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Diverse Populations of *Legionella pneumophila* Present in the Water of Geographically Clustered Institutions Served by the Same Water Reservoir

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We cultured potable water from seven institutions (six hospitals and one medical school) every 2 weeks for 6 months for *Legionella pneumophila*. All of the institutions were located close to each other and received water from the same freshwater source. Two institutions (the medical school and hospital F, a maternity hospital) never had *L. pneumophila* isolated from their potable water. The remaining five had 17 to 72% of their water samples positive for *L. pneumophila*. Most of the isolates were serogroup 1; however, in hospital B serogroup 5 accounted for 56% of the isolates. Oxford and OLDA monoclonal antibody subtypes of *L. pneumophila* serogroup 1 coexisted in four of the five institutions, while subtype France only was found in one institution. All 10 isolates from this institution lacked plasmids. The other four institutions had *Legionella* populations with plasmid profiles II, III, and VI. Two of these institutions also had isolates with no plasmids. The distribution of the plasmid types was significantly different for all institutions except C and D. The distribution of monoclonal antibody subtypes was significantly different for *L. pneumophila* isolates recovered from institutions C and D. There were no characteristics that distinguished the culture-positive institutions from the culture-negative areas. We conclude that diverse populations of *L. pneumophila* exist within these institutions despite their geographic proximity and identical potable water source.

Legionella pneumophila was first isolated in 1977 (28). At that time it was epidemiologically linked to an outbreak of pneumonia (Legionnaires' disease) among American Legion delegates attending a convention in Philadelphia, Pa. In retrospect, it was apparent that this microorganism had been responsible for an outbreak of nosocomial pneumonia at St. Elizabeth's Hospital in Washington, D.C., in 1965 (40). Since then, *L. pneumophila* has emerged as an important nosocomial pathogen (16, 29, 39, 45). Contamination of the potable water of a hospital by members of the family *Legionellaceae* has in many instances been linked to cases of nosocomial Legionnaires' disease (18, 33, 39), and eradication of the *Legionella* spp. from the water has led to cessation of cases of nosocomial Legionnaires' disease (4, 21). However, there are also instances in which members of the *Legionellaceae* are present in an institution's potable water and no disease occurs among its patients (31). This is especially true in relationship to community-acquired sporadic cases of Legionnaires' disease (1). In the study of Arnow et al., *L. pneumophila* was isolated from water in 32% of households in a Chicago, Ill., neighborhood; none of the 95 participants had pneumonia, and only 1 had an antibody titer to *L. pneumophila* of >1:256 (1).

Five health care institutions in the city of Halifax, Nova Scotia, Canada, have had at least one case of nosocomial Legionnaires' disease. Two of these have had a significant number of such cases. All of these institutions receive their potable water from the same source. Cooling towers are not used in Halifax, and from previous studies (26, 27) we have shown that contaminated potable water was the likely source of nosocomial legionellosis in institutions C and E. One institution, F, has never had cases of nosocomial Legionnaires' disease.

We designed this study to examine samples of potable water from these institutions for members of the *Legionellaceae* at regular intervals over an extended period of time. In addition, we included our medical school as a representative non-patient-care institution.

MATERIALS AND METHODS

The institutions. The characteristics of the institutions and their plumbing systems are given in Table 1. The relative locations of the institutions to one another and the mains that supply their water are shown in Fig. 1.

Water supply. The water supply to all institutions is from the same source—Pockwock, a freshwater lake 28 miles from the city of Halifax. Prior to 1978, water was obtained from a freshwater reservoir within the city of Halifax. The water main distribution system to each institution is shown in Fig. 1.

Collection of water samples for culture and chemical analysis. Five sites (three taps, one tub, and one shower) were chosen in each institution. Four of the five sites selected in institution E were from the North Wing. This wing was known to be positive for *L. pneumophila*. Water samples were obtained by turning on the hot- and cold-water taps so that the water flowed slowly. The hot- and cold-water systems were not sampled separately. Two hundred milliliters of water was then collected into a sterile bottle containing 0.1 ml of a 10% solution of sodium thiosulfite. Water samples from all institutions except C were collected on the same day of the week between 0730 and 0930 h. Sampling began in January 1990 and continued at 2-week intervals

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TABLE 1. Characteristics of the institutions studied

Characteristic[a]	Institution						
	A	B	C	D	E	F	G
Yr built	1977	1970	1945, 1967	1930, 1962	1950	1962	1964
Horizontal hot water tank	No	Yes	No	No	Yes	Yes	Yes
Avg temp of hot water (°C)	62	60	60	65	63[b]	46	60
Avg temp of hot water at faucet (°C)	48	43	50	54	60	39	60
Cases of Legionnaires' disease	Yes	Yes	Yes	Yes	Yes	No	No
No. of cases of Legionnaires' disease in last 8 yr	1	3	55	1	27	0	0
No. of beds	52	180	800	384	441	126	0
Type of institution	Rehabilitation center	Children's hospital	General hospital	General hospital	General hospital	Maternity hospital	Medical school

[a] Each institution's hot water system had total system recirculation.
[b] Superheats to 82°C once monthly; hyperchlorinates water once every 4 to 6 weeks.

until the end of June 1990. The cold water was also sampled as it entered each institution from the mains.

One sample of potable water from each institution was submitted for chemical analysis. This analysis was carried out by the Environmental Chemistry Laboratory of the Victoria General Hospital.

Culture of water for legionellae and amoebae. Water samples (50 ml) were centrifuged at 1,200 × *g* for 20 min. The supernatant was removed, leaving approximately 10% of the original volume, in which the sediment was resuspended. With a sterile pipette tip 0.1 ml of the suspension was removed and used to inoculate the surface of the following media: 5% sheep blood agar; buffered charcoal-yeast extract (BCYE) agar containing 0.1% alpha-ketoglutarate; (GIBCO Laboratories, Madison, Wis.); BYCE agar containing polymyxin, anisomycin, and vancomycin (PAV agar) (42). All plates were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide for 7 days and examined daily. Colonies that morphologically resembled *Legionella* colonies were subcultured onto blood and BCYE agar. Representative colonies of those that failed to grow on blood agar were examined by a direct fluorescent antibody technique (11) employing *L. pneumophila* serogroup 1 to 6 antisera (Mardx, Scotchplains, N.J.). One such colony from each plating was selected for subsequent characterization.

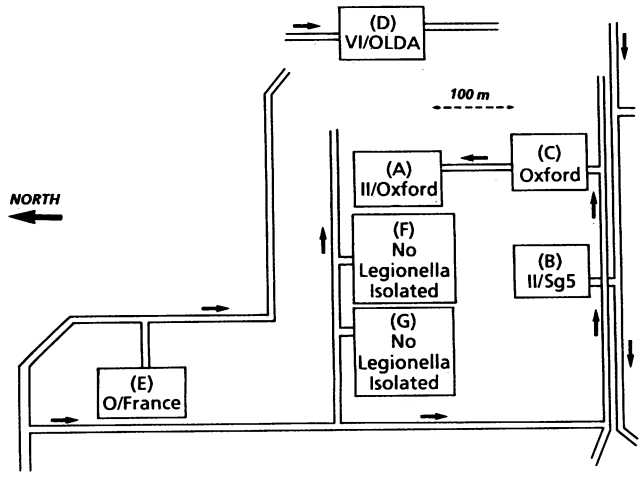

FIG. 1. Relative locations of each of the institutions (A to G) surveyed. The predominant plasmid profile (roman numeral) and MAb type of *L. pneumophila* isolates recovered from the potable water of the indicated institution are shown. In addition, the water mains supplying each institution are indicated. The direction of water flow is indicated by the arrows.

Samples of water, 150 ml in volume, were filtered through 0.45-μm-pore-size filters by using a vacuum pump. The filters were cut in half and placed cell side down on a 1% nonnutrient agar plate preinoculated with *Enterobacter aerogenes*. The plates were incubated at 37°C and examined every second day for amoeba growth by using ×100 magnification (44). Identification of 16 isolates of amoeba was based on characteristics described by Page (30). The size, shape, and number of pseudopodia produced and the character of the movement were noted by using a hanging drop preparation. Monopodial cylindrical amoebae showing steady movement were classed as "limax" amoebae, which includes the hartmannellids.

MAb typing. Isolates of *L. pneumophila* were typed according to their reactivity with a panel of monoclonal antibodies (MAbs) (23). These isolates were typed by Jean Joly, Universite Laval, Quebec City, Canada, who was unaware of the source of the isolates.

Plasmid profiles. Portions of the growth achieved after 48 h of incubation of the same isolates of *L. pneumophila* on BYCE agar were suspended in 0.5 ml of TE buffer (0.5 M Tris-HCl, pH 8.0, 0.02 M EDTA). After pelleting and resuspending in 25 μl of TE buffer, plasmid DNA was extracted from the cells by a modified alkaline sodium dodecyl sulfate procedure (14). The contents of the extracts were determined by electrophoresis with vertical 0.75% agarose gels followed by ethidium bromide staining. Strains with no detectable plasmids constituted profile 0; those carrying a 20-MDa plasmid were plasmid profile II. Profiles III and VI were composed of 96- and 72-MDa plasmids and 100-MDa plasmids, respectively.

Surveillance for cases of nosocomial Legionnaires' disease. All of the hospitals (institutions A to E) have infection control programs and infectious disease consultants. Institution G is not a patient care institute. A patient with nosocomial pneumonia was considered to have Legionnaires' disease if *L. pneumophila* was isolated from pulmonary tissue or respiratory secretions, a fourfold rise in antibody titer to >1:128 by an indirect fluorescent antibody technique was demonstrated, or a direct fluorescent antibody stain of respiratory secretions or pulmonary tissue for *L. pneumophila* was positive (27).

Statistical analysis. The distribution of the various plasmid types of *L. pneumophila* found in institutions A, B, C, D,

TABLE 2. Results of cultures of potable water from institutions A to G and results of characterization of isolates of *L. pneumophila* according to serogroup, MAb type, and plasmid content

Result	Institution						
	A	B	C	D	E	F	G
No. (%) of water samples							
Cultured	60	55	49	60	60	60	60
Positive for *L. pneumophila*	43 (72)	25 (45)	33 (67)	15 (25)	10 (17)	0	0
No. (%) of isolates							
Serogroup 1	42 (96.7)	11 (44)	33 (100)	15 (100)	10 (100)	0	0
Serogroup 5	1 (2.3)	14 (56)	0	0	0	0	0
Typed using MAbs	33	11	8	9	9	0	0
With MAb type:							
Oxford	30 (91)	2 (18)	6 (75)	2 (22)	0	0	0
OLDA	3 (9)	3 (32)	2 (25)	7 (78)	0	0	0
France	0	0	0	0	9	0	0
Examined for plasmids	37	23	29	9	10	0	0
With plasmid profile type:							
0	4 (10)	8 (35)	0	0	10 (100)		
II	30 (82)	10 (43)	10 (34)	3 (33)	0		
III	0	3 (13)	11 (38)	1 (11)	0		
VI	3 (18)	2 (8)	8 (28)	5 (56)	0		
$P < 0.004$							
$P < 0.000$							
$P < 0.001$							
$P < 0.000$							
$P < 0.000$							
$P < 0.01$							
$P < 0.004$							
$P < 0.23$							
$P < 0.000$							

and E was compared by the Fisher exact test for contingency tables (17a). Adjustment was made for multiple comparisons by the Bonferroni method (21a).

RESULTS

One case of nosocomial Legionnaires' disease was detected at institution C only during the 6 months of this study. Table 1 shows the number of cases of nosocomial Legionnaires' disease detected at each institution over the past 8 years.

Five of the seven institutions had *L. pneumophila* isolated from their potable water (Table 2). The percentage of samples positive ranged from 17% (institution E) to 72% (institution A). Most of the isolates were *L. pneumophila* serogroup 1; however, two institutions (A and B) had both serogroups 1 and 5 present. The number of *Legionella* colonies on PAV agar varied from 1 to 97, with a mean of 4 per plate. Only one colony from each sampling was analyzed. In preliminary studies we sampled up to 10 colonies per plate and found that they were homogeneous with regard to their plasmid content. During this and another study (25a) we found that many of the sampling sites consistently yielded *L. pneumophila* of one plasmid type only. MAb typing revealed three subtypes, OLDA, Oxford, and France (Table 2). Subtypes OLDA and Oxford occurred in institutions A, B, C, and D, while institution E appeared to be populated exclusively by subtype France. All 10 subtype France isolates were plasmid free. A variety of plasmid profiles were seen in the remaining institutions. The predominant profile varied for each institution. Plasmid profile II was most prevalent in institutions A and B, while profile III was more prevalent in institution C and profile VI was the predominant type in institution D. There was no obvious correlation between plasmid complement and MAb subtype in any of these institutions.

The two institutions (F and G) that did not have legionellae isolated were on the same branch of the water main system (Fig. 1). Likewise, the two institutions (A and C) with MAb subtype Oxford were on the same branch. These last two institutions had significant differences in the distribution of the plasmid content of their isolates ($P < 0.0000$). Plasmid type II constituted 82% of the 37 isolates typed from institution A compared with 34% of the 29 isolates typed from institution C ($P < 0.004$). Type III isolates were not found in institution A, while they accounted for only 38% of the 29 isolates typed from institution C ($P < 0.000$). Indeed, the only institutions that had a similar distribution of plasmid types in their *Legionella* isolates were institutions C and D. Institutions A, B, and E had distributions of plasmids that were significantly different from each other and from those of institutions C and D. The P values for the various comparisons are as follows: A versus B, 0.004; A versus C, 0.000; A versus D, 0.0017; A versus E, 0.000; B versus C, 0.0008; B versus D, 0.01; B versus E, 0.004; C versus D, 0.23; C versus E, 0.0000; and D versus E, 0.0000.

It is noteworthy that the distribution of MAb types was significantly different among the isolates typed from institutions C and D. Six of the eight isolates from institution C were MAb type Oxford, while seven of the nine from institution D were type OLDA ($P < 0.04$).

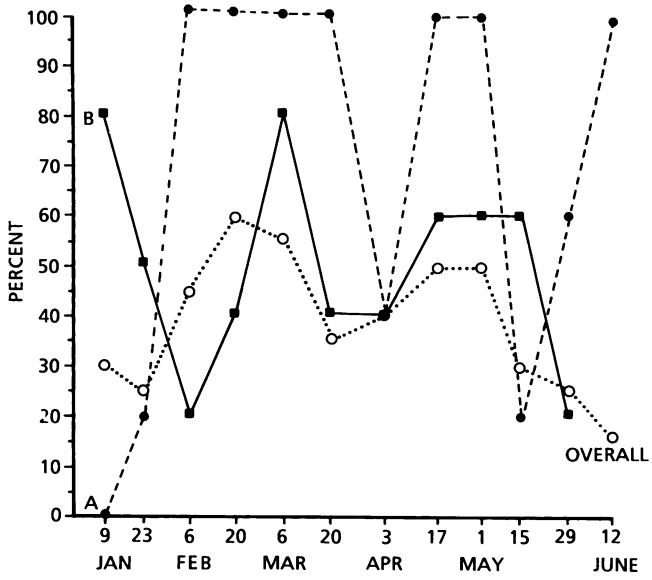

FIG. 2. Percentage of potable water samples from Halifax health care institutions positive for *L. pneumophila* at each sampling time. The overall curve is exclusive of institution C. A and B refer to institutions A and B.

By spreading 0.1 ml of water concentrate on BCYE plates, we were able to obtain a semiquantitative estimate of the number of CFU of *L. pneumophila* present at each sampling time. The values ranged from 1×10^3 to 97×10^3 CFU/liter.

Figure 2 shows the percentage of water samples positive for *L. pneumophila* at each sampling time. Between February and June 1990, two apparent blooms of contamination occurred, bridged by approximately 10 weeks of lower recovery rates (overall curve). This was also evident when each of the five institutions were considered separately (e.g., curves A and B).

The results of the chemical analysis of the water samples from each of the institutions are shown in Table 3. There were no major differences in any of the characteristics examined.

Amoebae were found in the water samples from all of the institutions, including isolation on five occasions from institution A, six occasions from institution B, five occasions from institution C, four occasions from institution D, two occasions from institutions E and G, and one occasion from institution F. Isolates from previously positive sites that appeared morphologically to be the same as previous isolates were not investigated further. Sixteen isolates were identified to the species level. Ten were limax amoebae. The remaining six were polypodial or monopodial with explosive activity. Limax amoebae were isolated from institutions A, B, C, D, and F.

DISCUSSION

We found that five of the seven institutions surveyed had legionellae in their potable water. Despite receiving this water from a common source, there were significant differences in the distribution of the plasmid profiles among the isolates of *L. pneumophila* from four of the five institutions with this microorganism in their potable water. Only institutions C and D had a similar distribution of plasmid types. Interestingly, 75% of the isolates that were MAb typed from institution C were OLDA, while 78% of those from institution D were type Oxford ($P < 0.04$). MAb subtype France was found only in institution E. Isolates displaying profiles 0, II, and VI occurred in institutions A and B, but the latter also harbored plasmid profile III isolates. All isolates from institution E carried no detectable plasmid DNA. Hence, each building appears to constitute a separate ecosystem. This is particularly so with institution E. Its water and patients (unpublished data) had only *L. pneumophila* serogroup 1 of MAb subtype France and plasmid profile 0. This pattern has persisted since 1983, the first year that we sampled water from this institution.

It is known that environmental isolates of *L. pneumophila* differ in virulence for guinea pigs and, by inference, for humans. Both plasmid carriage and MAb reactivity have been reported as being significant in this regard (5, 9, 15, 31). Brown and coworkers (9) suggested that plasmid-containing strains of *L. pneumophila* were less virulent than plasmidless isolates. Plouffe et al. (31) found that a plasmidless strain of *L. pneumophila* caused 24 of the 25 cases of nosocomial Legionnaires' disease at their hospital. In guinea pigs (5), the plasmidless isolate had a 50% lethal dose significantly lower than that of the isolate bearing a plasmid. As a part of their MAb typing study, Brindle et al. (8) observed that 95% of 41

TABLE 3. Results of chemical analysis of potable water from each institution

Characteristic[a]	Institution						
	A	B	C	D	E	F	G
Sodium	3.8	3.7	3.7	3.7	3.7	3.7	3.7
Potassium	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Calcium	14.0	14.0	14.0	14.0	14.0	14.0	14.0
Chloride	7.9	7.9	7.3	7.4	7.7	7.8	8.0
Nitrate + nitrite	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Iron	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Copper	0.04	0.09	0.03	0.05	0.01	0.01	0.06
pH	7.5	7.7	7.5	7.5	7.5	7.6	7.5
Total organic carbon	1.5	1.5	2.0	1.0	1.5	2.0	1.0
Conductivity (μΩ/cm)	92	94	91	90	93	92	94
Zinc	<0.01	0.02	0.01	0.05	0.01	0.01	0.03
Cadmium	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	
Magnesium	0.70	0.66	0.66	0.74	0.63	0.66	0.70
Hardness	37.8	37.6	37.6	38	37.5	37.6	37.8

[a] Unless otherwise indicated (and except for pH), values are reported in milligrams per liter.

clinical isolates of *L. pneumophila* serogroup 1 belonged to the Pontiac group. Stout et al. (38) reported that subtypes Bellingham and Philadelphia were most common among their environmental and patient isolates, respectively. The Pontiac and Philadelphia subtypes react with MAb2, whereas subtype Bellingham does not. This, coupled with the report from Paris (15) that MAb2 recognized 93.8% of 129 clinical isolates but only 35.3% of 85 environmental isolates, has led to the suggestion that reactivity with MAb2 be employed as a marker for virulence.

In the present study, plasmidless strains were detected among environmental isolates in three of the five culture-positive institutions. Institution E yielded plasmidless strains exclusively, whereas they accounted for only 10 and 35% of the strains in institutions A and B, respectively. Of the three serogroup 1 MAb subtypes detected in Halifax, only subtype France recognizes MAb2. Institution E, which had only 17% of its samples positive for *L. pneumophila* (Table 2) but recorded the second-highest number of patient infections (Table 1), was populated exclusively by MAb2-positive and plasmidless strains. In contrast, institution A had only one case of Legionnaire's disease, despite displaying a 72% positivity rate in its potable water. *L. pneumophila* isolates recovered from this institution were all MAb2 negative and predominantly plasmid containing (33 of 37). Thus, the implied relationship between the presence of plasmids and epitopes specific for MAb2 and virulence appears to have some support in our data. However, institution C had both high environmental contamination and patient infections but did not harbor any MAb2-positive or plasmidless strains. While some investigators (20) suggest that only genotypic characteristics be used to define strains of *Legionella*, it is evident that phenotypic characteristics are also useful in defining virulent isolates.

Factors other than bacterial characteristics may play a role in nosocomial legionellosis. For example, institution A is a rehabilitation hospital. Corticosteroid therapy, one of the most important risk factors for nosocomial Legionnaires' disease (25), is rarely used at this hospital. Hospital F is a maternity hospital where all nosocomial infections are uncommon (10). Institutions C, D, and E are all acute-care general hospitals where immunosuppressed patients are common. Hospitals C and E have had a problem with nosocomial Legionnaires' disease; hospital D does not. It is very unlikely that cases of Legionnaires' disease were missed at hospital D, since the same team of infectious disease physicians consult at all these hospitals.

Another factor influencing the spread of legionellae from the potable water to patients may be the physical environment. In residential water systems, hot-water temperatures of <48°C and the presence of electric heaters have been associated with *L. pneumophila* colonization (24). In hospital water systems, hot-water tank temperatures of <60°C, vertical configuration of the hot-water tank, and elevated calcium and magnesium concentrations of the water were factors associated with such colonization (43). The calcium concentration in *Legionella*-positive waters in the study of Vickers et al. (43) was 30 mg/liter, with a range of 3 to 48 mg/liter. In contrast, in our study, the calcium concentration ranged between 11 and 13 mg/liter. States et al. (37) found that organic carbon, turbidity, and concentration of zinc and copper correlated with *Legionella* isolation. At 1.2 to 1.5 mg/liter, the levels of total organic carbon in the waters of our institutions were lower than the 1.59- to 9.09-mg/liter values reported by these workers (37). These workers noted in a previous study that while high levels of a number of metals are toxic to *Legionella* spp., lower levels of iron, zinc, and potassium enhance growth of naturally occurring *L. pneumophila* (36). Thus, concentrations of iron of up to 50 mg/liter and zinc of up to 1 mg/liter are beneficial. Total organic carbon and turbidity are related to nutrient levels in the water (19). The ability of zinc to support *Legionella* growth has been noted by several investigators (32), and *L. pneumophila* is known to be tolerant to high copper levels (22).

We did not recover *L. pneumophila* from the cold water entering each institution from the mains. This may have been related to the small volume of water cultured; however, legionellae have been isolated from natural waters without concentration (6). It is also possible that organisms were in a stressed, unculturable condition at the time of entry to the hospitals and were detectable only after adaptation and amplification within the hospital water distribution systems (12, 17, 35). If this were so, then institutions F and G should also have been colonized. Our observation is in accord with the findings of Colbourne and Trew (13), who noted that only 2 (2.4%) of 62 monitoring taps in 21 supply areas in London, United Kingdom, were positive for *Legionella* spp. They were unable to isolate *Legionella* spp. from raw river water. In contrast, 314 of 1,538 (20%) water samples from hotels, hospitals, and other large buildings in the United Kingdom were positive for *Legionella* spp. (2). There is a suggestion, however, that the branch of the water distribution system may have had some as yet undefined influence on the *Legionella* types isolated. The two institutions that were negative for legionellae (F and G) were on the same branch. Likewise, the institutions (A and C) with MAb subtype Oxford predominating were on the same branch. However, the distribution of plasmid types was significantly different in these two institutions ($P < 0.0000$).

There is a seasonal influence on the rate of isolation of *Legionella* spp. from environmental waters. The isolation rate is higher in summer and autumn than during winter and spring (3, 41). Considerable fluctuation over time occurred in the positivity rates of the potable water in the various institutions in our study. However, given the small number of samples studied on each occasion and our semiquantitative technique, it is difficult to draw any conclusions from these fluctuations.

L. pneumophila multiplies in potable water that contains amoebae but not in filtered amoeba-free potable water (44). A possible role of amoebae in causing Legionnaires' disease associated with shower use has been reported (7). Amoebae were found in the potable water of all seven institutions in our study. Amoebae are natural hosts for legionellae (34) and are important determinants of the multiplication of *L. pneumophila* in some tap water cultures (44). However, it is evident from our study that amoebae occur in potable water in the absence of *L. pneumophila* and that even more importantly, the conjoint presence of *L. pneumophila* and amoebae does not mean that Legionnaires' disease is more likely to occur in an institution.

In conclusion, we have shown that in a small geographic area, five of seven institutions with the same potable water supply were colonized by *L. pneumophila*. There was considerable variation in the type of *Legionella* strains colonizing the different institutions. The difference in rates of disease in the colonized institutions suggests that there are still factors that are as yet unknown that influence the spread of *Legionella* spp. from potable water to humans.

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