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Impact of Chlorine and Heat on the Survival of *Hartmannella vermiformis* and Subsequent Growth of *Legionella pneumophila*

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***Hartmannella vermiformis*, a common amoebal inhabitant of potable-water systems, supports intracellular multiplication of *Legionella pneumophila* and is probably important in the transportation and amplification of legionellae within these systems. To provide a practical guide for decontamination of potable-water systems, we assessed the chlorine and heat resistance of *H. vermiformis*. *H. vermiformis* cysts and trophozoites were treated independently with chlorine at concentrations of 2.0 to 10.0 ppm for 30 min and then cocultured with *L. pneumophila*. Both cysts and trophozoites were sensitive to concentrations between 2.0 and 4.0 ppm and above (trophozoites somewhat more so than cysts), and 10.0 ppm was lethal to both forms. Hartmannellae treated with chlorine up to a concentration of 4.0 ppm supported the growth of legionellae. To determine whether heat would be an effective addendum to chlorine treatment of amoebae, hartmannellae were subjected to temperatures of 55 and 60°C for 30 min and alternatively to 50°C followed by treatment with chlorine at a concentration of 2 ppm. Fewer than 0.05% of the amoebae survived treatment at 55°C, and there were no survivors at 60°C. Pretreatment at 50°C appeared to make hartmannella cysts more susceptible to chlorine but did not further reduce the concentration of trophozoites.**

Several genera of free-living amoebae, including *Acanthamoeba*, *Vahlkampfia*, *Echinamoeba*, and most commonly *Hartmannella*, colonize potable-water systems (6, 24, 25). *Legionella pneumophila* occupies the same ecological habitat as the amoebae and parasitizes certain species of them (6, 8–10, 19, 20, 24, 25), as well as other protozoa, such as *Tetrahymena pyriformis* (7, 11) and *T. vorax* (22). *L. pneumophila* multiplies inside these organisms, thereby attaining high concentrations in certain freshwater systems, such as cooling towers (5, 24) and hot-water tanks (1, 3, 6, 24, 25).

In a previous study, we have shown that tap water-grown *L. pneumophila* is susceptible to free Cl_2 at 0.25 ppm (15). In potable-water systems like that of the City of Pittsburgh, where a free chlorine residual concentration of 0.75 to 1.5 ppm is maintained for extended periods in water leaving the treatment plant, legionellae might not survive unless some protection is afforded them. Hartmannellid amoebae are indigenous to this system, and legionellae are known to infect these organisms (3, 6, 18, 25). It is therefore likely that these amoebae provide a means by which intracellular legionellae may survive chlorine treatment and be transported to endpoints of distribution lines where conditions are favorable for growth. Knowledge of the chlorine and heat resistance of *Hartmannella vermiformis* may lead to improvements in the control of *L. pneumophila* contamination in potable-water systems.

In the present study, we determined the chlorine and heat resistance of *H. vermiformis* under laboratory conditions and treated hartmannellae with heat to determine whether this would make them more susceptible to subsequent chlorination. An assay based on most-probable-number analysis and the Poisson distribution (12) was developed to determine hartmannella survival following chlorine or heat treatment. The results of this study should be useful in designing treatment protocols for potable-water systems coinhabited by legionellae and hartmannellae.

MATERIALS AND METHODS

Organisms. (i) Bacteria. Stock cultures of *L. pneumophila* serogroup 1 (laboratory designation, E-28) and *Pseudomonas paucimobilis* (laboratory designation, E-29) were stored at −20°C in sterile tap water containing 15% (vol/vol) glycerol (Fisher Scientific, Pittsburgh, Pa.). These bacteria were isolated as described previously (25). Viable counts of *L. pneumophila* were determined by plating samples in duplicate on buffered charcoal-yeast extract (BCYE) medium. Heat-killed *P. paucimobilis* was used as a food source for *H. vermiformis*. Cultures of *P. paucimobilis* on BCYE medium were inoculated into sterile tap water containing 1.5% (wt/vol) yeast extract and 0.5% (wt/vol) ACES buffer (Sigma Chemical Co., St. Louis, Mo.), pH 6.9. Broth cultures were shaken continuously at 37°C for 4 days. *P. paucimobilis* was harvested by centrifugation, washed twice and suspended in sterile tap water to an optical density of 30 to 40 A_{450} units. This bacterial suspension was then autoclaved for 15 min and stored at 5°C for no more than 2 weeks. For use as a food source, the bacteria were diluted to a final concentration of 10^9 heat-killed CFU/ml.

(ii) Amoebae. Cultures of *H. vermiformis* were maintained in water cultures as previously described (22, 23), with a minor modification. The initial concentration of *H. vermiformis* was 10^3 cells per ml. Cultures were passaged into fresh medium every 4 or 5 days, unless they were used for

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chlorine or heat treatment experiments. For these experiments, cultures were either harvested after incubation for 1 day (for a population of >98% irregular, vacuolated trophozoites) or after 8 to 10 days (for a population of >98% round, nonvacuolated, double-walled refractile cysts). Hartmannellae were harvested by scraping the monolayer of cells with a cell scraper (GIBCO, Grand Island, N.Y.). The cells were centrifuged at 750 × *g* for 10 min and washed twice to minimize the chlorine demand. The pellet was resuspended in 5 ml of sterile tap water and adjusted to the desired concentration in sterile tap water. Concentrations of amoebae were determined with a hemocytometer and, where indicated, by the most-probable-number technique (described below).

Cl_2 studies. All of the glassware used was soaked in nitric acid overnight, rinsed with distilled, deionized water, treated with calcium hypochlorite for 24 h, and rinsed again with distilled, deionized water to eliminate any possible Cl_2 demand. Chlorine solutions were prepared for each experiment by adding 0.1 g of calcium hypochlorite to 100 ml of distilled, deionized water and diluted as required. Chlorine concentrations were checked before addition of cells, 5 min after the start of treatment, and at the end of experiments by the *N*-diethyl-*p*-phenylenediamine (Orbeco Analytical Systems, Inc., Farmingdale, N.Y.) method to determine whether any demand was present. Loss of free and total chlorine residuals never exceeded 20 and 10%, respectively, at the end of the experiments. Chlorine was neutralized with a filter-sterilized 10% solution of sodium thiosulfate (Sigma Chemical Co., St. Louis, Mo.). All experiments were conducted at a pH of 7.6 to 7.8 and a temperature of 20°C.

Cl_2 treatment of *H. vermiformis*. Calcium hypochlorite was added to 19-ml portions of autoclaved tap water to provide solutions containing chlorine at 2, 4, 10, and 20 ppm. Autoclaved tap water served as a control. One-milliliter portions of the suspensions of cysts and trophozoites were each added to the test and control solutions. Chlorine concentration was measured and subsequently neutralized after 30 min as described above.

Determination of hartmannella survival by most-probable-number analysis. Chlorine-treated and control samples of hartmannellae were serially diluted in 0.5-log increments with sterile tap water containing 10^9 heat-killed *P. paucimobilis* cells per ml. One-milliliter aliquots of each dilution were added in replicates of six to wells of a 24-well tissue culture plate (Corning Glass Works, Corning, N.Y.). The plates were sealed with tape to prevent dehydration and incubated at 37°C for 7 days. The wells were examined microscopically with an inverted microscope and scored as positive if a monolayer of hartmannella cysts and/or trophozoites was visible. An approximation of the viable mean number of cells per ml was determined by using the Poisson distribution (12).

Posttreatment of chlorine-treated cocultures. Cocultures were prepared from chlorine-treated samples by adding 1 ml of the treated sample to 9 ml of autoclaved tap water containing 10^9 heat-killed CFU of *P. paucimobilis* per ml and 3×10^3 CFU of *L. pneumophila* per ml.

Heat and Cl_2 treatment of *H. vermiformis*. Three-milliliter samples of *H. vermiformis* cysts and trophzoites (2.4×10^5 *H. vermiformis* organisms per ml) were heated separately to 50, 55, and 60°C for 30 min in a water bath, allowed to equilibrate to room temperature, added to 1 ml of a 2-ppm chlorine solution, and treated for 30 min. Hartmannella survivors were determined by the most-probable-number assay, and treated amoeba samples were then cocultured with *L. pneumophila*.

TABLE 1. Effect of chlorine on *H. vermiformis* trophozoites and cysts based on most-probable-number analysis

Chlorine concn (ppm)[a]	Mean no. of *H. vermiformis* organisms/ml (% survival) in following cultures:	
	1 day old[b]	10 days old[c]
0 (control)	6,900 (100)	4,500 (100)
2	454 (7)	2,200 (32)
4	1.1 (0.1)	3.5 (0.1)
10	<0.2 (<0.01)	<0.2 (<0.01)

[a] Contact time, 30 min.
[b] >98% trophozoites.
[c] >98% cysts.

Growth studies of infected and noninfected hartmannellae. Cultures of *H. vermiformis* were prepared as described above. Hartmannellae were counted with a hemocytometer on days 0, 1, 3, 5, and 7.

All experiments were repeated at least three times with less than a 10% difference in the results. The data from representative experiments are reported.

RESULTS

Effect of Cl_2 on *H. vermiformis*. Table 1 depicts the survival of hartmannella cysts and trophozoites following treatment with chlorine for 30 min. With chlorine at 2 ppm, trophozoites (1-day-old culture) were more susceptible than cysts (10-day-old culture). However, at Cl_2 concentrations of 4 ppm and above, trophozoites and cysts were equally susceptible. Figure 1 depicts the growth of non-chlorine-treated *L. pneumophila* in coculture with chlorine-treated *H. vermiformis*. Multiplication of *L. pneumophila* in the chlorine-treated cultures of *H. vermiformis* closely paralleled the extent of killing of amoebae by Cl_2 (Table 1).

Effect of heat treatment and combined heat-chlorine treatment on *H. vermiformis*. When hartmannella trophozoites or cysts were subjected to 55°C for 30 min, fewer than 0.05% of the starting concentration of 2.4×10^5 hartmannellae per ml survived. No survivors were detected in either sample treated at 60°C. Next, when the combined effects of heat and chlorine treatment on both forms of hartmannellae were investigated, the survival percentages of trophozoites and cysts after heat treatment at 50°C for 30 min followed by treatment with chlorine at 2 ppm for 30 min were 0.05 and 1.6%, respectively. While treatment at 50°C reduced the number of trophozoites to 0.04% of their starting concentration, it was not as effective, by itself, in reducing the number of cysts, as 16% survived.

Multiplication studies of infected and noninfected hartmannellae. Figure 2A shows that the concentration of *H. vermiformis* decreased by about 10-fold from days 3 to 7 after infection by legionellae. The percentage of trophozoites remaining in the infected batch was much higher than the number of true cysts (Fig. 2B). It may be that infected trophozoites of *H. vermiformis* have a reduced capacity for or are incapable of encysting. Many of these remaining trophozoites appeared to be structurally different from their healthy counterparts by optical microscopy (data not shown).

DISCUSSION

L. pneumophila is an important pathogen in potable-water systems and is more resistant to chlorine than are coliforms

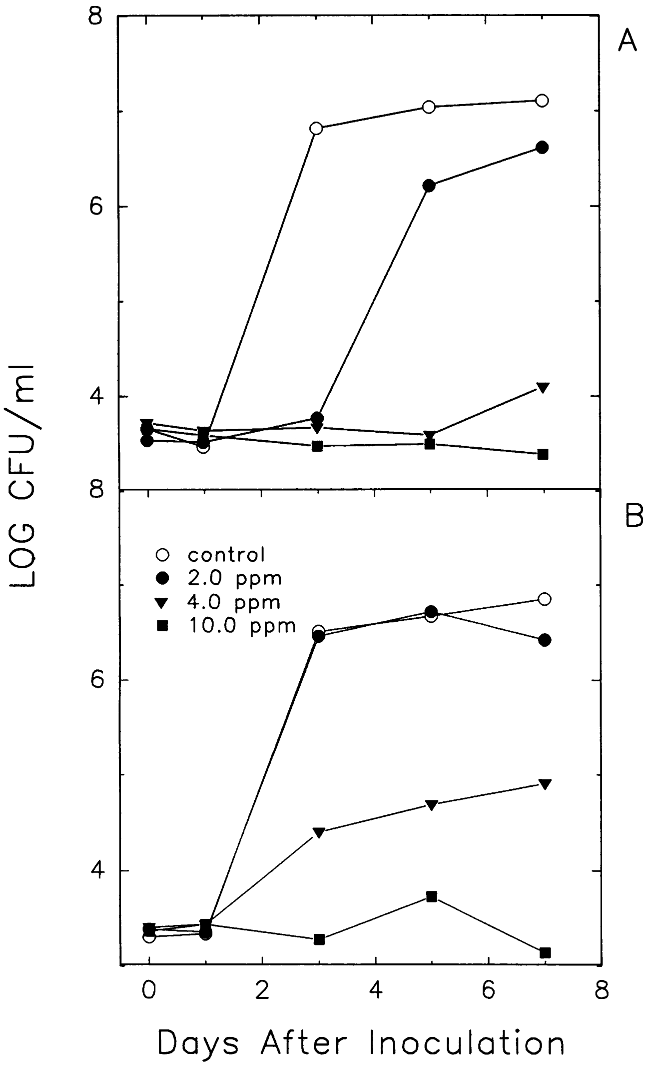

FIG. 1. Effect of chlorine treatment on the ability of *H. vermiformis* to support multiplication of *L. pneumophila* in coculture. Suspensions of *H. vermiformis* trophozoites [A] and cysts [B] were exposed to various concentrations of chlorine for 30 min, the chlorine was neutralized, and legionellae were then a added to the treated suspensions. (Note: the legionellae were not exposed to chlorine.)

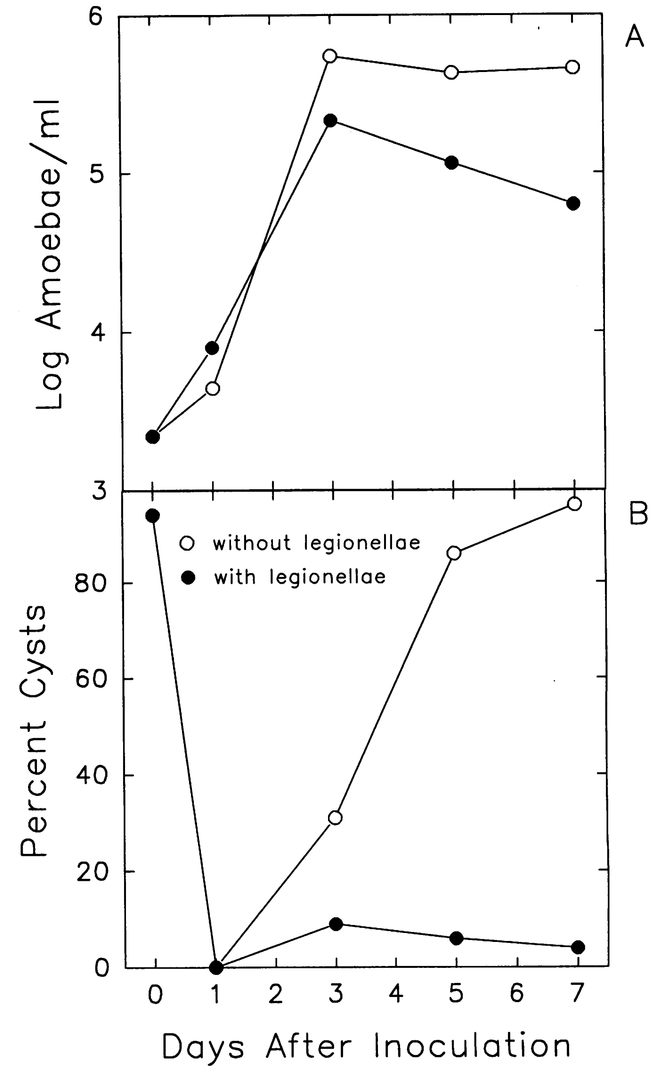

FIG. 2. (A) Growth of *H. vermiformis* (cysts and trophozoites) infected or not infected with *L. pneumophila*. (B) Percentages of *H. vermiformis* cysts in the above infected or noninfected cultures.

and other indicator organisms (15). The growth of legionellae in these systems is supported by several species of freshwater protozoa, including *H. vermiformis* (13, 24). Treatment protocols should therefore be designed to control the growth of both protozoa and legionellae.

The results of this study showed that cysts of *H. vermiformis* are only slightly more chlorine resistant than the trophozoite forms. Cysts were susceptible to 30 min of exposure to chlorine at concentrations of 4.0 to 10.0 ppm, whereas trophozoites were susceptible to chlorine at 2.0 to 4.0 ppm. Neither form survived treatment at a concentration of 10.0 ppm. These results differ from those obtained with *Acanthamoeba polyphaga*, the cysts of which survive chlorine concentrations of greater than 50.0 ppm for 18 h and are at least 50 times more resistant to chlorine than are trophozoites (4, 10).

We next examined the effect of heat on *H. vermiformis* to determine the efficacy of heat as an alternative or addendum to chlorine treatment. Exposure to 60°C for 30 min was lethal to hartmannella cysts and trophozoites. Heat treatment at a lower temperature, 50°C, followed by treatment with a free chlorine residual concentration of 2 ppm, was not significantly different from heat treatment alone (i.e., 0.04% trophozoites survival); however, a greater percentage of cysts survived heat treatment at 50°C (i.e., 16%) than when both treatments were employed. These findings suggest that treatment at 60°C in conjunction with a free chlorine residual concentration above 2 ppm could significantly reduce or eliminate *L. pneumophila* by controlling the population of *H. vermiformis* and perhaps other amoebal hosts.

In our studies of hartmannella growth in coculture with *L. pneumophila*, the total number of hartmannellae was reduced, as was the percentage of cysts in infected cultures compared with cultures of hartmannellae alone (Fig. 2). Preliminary electron microscopy studies of sectioned cocultures in our laboratory did not detect legionellae within cysts of *H. vermiformis*, although *L. pneumophila* organisms were abundantly present in trophozoites (14). Possibly, legionellae are transported through a potable-water system by the trophozoites rather than the cysts. We have been able to maintain cultures of *L. pneumophila* in tap water for several years. However, when we remove the amoebae from these cultures by filtration, the extracellular legionellae usually become nondetectable by culture in about 2 weeks. While some studies have shown that *L. pneumophila* can survive for long periods in sterile tap water (17, 21), differences between the experimental conditions of those studies and ours could account for these discrepancies. In both of these studies, the researchers started with high numbers of organisms, i.e., 10^6 to 10^8 CFU/ml. In addition, strain differences, incubation temperature, and inorganic and organic contents of the water may, individually or in concert, play roles in the long-term survival of these bacteria. Further, no one has periodically taken samples from these amoeba-free cultures and incubated them with food. When we did this, some hospital faucet samples containing free chlorine residual concentrations as high as 0.7 ppm became amoeba positive in 1 to 2 months. Other studies (8) have shown that when the starting numbers of *L. pneumophila* bacteria are closer to those we used, i.e., 10^3 to 10^5/ml, the bacteria become unculturable at day 7.

As pointed out by West et al. (26), culturability of legionellae may be affected by the nutrient shock of going from a nutrient-poor (tap water) to a nutrient-rich (BCYE) environment. Culturability is a complex issue and needs to be further explored.

In hospitals that demonstrate high numbers of legionellae, it is highly probable that amoebae exist. We have recently isolated amoebae from a cold-potable-water system. Therefore, successful control of legionellae in these systems involves control of the more chlorine-resistant amoebae. This idea has also been suggested by Kilvington and Price (10), King et al. (11), and Barker et al. (2) and was originally proposed by Rowbotham (19, 20). Use of heat is one way to control *H. vermiformis* in broth-grown cultures, as demonstrated recently by States et al. (23). Our results indicate that the water temperature should not drop below 55°C, preferably 60°C. If, alternatively, chlorine is used to control amoebae a free chlorine residual concentration of 4 ppm may not be sufficient. Under our conditions, fewer than 10 hartmannella survivors are necessary to support legionella growth. Although we have found hartmannellae to be sensitive to this concentration of chlorine, used alone it may not be a viable alternative to heat treatment. Increased corrosion and mechanical problems would almost certainly occur, creating a condition for the surviving *H. vermiformis* cells to grow and serve as hosts for the multiplication of *L. pneumophila* in the system.

In conclusion, our studies show that *H. vermiformis* trophozoites are slightly more sensitive to chlorine than are cysts but both are readily inactivated at free chlorine residual concentrations above 4 ppm. Although heat (i.e., 50°C) and chlorine (i.e., 2 ppm) are each effective in reducing the concentration of *H. vermiformis*, synergistic activity of both treatments occurs only with cysts. It is important to note that even under these conditions, some *H. vermiformis* organisms survived under our experimental conditions. In a real-life situation, many more amoebae may survive in biofilms, corrosion-induced inclusions in pipes, or other high-demand or cooler regions in a potable-water system.

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