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Intracellular Multiplication of *Legionella pneumophila* in Amoebae Isolated from Hospital Hot Water Tanks

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Abstract. We studied the ability of *Legionella* to multiply in potable water samples obtained from investigations of nosocomial legionellosis. Autochthonous *Legionella* multiplied in three of 14 hospital water samples after incubation at 35°C and 42°C. All three samples were from hot water tanks. Multiplication did not occur when a selected sample was filtered through a 0.45-μm membrane and reinoculated with indigenous *Legionella*. We isolated both *Legionella pneumophila* and one or more species of free-living amoebae, primarily members of the *Hartmannellidae*, from each of these hot water tank samples. Amoebae from a total of six hot water tank samples were used for cocultivation studies with *L. pneumophila*. All amoebae supported multiplication of *Legionella* in coculture at 35°C. Four of six isolates of amoebae supported multiplication of *Legionella* at 42°C, while none supported multiplication at 45°C. Gimenez staining and electron microscopy showed that *Legionella* multiplied intracellularly in amoebae. Control of these amoebae in potable water may prevent colonization and multiplication of *Legionella* in domestic hot water systems.

It is generally accepted that legionellae are ubiquitous in freshwater environments. Because the presence of legionellae is not always associated with disease, some environmental factors may allow these bacteria to multiply and achieve an undetermined infectious dose [5]. Several organisms, including protozoa, algae, and possibly non-legionellae bacteria have been reported to increase the concentration of legionellae when cultured together in laboratory tests [1, 3, 7, 9–11, 14, 19–21].

Several aspects of the interaction between legionellae and freshwater protozoa suggest that this is a natural relationship contributing to the multiplication of virulent legionellae in the environment. Previous studies have shown that only virulent strains of legionellae multiply in the ciliated protozoan, *Tetrahymena pyriformis* [4]. Several authors have reported that legionellae infect amoebae and ciliated protozoa and multiply intracellularly in a manner analogous to their growth in human monocytes [1, 4, 9]. In addition, ciliated protozoa and amoebae have been isolated from cooling tower water implicated as a reservoir in an outbreak of Legionnaires' disease. The number of *L. pneumophila* increased when both organisms were cultured together in laboratory tests [2]. Henke and Seidel detected amoebae and *L. pneumophila* in 38% to 88% of ground, drinking, or whirlpool waters tested [8]. Fresh environmental isolates of *Acanthamoeba* from these waters supported intracellular multiplication of *L. pneumophila* in laboratory experiments. Further evidence for the interaction of these organisms in the environment can be found in a recent study by Harf et al., in which amoebae isolated from river water and sediment were washed, lysed, and found to contain *L. pneumophila* [7].

Although intracellular multiplication of legionellae in protozoa has been established in vitro, the potential of such an interaction to increase the risk of legionellosis remains difficult to demonstrate. These studies were undertaken to determine (a) whether legionellae readily multiply in potable waters obtained from outbreaks of legionellosis and (b) whether protozoa are a factor in this amplification process.

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Table 1. Amoebae and *Legionella* isolated from hot water tank samples

Water sample	Temperature at collection	Source	Amoebae isolated	*Legionella* isolated
$SH_2$74	50°C	Hospital	*Echinamoeba exudans*	*L. pneumophila* (SG1)[a]
$SH_2$210	ND[b]	Hospital	*Hartmannella cantabrigiensis* *Hartmannella* spp.[c]	*L. pneumophila* (SG1)
TA10	51°C	Hospital	*Hartmannella cantabrigiensis* *Acanthamoeba culbertsoni* *Hartmannella* spp.[c]	None
TA17	52°C	Hospital	*Hartmannella vermiformis*	*L. pneumophila* (SG1)
PF355	45°–52°C	Hospital	*Hartmannella vermiformis*	*L. pneumophila* (SG1)
WS2	ND	Residential	*Hartmannella* spp.[c]	*L. pneumophila* (SG5)

[a] SG = Serogroup
[b] ND = Not determined
[c] * = Not identified at species level

Materials and Methods

Selection of potable water samples. Fourteen potable water samples were obtained from various sites in hospitals during three separate investigations of nosocomial legionellosis. Fifty-ml aliquots of these waters were incubated at 35°C and/or 42°C and cultured over a 15-day period to detect any increase in the number of indigenous legionellae. All specimens were cultured on selective and nonselective media according to the Centers for Disease Control guidelines for recovery of *Legionella* from water [6]. Because indigenous legionellae multiplied only in hot water tank samples, five of these samples from hospital hot water tanks were selected for further study. Four of these samples contained *L. pneumophila* (serogroup 1). We also included a sample described as a water-grown stock culture of *L. pneumophila* (serogroup 5). This sample was derived from a residential hot water tank and was maintained by periodic passage in sterile tap water as previously described [17, 25].

Isolation of amoebae. Amoebae were cultured by a modification of Singh's method for isolating soil amoebae with a bacterial associate [16]. Nonnutrient agar (1.5% agar and 0.5% NaCl) in 100-mm plates was inoculated with a loopful of *Escherichia coli* (ATCC #25922) prepared after incubation at 35°C for 2–3 days on blood agar plates. Aliquots of 25 μl of sediment were taken from the bottom of one-liter samples contained in Nalge polypropylene bottles and were inoculated onto each quadrant of a nonnutrient agar plate and incubated at 35°C in air. Amoebae were detected by examining the agar surface at a magnification of 100× with an inverted microscope. Cultures were examined for 14 days before being discarded as negative if amoebae were not detected. When amoebae were observed, a small section of the agar surface containing the protozoa was cut out and inverted on a nonnutrient agar plate freshly inoculated with *E. coli*. Amoebae isolates were maintained on these plates and subcultured weekly. Amoebae cultures are designated by the water sample from which they were isolated and, in some instances, contained more than one species of amoebae (Table 1).

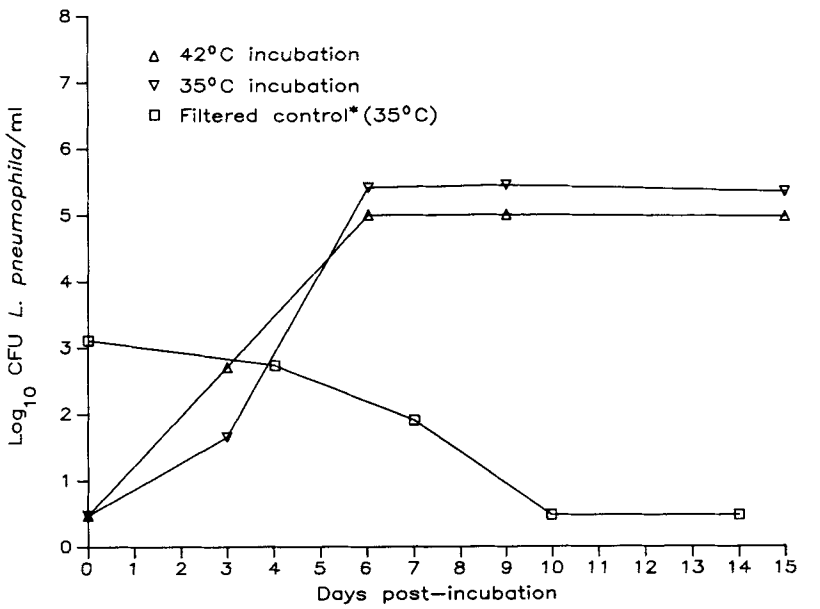
Identification of amoebae. All amoebae isolates were identified by T.K. Sawyer (Rescon Associates, Inc.) Identifications were based on length and width, cyst diameter, length : width ratios, and locomotive morphology, as described by Page [12]. To determine growth rates and the influence of salt concentration on cyst morphology, we incubated cultures at room temperature (20°–23°C) and at 32°C, and cultured on both distilled water agar and low salinity agar (0.3%).

Preparation of cocultures. All cocultures were prepared in 25-cm^2 tissue culture flasks. Three ml of molten nonnutrient agar was evenly distributed over the floor of the tissue culture flask and allowed to cool. The agar surface was overlaid with 0.5 ml of a suspension containing 1×10^{10} *E. coli* cells/ml, which had been killed by a 1-h exposure to cobalt-60 (8.75×10^3 rads/min) in a γ-cell irradiator.

The tissue culture flasks were inoculated with a small agar block from amoebae maintenance plates containing active trophozoites. The flasks were incubated at the desired temperature (35°, 42°, or 45°C), and the proliferation of amoebae was monitored with an inverted microscope. The cultures were incubated from 24 to 72 h until the amoebae had covered most of the agar surface. The numbers of amoebae were approximated by counting the number of trophozoites/field. The flasks were then overlaid with 10 ml of sterile tap water containing approximately 1×10^3 *L. pneumophila* cells/ml (serogroup 1, RI-243 strain). The number of CFU of *L. pneumophila*/ml was determined by culturing 0.1 ml of appropriate dilutions of the coculture supernatant on triplicate plates by the spread plate technique. All cocultures were plated on buffered charcoal yeast extract agar supplemented with α-ketoglutarate, glycine, polymixin B, anisomycin, and vancomycin (GPAVa). This selective medium prevented overgrowth by *E. coli* present in amoebal maintenance cultures [6]. These experiments were performed at least twice for each amoebal isolate.

Electron and light microscopy. Cocultures were harvested for Gimenez staining and electron microscopy after 48–72 h. Tissue culture flasks were briskly shaken to remove adherent amoebae, and the culture fluid was centrifuged in a Fisher 235B microcentrifuge for 30 s. The resulting pellet was then used to prepare smears for Gimenez staining as previously described [4].

When prepared for electron microscopy, the culture fluid was drawn off and the pellet resuspended in Trump's solution (glutaraldehyde, 1% vol/vol; formaldehyde, 4% vol/vol) and



*Filtered control was inoculated with *L. pneumophila* isolated from $SH_2$74 after a single passage on GPAVα agar.

Fig. 1. In situ multiplication of *L. pneumophila* in a hot water tank sample ($SH_2$74).

stored at 4°C overnight. The amoebae were washed twice in 0.2% collidine buffer. Fixed cells were resuspended in molten 2% agarose (45°C) and centrifuged into a cooled agar pellet. The pellets were cut into 1-mm³ sections, postfixed in 1% OsO_4 for 45 min, and stained en bloc with methanolic uranyl acetate for 2 h. The pellets were dehydrated with an alcohol series and infiltrated with propylene oxide and Maraglas plastic. The plastic was polymerized overnight in a 60°C oven. Sections were cut on an Ultracut E Microtome (Reichert) with a diamond knife, picked up on copper mesh grids, and poststained with lead citrate. The sections were then studied with a transmission electron microscope (Philips) at 40 kv.

Results

Legionella multiplied in one water sample from each of the three hospitals. All three of these samples were from hospital hot water tanks. Figure 1 shows the multiplication of indigenous *L. pneumophila* that occurred when one of the samples (SH_2-74) was incubated at 35°C and 42°C. No multiplication occurred when 50 ml of this water sample was passed through a 0.45-μm filter membrane, reinoculated with indigenous *L. pneumophila,* and incubated at 35°C. Autochthonous *Legionella* multiplied in the hot water tank samples from the other two hospitals in a similar manner (data not shown).

We found freshwater amoebae in five hospital hot water tank samples and the water-grown stock cultures. The species of amoebae isolated and the temperature of the water samples at the time of collection are shown in Table 1. All water samples for which temperatures were recorded were between 45°C and 52°C at the time of collection. At least two of the samples contained more than one species of amoebae. Seven of the nine strains identified are members of the *Hartmannellidae,* three of which were not identified at the species level. One hot water tank sample (TA10) contained *Acanthamoeba culbertsoni,* although these amoebae were greatly outnumbered by the more motile *Hartmannella.* No legionellae were isolated from this sample. The amoeba isolated from the water-grown stock culture (WS2) appears to be a new species of *Hartmannella.* This amoeba is very small (5 μm) and is morphologically similar to four species described in the literature; however, none of the identified species forms cysts.

Legionella pneumophila multiplied in coculture with amoebae from all of the samples when incubated at 35°C (Fig. 2). Each line in Fig. 2 represent a single assay; however, each amoebae isolate was tested at least twice with similar results. Each of the cocultures incubated at 35°C contained >50 trophozoites/30 mm². *L. pneumophila* did not multiply in the control flask containing irradiated *E. coli* and nonnutrient agar when incubated at 35°C.

Growth of *L. pneumophila* was supported by amoebae isolated from four of the six water samples when incubated at 42°C (Fig. 3). The two isolates that did not achieve concentrations of ≥50 tropho-

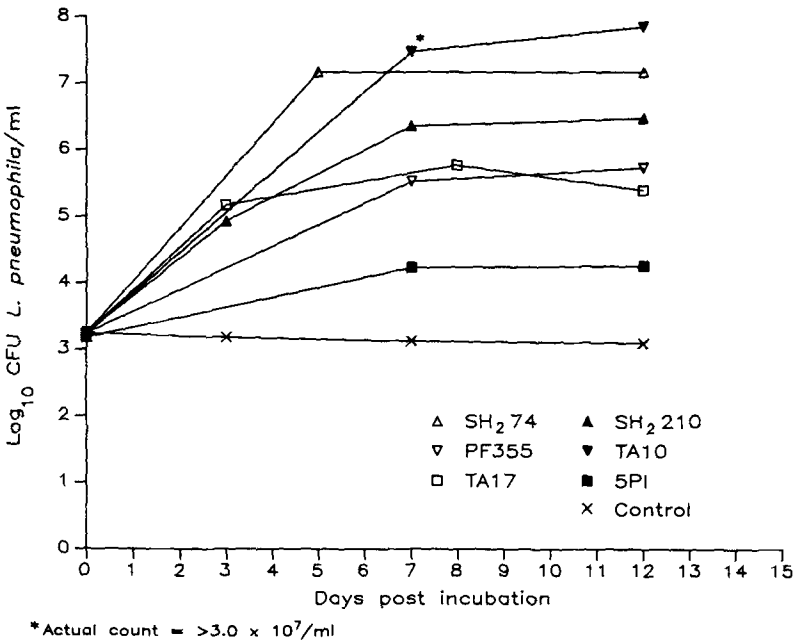


Fig. 2. Multiplication of *L. pneumophila* incubated with amoebal isolates at 35°C.

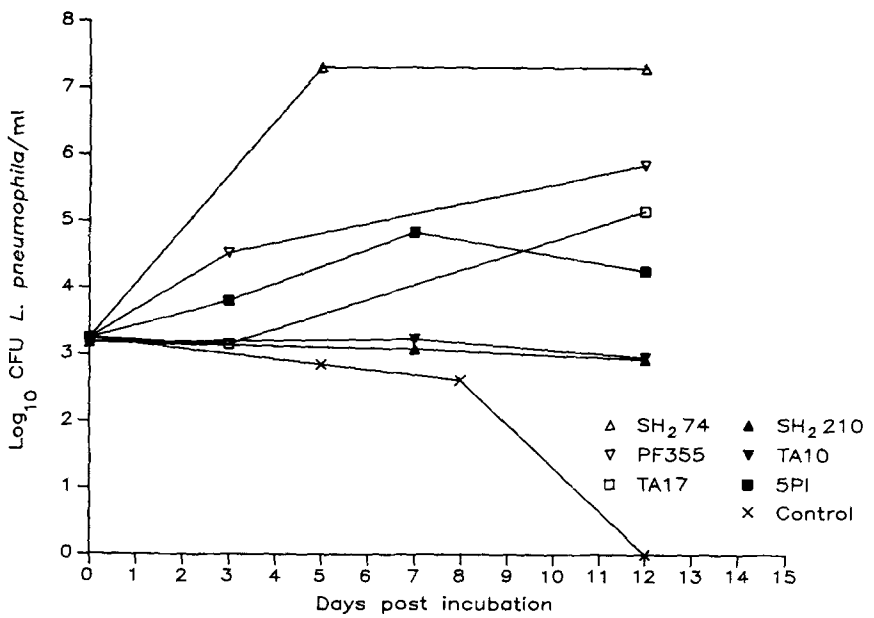


Fig. 3. Multiplication of *L. pneumophila* incubated with amoebal isolates at 42°C.

zoites/30 mm^2 failed to support multiplication of *Legionella*. The amoebae isolates that did not support the multiplication of *Legionella* were tested repeatedly to ensure that failure to detect multiplication was not due to test variability. The control flask incubated at 42°C did not support growth of *L. pneumophila*. All amoebae that supported growth of *Legionella* at 42°C were tested at 45°C. *L. pneumophilia* did not multiply in any of the cocultures at this temperature, although the amoebae grew moderately.

Both Gimenez staining and electron microscopy revealed *L. pneumophila* multiplying within amoebae. Electron micrographs of *Echinamoeba exudans* (SH_2-74) in various stages of infection with *L. pneumophila* are shown in Fig. 4.

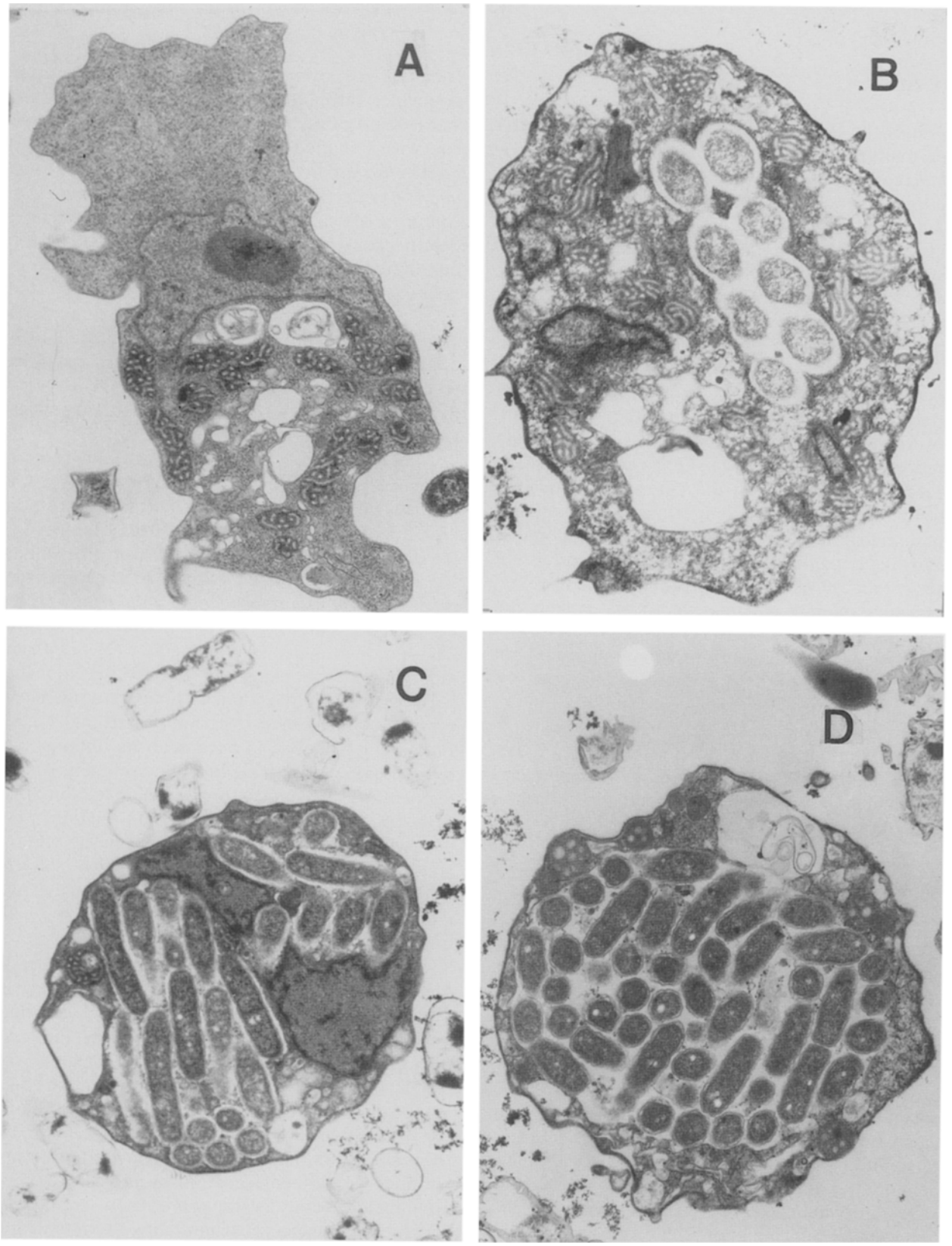


Fig. 4. Electron micrographs of *Echinamoebae exudans* ($SH_2$74) in various stages of infection with *L. pneumophila* (RI-243). (A) Amoebae (trophozoite stage) preparing to ingest a single *Legionella* cell (×12,400); (B) infected trophozoite containing eight *Legionella* cells within a vesicle (×14,375); (C) heavily infected trophozoite containing numerous *Legionella* cells in various arrangements in vesicles (×12,400); (D) amoeba with a single large vesicle containing numerous *Legionella* cells. Note loss of cytoplasmic integrity (×12,000).

Discussion

Each of the potable water samples that supported the multiplication of autochthonous *L. pneumophila* contained freshwater amoebae. In vitro experiments involving *L. pneumophila*, amoebae, and a bacterial associate (*E. coli*) in coculture showed that *L. pneumophila* multiply in the amoebae at 35° and 42°C, while only the amoebae proliferate at 45°C. All of the amoebal isolates supported a significant increase in the number of *Legionella* at 35°C, and four of six amoebal isolates supported significant increases at 42°C (<0.005, t test). Negative results, however, must be interpreted more cautiously. The two amoebal cultures that did not support multiplication of *Legionella* at 42°C were both predominantly *Hartmannella cantabridgiensis*. Preliminary data suggest that this species does not grow rapidly above ambient temperature (personal communication, T.K. Sawyer). Multiplication of *Legionella* in coculture did not occur with any of the amoebae at temperatures above 42°C. However, both bacteria and amoebae were isolated from hot water tanks at temperatures between 45°C and 52°C. We believe temperature variations within hot water tanks account for the difference between the temperatures at which *Legionella* multiplies in coculture and the higher temperatures of waters from which the organisms were isolated. One study has shown that, while temperatures at the center of a hot water tank were 60°C, sludge at the bottom of the tank was only 10°C [15].

The numerical data presented in this manuscript show that *Legionella* multiplies in the presence of these free-living amoebae and not without them. Because intracellular multiplication of *Legionella* in protozoa is a well-documented phenomenon, we relied on electron microscopy to demonstrate intracellular events associated with this infective process. The vesicles containing *Legionella* and the arrangement of the bacteria within the amoebae are consistent with published electron micrographs of protozoa infected with *L. pneumophila* [1, 4, 9]. The micrographs shown in Fig. 4 were taken of cocultures in which *Legionella* were multiplying and show intact *Legionella* cells that appear to be dividing within the amoebae. Microscopic examination by either electron microscopy, Gimenez staining, or both revealed intracellular *L. pneumophila* in all cocultures that supported growth of the bacterium.

All previous laboratory studies of *Legionella* and amoebae have been conducted with either *Acanthamoeba* or *Naegleria* spp. Although we isolated one species of *Acanthamoeba* from one of the hot water tanks, seven of nine amoebal isolates were identified as *Hartmannella* spp. The other isolate was identified as *Echinamoeba exudans* (formerly *Hartmannella exudans*). This strain of *E. exudans* has been deposited with the American Type Culture Collection (ATCC #50171). Hartmannellidae are considered nonpathogenic and are widely distributed in aquatic environments [24]. These amoebae are relatively small (trophozoites range from 5 to 32 μm long, cysts 5–11 μm wide). In a recent study, *Hartmannella, Acanthamoeba,* and *Echinamoeba* spp. were isolated from air samples and air conditioning filters; this implies a capacity for transmission of these amoebae through the atmosphere [24].

Previous studies of the ecology of *Legionella* in potable water systems have failed to identify a specific physical, chemical, or biological factor responsible for growth and survival of this bacterium in these environments [23]. However, several studies have correlated colonization or multiplication of legionellae in hot water systems with temperatures between 30° and 54°C, but not above 58°C [13, 18, 22]. Although these water samples were not examined for amoebae, one study revealed a relationship between microflora, sediment, temperature, and the presence of legionellae in hot water tanks [18]. Free-living amoebae in hot water distribution systems provide an intracellular environment at the temperatures necessary for the multiplication of legionellae. We believe that the in situ multiplication of *L. pneumophila* in the hot water tank samples we studied is due to the free-living amoebae isolated. Further evidence that amoebae are responsible for multiplication of legionellae in water-grown cultures is presented by Wadowsky et al. (in press).

Efforts to control legionellosis by eradicating the bacterium in source water are impractical because, in addition to being ubiquitous, legionellae multiply in the water systems of buildings. Previous attempts to eliminate *Legionella* by chemical and physical procedures on the basis of laboratory data have had limited success [15]. If amoebae provide the primary means by which legionellae multiply, then hot water tanks in which temperatures are stratified would provide an ideal site for both the multiplication and distribution of these organisms. Our findings suggest that inhibiting the interaction of these organisms may be a practical approach to preventing multiplication of legionellae in potable water systems. We are currently evaluating the effect of disinfectants on legionellae and protozoa.

We found that autochthonous *L. pneumophila*

multiplied in some potable water samples obtained during investigations of nosocomial legionellosis. All samples which supported the multiplication of *L. pneumophila* were from hot water tanks and contained free-living amoebae. These amoebae, when cultured with *Legionella* in vitro, amplified the numbers of the bacterium at temperatures ≤42°C. These results describe circumstances by which the risk of nosocomial legionellosis could increase because of amplification of legionellae in hot water distribution systems. Further epidemiologic studies linking the presence of both *Legionella* and amoebae to disease are needed to prove this association.

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