Influence of amoebae and physical and chemical characteristics of water on presence and proliferation of Legionella species in hospital water systems

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The reservoir for hospital-acquired Legionnaires’ disease has been shown to be the potable water distribution system. The objectives of the present study were as follows: (1) to examine the possible relationship between physical-chemical characteristics of water such as temperature, pH, hardness, conductivity, and residual chlorine and the presence of amoebae as growth-promoting factors for Legionella species and (2) to determine eradication measures for water distribution systems to seek ways of reducing the risk of legionellosis. Ten hospitals in southwest France took part in this study. Water samples were collected from 106 hot water faucets, showers, hot water tanks, and cooling towers. Two analyses were performed to analyze the association between water characteristics and (1) the presence of Legionella species and (2) the proliferation of Legionella species. Of the 106 water samples examined, 67 (63.2%) were positive for Legionella species. Amoebae were detected in 75 of 106 (68.9%) samples and in 56 of 67 (86.6%) Legionella species-positive samples (P < 10⁻⁵). In these positive samples, conductivity was lower than 500 μΩ⁻¹ cm⁻¹ in 58.2% (P = .026), temperature was below 50°C in 80.6% (P = .004), and hardness was significantly higher (P = .002) than in Legionella species-negative samples. Neither Legionella species nor amoebae were isolated from any sampling point in which the water temperature was above 58.8°C. Multivariate analysis shows that high hardness and presence of amoebae were strongly correlated statistically with the presence of Legionella when showers, tanks, pH, and temperature promoted their proliferation. This study shows the importance of water quality evaluation in assessing environmental risk factors and in selecting the most appropriate prevention and control measures in hospital water systems. (Am J Infect Control 2006;34:520-5.)

Hospital-acquired Legionnaires’ disease not only can be treated but also can be prevented by taking adequate measures in the potable water distribution system. Success of eradication depends on the information obtained by analyzing the ecology of Legionella species in the water distribution system.

We examined the current contamination of Legionella species in hospital water systems in southwest France. First, we investigated how the presence and proliferation of Legionella species might be due to the physical-chemical characteristics of water (temperature, pH, hardness, conductivity, residual chlorine) and to the presence of amoebae as growth-promoting factors for Legionella species. Many reports show the strong statistical correlation between Legionella species and amoebae, and water temperature is a decisive factor in Legionella colonization. The second objective was to determine eradication measures to reduce the risk of legionellosis in water distribution systems.

**METHODS**

Study sites and sample collection

Ten hospitals in southwest France took part in this study. Water samples were collected from hot water faucets, showers or hot water tanks, and cooling
towers. The temperature was also measured after allowing the hot water alone to flow from the selected faucet for 5 minutes until the temperature was constant. A 1-L sample was collected for *Legionella* species assessment in sterile glass bottles containing 5 mg/L of sodium thiosulfate to remove any residual disinfectant. A second 1-L sample was collected in sterile glass bottles for amoebae determination. A third sample of 250 mL was collected in sterile glass bottles for physical and chemical examination.

**Legionella** species examination

*Legionella* species were determined by the following method: 1-L water samples were concentrated by filtration through a 0.22-μm pore-size polycarbonate filter (Millipore SA, St. Quentin en Yvelines, France; réf GTTP04700). The intact filter membranes were resuspended in 5 mL of the original water samples and sonicated for 30 seconds (Bransonic model 3200; Branson Cleaning Equipment Company, Shelton, CT). A 2-mL volume of the suspension was placed in a 50°C water bath and incubated for 30 minutes. A second 2-mL volume of the suspension was acidified for 5 minutes with buffered HCl (Prolabo)-KCl (Merck) solution (final pH, 2.0). One milliliter did not undergo any treatment; 0.2 mL of the original water, 0.2 mL of a decimolar dilution of the original water, 0.1 mL of untreated samples, 0.1 mL of heat-treated samples, and 0.2 mL of acid-treated samples were spread on plates of GVPC Selective medium (charcoal, yeast extract, glycin, vancomycin, polymyxin, cycloheximidine; Oxoid SA, Dardilly, France). The 5 plates were incubated at 37°C in a humid 2.5% CO₂ environment for 10 days and read at days 3, 5, and 10. All colonies consistent with *Legionella* species according to the detection limit of the method (50 cfu/L). The *χ*² test was used to compare proportions of contamination over categorized variables. Student *t* test was used to compare mean values of continuous variables according to the presence of *Legionella* species in the different devices. Statistical interactions were also evaluated, but none of them were highlighted. Two different models were chosen to study associations (1) between water characteristics and presence of *Legionella* species in all samples and (2) between water characteristics and *Legionella* species concentrations in samples with presence of *Legionella* species. The associations between the presence of *Legionella* species and the predictive variables (presence of amoebae, circulating water temperature, conductivity, hardness, free chlorine, total residual chlorine, and pH) were investigated by multivariate logistic regression analysis using a forward likelihood ratio procedure (Wald test). A second analysis was performed on the samples containing Legionella (up to the detection limit of the method) to study the variation of log-transformed concentrations of Legionella and the same predictive variables. Multivariate linear regression analysis using a forward likelihood ratio procedure was used (Wald test) to evaluate the importance of independent variables in relation to the increase in *Legionella* species.

**Physical and chemical analysis**

Water temperature, free chlorine, and total residual chlorine contents were determined at the time of collection. Chlorine determinations were performed colorimetrically (Chlor-Test, Merck KGaA, 64271 Darmstadt, Germany). Standard techniques were used to measure all physical and chemical parameters (pH, hardness, conductivity). Free chlorine and total residual chlorine were measured to know whether the water systems were disinfected by chlorine before collection because disinfection could influence the results.

**Statistical analysis**

Data were entered into an Excel spreadsheet (Microsoft Corp, Redmond, WA), and statistical analyses were carried out using Intercooled Stata 7 for Windows (StataCorp LP). A first analysis was performed by considering the presence or the absence of *Legionella* species according to the detection limit of the method (50 cfu/L). The *χ*² test was used to compare proportions of contamination over categorized variables. Student *t* test was used to compare mean values of continuous variables according to the presence of *Legionella* species in the different devices. Statistical interactions were also evaluated, but none of them were highlighted. Two different models were chosen to study associations (1) between water characteristics and presence of *Legionella* species in all samples and (2) between water characteristics and *Legionella* species concentrations in samples with presence of *Legionella* species. The associations between the presence of *Legionella* species and the predictive variables (presence of amoebae, circulating water temperature, conductivity, hardness, free chlorine, total residual chlorine, and pH) were investigated by multivariate logistic regression analysis using a forward likelihood ratio procedure (Wald test). A second analysis was performed on the samples containing Legionella (up to the detection limit of the method) to study the variation of log-transformed concentrations of Legionella and the same predictive variables. Multivariate linear regression analysis using a forward likelihood ratio procedure was used (Wald test) to evaluate the importance of independent variables in relation to the increase in *Legionella* species.
Table 1. Frequency of sites contaminated by Legionella species

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>Not contaminated N (%)</th>
<th>Contaminated N (%)</th>
<th>Total N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot water tanks</td>
<td>13 (39.4)</td>
<td>20 (60.6)</td>
<td>33</td>
</tr>
<tr>
<td>Cooling towers</td>
<td>5 (50.0)</td>
<td>5 (50.0)</td>
<td>10</td>
</tr>
<tr>
<td>Showers</td>
<td>12 (32.4)</td>
<td>25 (67.6)</td>
<td>37</td>
</tr>
<tr>
<td>Faucets</td>
<td>9 (34.6)</td>
<td>17 (65.4)</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>39 (36.8)</td>
<td>67 (63.2)</td>
<td>106</td>
</tr>
</tbody>
</table>

Although other important factors such as the age and physical composition of the water distribution systems could not be included in the model because this information was not available for every hospital, we took into account the intrahospital correlation for both analyses by using the random-effects model.18,19

RESULTS

Description

Of the 106 water samples examined, 67 (63.2%) were positive for Legionella species: 60.6% of hot water tanks, 67.6% of showers, and 65.4% of faucets were contaminated. Table 1 shows the distribution of the contaminated sites. Of these water samples positive for Legionella species, 24 (35.8%) contained between 50 and 10^3 cfu/L, 34 (50.7%) between 10^3 and 10^5 cfu/L, and 9 (13.5%) above 10^5 cfu/L.

Tables 2 and 3 show the physicochemical characteristics of the water and the presence of amoebae in Legionella species-positive and -negative samples. Amoebae were detected in 73 of 106 (68.9%) samples examined and in 58 of 67 (86.6%) Legionella species-positive samples (P < .002). In these positive samples, conductivity was below 500 μΩ⁻¹·cm⁻¹ in 58.2% (P = .026), temperature below 50°C in 80.6% (P = .004), and hardness was significantly higher (P = .002) than in Legionella species-negative samples. Temperature ranged from 15°C to 72°C (mean, 41°C; 95% CI: 38.4°C-43.7°C). Legionella species isolation frequencies in water samples with 2 temperature ranges were 80.6% (15°C-49.9°C) and 19.4% (50°C-58.8°C).

Legionella species and amoebae were not isolated from any sampling point in which the water temperature was above 58.8°C. Free chlorine and total residual chlorine were not examined in the analysis because their concentrations were very low and not different from one sample to another. In the 2 models studied, predictive variables in the presence of Legionella species were very different from predictive variables in the multiplicity of Legionella species.

Analysis of predictive variables in Legionella species-positive and in Legionella species-negative samples

First, predictive variables associated with the presence of Legionella species were selected (Wald test; P < .25): temperature (P = .004), presence of amoebae (P < 10⁻⁴), hardness (P = .002), and conductivity (P = .029). The association between the presence of legionella and these predictive variables was determined by logistic regression analysis. After applying a forward likelihood ratio procedure, the final model included predictive variables such as hardness and presence of amoebae (Tables 4 and 5). The risk of having Legionella species-positive samples was significantly higher when hardness increased by 0.2 mmol/L (P = .031; OR, 1.16; 95% CI: 1.01-1.33). If hardness increased by 2 mmol/L, the odds ratio estimate was 4.4. The risk of finding Legionella species-positive samples was significantly higher when amoebae were present (P < 10⁻⁴; OR, 16.29; 95% CI: 3.81-69.74).

Analysis of variation of log-transformed concentrations of Legionella species and the same predictive variables in Legionella species-positive samples

First, the predictive variables associated with log-transformed concentrations of Legionella species were selected (F test; P < .25): sampling sites (P = .044), temperature (P = .033), hardness (P = .163), and pH (P = .029). The association between log-transformed concentrations of Legionella species and these predictive variables was determined by linear regression analysis. After applying a forward likelihood ratio procedure, the final model included the predictive variables sampling site, pH, and temperature (Tables 6 and 7). High Legionella concentrations were significantly associated with showers and tanks (P = .016 and P = .012, respectively), whereas this was not the case with faucets and cooling towers. Legionella concentrations increased significantly with pH (P = .026) and decreased significantly as the temperature rose above 50°C (P = .012).

DISCUSSION

Legionella species and amoebae were common in the hospital water distribution systems studied here (63.2% and 68.9%, respectively), thus confirming other investigations with various water samples from hospital plumbing systems.12,13 Water quality had an important role on the presence and proliferation of Legionella species. Certain water characteristics such as hardness or the presence of amoebae were associated with the presence of Legionella species but did not promote their
proliferation. Conversely, temperature and pH were not correlated with the presence of *Legionella* species but did promote their proliferation. For example, the presence of amoebae may introduce *Legionella* species into the environment then the *Legionella* species may multiply if certain conditions are met such as temperature between 25°C and 50°C. Moreover, high hardness and the presence of amoebae are strongly correlated statistically with the presence of *Legionella* species when showers, tanks, pH, and temperature promote their proliferation.

An important factor related to the multiplication of Legionella is the temperature of the water, which is a major determinant of Legionella colonization. Linear regression analysis indicated an inverse relationship between circulating hot water temperature and the presence of Legionella in hot water systems, so circulating hot water must reach 50°C before a significant reduction in the isolation of Legionella can be achieved. Neither Legionella nor amoebae were found when circulating water temperature was above 58.8°C. Because Legionella growth is maximal between 25°C and 45°C, lagging cold water piping to maintain the temperature below 20°C is mandatory. It might be expected that raising the water temperature to 60°C and flushing all faucets for 15 minutes would create a temperature/time situation in which Legionella would be eliminated from the hot water systems, including the faucets. However, because some patients might suffer scalding, it is essential to install mixer faucets. The French Department of Health provides guidelines for preventing the multiplication of Legionella in hospital water systems. Hot water should be stored at 60°C, and returning water should be maintained at 50°C. Water from any faucet in the circuit should reach 50°C after 1 minute of flushing. Cold water systems should be maintained below 20°C to obviate excessive growth within the systems. When they occur, cases of infection are due to the operating temperature of hot water systems being below that of the suggested guidelines. The maintenance of high-operating temperatures has been largely successful in the elimination of *Legionella* species from hot water systems. However, *Legionella* species can persist despite heat treatment. The failure

<table>
<thead>
<tr>
<th>Physical-chemical and presence of amoebae</th>
<th>Presence of Legionella species</th>
<th>N (%)</th>
<th>N (%)</th>
<th>N (%)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoebae</td>
<td>N (%)</td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>absence</td>
<td>24 (61.5)</td>
<td>9 (13.4)</td>
<td>33 (31.1)</td>
<td></td>
<td>&lt;10^-6</td>
</tr>
<tr>
<td>presence</td>
<td>15 (38.5)</td>
<td>58 (86.6)</td>
<td>73 (68.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conductivity (µS/cm)</td>
<td>N (%)</td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>&lt;500</td>
<td>31 (79.5)</td>
<td>39 (58.2)</td>
<td>70 (66.0)</td>
<td></td>
<td>.026</td>
</tr>
<tr>
<td>≥500</td>
<td>8 (20.5)</td>
<td>28 (41.8)</td>
<td>36 (34.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>N (%)</td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>21 (53.8)</td>
<td>54 (80.6)</td>
<td>75 (70.7)</td>
<td></td>
<td>.004</td>
</tr>
<tr>
<td>≥50</td>
<td>18 (46.2)</td>
<td>13 (19.4)</td>
<td>31 (29.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>39 (36.8)</td>
<td>67 (63.2)</td>
<td>106 (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*χ² test.

**Table 3.** Mean values* and range of physicochemical characteristics of water in *Legionella* species-positive and -negative samples

<table>
<thead>
<tr>
<th>Physical-chemical characteristics</th>
<th>Presence of Legionella species</th>
<th>Mean* 95% CI</th>
<th>Mean* 95% CI</th>
<th>Mean* 95% CI</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>Negative</td>
<td>44.4</td>
<td>39.0-49.7</td>
<td>39.0</td>
<td>36.3-41.8</td>
</tr>
<tr>
<td>Hardness (mmol/L)</td>
<td>Negative</td>
<td>1.72</td>
<td>1.44-2.02</td>
<td>2.48</td>
<td>2.2-2.76</td>
</tr>
<tr>
<td>pH</td>
<td>Negative</td>
<td>7.8</td>
<td>7.7-8.0</td>
<td>7.7</td>
<td>7.6-7.9</td>
</tr>
<tr>
<td>Conductivity (µS/cm²)</td>
<td>Negative</td>
<td>610</td>
<td>390-831</td>
<td>708</td>
<td>562-855</td>
</tr>
</tbody>
</table>

*Values represent arithmetic mean.

**Table 2.** Frequency of physicochemical characteristics of water and presence of amoebae in *Legionella* species-positive and -negative samples

*NS, not significant; S, significant.*

NS, not significant NS; S, significant.

*Values represent arithmetic mean.

**Student t test.**
of the heat treatment has also been attributed to the presence of dead ends of pipe work not reached by the hot water used for decontamination or because some outlets are not frequently used. Moreover, additional control procedures and specific design work are required, such as microfiltration, to minimize the risk of legionellosis in units used for severely immunosuppressed patients.

In 1980, Rowbotham reported that *Legionella pneumophila* was a pathogen for some amoebae. Since then, many reports have confirmed the role of amoebae in promoting the growth of *Legionella* in man-made water environments. In 1990, Breiman et al. showed that, during an outbreak of Legionnaires’ disease that was epidemiologically associated with showering, the presence of amoebae in water samples was predictive of the presence of the epidemic strain of *Legionella pneumophila*. In our study, the presence of *Legionella* species and amoeba was significantly associated (\( P < 10^{-4} \)). The complex biologic relationship between Legionella and amoebae, which is partly temperature dependent, is now well understood. Moreover, the ability of *Legionella* species and free-living protozoa to grow and survive over a wide range of temperatures has been demonstrated. To combat this ecologic interaction in our hospitals, both amoebae and Legionella should be eliminated using water stored at 60°C.

In agreement with the findings of Zanetti et al., Legionella were found at low levels of water hardness and therefore at low concentrations of calcium and magnesium, and their presence was significantly associated with hardness (\( P = .031 \)). Softened water could be used to decrease the risk of finding Legionella in water systems, providing that the water softeners used are regularly cleaned and disinfected. In fact, bacteria frequently contaminate their salt tubs, which could in turn represent a source of water contamination.

In our study, showers and water tanks were significantly more contaminated with Legionella than faucets. Because contamination of showerheads is strongly linked to the presence of Legionella in water heaters and because the presence of these bacteria in the heater is the most important predictor of peripheral outlet contamination, it is important to control water quality in showers, water tanks, and heaters regularly. The flexible tubing of showers, tanks, and heaters is known to promote the stagnation of water and the proliferation of Legionella when not sufficiently used. It has long been known that it is necessary to take off faucet aerators because obstruction to water flows at the faucet level (presence of an aerator) has been linked to contamination of peripheral outlets. In our study, faucets were less contaminated, but we were unable to demonstrate any association between contamination of faucets and the presence of faucet aerators. Cooling towers were also less contaminated, perhaps because,
since 1999, French bylaws have decreed compulsory measures for controlling cooling towers. Although copper tubes in plumbing systems have been linked to contamination of water systems, we were unable to demonstrate such an association in this study. Moreover, because no attempts were made to identify any other bacteria, it is not possible to exclude a relationship between the presence of Legionella and other bacterial species.

In conclusion, this study demonstrates the importance of assessing water quality when evaluating environmental risk factors and in selecting the most appropriate prevention and control measures in hospital water systems. This is important because contamination within these settings has been clearly linked to outbreaks of Legionnaires’ disease.

References