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Legionella Contamination in Hot Water of Italian Hotels

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A cross-sectional multicenter survey of Italian hotels was conducted to investigate *Legionella* spp. contamination of hot water. Chemical parameters (hardness, free chlorine concentration, and trace element concentrations), water systems, and building characteristics were evaluated to study risk factors for colonization. The hot water systems of Italian hotels were strongly colonized by *Legionella*; 75% of the buildings examined and 60% of the water samples were contaminated, mainly at levels of $\geq 10^3$ CFU liter^{-1}, and *Legionella pneumophila* was the most frequently isolated species (87%). *L. pneumophila* serogroup 1 was isolated from 45.8% of the contaminated sites and from 32.5% of the hotels examined. When a multivariate logistic model was used, only hotel age was associated with contamination, but the risk factors differed depending on the contaminating species and serogroup. Soft water with higher chlorine levels and higher temperatures were associated with *L. pneumophila* serogroup 1 colonization, whereas the opposite was observed for serogroups 2 to 14. In conclusion, Italian hotels, particularly those located in old buildings, represent a major source of risk for Legionnaires' disease due to the high frequency of *Legionella* contamination, high germ concentration, and major *L. pneumophila* serogroup 1 colonization. The possible role of chlorine in favoring the survival of *Legionella* species is discussed.

Bacteria of the genus *Legionella* normally inhabit freshwater or wet soil, but the major reservoirs are man-made aquatic environments, particularly warm water systems (13, 20, 22, 41, 51). In freshwater, legionellae survive as intracellular parasites of free-living protozoans, which are their natural hosts (1, 9, 25, 49). In building water systems, legionellae are also associated with biofilms that provide shelter and nutrients and support their survival and multiplication even outside a host cell (32, 38, 39). These peculiar abilities to grow are responsible for the frequent contamination of artificial water systems, as well as difficulties in eradicating legionellae from contaminated water systems and the lack of biocide efficacy (8, 11, 21, 24, 31, 34). The accumulation of bacteria on pipeline surfaces and biofilm formation are influenced by many factors, such as an inadequate flow rate or stagnation of the water, surface materials and roughness, the concentration and quality of nutrients and disinfectants, temperature, and the hydraulics of the system (5, 46, 61). A better understanding of the risk factors for *Legionella* colonization in artificial water systems could help in the development of control strategies for prevention of legionellosis. For instance, *Legionella* contamination of domestic hot water was found to be associated with a centralized system, a greater distance from the heat source, and an older water plant, whereas copper in the water had a protective effect (10).

Different distributions of *Legionella* species and serogroups have been observed depending on the water type. Significant amounts of *Legionella* spp. were detected in groundwater and potable water, whereas *Legionella pneumophila* was found to be adapted to warm water systems, in which this organism multiplies most efficiently (18, 36, 37). The relative distributions of different *Legionella* species may have a substantial impact on public health as the majority of Legionnaires' disease is caused by *L. pneumophila*, and within this species the most prevalent serogroup is serogroup 1 (26).

Tourism is a major industry in many European countries and is sensitive to health threats. In 1987, a European surveillance scheme for travel-associated Legionnaires' disease was introduced to detect cases (19, 28) and control and prevent *Legionella* infections associated with tourism in both indigenous and foreign persons (15, 44). In 2002, the highest number of reported cases in tourists was associated with travel in Italy (132 of 676 cases), where 35 clusters of travel-associated Legionnaires' disease were identified from July 2002 to October 2003, mainly involving hotels and residences located in 14 Italian regions (47).

Due to the high prevalence of cases associated with travel in Italy, we decided to estimate *Legionella* spp. contamination in a representative number of Italian hotels. A multicenter investigation was carried out to identify *Legionella* spp. in hot water

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samples and to study risk factors associated with microbial contamination in Italian hotels. The aims of this study were to quantify the frequency and severity of contamination, to evaluate the characteristics of buildings and water distribution systems associated with *Legionella* colonization and their relative roles in favoring or protecting against contamination, and to suggest preventive measures to limit colonization. Other aquatic bacteria may compete with *Legionella* for growth in the aquatic environment (23, 55), so we also evaluated *Pseudomonas* spp. colonization and total microbial counts at 36°C and 22°C. Finally, risk factors for the presence of different *Legionella* species and serogroups with special reference to *L. pneumophila* serogroup 1 were examined due to the potential impact on public health.

MATERIALS AND METHODS

Sample collection. From September 2003 through July 2004, 119 water samples were collected from 40 hotels located in five towns (Milan, Modena, Bologna, Naples, and Bari) that are representative of different Italian regions (northern, central, and southern Italy). The hotels were selected on the basis of the water distribution systems in the cities, the characteristics of the buildings, and hotel cooperation. Three to five water samples were taken from each hotel, depending on the number of rooms. Hot water samples were collected from bathroom outlets (showerheads or bath taps) in three sterile glass bottles (1 liter each) without flaming after a brief flow time to eliminate cold water inside the tap or the flexible pipe of the shower. In order to neutralize residual free chlorine, sodium thiosulfate was added to sterile bottles for bacteriological analysis, whereas acid-preserved glass bottles were used for chemical determinations. The methods used for sample processing and the storage conditions have been described elsewhere (10).

Microbiological analysis. Legionellae were isolated by using the procedure described in the Italian guidelines (4). Culture and identification of *L. pneumophila* were carried out by using the ISO 11731 method (3).

Briefly, 2 liters of a water sample was concentrated by membrane filtration (0.2-μm-pore-size polyamide filter; Millipore, Billerica, MA). The filter membrane was resuspended in 10 ml of the original sample water (15). Two 0.1-ml aliquots of the original and concentrated specimens (heat treated and untreated, diluted 1:10 and undiluted) were spread on duplicate plates containing MWY selective medium (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). The plates were incubated at 36°C in a humidified environment with 2.5% CO_2 at least for 10 days and examined beginning on day 5 with a dissecting microscope. All colonies on plates containing ≤10 colonies and 10 to 20 random colonies from other plates were subcultured on BCYE (with cysteine) and CYE (cysteine-free) media (Oxoid) for ≥2 days. Colonies grown on BCYE were subsequently identified by an agglutination test (*Legionella* latex test; Oxoid). This test allows separate identification of *L. pneumophila* serogroup 1 and serogroups 2 to 14 and detection of seven species of non-*L. pneumophila* legionellae (polyvalent) which have been implicated in human disease, *L. longbeachae*, *L. bozemanii* 1 and 2, *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei*, and *L. anisa*. For selected *L. pneumophila* isolates, serotyping was also performed by agglutination with commercial specific monoclonal antibodies (Pro-lab Diagnostics, Canada). The results were expressed as CFU $liter^{-1}$, and the detection limit of the procedure was 25 CFU $liter^{-1}$ (mean of two plates).

Accuracy and recovery performance were periodically established by using water samples supplemented with different *Legionella* species and concentrations distributed in a quality program organized by the National Health Institute, as well as commercial certified specimens (Oxoid).

The total microbial counts at 36°C and 22°C were determined twice by the pour plate method on plate count agar (Oxoid). The plates were incubated at 36°C for 48 h or at 22°C for 72 h.

In order to isolate *Pseudomonas* spp., 100-ml and 10-ml water samples were filtered through 0.45-μm-pore-size membranes (Millipore, Billerica, MA). If the number of bacteria was high, suitable dilutions were prepared. The membranes were placed on *Pseudomonas* CFC agar (Oxoid) and incubated at 30°C for 48 h. Each type of oxidase-positive colony was counted.

Chromosomal PFGE analysis. *L. pneumophila* environmental isolates were subjected to pulsed-field gel electrophoresis (PFGE) using a contour-clamped homogeneous electric field system (CHEF MAPPER; Bio-Rad, United States). Patterns were obtained by SfiI (Promega, United States) digestion using self-made reagents produced by a slight modification of a previously described protocol (50). The running conditions were 200 V for 27 h at 14°C with switch times of 1 s (initial) and 35 s (final). After ethidium bromide staining, the gels were photographed with a UV light source. According to Tenover criteria, a pattern designation was assigned if the electrophoretic profile differed by more than three bands (52).

Macrorestriction patterns were analyzed using the Bionumerics software (version 3.0; Applied Maths, Kortrijk, Belgium) to generate a similarity dendrogram. A similarity matrix was created by using the band-based Dice similarity coefficient, and the unweighted pair group method was used to cluster strains (16).

Physical and chemical analyses. Water temperature and the residual free chlorine content (colorimetric DPD method; Microquant; Merck, Darmstadt, Germany) were determined at the time of sample collection. Standard techniques were used to measure oxidizability (2) and water hardness (method 2040; IRSA-CNR, Rome, Italy). Concentrations of calcium, magnesium, iron, manganese, copper, and zinc were determined with a flame atomic absorption spectrophotometer (model 5000; Perkin-Elmer, Wellesley, MA) by using acidified samples (1% HNO_3) concentrated by boiling (12).

Risk factors. A detailed standardized questionnaire was developed to evaluate risk factors possibly associated with colonization. The first part of this questionnaire collected information on the building, including the number of floors, the number of hotel rooms, the number of bathrooms, the building age, the type of water supply, and the disinfection systems used. The second part collected information on the heating system (central or independent, electric or gas heater), the distance of the sample site from the water distribution point, the existence of a tank and its volume, the age of the system, the frequency of service, and the existence and characteristics of a softening system and a water recycling system. The normal water temperature was also recorded.

Statistical analysis. All statistical calculations were made with SPSS/pc (SPSS Inc., Chicago, IL). Logarithmic transformations were used in statistical analyses to normalize the nonnormal distributions, and the results were expressed as geometric means. The bacteriological data were converted into $\log_{10}(x + 1)$. When possible, variables were categorized into dichotomous variables. The results were analyzed by correlation analysis, by a t test, by a one-way analysis of variance, and by a χ^2 test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to assess categorical risk variables associated with microbial contamination. Variables that were significant as determined by the univariate analysis were entered into a multiple logistic regression model. By using conditional logistic regression models, independent predictors of colonization were established. Variables were retained in the model if the likelihood ratio test was significant ($P < 0.05$).

RESULTS

Table 1 shows the general characteristics of the water examined in terms of water supply and distribution system. The typical features of Italian hotels include limited numbers of rooms and floors and old age of the building (up to 800 years). The water supply is mainly groundwater, and all water is disinfected by chlorine in different chemical forms irrespective of the water origin. A clear preponderance of metal plumbing, gas water heaters, and water recirculation systems was recorded.

A total of 30 hotels (75.0%) and 72 water samples (60.5%) were contaminated by at least one *Legionella* species, and there were no significant differences for different geographic areas (Table 2). For 18 hotels, all 44 water samples were positive, while for the other hotels some sites were positive and some were negative (21 of 38 sites were positive). *Pseudomonas* spp. were isolated from 18 hotels (45.0%) and 33 (27.7%) water samples; no sample was positive in Bari, whereas 63.6% of the samples in Modena were positive ($P < 0.001$).

L. pneumophila was the most frequently isolated species (87% of the isolates). Among the positive hotels, nine were colonized by *L. pneumophila* serogroup 1 (20 positive samples and 5 negative samples) and 15 were colonized by *L. pneumophila* serogroups 2 to 14 (31 positive samples and 10 negative

TABLE 1. Main characteristics of the building and water supplies in the 40 hotels examined

Characteristic	Value
No. of rooms	68.7 ± 46.4 (10–189)[a]
No. of floors	3.7 ± 1.8 (1–10)[a]
Age of building (yr)	88.8 ± 201.6 (2–800)[a]
Age of water distribution system (yr)	9.0 ± 8.4 (1–32)[a]
Water system characteristics	
Water type	
Groundwater	20 (50.0)[b]
Mixture water	20 (50.0)[b]
Disinfection	
Cl dioxide	10 (25.0)[b]
Na hypochlorite	10 (25.0)[b]
Cl dioxide + Na hypochlorite	20 (50.0)[b]
Plumbing material	
Metal	25 (62.5)[b]
Plastic	5 (12.5)[b]
Both metal and plastic	10 (25.0)[b]
Heater type	
Electric	3 (7.5)[b]
Gas, steam	2 (5.0)[b]
Gas, water	35 (87.5)[b]
Softening system	
Absent	26 (65.0)[b]
Present	15 (35.0)[b]
Hot water recirculation	
Absent	15 (37.5)[b]
Present	25 (62.5)[b]

[a] Mean ± standard deviation (range).
[b] Number of hotels (percent).

samples). Mixed *Legionella* cultures were obtained for the remaining six hotels. In two hotels three sites were colonized by *L. pneumophila* (all serogroups), and two sites were colonized by either serogroup 1 or serogroups 2 to 14. In two hotels five sites were colonized by serogroup 1 plus unknown species, and four sites were colonized by serogroup 1. In two hotels six sites were colonized by serogroups 2 to 14 plus unknown species, one site was colonized by serogroups 2 to 14, and one site was negative. As shown in Table 3, the mean number of legionellae for positive samples was 1.9×10^3 CFU liter^{-1} (geometric mean), and 19.4% of these samples contained $\geq 10^4$ CFU liter^{-1}. *L. pneumophila* serogroup 1 was isolated from 33 points, mainly at levels of $\geq 10^3$ CFU liter^{-1}. *L. pneumophila* serogroup 1 correlated negatively with *L. pneumophila* serogroups 2 to 14 ($r = -0.309$, $P < 0.001$) and positively with the unknown species ($r = 0.205$, $P < 0.05$). The geometric mean level of *Pseudomonas* spp. was 176 CFU liter^{-1}, and the values ranged from 2 to 10^5 CFU liter^{-1}.

Twenty-two *L. pneumophila* isolates collected from 15 different hotels in three cities (Naples, Modena, and Bologna) were characterized further, divided into four different serogroups (serogroups 1, 3, 6, and 9), and subsequently typed by the PFGE method. The similarity dendrogram derived from SfiI macrorestriction analysis is shown in Fig. 1. The patterns obtained contained 8 to 12 fragments. Isolates with patterns that differed by no more than three bands had similarity coefficients of ≥0.80, and this cutoff was used to define pulsotypes. Sixteen different pulsotypes were identified, including 11 pulsotypes that contained a single isolate. No genetic similarities were found among isolates collected in different hotels. With regard to the correlation between serogroup and genotype, all the serogroups appeared to be particularly heterogeneous, with representatives found in multiple pulsotypes. It is interesting that pulsotypes 6 and 11 were represented by two isolates belonging to different serogroups (serogroups 3 and 6 and serogroups 6 and 9, respectively).

No difference in building and water characteristics was observed between colonized and *Legionella*-free hotels, except for a lower zinc concentration in the water (geometric means, 158.2 and 498.5 μg liter^{-1}[$P < 0.001$]). Similarly, colonized points did not differ from uncontaminated water, except for zinc concentration and total hardness (geometric means, 20.8 and 31.9°F [$P < 0.001$]) due to lower levels of both calcium and magnesium. *Pseudomonas*-positive hotels were older than *Pseudomonas*-free structures (53.1 versus 14.6 years [$P < 0.001$]), and the free chlorine concentrations were lower (10.1 versus 69.0 μg liter^{-1} [$P < 0.05$]). The total microbial counts for *Pseudomonas*-positive sites were also higher than the total microbial counts for *Pseudomonas*-free structures at both 36 and 22°C (596 versus 91 CFU ml^{-1} [$P < 0.001$] and 142 versus 35 CFU ml^{-1} [$P < 0.05$, respectively]).

In contrast, there were differences according to *Legionella* species and serogroups (Table 4). The zinc, calcium, and magnesium concentrations and the total hardness were significantly lower in *L. pneumophila* serogroup 1-colonized samples than in serogroup 2 to 14-colonized samples. The copper concentrations and oxidizability were lower in serogroup 2 to 14-contaminated samples than in uncontaminated samples. Trends toward greater hotel age for serogroup 2 to 14-colonized water and higher free chlorine concentrations in serogroup 1-contaminated water were also observed, but the differences did not reach statistical significance. The manganese concentration was significantly higher in serogroup 1-contaminated water than in serogroup 2 to 14-contaminated water, as determined by the Student *t* test (3.46 and 2.03 μg liter^{-1}, respectively [$P < 0.05$]). When a nonparametric test (Kruskal-Wallis) was applied, the results did not change.

The risk factors for microbial contamination were analyzed by means of univariate logistic, regression on dichotomous variables (Table 5). A hotel age of >20 years was positively associated with a risk for *Legionella* contamination (OR = 2.70, $P < 0.01$), whereas an operating temperature of >60°C significantly inhibited *Legionella*. Furthermore, a sample temperature of >55°C, a total hardness of >20°F, an oxidizability of

TABLE 2. Distribution of *Legionella* and *Pseudomonas* isolates in the areas examined[a]

Location	No. positive/total no. (%)			
	Legionella spp.		*Pseudomonas* spp.	
	Hotels	Samples	Hotels	Samples
Total	30/40 (75.0)	72/119 (60.5)	18/40 (45.0)	33/119 (27.7)
Milan	4/5 (80.0)	11/17 (64.7)	1/5 (20.0)	3/17 (17.6)
Modena	7/10 (70.0)	12/22 (54.5)	8/10 (80.0)	14/22 (63.6)
Bologna	7/10 (70.0)	28/44 (63.6)	7/10 (70.0)	12/44 (27.3)
Naples	3/5 (60.0)	6/16 (37.5)	2/5 (40.0)	4/16 (25.0)
Bari	9/10 (90.0)	15/20 (75.0)	0/10 (0.0)	0/20 (0.0)

[a] For *Legionella* spp. the χ^2 values for hotels and samples were 2.13 (not significant) and 5.93 (not significant), respectively; for *Pseudomonas* spp. the χ^2 values for hotels and samples were 16.97 ($P < 0.002$) and 22.75 ($P < 0.001$), respectively.

TABLE 3. Characteristics of *Legionella* contamination in the hot water samples examined

Parameter	*Legionella* spp.	*L. pneumophila* serogroup 1	*L. pneumophila* serogroups 2 to 14	Unknown *Legionella* spp.
No. of positive samples/total no. (%)	72/119 (60.5)	33/119 (27.7)	41/119 (34.5)	11/119 (9.2)
No. of samples with $\geq 10^3$ CFU liter^{-1}/total no. (%)	45/72 (62.5)	25/33 (75.8)	20/41 (48.8)	10/11 (90.9)
No. of samples with $\geq 10^4$ CFU liter^{-1}/total no. (%)	14/72 (19.4)	9/33 (27.3)	4/41 (9.7)	3/11 (27.3)
Geometric mean count (CFU liter^{-1})[a]	1.9×10^3	2.7×10^3	1.1×10^3	4.3×10^3
Median count (CFU liter^{-1})[a]	2.4×10^3	4.5×10^3	700	4.5×10^3
Count range (CFU liter^{-1})[a]	25–5.8×10^4	25–3.1×10^4	50–5.5×10^4	600–4.8×10^4

[a] Only positive samples were included.

>3.0 mg liter^{-1} of O_2, a copper concentration of >50 μg liter^{-1}, and a zinc concentration of >200 μg liter^{-1} were protective. The results were similar when we introduced into the model positive/negative hotels instead of positive/negative sites, but the statistical significance disappeared for copper concentration and hardness (data not shown).

A higher free chlorine concentration and lower hardness were positively associated with *L. pneumophila* serogroup 1 colonization, whereas the opposite trend was observed in samples colonized by *L. pneumophila* serogroups 2 to 14 (Table 5). The results did not substantially change when we omitted samples from which serogroup 1 and/or serogroups 2 to 14 were isolated in combination with other species or serogroups (data not shown). The 11 unknown *Legionella* strains were negatively associated with total hardness (OR = 0.021, 95% CI = 0.003 to 0.18, $P < 0001$) and with zinc concentration (OR = 0.29, 95% CI = 0.08 to 1.03, $P < 0.05$) and were never found when the system temperature was >60°C and the sample temperature was >55°C. The only factor negatively associated with *Pseudomonas* contamination was the free chlorine level.

When the same parameters (excluding operating temperature) were introduced into a multivariate conditional logistic regression model (Table 6), a hotel age of >20 years remained independently associated with an increased risk for *Legionella* spp. colonization, whereas water oxidizability of >3 mg liter^{-1} O_2 and total hardness of >20°F were protective. Chlorine levels of >100 μg liter^{-1} and hardness of ≤20°F were predictive of *L. pneumophila* serogroup 1 colonization, whereas greater hotel age and greater hardness were predictive of *L. pneumophila* serogroup 2 to 14 colonization. When we introduced positive hotels versus negative

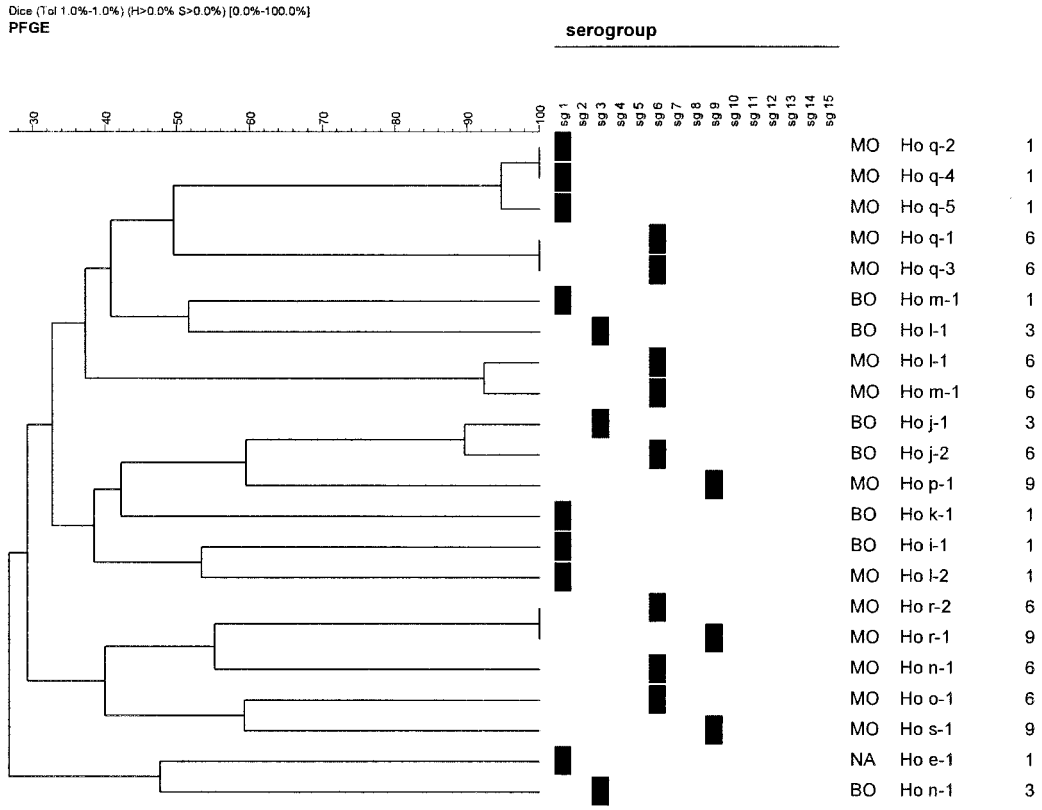

FIG. 1. Dendrogram showing similarities between 22 environmental isolates of *L. pneumophila* based on PFGE results. Isolates with Dice similarity coefficients of ≥0.80 are considered identical. Solid boxes indicate the serogroups (sg) for isolates. The columns on the right indicate the town (MO, Modena; BO, Bologna; NA, Naples), the hotel (Ho) and sample examined, and the *L. pneumophila* serogroup.

TABLE 4. Variables (geometric means) associated with *Legionella* contamination as determined by analysis of variance

Parameter	No *Legionella* ($n = 47$)	*L. pneumophila* serogroup 1 ($n = 25$)	*L. pneumophila* serogroups 2 to 14 ($n = 33$)	Other *Legionella* spp. ($n = 14$)	F (P)
Zinc concn (μg/liter)	290.1	97.0[a]	212.8	131.2	5.62 (<0.001)
Calcium concn (mg/liter)	93.9	55.4[a]	83.3	35.0[a,b]	12.94 (<0.001)
Magnesium concn (mg/liter)	18.7	11.3[a,b,c]	17.6	6.6[a]	19.14 (<0.001)
Total hardness (°F)	31.9	18.4[a,b]	29.7	11.4[a,b]	14.99 (<0.001)
Oxidizability (mg/liter O_2)	1.23	0.78	0.45[a,c]	1.45	4.38 (<0.01)
Copper concn (μg/liter)	18.4	10.6	7.1[a,c]	30.7	4.88 (<0.005)
Hotel age (yr)	28.3	22.1	42.1	55.2	1.63 (ns)[d]
Free chlorine concn (μg/liter)	25.7	41.3	17.7	14.6	0.73 (ns)
Manganese concn (μg/liter)	2.55	3.46	2.03	4.13	1.64 (ns)

[a] Significantly different from the value for no *Legionella* as determined by post hoc analysis (Bonferroni's test).
[b] Significantly different from the value for *L. pneumophila* serogroups 2 to 14 as determined by post hoc analysis (Bonferroni's test).
[c] Significantly different from the value for other *Legionella* spp. as determined by post hoc analysis (Bonferroni's test).
[d] ns, not significant.

hotels into the model as a dependent variable, only hotel age remained significantly associated with an increased risk of colonization (OR = 8.00, 95% CI = 1.42 to 44.92, $P < 0.05$). *Pseudomonas* contamination remained negatively associated with the free chlorine concentration (OR = 0.26, 95% CI = 0.09 to 0.74, $P < 0.05$).

DISCUSSION

Sporadic cases and outbreaks of travel-associated Legionnaires' disease are common in Italy, which tops the list of European countries for the number of reported cases (45). Hotels are the most frequent sources of cases, probably be-

TABLE 5. Association of water and building parameters with *Legionella* and *Pseudomonas* contamination (univariate regression analysis)

Characteristic	Value	No. (%)			
		All *Legionella* spp.	*L. pneumophila* serogroup 1	*L. pneumophila* serogroups 2 to 14	*Pseudomonas* spp.
Hotel age (yr)	≤20 ($n = 51$)	24 (47.1)	16 (31.4)	7 (13.7)	11 (21.6)
	>20 ($n = 68$)	48 (70.6)	17 (25.0)	34 (50.0)	22 (32.4)
OR (95% CI)		2.70 (1.26–5.76)[a]	0.73 (0.32–1.63)	6.29 (2.49–5.9)[b]	1.74 (0.75–4.02)
Operating temp (°C)	≤60 ($n = 109$)	71 (65.1)	32 (29.4)	41 (37.6)	30 (27.5)
	>60 ($n = 10$)	1 (10.0)	1 (10.0)	0	3 (30.0)
OR (95% CI)		0.06 (0.007–.49)[b]	0.27 (0.03–2.20)	0.62 (0.54–0.72)[c,d]	1.13 (0.27–4.65)
Sampling temp (°C)	≤55 ($n = 105$)	67 (63.8)	29 (27.6)	40 (38.1)	29 (27.6)
	>55 ($n = 14$)	5 (35.7)	4 (28.6)	1 (7.1)	4 (28.6)
OR (95% CI)		0.31 (0.10–1.00)[c]	1.05 (0.31–3.61)	0.12 (0.02–0.99)[c]	1.05 (0.30–3.61)
Total hardness (°F)	≤20 ($n = 29$)	23 (79.3)	18 (62.1)	5 (17.2)	5 (17.2)
	>20 ($n = 90$)	49 (54.4)	15 (16.7)	36 (40.0)	28 (31.1)
OR (95% CI)		0.31 (0.12–0.84)[c]	0.12 (0.05–0.31)[b]	3.20 (1.12–9.16)[c]	2.17 (0.75–6.27)
Free chlorine concn (μg liter^{-1})	≤100 ($n = 79$)	46 (58.2)	17 (21.5)	32 (40.5)	28 (35.4)
	>100 ($n = 40$)	26 (65.0)	16 (40.0)	9 (22.5)	5 (12.5)
OR (95% CI)		1.33 (0.60–2.93)	2.43 (1.06–5.57)[c]	0.42 (0.18–1.00)[c]	0.26 (0.09–0.74)[a]
Oxidizability (mg liter^{-1} O_2)	≤3.0 ($n = 91$)	61 (67.0)	24 (26.4)	39 (32.9)	27 (29.7)
	>3.0 ($n = 28$)	11 (39.3)	9 (32.1)	2 (7.1)	6 (21.4)
OR (95% CI)		0.32 (0.13–0.76)[a]	1.32 (0.53–33.2)	0.10 (0.02–0.46)[b]	0.65 (0.24–1.77)
Copper concn (μg liter^{-1})	≤50 ($n = 108$)	69 (63.9)	31 (28.7)	40 (37.0)	31 (28.1)
	>50 ($n = 11$)	3 (27.3)	2 (18.2)	1 (9.1)	2 (18.2)
OR (95% CI)		0.21 (0.05–0.85)[a]	0.55 (0.11–2.70)	0.17 (0.02–1.38)	0.55 (0.11–2.70)
Zinc concn (μg liter^{-1})	≤200 ($n = 48$)	43 (78.2)	23 (41.8)	19 (34.5)	11 (20.0)
	>200 ($n = 57$)	29 (45.3)	10 (15.6)	22 (34.4)	22 (34.4)
OR (95% CI)		0.23 (0.10–0.52)[b]	0.26 (0.11–.61)[b]	0.99 (0.46–2.12)	2.09 (0.91–4.84)

[a] $P < 0.01$.
[b] $P < 0.001$.
[c] $P < 0.05$.
[d] Data for the cohort *L. pneumophila* serogroups 2 to 14 absent.

TABLE 6. Multiple logistic regression of building and water characteristics associated with *Legionella* contamination

Characteristic	OR (95% CI)		
	All *Legionella* spp.	*L. pneumophila* serogroup 1	*L. pneumophila* serogroups 2 to 14
Hotel age > 20 yr	2.26 (0.91–5.64)[a]	0.93 (0.32–2.66)	4.02 (1.49–10.81)[b]
Oxidizability > 3.0 mg liter^{-1} O_2	0.24 (0.07–0.78)[a]	0.61 (0.17–2.17)	0.21 (0.04–1.01)
Copper concn > 50 μg liter^{-1}	0.23 (0.05–1.03)	0.66 (01.2–3.77)	016 (0.02–1.47)
Total hardness > 20°F	0.15 (0.04–0.53)[b]	0.09 (0.03–0.25)[c]	3.33 (1.02–10.85)[a]
Chlorine concn > 100 μg liter^{-1}	2.08 (0.83–5.20)	3.91 (1.43–10.66)[b]	0.48 (018–1.46)

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

cause large buildings provide a more hospitable environment than small facilities because the more extensive piping network provides a larger surface with variable temperatures and biofilm accumulation (58). Our investigation confirmed that hot water systems of Italian hotels are strongly colonized by *Legionella* spp., as 75.0% of the buildings examined and 60.5% of the water sites sampled were colonized; *Pseudomonas* spp. are also common colonizers. Genotyping data obtained for 22 isolates revealed high genetic heterogeneity; all of the buildings were colonized by different strains, and in two hotels in Modena different pulsotypes were recovered at the same time. The frequencies of colonization, however, were similar in the different geographic areas, which included towns located in northern, central, and southern Italy, despite the fact that reports of Legionnaires' disease have occurred mainly in northern Italy (48). This suggests that diagnosis and/or notification may be underestimated in southern Italian regions, although recently in the Puglia region, where Bari is located, three travel-associated clusters were reported (47).

Unfortunately, we could not document the temporal variability of colonization by repeatedly sampling the same sites in each hotel. In studies in which sampling was repeated in each building over a period of 1 year, the workers found differences between seasons in terms of counts but not in terms of serogroup distribution, which remained fairly constant over time, at least in Italian private healthcare facilities (34). Indeed, in our study contaminating concentrations were not associated with the possible risk factors, suggesting that levels may be less important than types. Nevertheless, in our hotels the concentrations were noteworthy; 62.5% of the concentrations exceeded 10^3 CFU liter^{-1}, a threshold for considering preventive measures according to Italian guidelines. During environmental investigations of water at Italian locations where clusters of travel-associated Legionnaires' disease occurred, the *Legionella* concentration was $>10^4$ CFU liter^{-1} in 52% of the buildings examined (47). In our study, the concentrations in 35% of the hotels exceeded this level, and more than 90% of the isolates belonged to *L. pneumophila*, suggesting that the risk for cases in contaminated Italian hotels is worrisome.

If we consider that cultural methods generally underestimate the presence of *Legionella*, the real level of hotel contamination could be greater. Recently, a real-time LightCycler PCR assay has been proposed for investigation of *Legionella* contamination in potable water systems (35, 57). Compared to cultures, this method is much more rapid, has higher sensitivity, and detects even nonculturable legionellae. However, possible inclusion of nonviable cells and nonlegionella DNA and shielding of legionellae sequestered within protozoans from the PCR are limitations of the PCR assay, which at present does not detect non-*L. pneumophila* species. Cultural methods remain the "gold standard" for associating environmental isolates with clinical isolates and for estimating the virulence of the *Legionella* spp. detected.

Another aspect which deserves special attention is the high frequency of *L. pneumophila* serogroup 1, which was isolated from 45.8% of the contaminated sites and from 32.5% of the hotels examined, with differences according to geographic area. Furthermore, the levels of serogroup 1 were higher than the levels of the other isolates. In our previous study of domestic contamination in the same cities, *L. pneumophila* serogroup 1 was found only in 18.2% of positive hot water samples, and residents living in *Legionella*-positive homes did not have a significantly higher risk of disease than residents living in *Legionella*-free buildings (10).

Worldwide, *L. pneumophila* serogroup 1 is the most common agent of Legionnaires' disease, accounting for about 80 to 90% of the reported cases (6, 26, 59) and approximately 70% of the European travel-associated cases (44). In contrast, *L. pneumophila* serogroups 2 to 14, although accounting for more than 50% of the isolates obtained from man-made aquatic systems, account for only 15 to 20% of community cases. The discrepancy between environmental isolates and clinical expression of disease has recently been observed by Doleans et al. (17), who suggested that there are differences in virulence rather than greater abundance in water distribution systems.

In Italy, as in other European countries, approximately 20% of the total cases and 25% of the community cases of Legionnaires' disease occur in travelers, and the main sources are hotels (30, 43). This proportion suggests that the risk for travelers is particularly high, as people do not normally spend 20 to 25% of their time traveling. The high frequency of *L. pneumophila* serogroup 1 in the hotels examined could at least partially explain the elevated number of travel-associated cases in Italy. It would be interesting to place serogroup 1 strains into monoclonal antibody subgroups, as travel-associated cases were found to be caused predominantly by monoclonal antibody 3/1-positive *L. pneumophila* serogroup 1 (26). In any case, there should be a major effort to avoid or reduce *L. pneumophila* serogroup 1 colonization in accommodation sites. In addition, 8 of 14 *L. pneumophila* serogroup 2 to 14 isolates belonged to serogroup 6, a serogroup recently involved in

outbreaks of Legionnaires' disease and Pontiac fever in the United States (7).

In our study of the risk factors for *Legionella* contamination, we did not observe major differences between colonized and *Legionella*-free hotels, at least for the parameters measured, and hotel age was the only risk factor which remained positively associated with contamination after a multiple logistic regression was applied. In Italy, the existence of very old buildings is a further reason explaining the high frequency of travel-associated cases. Conversely, we could not find a clear association with heating system age, like that documented at the domestic level, most probably due to inaccurate reporting of this information. Hotel contamination was not associated with pipe material, water origin, type of chlorine used for water disinfection, type of heater, tank capacity, number of floors and/or rooms, or distance between the hot water reservoir and the outlet sites sampled. The role of copper in the water in reducing the risk of *Legionella* contamination was less evident than the role found at the domestic level, probably due to the limited number of water samples with copper concentrations of >50 μg liter^{-1} (11 samples). The zinc concentration was particularly high, probably due to plumbing corrosion, but we did not find any influence of zinc on *Legionella* contamination after applying a multivariate analysis. All these data confirm that *Legionella* colonization in complex water distribution systems is a deep-rooted phenomenon and that the organisms are able to persist and increase with time independent of the various water and system characteristics and of the cleanliness and regular preventive maintenance measures (54).

When the role of water temperature was examined, protection against *Legionella* colonization was observed only when the system temperature was >60°C and, to a lesser extent, when the outlet temperature was >55°C. *L. pneumophila* was not isolated from water samples at temperatures of >55°C in Finnish apartment buildings (60). The problem is interesting as the majority of countries set limits for hot water temperature at the storage site of around 50°C (in Italy, 48 ± 5°C is mandatory temperature for public buildings) to avoid scalding (27, 29). To control *Legionella*, hotels should set the storage temperature at 60°C and the delivery temperature at 55°C, but this apparently simple solution has two drawbacks: the increased cost of heating and, even more relevant from a public health point of view, the need to install appropriate water mixers at outlets to protect people from hot water burns.

Interestingly, the temperature effect was less relevant and statistically not significant for *L. pneumophila* serogroup 1 contamination, suggesting that serogroup 1 is more resistant to higher temperatures. Furthermore, *L. pneumophila* serogroup 1 was not influenced by hotel age or oxidizability, and the protective effect of copper was limited and not significant.

These are not the only differences that characterize *L. pneumophila* serogroup 1 compared with the other legionellae. *L. pneumophila* serogroup 1 appeared able to survive when the free chlorine concentration was higher (>100 μg liter^{-1}), conditions which inhibited both *L. pneumophila* serogroups 2 to 14 and *Pseudomonas* spp. In our study, the free chlorine concentration in water in Bari was higher than that in the other cities examined (geometric mean, 207 μg liter^{-1}; range, 100 to 500 μg liter^{-1}), and the hotels in Bari had the highest frequency of *L. pneumophila* serogroup 1 isolates (45% of samples) and the lowest levels of *Pseudomonas* contamination. In contrast, in Modena, where the free chlorine concentration was low (mean, 0.76 μg liter^{-1}; range, 0 to 100 μg liter^{-1}), only four samples (18.1%) were contaminated by *L. pneumophila* serogroup 1, but *Pseudomonas* was found particularly frequently (Table 2). This peculiar situation was also observed at the domestic level, where *L. pneumophila* serogroup 1-contaminated water had a free chlorine level of 78.0 μg liter^{-1}, compared with 2.6 μg liter[1] in water contaminated by serogroups 2 to 14 (10). Our experience with water decontamination in a large hospital showed that shock chlorination with sodium chlorine substantially reduced the number of legionellae immediately after treatment, but the proportion of samples contaminated by *L. pneumophila* serogroup 1 increased from 2 to 30% (unpublished data).

Many microorganisms of aquatic origin, such as *Pseudomonas*, produce bacteriocins that may actually be inhibitory to *Legionella* spp. (9). Thus, chlorine levels that eliminate these microorganisms could increase the population of indigenous legionellae. Furthermore, *Legionella* spp. are more resistant to chlorine than other bacteria because they can enter a viable but nonculturable state, as observed using PCR methods (42), can be protected by amoebae, and/or can survive in pipe biofilms (23, 38). In an experimental study of microbial changes during and after application of various disinfection treatments, amoebae resisted all the treatments and probably acted as reservoirs for *L. pneumophila*, allowing quick recolonization of the system once the treatments were interrupted (53). As multiplication in protozoans can differ depending on the *Legionell*a species (21, 40), it is conceivable that *L. pneumophila* serogroup 1 is more able than other serogroups to survive within protozoans or biofilms under stressful environmental conditions, such as high temperature and high chlorine levels. These observations should be noted by people responsible for water disinfection at the municipal and/or building level. Further studies are needed to establish the cutoff value for the residual free chlorine concentration not associated with survival of virulent legionellae, such as serogroup 1.

We found that total hardness protected *L. pneumophila* serogroup 1 and unknown species. As hotels frequently use water softening systems, the high levels of *L. pneumophila* serogroup 1 colonization of hotel water may also depend on this technical device. Hardness is generally considered a risk factor for *Legionella* colonization, and in our investigation this was confirmed for *L. pneumophila* serogroups 2 to 14. A negative correlation with hardness and chemical oxygen demand has been described in other studies, but *Legionella* species were not examined (33). As a lower number of bacteria were found in dental unit water systems using soft water than in dental unit water systems using hard water (56), we suggest that less microbial competition or modification of biofilm formation and/or composition in soft water might represent a risk factor for *L. pneumophila* serogroup 1 colonization more than hardness per se. Finally, higher manganese and lower zinc concentrations were typical features of water colonized by either *L. pneumophila* serogroup 1 or unknown species compared to water colonized by *L. pneumophila* serogroups 2 to 14 (Table 4). Different distributions of *Legionella* species and serogroups according to heater type, water temperature,

and chlorine and manganese concentrations were also observed at the domestic level.

The reasons for these trends are currently unknown, but our data suggest that *Legionella* species and serogroups may have different ecological niches and/or that their ability to survive in man-made water environments varies with the environmental conditions. In particular, *L. pneumophila* serogroup 1 shows a special ability to colonize "cleaner" water systems and to survive under more stressful conditions, such as higher temperature and chlorine levels, which are not consistent with the survival of other serogroups.

In conclusion, Italian hotels, particularly those located in old buildings, represent a major source of risk for Legionnaires' disease due to the high frequency of *Legionella* contamination, high germ concentration, and major *L. pneumophila* serogroup 1 colonization. Our results do not suggest specific new measures to control *Legionella* contamination except for the protective role of temperatures of >60°C. However, the variability of contaminating species seems to depend on different environmental risk factors, such as chlorine content and hardness, which should be controlled to reduce at least the most virulent *L. pneumophila* serogroups as the risk of disease may substantially change.

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