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Proliferation of *Legionella pneumophila* as an Intracellular Parasite of the Ciliated Protozoan *Tetrahymena pyriformis*

BARRY S. FIELDS,[1,2] EMMETT B. SHOTTS, JR.,[1]* JAMES C. FEELEY,[2] GEORGE W. GORMAN,[2] AND WILLIAM T. MARTIN[2]

Department of Medical Microbiology, College of Veterinary Medicine, The University of Georgia, Athens, Georgia 30602,[1] and Respiratory and Special Pathogens Laboratory Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333[2]

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In a series of experiments, we have determined that *Legionella pneumophila* will proliferate as an intracellular parasite of the ciliated holotrich *Tetrahymena pyriformis* in sterile tap water at 35°C. After 7 days of incubation, serpentine chains of ~10^3 *L. pneumophila* cells were observed throughout the cytoplasm of the protozoan infected initially with 1 to 30 *L. pneumophila* cells. The overall *L. pneumophila* population increased from ca. 1.0×10^2 to ca. 5.0×10^4 cells per ml in the coculture within this time frame. The interactions between the protozoan and the bacterium appear to depend upon their concentrations as well as temperature of incubation. *L. pneumophila* did not multiply in sterile tap water alone, in suspensions of lysed *T. pyriformis*, or in cell-free filtrates of a *T. pyriformis* culture. In addition to establishing an ecological model, we found that addition of *T. pyriformis* to environmental specimens served as an enrichment method that improved isolation of legionella from the specimens.

Legionella pneumophila has been isolated from cooling towers, evaporative condensers, streams, lakes, hospital shower heads, and sources of potable water (4, 7, 9). The ubiquitous distribution of *L. pneumophila* would appear to contradict the stringent conditions required to grow the bacterium in the laboratory (6). These observations have led some researchers to suggest that *L. pneumophila* is not a free-living aquatic bacterium (13). Tison et al. reported an association of blue-green algae (cyanobacteria) and *L. pneumophila* and proposed that the bacterium was utilizing algal extracellular products as its carbon and energy sources (13).

Rowbotham allowed amoebae of the genera *Acanthamoeba* and *Naegleria* to feed on *L. pneumophila* on an agar surface (12) and observed that they became heavily infected or were lysed. From this he hypothesized that these amoebae could be natural hosts for *L. pneumophila* and could be a potential enrichment mechanism for the bacterium in nature. Tyndall and Domingue subsequently reported the ability of *L. pneumophila* to infect the free-living amoebae *Naegleria lovaniensis* and *Acanthamoeba royreba* on nonnutrient agar plates (14).

This study was initiated to determine whether *L. pneumophila* could react similarly with ciliated protozoa in an aquatic environment. We selected the protozoan *Tetrahymena pyriformis* for study because it (i) is found ubiquitously in the same types of water as *Legionella* spp. (1), (ii) feeds primarily on bacteria, (iii) is easily maintained in axenic culture, (iv) is extremely motile, and (v) is readily available because of its wide use in biological research (10).

MATERIALS AND METHODS

Organisms and preparation of inocula. The *L. pneumophila* serogroup 1 (Philadelphia 1 strain) used in this study had been maintained by guinea pig passage and had never been grown on bacteriological agar. When needed, portions of infected guinea pig spleen tissue which had been quick frozen and stored at −70°C were inoculated onto a buffered charcoal-yeast extract agar plate and incubated for 3 days at 35°C under air and 2.5% CO_2 (11).

The *T. pyriformis* cultures were from a University of Georgia stock strain (no. 500, Midwest Culture Service) grown in Elliot medium no. 2 at 25°C (5). When needed, *T. pyriformis* organisms were collected on a 0.8-μm filter membrane (Millipore Corp., Bedford, Mass.) by gravity filtration, washed, and suspended in sterile tap water. In the experiment designed to determine optimum coculture conditions, some *T. pyriformis* organisms were grown in a suspension of heat-killed *Escherichia coli* (CDC no. A8188) in sterile tap water, and cells were collected as described above.

Microscopy. We examined all cocultures daily with a Zeiss microscope. Slide preparations consisted of a drop of the coculture smeared on a glass slide and stained by the Gimenez technique (8) or with immunofluorescent antiserum to *L. pneumophila* serogroup 1 (3).

Determination of interaction of legionella and protozoa. Two identical tests were performed, each consisting of one coculture, a suspension of *L. pneumophila* (control 1), and a suspension of *T. pyriformis* (control 2). Equal concentrations of the appropriate organisms were suspended in 25 ml of sterile tap water and incubated at 35°C. The numbers of extracellular *L. pneumophila* present at 0, 3, and 7 days were determined by removing 0.1-ml samples from each suspension and culturing them in triplicate on buffered charcoal-yeast extract agar plates. The plates were incubated at 35°C under air and 2.5% CO_2, and the number of CFU was counted after 7 days.

The number of *T. pyriformis* present at these times was determined by placing 0.1 ml of the suspensions on a glass slide and counting the protozoa by visual inspection with a Bausch & Lomb dissecting microscope, (×8 to ×40 magnification).

Determination of nutritional sources. A 2-week-old culture of *T. pyriformis* grown in 25 ml of Elliot medium no. 2 at 25°C was lysed by three consecutive freezings (−20°C) and thawings (25°C). The fragmented cell suspension was centrifuged at 1,500 rpm for 10 min. The supernatant was removed

* Corresponding author.

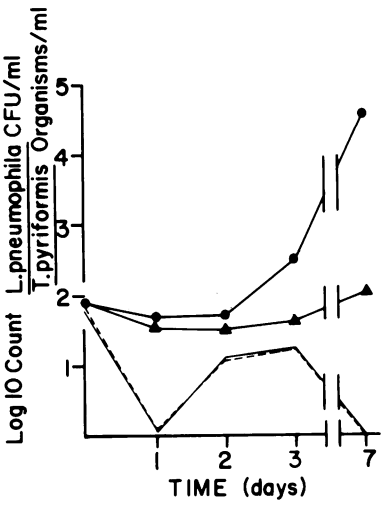

FIG. 1. Effects of coculture on the growth of *L. pneumophila* and *T. pyriformis* at 35°C. Symbols: ●, *L. pneumophila* in coculture; ▲, *L. pneumophila* control; - - -, *T. pyriformis* in coculture; ——, *T. pyriformis* control. Values for this figure are compiled data of two trials.

and filtered through a 0.45-μm Nalgene filter assembly. The resulting pellet of fragmented cells was suspended in 22.5 ml of sterile tap water by vortex blending. An inoculum of *L. pneumophila* (10^3/ml) was added to 22.5 ml of each of the following: the suspension of lysed cells, the filtered supernatant, sterile tap water, and uninoculated Elliot medium no. 2. All were incubated and sampled as previously described to determine the number of CFU of *L. pneumophila*.

Effects of temperature. A suspension of 2.5 ml containing ca. 10^3 *T. pyriformis* organisms per ml was added to 22.5 ml of sterile tap water in each of four test vessels and incubated for 7 days at 25, 30, 35, or 40°C, respectively. The numbers of protozoa present were determined as described above.

Cocultures were prepared by adding 2.5 ml of a suspension containing 10^3 *T. pyriformis* organisms per ml to 22.5 ml of a suspension which contained 2.0×10^2 *L. pneumophila* cells per ml in sterile tap water. One vessel was incubated for 7 days at each of the following temperatures: 25°C, 30°C, 35°C, 40°C. The suspensions were sampled to determine the number of organisms as described above.

Optimization of test conditions. From the results of the first three experiments, we hypothesized that increased multiplication of legionella in *T. pyriformis* coculture could be achieved by (i) prefeeding the *T. pyriformis* on killed bacteria, (ii) increasing the ratio of *T. pyriformis* to legionella, and (iii) preincubating the coculture at 25°C for 2 h before shifting it to 35°C. This was accomplished by adding 24 ml of 10^3 *L. pneumophila* cells per ml in sterile tap water to each of four flasks. One flask (a) was seeded with 1.0 ml of *T. pyriformis* at 10^2 organisms per ml that were grown in a suspension of 1 ml of killed *E. coli* (10^7/ml) in sterile water; two flasks (b, c) each were seeded with *T. pyriformis* at 10^2 organisms per ml grown in Elliot medium no. 2 at 25°C; and the fourth flask (d) was seeded with *T. pyriformis* grown in Elliot no. 2 medium to 10^3 organisms per ml. All cocultures were immediately incubated at 35°C except the coculture in flask c that was incubated at 25°C for 2 h before being shifted to 35°C. Suspensions were sampled to determine the number of organisms as described above.

Enrichment for naturally occurring *L. pneumophila*. Samples (25 ml) from each of 10 water samples associated with an outbreak of Legionnaires disease and stored for 2 to 3 weeks at room temperature (25°C) were inoculated with 0.1 ml of a suspension containing 2.0×10^6 *T. pyriformis*. The samples were incubated at 25°C for 2 h and then held at 35°C for the remainder of the experiment. The samples were cultured for 0, 3, and 7 days by (i) culturing 0.1 ml on each of three plates of BCYE agar and (ii) culturing 0.1 ml on each of three plates of BCYE agar after acid treatment (2). Data were analyzed by the binomial test for determination of statistical significance.

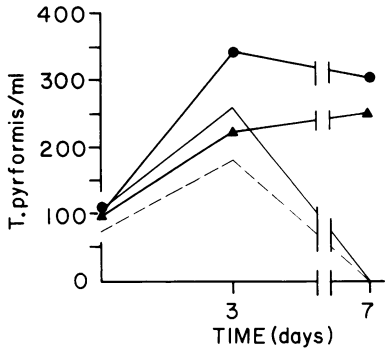

FIG. 2. Effects of temperature on the growth of *T. pyriformis* in axenic culture. Symbols: ●, 25°C; ▲, 30°C; ——, 35°C; - - -, 40°C.

RESULTS

The number of *L. pneumophila* CFU increased by 3 to 4 logs within 1 week in each of the cocultures (Fig. 1). However, *L. pneumophila* did not multiply in axenic culture in sterile tap water. The *T. pyriformis* organisms in both cocultures and axenic cultures were destroyed over a 7-day period. Neither the suspension of lysed *T. pyriformis* cells nor the cell extract of the spent *T. pyriformis* supported growth of *L. pneumophila*. *T. pyriformis* grew best at 25°C in axenic culture and in coculture with *L. pneumophila*. Higher temperatures reduced the ability of *T. pyriformis* to multiply and survive (Fig. 2). Maximum multiplication of *L. pneumophila* occurred in coculture with *T. pyriformis* at 35°C (Fig. 3).

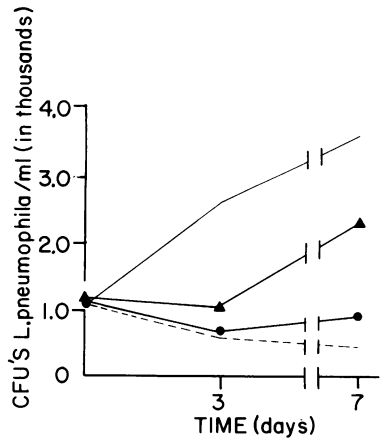

FIG. 3. Effects of temperature on the growth of *L. pneumophila* in coculture with *T. pyriformis*. Symbols: ●, 25°C; ▲, 30°C; ——, 35°C; - - -, 40°C.

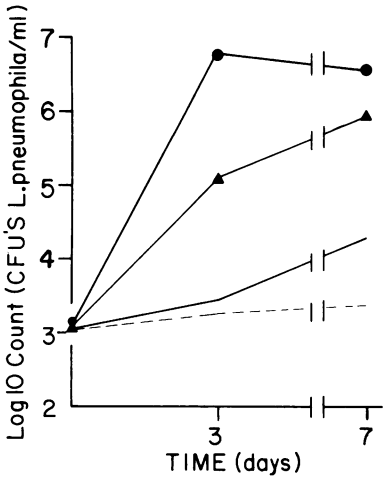

FIG. 4. Effects of various factors on the growth of *L. pneumophila* in coculture with *T. pyriformis*. Symbols: ●, increased concentration (10^3/ml) of *T. pyriformis* (broth grown) at 35°C; ▲, shift in temperature of incubations (25 to 35°C); ——, *T. pyriformis* (broth grown) at 35°C; - - -, *T. pyriformis* (bacteria grown) at 35°C.

Broth-grown protozoa supported greater multiplication of *L. pneumophila* (1 log) than the suspension of bacteria-fed protozoa (Fig. 4). *L. pneumophila* CFU increased by 5 logs when the temperature of incubation of the coculture was changed, whereas increasing the concentration of *T. pyriformis* by 1 log resulted in a final concentration of 6 logs of *L. pneumophila* per ml (Fig. 4).

Using acid treatment and direct plating procedures before enrichment, we found that 5 of the 10 environmental water samples containing naturally occurring *Legionella* species were positive for the bacterium. After 7 days of incubation with *T. pyriformis*, an additional 4 samples were found positive for *Legionella* species, resulting in 9 of 10 positive samples ($P = 0.0625$).

Immunofluorescent staining repeatedly revealed clusters of 10 to over 100 brightly staining bacterial cells both within the cytoplasm of the protozoa and free in the menstruum of the coculture. The appearance of these clusters of cells was simultaneous with the onset of multiplication of *L. pneumophila*.

Gimenez staining (8) of test samples indicated that these clusters of *L. pneumophia* cells were contained in food vacuoles of *T. pyriformis*. These same preparations also revealed many *T. pyriformis* organisms heavily infected with *L. pneumophila* throughout their cytoplasm (Fig. 5 and 6). Some heavily infected protozoa appeared as a sphere of bacterial cells and were often partially ruptured.

DISCUSSION

Our study showed that *L. pneumophila* can infect and multiply within protozoa in an aquatic environment. Detection of large numbers of *L. pneumophila* in both food vacuoles and cytoplasm of *T. pyriformis* and the inability of legionella to multiply on extracellular products or lysed *T. pyriformis* cells support this conclusion. We also determined that temperature had an influence on the overall population of legionella in coculture with *T. pyriformis*. The fact that increased multiplication of *L. pneumophila* occurred when cocultures were incubated at 35°C may have been due to the compromised condition of the protozoa. The initial drop in *T. pyriformis* concentration after 24 h was attributed to the osmotic shock which occurred when the organisms were removed from the culture medium and placed in the sterile tap water. Microscopic examination of these cocultures revealed that many protozoa were greatly enlarged or had ruptured a short time after being transferred to the tap water.

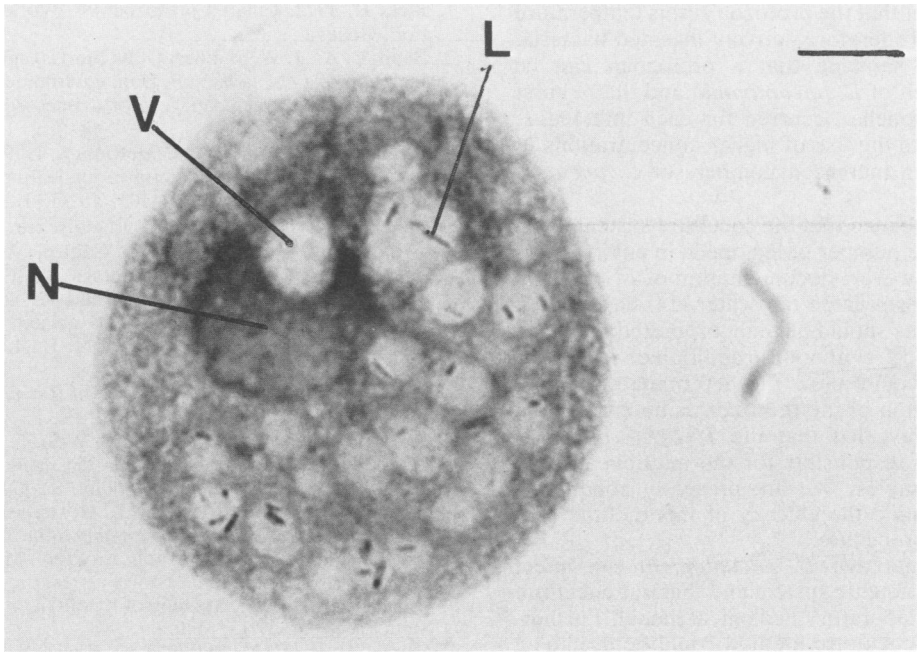

FIG. 5. *T. pyriformis* containing small numbers of *L. pneumophila* within vacuoles. Gimenez stain. N, Nucleus; L, *L. pneumophila*; V, vacuole. Bar, ca. 10 μm.

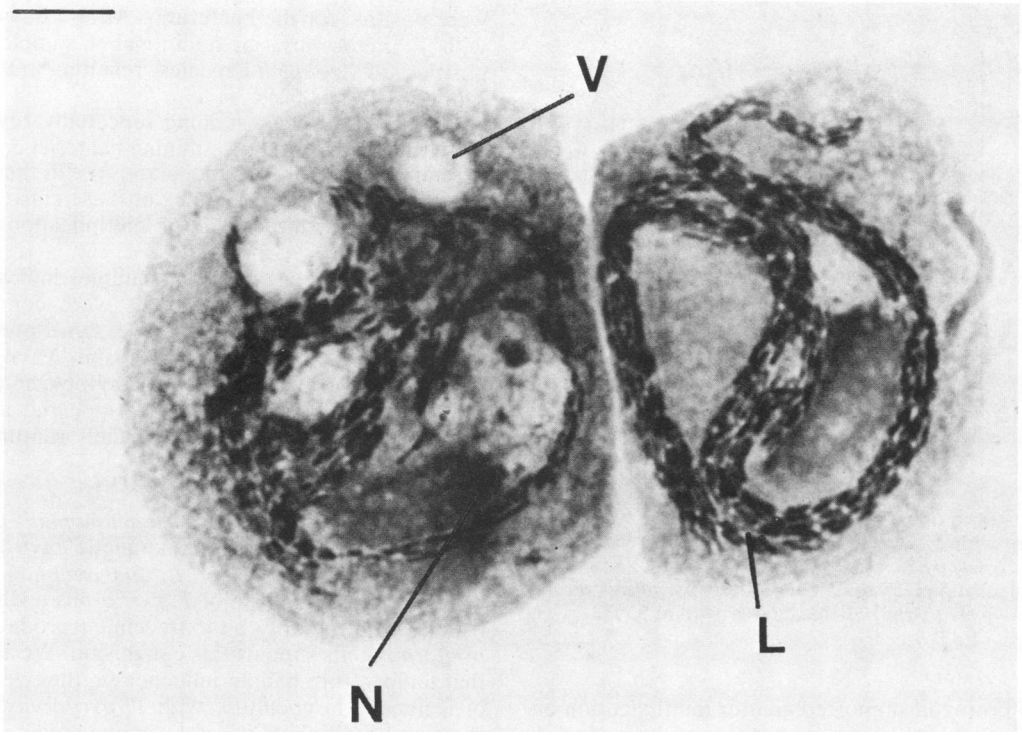

FIG. 6. *T. pyriformis* heavily infected with *L. pneumophila*. *L. pneumophila* are in serpentine chains throughout the cytoplasm of the protozoa. Gimenez stain. L, *L. pneumophila*; N, nucleus; V, vacuole. Bar, ca. 10 μm.

However, surviving protozoa did continue to multiply for a period of less than 7 days. Because incubation at 35°C will eventually destroy the *Tetrahymena* cells, it is difficult to determine if their death is the result of temperature, infection by *L. pneumophila*, or both. In contrast, the increase in the overall population of legionella that resulted when the protozoa were held at 25°C for 2 h before being shifted to 35°C can be explained by the fact that the protozoa at this temperature were very healthy and therefore actively ingested bacteria. Our preliminary test showing that a protozoan can be infected by a single cell of *L. pneumophila* and that a burst size of 10^2 to 10^3 legionella occurred for each infected *T. pyriformis* cell suggests the use of higher concentrations of *T. pyriformis* to obtain increased numbers of *L. pneumophila*.

Our study also determined that the coculture system could be used to increase the number of legionella in environmental water samples. However, documentation of *L. pneumophila* multiplying in unsterilized tap water (14) has made it apparent that these tests should have incorporated duplicate samples incubated at 35°C without the addition of *T. pyriformis*. These controls would allow the determination of the singular effect of addition of the protozoa as an enrichment source. Microscopy revealed that the *T. pyriformis* cells were at least partially responsible for the multiplication of legionella in these samples. We are presently conducting further tests to determine the efficacy of this method as a standard enrichment procedure.

Our study documents that *L. pneumophila* can infect ciliated protozoa in an aquatic system and that our coculture system can serve as a laboratory ecological model. Furthermore, we suggest the coculture method could be used as a laboratory enrichment for legionella in environmental samples.

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