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Multiplication of *Legionella pneumophila* in Unsterilized Tap Water

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Naturally occurring *Legionella pneumophila*, an environmental isolate which had not been grown on artificial medium, was tested for the ability to multiply in tap water. A showerhead containing *L. pneumophila* and non-*Legionellaceae* bacteria was immersed in nonsterile tap water supplying this fixture. Also *L. pneumophila* and non-*Legionellaceae* bacteria were sedimented from tap water from a surgical intensive care unit. This bacterial suspension was inoculated into tap water from our laboratory. The legionellae in both suspensions multiplied in the tap water at 32, 37, and 42°C. The non-*Legionellaceae* bacteria multiplied at 25, 32, and 37°C. A water sample which was collected from the bottom of a hot water tank was found to contain *L. pneumophila* and non-*Legionellaceae* bacteria. These legionellae also multiplied when the water sample was incubated at 37°C. These results indicate that *L. pneumophila* may multiply in warm water environments such as hot water plumbing fixtures, hot water tanks, and cooling towers.

Water and moist environments may be the natural habitats for *Legionella pneumophila*. These bacteria have been isolated from natural waters such as lakes, ponds, and streams (4, 5, 11), from cooling towers of air conditioning systems (8), and from the plumbing systems of hospitals and hotels (2, 15).

Tison et al. (14) have reported growth of *L. pneumophila* with a mean doubling time of 2.7 h in coculture with cyanobacteria under conditions of illumination. Only slight growth of the legionellae occurred when photosynthesis was inhibited by either dark incubation or exposure to a chemical inhibitor of photosystem II. These investigators concluded that the high rate of multiplication of *L. pneumophila* was dependent on active photosynthesis of the cyanobacteria. Accordingly, under conditions of darkness, which occur in plumbing systems, the cyanobacteria may not be able to support the growth of *L. pneumophila*. Factors other than cyanobacterial photosynthesis may be involved in providing the nutrients for the growth of the legionellae in tap water in plumbing systems. The work of Highsmith et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, Q71, p. 231) suggests that *L. pneumophila* may be able to grow in sterile water that is used for intravenous injection. These investigators found that, after incubation for 6 weeks at 25°C after inoculation with *L. pneumophila*, bottles of the water became turbid. Examination by Gram staining, phase-contrast microscopy, and direct immunofluorescence indicated that there had been an increase in the number of legionellae. However this observation was not confirmed by culturing the water on Feeley-Gorman medium. In contrast, a study on suspensions of *L. pneumophila* in sterile distilled and tap water showed long-term survival of the bacteria and no evidence of multiplication (13). These studies did not approximate natural conditions in that laboratory stock strains of the legionellae were used and the effect of naturally occurring water bacteria, ones that had not been grown on artificial medium, was not assessed.

Recently we developed a selective medium for the isolation of *Legionellaceae* from environmental specimens (17). This medium enabled us to recover quantitatively *Legionella* from specimens which also contained non-*Legionellaceae* bacteria. The non-*Legionellaceae* bacteria in most of the environmental samples from plumbing fixtures and hot water tanks were completely or almost completely inhibited by the medium. The medium has permitted the successful isolation of *Legionella* from environmental samples locally by A. Brown and V. Yu at the Oakland Veterans Administration Hospital and by S. Kominos at Mercy Hospital and in Los Angeles, Calif., by P. Edelstein at the Wadsworth Veterans Administration Hospital (personal communications). Using this medium in the present study, we have been able to determine the ability of naturally occurring *L. pneumophila* to survive and multiply in nonsterile tap water.

MATERIALS AND METHODS

Medium. A modification of a selective medium, glycine-vancomycin-polymyxin B (GVP) agar, was used for the isolation of *L. pneumophila*. The selective medium was composed of buffered charcoal-yeast extract (BCYE) agar (10) containing 0.3% glycine, 5 μg of vancomycin per ml, and 100 U of polymyxin B per ml (17). In this study, bromthymol blue and bromcresol purple, each at a final concentration of 0.001%, were added to the selective medium to permit differentiation of members of the *Legionellaceae* family (16). This medium will be referred to as differential GVP (DGVP) agar.

Water suspensions of *L. pneumophila*. A showerhead from a hospital was used as a source of naturally occurring legionellae for the preparation of one of the bacterial suspensions that was used in the multiplication experiments. We had found that the plumbing system of this hospital was contaminated with *L. pneumophila* serogroup 1. Cultures on DGVP agar of swab specimens of the showerhead had yielded on each plate over 300 colonies with a morphology that was typical for *L. pneumophila*. A total of 145 of these colonies were tested on dye-containing BCYE agar and unsupplemented BCYE agar (no addition of cysteine and ferric pyrophosphate) as described below. All of the colonies grew only on BCYE agar, a finding which was consistent with their being *L. pneumophila*. Five of these colonies were serogrouped as described below and found to be *L. pneumophila* serogroup 1. The showerhead was immersed in cold tap water which had been collected from the hospital and was stored at 5°C. Before the removal of samples, this showerhead tap water was agitated on a magnetic stirrer for 15 min.

A second suspension of bacteria which had been obtained from tap water in a surgical intensive care unit (SICU) was supplied by A. Brown and V. Yu of the Veterans Administration Hospital in the University Health Center of Pittsburgh. The bacteria had been concentrated by them from tap water by centrifugation. The sedimented bacteria were then resuspended in tap water at one-hundredth the original volume. Using DGVP, they had isolated both *L. pneumophila* and *Legionella micdadei* from this suspension (*Tatlockia micdadei* [6] and *Legionella pittsburgensis* [10] have also been proposed as taxonomic designations for *L. micdadei*). We confirmed their results, finding 410 colony-forming units of *L. pneumophila* and 30 colony-forming units of *L. micdadei* per ml. This suspension was stored at 5°C. For the growth experiment, 1 ml of this suspension was inoculated into 54 ml of tap water which had been obtained from our laboratory. This diluted suspension was designated "SICU suspension."

Water, collected from the bottom of a hot water tank in a gymnasium, was found by us to contain 50 colony-forming units of *L. pneumophila* serogroup 1 per ml. In the growth experiments, this water sample was incubated without any addition of bacteria.

Incubation and culturing of water suspensions. Portions (10 ml) of the showerhead and SICU suspensions and 5-ml portions of the hot water tank sample were dispensed into 18- by 150-mm culture tubes. The showerhead and SICU suspension were incubated at 25, 32, 37, 42, and 45°C. The hot water tank sample was incubated at 37°C which was the temperature of the sample at the time of collection and presumably the temperature at the bottom of the tank.

The suspensions were cultured on duplicate plates by the spread plate technique with an inoculum of 0.1 or 0.2 ml. Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) was used to obtain a count of non-*Legionellaceae* bacteria, and DGVP agar was used for a count of the legionellae. Final colony counts were obtained after incubation of the plates for 8 days at 37°C.

Identification of *Legionella* in growth experiments. Colonies were presumptively identified as *L. pneumophila* if they appeared on DGVP agar after 3 days of incubation at 37°C, had a characteristic cut glass appearance and purplish or green irridescence (17), and developed a faint olive green coloration. Selected colonies were confirmed by subculture to BCYE agar containing bromthymol blue and bromcresol purple (16) and to unsupplemented BCYE agar which did not contain L-cysteine or ferric pyrophosphate. Colonies which grew only on the dye-containing BCYE agar were considered to be legionellae. Confirmation was done by testing bacteria from these colonies by the direct fluorescent antibody procedure (1) with reagents that were kindly supplied by the Biological Products Division of the Bureau of Laboratories at the Centers for Disease Control, Atlanta, Ga.

In the multiplication experiments, we tested 23 typical colonies from the plates that were inoculated with the showerhead suspension and 20 from the plates that were inoculated with the SICU suspension. All of these colonies were found to be *L. pneumophila*.

RESULTS

Initially a preliminary experiment was done with the showerhead suspension to assess the ability of naturally occurring *L. pneumophila* to survive in tap water at 5, 25, 37, 42, and 45°C. This experiment showed that the bacterium was able to grow at 37 and 42°C.

A second experiment was designed to evaluate more definitively the effect of temperature on the multiplication of naturally occurring *L. pneumophila*. The showerhead and SICU suspensions were used in this evaluation. Both suspensions were incubated for 35 days. The growth curves of *L. pneumophila* obtained from both of these samples confirmed that naturally occurring *L. pneumophila* multiplied in tap water. The results of this experiment suggested a temperature range of 32 to 42°C for the multiplication of *L. pneumophila* in tap water (Fig. 1). In both samples the greatest increase in viable counts of legionellae, nearly 3 log units, occurred at 37 and 42°C, whereas a smaller increase of about 2 log units occurred at 32°C. The most rapid rate of multiplication during the exponential growth phase was observed at 42°C. Little, if any, growth was obtained at 25°C in both suspensions. The viable counts of *L. pneumophila* may be imprecise for the showerhead suspension at this temperature. Flavobacteria

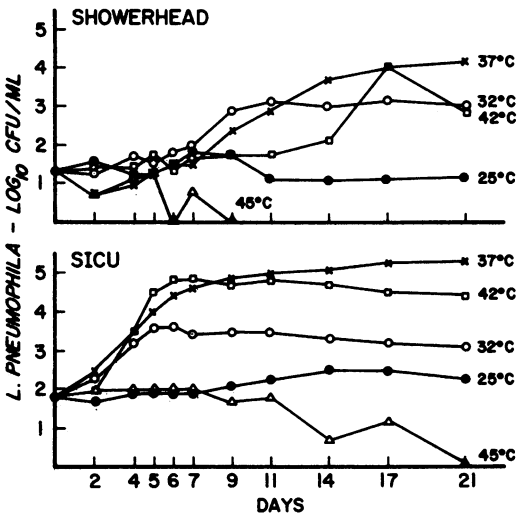

FIG. 1. *L. pneumophila* multiplication in unsterilized tap water.

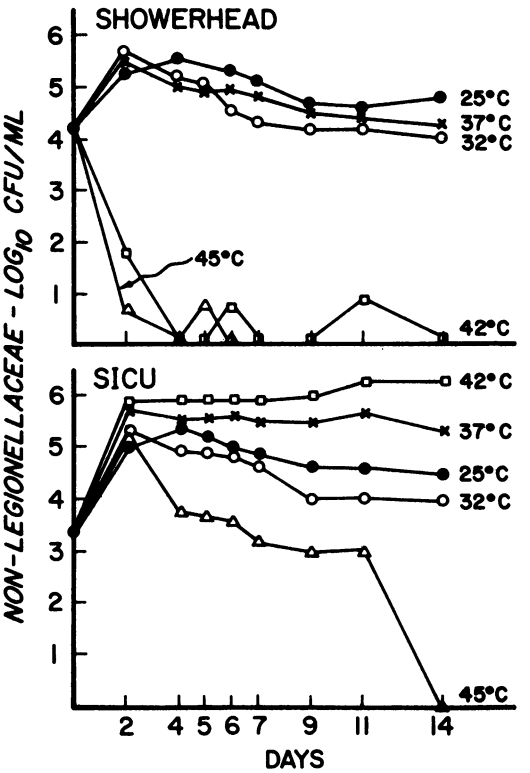

FIG. 2. Non-*L. pneumophila* bacterium multiplication in unsterilized tap water.

which were able to grow on DGVP agar multiplied at this temperature. The large number of colonies of flavobacteria on the plates may have obscured colonies of the legionellae.

As mentioned above, *L. micdadei* had been found previously in the SICU suspension. However we did not recover these legionellae in this experiment.

The major differences that were noted between the showerhead and SICU suspensions were the length of the lag period of growth and the survival of *L. pneumophila* at 42°C after multiplication. The legionellae in the showerhead suspension exhibited a relatively longer lag period, 5 to 7 days at 32 and 37°C and 14 days at 42°C. In the SICU suspension, the legionellae survived at 25, 32, 37, and 42°C for up to 35 days, the last day of culturing. However, they began to die off after 17 days at 42°C in the showerhead suspension. These dissimilarities between the two suspensions may reflect differences in the strains of *L. pneumophila* or differences in the kinds of non-*Legionellaceae* bacteria that were present and having an effect on the legionellae (or in both).

We also measured the multiplication of the non-*Legionellaceae* bacteria in the showerhead and SICU suspensions (Fig. 2). These bacteria in both suspensions multiplied during the first 2 days of incubation at 25, 32, and 37°C. After a gradual decrease in numbers of 0.5 to 1 log, the viable count remained relatively constant for at least 35 days. However at 42 and 45°C, the non-*Legionellaceae* bacteria in the two suspensions behaved differently. They did not multiply in the showerhead suspension at these two temperatures. Instead the viable count decreased rapidly within 2 to 4 days. In the SICU suspension they multiplied at both temperatures during the first 2 days of incubation. The viable numbers remained constant at 42°C for at least 35 days, but declined at 45°C with a rapid decrease occurring between 11 and 14 days of incubation. These results showed that each suspension contained different kinds of non-*Legionellaceae* bacteria.

The water sample from the hot water tank was incubated at 37°C to determine whether the legionellae in this sample could similarly multiply. The results showed multiplication of *L. pneumophila* within 7 days of incubation and a maximum increase of about 2 log units (Table 1). The results in Table 1 also show there was good agreement between the colony counts of duplicate plates, indicating that the spread plate technique provided good replicability for the quantitation of *L. pneumophila*.

DISCUSSION

We have demonstrated that *L. pneumophila* can grow in tap water at 32 to 42°C with an optimum temperature for growth of 37°C. Other investigators (13) may have failed to demonstrate this phenomenon since they used pure

TABLE 1. Multiplication of *L. pneumophila* in water from hot water tank

Time (days)	Dilution cultured[a]	Plate count	Avg CFU[b] ml^{-1}
0	Undiluted	50, 52	51
7	Undiluted	182, 197	1,895
14	10^{-1}	101, 126	11,350
21	10^{-2}	101, 92	9,650
28	10^{-2}	137, 123	13,000

[a] On day 0, 1.0 ml was cultured on each duplicate plate, on subsequent days, 0.1 ml was cultured on each duplicate plate.

[b] Colony-forming units of *L. pneumophila*.

cultures of *L. pneumophila* that had been maintained on artificial medium, employed sterile instead of nonsterile tap water, and incubated the samples at room temperature. Mixed cultures and nonsterile tap water could not be used since the non-*Legionellaceae* bacteria would overgrow the legionellae. Our development of a selective medium for *Legionellaceae* made the present study possible, i.e., culturing tap water suspensions in which *L. pneumophila* was a minor constituent of the bacterial population.

It should be noted that the efficiency of plating of DGVP agar for environmental strains is not known. Although no inhibition of laboratory stock strains was found (17), strains which are adapted to growing in water may vary in their ability to grow on this selective medium. Thus the plate counts obtained in this study may be an underestimation of the viable *L. pneumophila* that were present. Nevertheless the use of DGVP agar has enabled us to obtain evidence that this bacterium can grow in tap water. Our reported increase may be lower than the true one.

Slight, if any, increase in *L. pneumophila* was observed at 25°C, and no marked decline in the viable count occurred at this temperature during the 35 days of incubation of the showerhead and SICU suspensions. These results indicate that either the legionellae were not multiplying or cryptic growth occurred at this temperature, i.e., the multiplication and death rates were similar.

Our findings indicate that *L. pneumophila* may not merely contaminate cooling towers, tap water, and plumbing fixtures but also may grow in these environments, particularly at warm temperatures. The availability of oxygen may have been a growth rate-limiting parameter under our experimental conditions. In cooling towers and in small volumes of water around showerheads, valves, pipes, etc., large air-water surface interfaces would be available for the entry of oxygen into the water medium. In the tubes used in our experiments, the surface available for the diffusion of oxygen into the water would be much lower. It is possible that growth of the bacteria may occur even more rapidly in cooling towers and plumbing fixtures.

The plumbing systems of hospitals in this country may especially be a favorable environment for the legionellae. Hospitals generally have a circulating hot water system, and the Joint Commission on Accreditation of Hospitals has required that hot water emanating from a shower should be no higher than 49°C (7). The situation is compounded by the fact that some hospitals may maintain the hot water at even a lower temperature for energy conservation. One hospital in this Health Center has kept its hot water at 43°C. As a result of these factors, plumbing fixtures in hospitals may attain temperatures that are above 25°C and at or lower than 42°C, thereby permitting the multiplication of *L. pneumophila*. We therefore were not surprised to recover the legionellae in plumbing fixtures, tap water, and water and sediment at the bottoms of the hot water tanks in three hospitals (18).

Experiments are in progress to determine the nature and the source of growth-supporting nutrients for the legionellae in tap water. We postulate that the nutrients are either present in tap water per se or are being supplied by non-*Legionellaceae* bacteria. In the present study, these bacteria were present in both the tap water and the initial inocula of naturally occurring *L. pneumophila*. Other workers have shown that the growth of *L. pneumophila* is supported by cyanobacteria (14) and that these legionellae can multiply in free-living amoebae which are frequently found in water environments (12). It therefore would not be surprising to find that non-*Legionellaceae* bacteria can support the growth of *L. pneumophila*.

Our findings provide a method for studying water-grown *L. pneumophila* and comparing these bacteria to those grown on artificial media, in eggs, and in animals. For example, virulence and susceptibility to disinfectants may be determined. Ormsbee et al. (9) have already shown that prolonged cultivation on an artificial medium reduces the virulence of *L. pneumophila* for guinea pigs. Other investigators have shown that water-grown strains of *Pseudomonas aeruginosa* are more resistant to disinfectants than those grown on artificial medium (3). Therefore, differences between water-grown and artificial medium-grown legionellae in susceptibility to disinfectants would not be unexpected.

About 5% of the environmental samples that we have tested contained non-*Legionellaceae* bacteria which produced overgrowth on DGVP agar. This overgrowth could have prevented the

detection of small numbers of *Legionella*. The rapid die-off of the non-*Legionellaceae* bacteria in the showerhead suspension at 42 and 45°C suggests that incubation at these temperatures should be tested as a means for the selective isolation of *Legionella*. *L. pneumophila* grows at 42°C and survives better than the non-*Legionellaceae* bacteria at 45°C. In preliminary experiments with incubation at 42°C for 1 week, we have been able to isolate *L. pneumophila* from two environmental samples, a suspension of bacteria which had been concentrated from 20 liters of reservoir water by centrifugation and one from a swab sample from a cooling tower. No legionellae could be recovered from the untreated samples. However, selective treatment at 42°C may be inappropriate for specimens containing *P. aeruginosa* and other bacteria which multiply at 42°C. Exposure of these samples for 24 h at 45°C or 30 min at 50°C are additional selective measures which we are presently evaluating. Preliminary results showed that treatment of a transtracheal aspirate and a sputum at either of these two temperatures killed the *Klebsiella* and *P. aeruginosa* that were present. As a result *L. pneumophila* was recovered from both clinical specimens. Further work is being done with additional environmental samples and clinical specimens to evaluate the usefulness of this treatment at elevated temperatures for the isolation of *Legionella* species.

Our inability to isolate *L. micdadei* from the SICU water is unexplained. Since original culturing of the stock specimen had shown that this bacterium was present, we had expected that it might also grow in tap water. We hypothesize that (i) a sampling error had occurred, i.e., the portion of the SICU suspension used in this study did not contain *L. micdadei* since this bacterium was present in low numbers; (ii) *L. micdadei* does not grow or survive well in tap water; or (iii) *L. micdadei* does not compete well with *L. pneumophila* or non-*Legionellaceae* bacteria in tap water.

In conclusion, with the use of a selective medium, we have shown that *L. pneumophila* is able to multiply in nonsterile tap water at a relatively wide temperature range.

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