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Isolation of Protozoa from Water Associated with a Legionellosis Outbreak and Demonstration of Intracellular Multiplication of *Legionella pneumophila*

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At the site of a legionellosis outbreak, amoebae and two ciliates, *Tetrahymena* sp. and *Cyclidium* sp., were isolated from cooling-tower water containing *Legionella pneumophila*. The *Tetrahymena* sp. and the amoebae repeatedly showed the ability to support intracellular multiplication of *L. pneumophila*. Both were isolated from cooling towers specifically implicated as the source for the spread of legionellosis. These protozoa may be reservoirs supporting the survival and multiplication of virulent legionellae in cooling-tower water.

The ingestion of *Legionella pneumophila* by protozoa and the subsequent intracellular multiplication of the bacterium have been reported with amoebae (1, 9) and the ciliate *Tetrahymena* sp. (5). The ability of *L. pneumophila* cells to reside inside free-living protozoa offers a possible mechanism by which these bacteria can persist through adverse conditions and propagate rapidly in favorable conditions. This protective mechanism may explain, at least in part, some of the negative results reported when several biocides were used to treat cooling towers for *L. pneumophila* (4, 7, 10). Also, it could offer a mechanism for legionellae to survive the winter in cooling-tower water and reappear in the spring. To date, epidemic investigations of legionellosis have not included studies to determine if protozoa are present in environmental water samples collected during an outbreak. Since the efficiency of control measures might be influenced significantly by the presence of protozoa that support intracellular multiplication of *Legionella* spp., we undertook a project to determine the presence or absence of such free-living protozoa in water implicated as a source for the dissemination of *L. pneumophila* causing legionellosis.

A simple method for culturing protozoa from water samples was developed and used for testing water collected at the site of an outbreak investigated by Garbe et al. (5a). Samples of 10 ml of unconcentrated water from each of four cooling towers and 10 ml of concentrated (10×) potable water from 18 water faucets were put into sterile individual 50-ml glass jars. A block of nutrient agar (approximately 2 cm^3) was added to each jar. Then the inoculated jars were incubated at 25°C in air and examined with a dissecting microscope (×60 to ×100) for protozoa twice per week.

The water samples were cultured concomitantly for *L. pneumophila* by using buffered charcoal-yeast extract agar (8) containing 0.1% alpha-ketoglutarate (BCYEα) medium and GPAV5α medium, (BCYEα agar plus 0.3% [wt/vol] glycine, 100 U of polymyxin B per ml, 5 μg of vancomycin per ml and 80 μg of anisomysin per ml). When overgrowth by contaminants occurred, water was acid treated before being inoculated onto media (2).

Ciliates and amoebae that were detected were characterized by microscopic observations of live cultures and slide preparations stained with Catton-Lwoff silver impression (3) and trichrome stains, respectively (6, 11).

Axenic cultures of ciliates were obtained by repeated passages in either Elliot medium supplemented with penicillin (10 μg/ml), streptomycin (66 μg/ml), tetracycline (100 μg/ml), trimethoprim (1 μg/ml), rifampin (5 μg/ml), and sulfamethoxazole (19 μg/ml) or sterile tap water containing penicillin (10 μg/ml), streptomycin (66 μg/ml), and heat-killed *Escherichia coli* CDC A9999C3. Background bacterial flora could not be eradicated from samples containing amoebae because the amoebae were sensitive to the antimicrobial agents. The acid treatment of water and use of the GPAV5α medium had to be relied on exclusively for minimizing background flora when the samples from cocultures were plated.

The ability of protozoa to ingest *L. pneumophila* was determined microscopically. Steps in this procedure were (i) incubation of live serogroup 1 *L. pneumophila* cells with fluorescein isothiocyanate-labeled antiserum to serogroup 1 *L. pneumophila*, (ii) removal of excess antibody, (iii) suspension of the cells in sterile tap water, (iv) addition of 0.1 ml of the bacterial suspension to the protozoa test culture, (v) incubation of the bacteria-protozoa mixture at 25°C, and (vi) use of an epi-illumination fluorescence microscope to periodically observe fluorescing ingested bacteria in the vacuoles of the protozoa.

Intracellular multiplication of *L. pneumophila* was deter-

TABLE 1. Protozoa and legionellae isolated from water collected from four cooling towers at the site of a legionellosis outbreak

Cooling tower	Protozoa	Serogroups of *L. pneumophila*
A	*Tetrahymena* sp.[a,b]	1 and 3
	Amoebae[b]	
B	*Cyclidium* sp.[c]	1
C	Amoebae[b]	1
D	None	1 and 5

[a] Isolated before and after initial chlorination and flushing of the tower.
[b] Supported intracellular multiplication of *L. pneumophila* in repeated cocultures.
[c] Initially supported intracellular multiplication, but failed to do so on repeated coculture.

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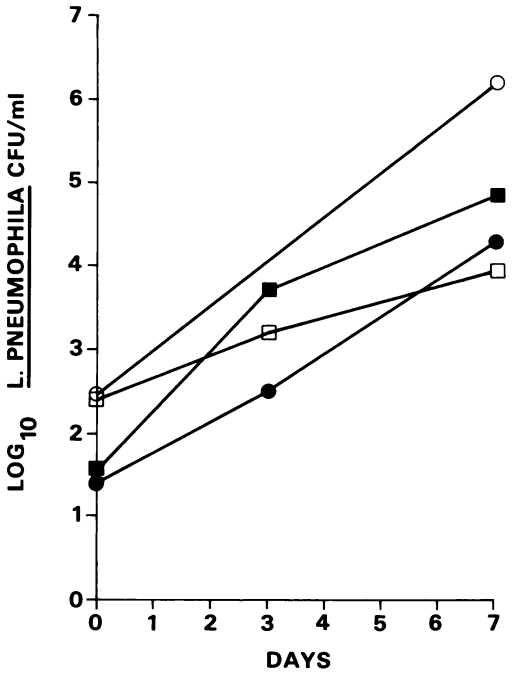

FIG. 1. Multiplication of *L. pneumophila* in coculture with protozoa from cooling towers. Symbols: □, coculture with *Tetrahymena* sp. from tower A before chlorination; ○, coculture with *Tetrahymena* sp. isolated after chlorination; ●, coculture with amoebae from tower A; ■, coculture with amoebae from tower C. All controls without protozoa diminished in CFU from the base-line count (zero time).

mined by modifying and using the coculture system described by Fields et al. (5). Ciliates were concentrated by gravity filtrations through a 1.0-μm-pore Nuclepore filter and suspended in sterile tap water containing approximately 10^3 cells of *L. pneumophila* per ml. The Gimenez stain was also used to observe *L. pneumophila* in the cells (5). Amoebae were either harvested after incubation at 25°C in the agar block medium or grown on a lawn of *Klebsiella pneumoniae* on maltose extract agar before mixing with approximately 10^3 cells of *L. pneumophila* per ml for the coculture system. All cocultures were incubated at 35°C and subcultured after 0, 3, and 7 days onto triplicate plates of BCYEα and GPAV5α agars. The *L. pneumophila* CFUs were recorded after incubation of the plates at 35°C for 4 days, and the average counts were compared with base-line counts (0 h) to detect any increases in growth.

Two types of protozoa and two serogroups of *L. pneumophila* were isolated from two of four cooling towers (Table 1). All 18 potable water samples were negative for protozoa. Towers A and B were implicated epidemiologically as the sources for the spread of *L. pneumophila* for the outbreak (5a). Before batch chlorination, *L. pneumophila* serogroups 1 (predominant) and 3 and a *Tetrahymena* sp. were isolated from tower A. After the tower was flushed, *L. pneumophila* serogroup 1, *Tetrahymena* sp., and a predominant amoeba resembling *Naegleria* sp. were isolated. Another ciliate, *Cyclidium* sp., was isolated from cooling tower B, which contained *L. pneumophila* serogroup 1. An amoeba resembling the one isolated from tower A was also isolated from tower C, which contained *L. pneumophila* serogroup 1. Cooling tower D contained *L. pneumophila* serogroups 1 and 5, but no protozoa were isolated from it.

Except for the *Cyclidium* sp., all of the protozoa isolated

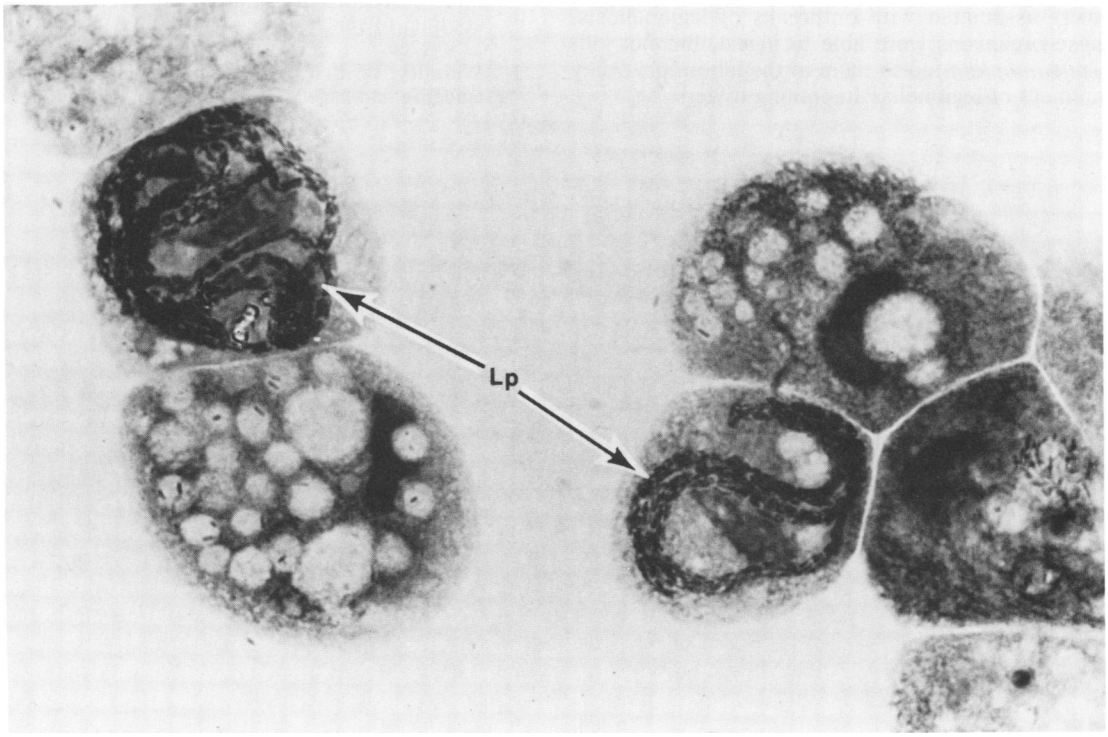

FIG. 2. Intracellular multiplication of *L. pneumophila* in *Tetrahymena* sp. isolated from cooling tower A. Lp, *L. pneumophila* multiplying intracellularly in *Tetrahymena* sp. Magnification, approximately ×3,750.

from the cooling towers were able to ingest *L. pneumophila* and consistently support intracellular multiplication of the bacterium. Within 7 days, there was at least an increase of 100 *L. pneumophila* cells per ml in coculture with *Tetrahymena* sp. or the amoeba (Fig. 1). The micrograph (Fig. 2) of the coculture of *Tetrahymena* sp. isolated from cooling tower A and *L. pneumophila* shows that *L. pneumophila* cells are growing intracellularly in high numbers. The numbers of *L. pneumophila* declined when no protozoa were added to the coculture system.

The precise sensitivity of our procedure to isolate protozoa would be extremely hard to determine under field conditions. However, since an inoculum of 10^2 *Tetrahymena* sp. per ml is sufficient for the propagation of this protozoan in laboratory studies, the sensitivity of the isolation technique should be $\geq 10^2$ *Tetrahymena* sp. per ml.

The combination of the lowest numbers of *L. pneumophila* and *Tetrahymena* cells that can result in intracellular multiplication of *L. pneumophila* is 1 to 30 *L. pneumophila* cells in a suspension of at least 100 *Tetrahymena* cells per ml (5). Thus, environmental conditions supporting intracellular multiplication could exist in epidemic sources such as cooling tower water.

Experiments showing the carry-over of ingested legionellae from one environment or season to another have not been feasible owing to technical difficulties in isolating the protozoa containing legionellae from legionellae suspended in the water.

Amoebae may persist better in cooling towers than *Tetrahymena* sp. because amoebae can revert from the trophozoite stage to the cyst stage and vice versa as environmental conditions change. However, since *Tetrahymena* organisms are ubiquitous in surface water, they must also have sufficient abilities to survive detrimental conditions.

In conclusion, our study documents that protozoa (*Tetrahymena* sp. and amoebae) were present in the water of two cooling towers associated with outbreaks of legionellosis. Because these organisms were able to ingest, but not kill, virulent *L. pneumophila* fed to them in the laboratory, they may be amplifiers of legionellae in cooling towers.

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