples taken from the same building (Table 1). No statistically significant differences were seen between the five hospital units and no evidence was found of the distance from the boiler affecting the levels of legionellae. The isolation of *P. aeruginosa* was more frequent than in other establishments (40% of the systems and 12·5% of samples), but the colonization was again limited to single outlets rather than involving the whole system. The other Pseudomonadaceae belonged to the species *P. stutzeri* (15·6% of samples), *Flavimonas oryzihabitans* (12·5%) and *C. acidovorans* (9·4%).

Considering the overall results obtained from the three different types of establishments, both the total legionellae and *L. pneumophila* appear to be inversely correlated to the concentrations of copper, calcium and magnesium, and

Table 1 Microbiological characteristics of hot water distribution systems in relation with the different settings (apartments, hotels and hospitals)

	Apartments with centralized system		Apartments with independent system		Hotels			Hospitals		
	Positive samples no. (%)	Geometric mean (range)*	Positive samples no. (%)	Geometric mean (range)*	Positive distribution systems no. (%)	Positive samples no. (%)	Geometric mean (range)*	Positive distribution systems no. (%)	Positive samples no. (%)	Geometric mean (range)*
L. pneumophila (CFU l^{-1})	13 (41·9)	30·5 (25–9·7 × 10^4)	0	–	7 (63·6)	28 (60·9)	127·26 (25–5·5 × 10^4)	5 (100)	30 (93·7)	2·4 × 10^3 (200–1·0 × 10^5)
≤10^3	3 (9·7)					4 (8·7)			8 (25·0)	
10^3–10^4	4 (12·9)					18 (39·1)			14 (43·8)	
≥10^4	6 (19·4)					6 (13·0)			8 (25·0)	
L. non-*pneumophila* (CFU l^{-1})	4 (12·9)	2·6 (400–3·3 × 10^3)	1 (3·6)	1·5	3 (27·7)	10 (21·7)	6·44 (10^3–4·7 × 10^4)	3 (60·0)	10 (31·2)	5·1 (25–2·7 × 10^3)
≤10^3	1 (3·2)					1 (2·2)			7 (21·9)	
10^3–10^4	3 (9·7)					7 (15·2)			3 (9·4)	
≥10^4	0					2 (4·3)			0	
Pseudomonadaceae (CFU 100 ml^{-1})	14 (45·2)	7·9 (4–5·5 × 10^3)	16 (57·1)	28·9 (14–8·2 × 10^3)	10 (90·9)	27 (58·7)	11·03 (1–2·1 × 10^3)	3 (60·0)	6 (18·7)	1·7 (1–3·5 × 10^3)
P. aeruginosa (CFU 100 ml^{-1})	3 (9·7)	1·5 (26–180)	2 (7·1)	1·4 (40–610)	3 (27·7)	5 (10·9)	1·59 (21–200)	2 (40·0)	4 (12·5)	1·3 (1–104)
Total count at 36°C (CFU ml^{-1})	31 (100)	60·4 (4–1·0 × 10^4)	28 (100)	60·0 (1–5·1 × 10^3)	11 (100)	46 (100)	127·13 (2–6·7 × 10^3)	5 (100)	32 (100)	23·6 (4–160)
Total count at 22°C (CFU ml^{-1})	31 (100)	22·1 (1–3·8 × 10^3)	28 (100)	23·1 (1–3·3 × 10^3)	11 (100)	45 (97·8)	28·01 (1–930)	5 (100)	32 (100)	31·6 (4–315)

*Only positive samples.

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Table 2 Physical and chemical characteristics of examined hot water in relation to *Legionella* colonization

	Positive samples for *Legionella* spp. (no. 76)	Negative samples for *Legionella* spp. (no. 61)	Positive samples for *L. Pneumophila* (no. 71)	Negative samples for *L. Pneumophila* (no. 66)	Simple correlation coefficient *r* (no. 137)	
					Legionella spp.	*L. pneumophila*
Temperature (°C)	48·9 ± 5·1	49·7 ± 5·9	48·8 ± 5·3	49·8 ± 5·7		
pH	7·2 ± 0·3**	6·9 ± 0·3	7·2 ± 0·3***	6·9 ± 0·3	0·28**	0·37***
Ca (mg l^{-1})	58·4 ± 29·3***	83·2 ± 44·3	56·9 ± 29·7***	82·5 ± 42·4	−0·35***	−0·33***
Mg (mg l^{-1})	11·1 ± 5·6***	16·3 ± 7·1	10·9 ± 5·7***	16·1 ± 6·8	−0·42***	−0·39***
Fe (μg l^{-1})	39·4 ± 72·0	36·0 ± 87·2	41·5 ± 74·6	33·9 ± 82·4		
Mn (μg l^{-1})	6·4 ± 9·3	5·3 ± 9·9	6·6 ± 9·7	5·2 ± 9·4		
Cu (μg l^{-1})	17·4 ± 14·5***	29·0 ± 24·9	16·5 ± 13·9***	28·9 ± 24·3	−0·29**	−0·28***
Zn (μg l^{-1})	238·4 ± 296·2	189·38 ± 210·1	220·9 ± 284·5	200·5 ± 216·7		
TOC (mg l^{-1})	0·9 ± 0·6*	0·5 ± 0·3	0·9 ± 0·6*	0·5 ± 0·3	0·29*	0·29*

Values are presented as median ± S.D.
*$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 3 Distribution of positive samples for *Legionella* and Pseudomonadaceae according to Cu concentrations

	Positive samples (%)			
Cu (μg l^{-1})	<10	10–25	25–40	>40
L. pneumophila	64·2	56·4	38·1	29·2
L. non-*pneumophila*	20·3	7·7	28·6	25·0
Pseudomonadaceae	41·5	41·0	57·1	54·2

directly proportional to the pH and TOC. No correlation was seen with the other microbiological parameters (total bacteria count and Pseudomonadaceae). However, a correlation was found between the Pseudomonadaceae and the total counts at 22°C ($r = 0·57$; $P < 0·001$) and at 36°C ($r = 0·62$; $P < 0·001$). The level of *Legionella* contamination was not affected by the temperature of the water, whereas the temperature of the samples positive for Pseudomonadaceae was much lower compared with negative samples (48·2 ± 5·2 vs 50·3 ± 5·6°C; $P < 0·05$). Unlike legionellae, the Pseudomonadaceae showed no association with the concentrations of copper, calcium and magnesium. Table 2 gives the correlation coefficients of the various physical and chemical parameters where statistically significant differences were seen in relation to the contamination by legionellae. The levels of copper, calcium and magnesium were significantly lower in the samples positive for legionellae, while the opposite was observed in the case of iron, zinc and manganese, although the differences were not statistically significant. Table 3 shows the distribution of the samples positive for legionellae and Pseudomonadaceae in relation to the different concentrations of copper. The trend confirms the inverse association between copper and *L. pneumophila*, whereas the metal does not appear to have affected the Pseudomonadaceae and *L.* non-*pneumophila*.

DISCUSSION

The presence of legionellae was detected with a high frequency and at high levels in the hot water distribution network of Bologna. As many as 40·0% of the systems examined were positive for legionellae, in particular *L. pneumophila* (33·3%) which, when present, colonized the whole distribution systems. The Pseudomonadaceae, on the other hand, were found frequently but nearly always at low concentrations and in association with lower water temperatures, affecting single sample points rather than the whole system. The different types of buildings gave rise to varying situations: the greatest colonization was found in the hot water of hospitals both in terms of percentage of positive samples (93·7%) and plate counts (geometric mean: $2·8 \times 10^3$ CFU l^{-1}); they were followed by the hotels (63·6%, geometric mean: 156·5 CFU l^{-1}) and the private apartments with centralized heating systems (54·8%, geometric mean: 116·8 CFU l^{-1}). Statistically significant differences were recorded between the level of *Legionella* hot water colonization and the three types of buildings, both for the total legionellae and *L. pneumophila* (ANOVA). No significant differences were seen between the building types and the Pseudomonadaceae. The lowest risk of *Legionella* colonization was found in the independently controlled heating systems (only one positive sample of 28). These results confirm the reports of other authors who observed 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), 27–43% in hotels (Ranieri *et al.* 2002; Borella *et al.* 2003b) and between 6 and 30% in private residences (Alary and Joly 1991; Stout *et al.* 1992; Marrie *et al.* 1994; Zacheus and Martikainen 1994).

The higher levels of legionellae in centralized systems can be explained by the stagnation of the water in the storage

tanks and within any closed pipes; such stagnation enhances the formation of biofilm. In addition, the recirculation of the water tends to promote the build up of organic matter and produces a fairly uniform contamination throughout the whole system; the distance from the boiler did not, in fact, seem to affect the level of colonization, which was instead directly correlated to the TOC. In hotels and above all in hospitals stagnation is facilitated by the intermittent use of the hot water and also by the greater complexity of the systems, which have often undergone restructuring, sometimes resulting in the closure of pipelines and the creation of dead-ends.

Legionella pneumophila was the most frequently encountered species, being detected in 25 of 75 buildings (33·3% of the total buildings and 53·2% of the buildings with centralized systems): SG 3 in 17·3% of the systems, SG 6 and SG 9 in 5·3% each, SG 1 in 4·0%, SG 8 in 2·7%. The concentrations of this species reached values $>10^3$ CFU l^{-1} in 40·9% of the samples and $\geq 10^4$ CFU l^{-1} in 14·6%. There are no hard scientific data correlating the concentration level of legionellae in water with disease. Consequently there is a controversy about the importance of quantitative assays of legionellae. However the concentrations found in this study not only exceed the threshold of risk estimated by several authors (Meenhorst *et al.* 1985; Patterson *et al.* 1994) but are also higher than the safety levels established in the regulations now in force in many European Countries regarding the control of legionellosis (Italy: Ministero della Salute 2000; UK: Health and Safety Executive 2000; France: Ministere de l'Emploi et de la Solidarité 2002). This situation calls for the prompt application of decontamination measures, especially in hospitals, although the efficiency of such action cannot always be guaranteed considering that legionellae may be protected from disinfection due to their growing within protozoa and biofilm. It is imperative, therefore, to identify and act on the factors leading to the development of biofilm, and thus legionellae, in the water network.

The analysis of the various physical and chemical parameters on the growth of legionellae highlighted certain aspects able to exert an inhibiting action. The water samples contaminated by legionellae showed a higher pH and a lower content of copper, calcium and magnesium. This would seem to suggest that the pH affects the solubilization of copper, a substance often present in the waterlines. A statistically significant inverse correlation was in fact seen between the pH and the concentrations of copper, a metal that has been demonstrated to represent an inhibiting factor on the development of *L. pneumophila* in several experimental and *in situ* studies (Rogers *et al.* 1994; Stout *et al.* 1998; Kusnetsov *et al.* 2001). Furthermore, the positive association with pH confirms the reports of other authors (Yamamoto *et al.* 1992; Marrie *et al.* 1994; Kusnetsov *et al.* 2003). The inverse correlation observed between legionellae and calcium and magnesium is somewhat more difficult to explain. Some authors have published conflicting reports about such association (States *et al.* 1987; Vickers *et al.* 1987; Stout *et al.* 1992; Marrie *et al.* 1994). It is unlikely, then, that the development of legionellae can be affected by the levels of calcium and magnesium, a supposition further supported by the fact that no differences were observed between softened and unsoftened waters. It would therefore appear that copper is the trace element that represents a limiting factor for the development of *L. pneumophila*, an action that appears to be specific for this species given that the same effect was not seen for the other *Legionella* species or for the Pseudomonadaceae. A possible explanation could be that the metal is able to effectively penetrate the biofilm which provides the basis for the colonization of water distribution systems (Rogers *et al.* 1994; Borella *et al.* 2003a). Taking into account the present findings, research will now be carried out on the use of copper and silver ions electrolytically released in the water to control *L. pneumophila* in hot water systems.

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