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PRESENCE OF PATHOGENIC MICROORGANISMS IN POWER PLANT COOLING WATERS

Report for October 1, 1979, to September 30, 1981

R. L. Tyndall

ENVIRONMENTAL SCIENCES DIVISION
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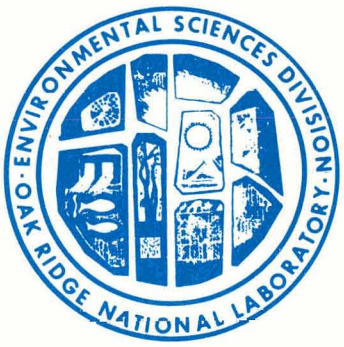

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REPORT FOR OCTOBER 1, 1979, to SEPTEMBER 30, 1981

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ENVIRONMENTAL SCIENCES DIVISION
Publication No. 2060

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Task: Pathogenic Microorganisms in Closed-Cycle Cooling Systems

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ABSTRACT

TYNDALL, R. L. 1982. Presence of pathogenic microorganisms in power plant cooling waters: Report for October 1, 1979, to September 30, 1981. ORNL/TM-5823; NUREG/CR-2980. Oak Ridge National Laboratory, Oak Ridge, Tennessee. 42 pp.

Cooling waters from eleven geographically disparate power plants were tested for the presence of Naegleria fowleri and Legionella pneumophila (LDB). Control source waters for each plant were also tested for these pathogens. Water from two of the eleven plants contained pathogenic Naegleria, and infectious Legionella were found in seven of the test sites. Pathogenic Naegleria were not found in control waters, but infectious Legionella were found in five of the eleven control source water sites. Concentrations of nitrite, sulfate, and total organic carbon correlated with the concentrations of LDB. A new species of Legionella was isolated from one of the test sites. In laboratory tests, both Acanthamoeba and Naegleria were capable of supporting the growth of Legionella pneumophila.

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SUMMARY

Cooling waters of eleven nuclear power plants and associated control source waters were studied for the presence of Legionnaires' Disease Bacterium (LDB) and thermophilic free-living amoebae. Concentrations of LDB were determined microscopically by fluorescent antibody analysis, and infectious LDB was demonstrated by guinea pig inoculation. Presence of pathogenic Naegleria was demonstrated by mouse inoculations. In general, the artificially heated waters showed only a slight increase (i.e., ≤10-fold) in concentrations of LDB relative to source water. In a few cases, source waters had higher levels than heated waters. Infectious LDB was found in seven of eleven test waters and five of eleven source waters. A new species of Legionella was isolated from one of the test sites. Concentrations of LDB in source and test water correlated with the concentration of nitrite, sulfate, and total organic carbon. While all but one test site was positive for thermophilic free-living amoebae, only two test sites were positive for pathogenic Naegleria fowleri. Pathogenic Naegleria were not found in control source waters. Laboratory studies demonstrate that both Acanthamoeba and Naegleria species could support the growth of various Legionella species.

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CONCLUSIONS AND RECOMMENDATIONS

As a result of our studies on the association between thermal additions and the presence of infectious Legionella and pathogenic Naegleria, we conclude that:

(1) Infectious LDB can be detected in water from some cooling tower basins, even though the concentrations of LDB may not be markedly enhanced over those found in source waters.

(2) Infectious LDB can be detected in some source waters that are probably the source of infectious LDB in many of the cooling tower waters.

(3) Most cooling tower waters contain free-living amoebae; a few contain pathogenic Naegleria.

(4) Certain free-living amoebae are capable of supporting the growth of LDB in laboratory tests.

Based on our study, we make the following recommendations:

(1) Consider use of protective devices for plant personnel in close contact with cooling water shown to contain infectious LDB or pathogenic amoebae.

(2) Consider managing public use of and/or exposure to cooling waters known to contain infectious LDB or pathogenic amoebae.

(3) Develop more rapid screening assays for the presence of infectious LDB and pathogenic amoebae so that monitoring for these pathogens can be more easily effected.

(4) Commence aerosol samplings of cooling tower plumes to relate the concentration and infectivity of airborne LDB and amoebae with that found in basin water so that possible public health consequences of the cooling tower plumes can be assessed.

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INTRODUCTION

The recent study on the presence of pathogenic Naegleria in power plant cooling water sponsored by NRC showed that artificial heating of water by power plants can facilitate the propagation and/or persistence of these pathogens (Tyndall et al. 1980). Pathogenic Naegleria can cause rapid, fatal meningoencephalitis. Some Acanthamoebae, also associated with thermally altered waters, can be infectious for man. Both Naegleria and Acanthamoebae are free-living amoebae capable of growing in a dissolved organic media or feeding on gram-negative bacteria.

Recent studies also indicate an association of Legionnaires' Disease Bacterium (LDB) with cooling systems (Tyndall 1982, Dondero et al. 1980). The etiological agent of Legionnaires' Disease is a gram-negative, weakly oxidase-positive, catalase-positive, rod-shaped to filamentous bacteria (McDade et al. 1977, Brenner et al. 1979, Chandler et al. 1978). Detailed serologic studies of cell surface antigens (Cherry et al. 1978), biochemical studies of guanine-cytosine ratios, and DNA homology, as well as gas-liquid chromatographic studies of cellular lipids (Moss et al. 1977), all indicate LDB is not related to other known bacteria. Consequently, it has been classified as a new genus, Legionella, and as a new species, Legionella pneumophila (Brenner et al. 1979).

Legionella pneumophila isolates are antigenically distinguished into six groups called serogroups. These have been designated: serogroup 1, as represented by the Knoxville isolate; serogroup 2 (Togus); serogroup 3 (Bloomington); serogroup 4 (Los Angeles); serogroup 5 (Dallas); and serogroup 6 (Chicago) (McKinney et al. 1978, England et al. 1980, McKinney et al. 1980). In addition to the six serogroups of L. pneumophila there are six other known species of Legionella, i.e., L. gormanii, L. micdadei, L. longbeachae, L. dumoffii, L. bozemanii, L. jordanis (Brenner et al. 1980, Herbert et al. 1980, Morris et al. 1980, and McKinney et al. 1980, McKinney et al. 1981, Cherry et al. 1982). Of the six L. pneumophila serotypes and the six other species of Legionella, all but one were isolated from clinical samples. L. jordanis was isolated from environmental samples (Cherry et al. 1982). In spite of the probable environmental source of most Legionella infections, relatively few studies have characterized the distribution of infectious Legionella in environmental samples.

Studies of the source of infection of some Legionnaires' disease outbreaks at times implicated cooling towers associated with air conditioning systems as the dispersal vehicle. Known outbreaks occurred in hospitals, hotels, a university student center, and the New York garment district. It thus appears that closed-cycle cooling systems may provide optimal habitats for propagation of not only pathogenic amoeba but LDB as well. The largest cooling tower systems, i.e., those of electrical generating stations, had not been previously examined as a source of LDB. A detailed study of cooling towers was thus warranted to determine the potential risk and the features of cooling towers that may

contribute to the growth of the bacterial pathogen. In addition, the interaction of Legionella with Naegleria and Acanthamoebae was studied since these microbes share the same habitat and the amoebae feed on gram-negative rods. By better understanding factors affecting the presence of LDB, we can devise methods for controlling the bacterium.

MATERIALS AND METHODS

Test sites chosen for the study were nuclear power plants with either mechanical- or natural-draft cooling towers. Plants tested included sites A, B, C, D, and E which have natural-draft towers and sites F, G, H, I, J, and K which are cooled with mechanical-draft towers. While all the sites were analyzed primarily for LDB, many sites were also tested for the presence of pathogenic Naegleria. Nearby source waters receiving solar thermal additions only were also tested for the presence of LDB and amoebae. Due to the proprietary nature of the results in this report, the sites are coded and incompletely described.

LDB Analysis

Towers were sampled in cooperation with the environmental control specialist at each facility. At both test and control sites, 3.8-L (1-gal) samples were taken in clean containers from tower basin water without stirring the underlying bottom sediment. The ambient source water samples were collected upstream from plant intakes and were not influenced by plant discharge. After collection, the water was brought to the laboratory for analysis.

Water was centrifuged (Sorvall SS3) at 7000 g's for 30 min at room temperature to concentrate suspended microorganisms. Aliquots of the centrifugates were examined by the direct fluorescent antibody test (DFA) using antisera specific for LDB. The degree of fluorescence was estimated subjectively from 1+ to 4+, with 4+ denoting the brightest fluorescence. Subsamples (0.010-mL) were pipetted into predesignated 7-mm-diam wells on toxoplasmosis slides (Cel-Line Associates, Minotola, N.J.). Smears were air dried, heat fixed, and stained with specific fluorescent antibodies for serogroups 1, 2, 3, and 4; i.e., Knoxville, Togus, Bloomington, and Los Angeles of L. pneumophila. The initial analysis used polyvalent antisera containing antibodies against the four major serogroups of L. pneumophila. Negative controls were fluorescein-conjugated sera prepared from the preimmunization sera of rabbits later immunized with the Knoxville strain (serogroup 1) of L. pneumophila. Test and control antisera were supplied by the Center for Disease Control (Atlanta, Georgia). Samples were viewed by epifluorescence microscopy. The number of fluorescing cells with morphological characteristics of L. pneumophila was counted in 30 to 60 fields at 1000X magnification, and the cell count normalized to the number per liter of unconcentrated sample. The degree of variation in determining concentrations of LDB by this method was tested by counting

triplicate samples taken at 36 separate locations. The results showed a coefficient of variation ($P > 0.98$) of 30 to 59% (Solomon et al., unpublished results).

Two milliliters of 400X concentrations of the 1981 samples were injected intraperitoneally (IP) into uncompromised American breed guinea pigs. The guinea pigs were observed daily for a rise in temperature and for evidence of overt illness. When the animals became ill or moribund, they were sacrificed and necropsied. Peritoneal fluids and spleen and liver tissues were plated on charcoal yeast extract (CYE) agar (Feeley et al. 1978) with or without the addition of brom creosol purple and brom thymol blue (Vickers et al. 1981). Tissues were also plated on yeast extract (YE) agar, brain heart infusion (BHI) agar, and blood agar plates. Freshly cut surfaces of the liver and spleen and smears from swabs of the peritoneal wall or viscera were also imprinted onto microscope slides and examined by DFA staining with conjugates for serogroups 1, 2, 3, and 4 of L. pneumophila.

MICROBIAL AND CHEMICAL ANALYSIS

Samples consisting of 100 to 400 mL of water were filtered through 1.2-μm cellulose membranes. These filters were inverted and placed onto nonnutrient agar plates seeded with a lawn of live Escherichia coli. The plates were incubated at 45°C for analyses of amoebic growth. Other water samples were filtered through 0.45-μm membrane filters kept chilled for chemical analyses by the Analytical Chemistry Division at Oak Ridge National Laboratory. Water samples were also analyzed for number of total aerobic bacteria using an Orion Diagnostica Easicult-TTC test kit.

The agar plates were incubated for 3 to 7 d or until growth of amoebae was observed. Amoebae that grew out at 45°C were tested for their ability to flagellate. All amoebae-flagellates were tested for pathogenicity by intranasal inoculation into weanling ICR mice. Moribund mice were sacrified and the brain tissue was plated on coliform-seeded agar plates. Pieces of brain were also inoculated directly into axenic medium for growth of amoebae.

The chilled, filtered water was analyzed for levels of nitrites, nitrates, sulfates, phosphorus, chlorine, and total organic carbon. Total aerobic bacteria per milliliter of test water were determined by colony counts of the test plates.

AMOEBAE AND LDB INTERACTION

A Los Angeles serotype of LDB originally isolated from a cooling pond at the Savannah River Laboratory was obtained through the courtesy of Dr. Carl Fliermans (Savannah River Laboratory, Aiken, South Carolina). A Knoxville serotype of LDB was isolated from cooling tower water (unpublished results). An isolate of Naegleria lovaniensis

(Stevens et al. 1980) was also obtained from thermally altered waters at the Savannah River Laboratory site, as was Acanthamoeba strain 5334. Acanthamoeba royreba was isolated from cultured human choriocarcinoma cells (Tyndall et al. 1979, Willaert et al. 1978).

The LDB was maintained by passage in yeast extract media (Feeley et al. 1978). Naegleria and Acanthamoeba were maintained by passage in casitone-based media (Willaert 1971) with or without NaCl (CGV and CGVS, respectively) and with 5% fetal calf sera, penicillin, and streptomycin (50 units and 50 mg mL^{-1}, respectively). When amoebae were cocultivated with LDB, the antibiotics were omitted from the casitone media. Cultures of either A. royreba or N. lovaniensis containing approximately 3 x 10^6 cells in 25-cm^2 tissue culture bottles were inoculated with 1 x 10^6 cells of either Knoxville or Los Angeles serotype of LDB. Some cultures were maintained by change of media every 5 d (4.5 mL of fresh media + 0.5 mL of original media). Other cultures were harvested 5 and 24 h after LDB exposure. The cells were suspended in the 5.0 mL of culture fluid, frozen at -70°C, thawed, and titrated in yeast extract broth to determine concentrations of viable LDB. In some cases nonnutrient agar plates were spread with a lawn of either E. coli or LDB and used as a food source for various amoebae. Equivalent Klett units of LDB and E. coli were used, and the centers of the test agar plates were inoculated with approximately 1 x 10^5 amoebae.

Amoebic, and in some cases LDB, pathogenicity was tested by intranasal inoculation of 5- to 6-week-old ICR mice. Mouse temperatures were monitored daily with a rectal thermometer. The mice were inoculated with approximately 1 x 10^6 amoebae and/or 1 x 10^6 LDB. On necropsy, lung and brain tissues were removed, examined, and placed on E. coli-seeded, nonnutrient agar plates to determine the presence of viable amoebae. Pathogenicity of LDB was also tested by intraperitoneal inoculation of male American breed guinea pigs with 10^9 and 10^{10} bacteria. Rectal temperatures were monitored daily. When necropsied, tissues of infected guinea pigs were plated on CYE agar or placed in YE broth for isolation of viable LDB. Tissue imprints were made on glass slides, allowed to air dry, and then heat fixed.

The amoeba/LDB preparations were heat fixed on glass slides, stained with 5% Giemsa for 20 min, washed in phosphate-buffered saline (PBS), and counterstained with fluoroscein-labeled, anti-LDB antisera. The preparations were then viewed microscopically with transmitted and epifluorescent bright-field illumination. Tissue imprints were similarly treated with fluoroscein-labeled, anti-LDB antisera.

RESULTS

Considering the concentrating action of cooling towers, the majority of towers tested in summer 1980 showed no marked elevation of LDB concentrations relative to their source water. Sites A, C, D, H, J, and K showed only a threeto tenfold increase of LDB relative to source water (Table 1). While the tower water at site F showed a

Table 1. Combined concentration[a] of the four major serogroups of *L. pneumophilla* in cooling tower water and ambient control source waters

Site	Summer 1980	Spring/summer 1981	Fall 1981
A-test	1.0×10^5	7.6×10^5	6.9×10^5
A-control	2.6×10^4	4.0×10^4	7.6×10^4
B-test	6.7×10^4	3.0×10^5	8.0×10^4
B-control	4.0×10^5	$<1.3 \times 10^4$	7.3×10^4
C-test	8.1×10^5	6.3×10^5	6.8×10^4
C-control	2.7×10^5	1.5×10^5	1.0×10^5
D-test	1.7×10^5	8.0×10^5	1.7×10^5
D-control	1.5×10^4	4.0×10^5	1.3×10^5
E-test	4.4×10^4	4.4×10^4	1.1×10^5
E-control	4.2×10^4	4.2×10^4	6.4×10^4
F-test	1.7×10^6	1.0×10^5	4.8×10^5
F-control	$<1.3 \times 10^4$	4.1×10^5	7.3×10^4
G-test[a]	5.4×10^5	5.7×10^5	1.5×10^4
G-control	6.0×10^5	5.4×10^5	3.9×10^4
H-test	4.0×10^5	1.0×10^5	7.3×10^6
H-control	4.0×10^4	1.7×10^5	9.2×10^5
I-test	1.3×10^5	8.0×10^4	1.6×10^5
I-control	3.8×10^5	2.6×10^5	2.2×10^5
J-test	1.0×10^5	4.8×10^5	6.4×10^5
J-control	1.3×10^4	1.6×10^5	3.6×10^4
K-test	1.3×10^5	NT[c]	8.7×10^5[d]
K-control	2.4×10^4	NT	1.1×10^5[d]

[a] LDB/liter of water.

[b] Site of isolation of new species of *Legionella* (i.e., *Legionella oakridgensis*).

[c] NT = not tested.

[d] Sample collected and tested in January 1982.

somewhat greater increase (≥100-fold), nevertheless, the LDB levels were below the $\geq 10^8$ LDB/L concentrations in towers implicated in some Legionnaires' Disease outbreaks. The source water for test sites B, E, G, and I had concentrations of LDB similar to or slightly higher than those found in the cooling tower water.

The concentrations of the four major serogroups of L. pneumophila found at the same sites in 1981 were similar to those found in 1980 (Table 1). Sites A, B, D, E, and J had concentrations only two to thirty times that of source water. Other test sites had levels of L. pneumophila similar to or slightly lower than source water.

In spite of relatively low concentrations of the four major serogroups of L. pneumophila, however, the majority of test sites and some ambient source waters were positive for the presence of infectious LDB as indicated by guinea pig inoculations. Infectious LDB was demonstrated in the cooling tower water of seven of eleven test sites (Tables 2 and 3). However, only two of the seven positive sites yielded infectious LDB from both summer and fall samples. Water concentrates from five of eleven control ambient sources were also positive for infectious LDB and probably accounted for the presence of infectious LDB in many of the cooling tower samples positive for infectious LDB (Tables 2 and 3). Infectivity did not obviously relate with the number of LDB inoculated.

Two separate samples of cooling tower water concentrates from site G also yielded Legionella-like isolates not typeable with known LDB antisera. A guinea pig inoculated with one of the samples showed a temperature rise of 1.4°C and was sacrificed the fourth day after injection. Only untypeable Legionella-like bacteria were isolated from spleen tissues plated on CYE agar. The guinea pig inoculated with the second sample showed a temperature rise of 0.8°C and was sacrificed on the fifth day after injection. Legionella-like bacteria, untypeable with antisera made against known Legionella species, were isolated from both spleen and liver tissues plated on CYE agar.

None of the five isolates [denoted Oak Ridge (OR) isolates] could be grown on blood or BHI agar. Growth on YE agar was variable. The isolates grew on YE agar, producing the typical brown pigment. In general, all the isolates grew poorly on CYE agar plates. The colonies were slow to appear (≥3 d), and colony size did not greatly increase with time. The isolates grew more profusely on CYE slants relative to plates, suggesting a limited air supply is preferable. This was also indicated by the propensity of some isolates for growth at the bottom of YE broth tubes. Growth of all the isolates on CYE agar containing brom cresol purple and brom thymol blue produced a green colony as opposed to the bluish green color of L. pneumophila.

The gram staining characteristics of the isolates were similar to those seen with other species of Legionella. Weakly gram-negative rods of varying lengths were apparent on microscopic examination of all the isolates. Antisera (obtained through the courtesy of the reagent

Table 2. Presence of infectious LDB in cooling tower waters and ambient control source waters sampled in spring/summer 1981

Site	No. LDB injected	Infectious for guinea pigs	Serotype isolated
A-test	7.6×10^5	Neg	NA[a]
A-control	7.2×10^4	Neg	NA
B-test	3.0×10^5	Neg	NA
B-control	$<10^4$	Neg	NA
C-test	6.3×10^5	Pos	Knox.[b]
C-control	1.5×10^5	Pos	Bloom.
D-test	9.2×10^5	Neg	NA
D-control	4.1×10^5	Neg	NA
E-test	4.4×10^4	Pos	Chic., Knox.
E-control	4.2×10^4	Pos	Chic.
F-test	1.7×10^5	Neg	NA
F-control	2.6×10^5	Neg	NA
G-test	5.5×10^4	Pos	OR
G-control	3.0×10^5	Tox[c]	NA
H-test	5.3×10^4	Neg	NA
H-control	2.1×10^5	Neg	NA
I-test	2.4×10^4	Neg	NA
I-control	5.1×10^4	Neg	NA
J-test	3.9×10^5	Pos	Chic., LA
J-control	7.9×10^4	Pos	Knox., LA

[a]NA = not applicable.

[b]Knox. = Knoxville, Bloom. = Bloomington, Chic. = Chicago, and LA = Los Angeles.

[c]Tox = Animals died within 24 h from toxicity of inoculum.

Table 3. Presence of infectious LDB in water concentrates from cooling towers and ambient control source waters sampled in fall 1981

Site	Concentration of LDB injected	Infectious for guinea pigs	Serotype isolated
A-test	6.9×10^5	Pos	Chic., Knox.[a]
A-control	7.6×10^4	Neg	NA[b]
B-test	8.0×10^4	Pos	Knox.
B-control	7.3×10^4	Pos	Knox.
C-test	4.2×10^4	Cont.[c]	NA
C-control	4.2×10^4	Neg	NA
D-test	1.7×10^5	Neg	NA
D-control	1.3×10^5	Neg	NA
E-test	1.1×10^5	Neg	NA
E-control	6.4×10^4	Neg	NA
F-test	4.8×10^5	Neg	NA
F-control	7.3×10^4	Neg	NA
G-test	1.5×10^4	Pos	OR, LA
G-control	3.9×10^4	Pos	LA
H-test	7.3×10^6	Neg	NA
H-control	9.2×10^5	Neg	NA
I-test	1.6×10^5	Neg	NA
I-control	2.2×10^5	Neg	NA
J-test	6.4×10^5	Pos	Knox., LA
J-control	3.6×10^4	Neg	NA
K-test[d]	8.7×10^5	Pos	Knox.
K-control	1.1×10^5	Neg	NA

[a]Chic. = Chicago, Knox. = Knoxville, OR = L. oakridgensis, and LA = Los Angeles.

[b]NA = not applicable.

[c]Cont. = contaminated, i.e., animals showed temperature rise and signs of distress by day two after inoculation, and on sacrifice the agar plates were overgrown with bacteria other than LDB.

[d]Samples collected and tested in January 1982.

branch, CDC) specific for the six serotypes of L. pneumophila and the six other known Legionella species did not react with the OR isolates. Conversely, antisera prepared against an OR isolate (obtained through the courtesy of Dr. W. Cherry, CDC) reacted maximally with all OR isolates (Table 4). Analysis of heated and ambient source water concentrates from the various other locations showed a wide distribution and concentration of the Oak Ridge species of Legionella (L. oakridgensis) (Table 5), not unlike that of the four major serotypes of L. pneumophila combined (Table 1).

While all of the sites tested, except site H, were positive for the presence of thermophilic amoeba, only site D was positive for pathogenic Naegleria (Table 6). The Naegleria isolates were pathogenic for mice, with all inoculated animals succumbing within 7 d after intranasal inoculation. The amoebae were readily reisolated from infected brain tissues. The pathogen had also been isolated previously from site G. Pathogenic Naegleria were not detected in either source or cooling water from the other sites tested (Tables 6 and 7). Thermophilic amoebae, other than pathogenic Naegleria, were abundant in most other sites except in the water from site H (Table 6).

The chemical characteristics of both heated and ambient source waters were analyzed statistically relative to the concentrations of LDB (Tables 8 and 9). The SAS (Helwig and Council 1979) procedure "Proc Stepwise" was used to perform a stepwise regression analysis. The results of the forward, backward, and stepwise variable selection methods were compared for consistency of variable selection. The logarithm of LDB population density was the dependent (regressor) variable, and the independent (predictor) variables available for selection by the procedure included sample temperature, pH, conductivity, nitrate, nitrite, phosphate, sulfate, chloride, and total organic carbon. The results of the analysis are given in Table 10: The concentrations of nitrite, sulfate, and total organic carbon explained the presence of 55% of the variance of LDB population densities ($P < 0.01$).

The abundance of free-living amoebae, other than pathogenic Naegleria, in most cooling waters may be of significance, considering the ability of some such amoebae to interact with LDB either destructively or supportively. When approximately 1.0×10^5 cells of Naegleria lovaniensis or Acanthamoeba royreba were placed in the center of nonnutrient agar plates spread with a lawn of E. coli or LDB, the outgrowth of amoebae from the original point of application showed that amoeba growth and migration occurred in both cases but was diminished by day 3 and 4 on the lawn of LDB (Table 11). The Acanthamoeba migrated more slowly than Naegleria on both the LDB- and E. coli-seeded plates.

When LDB was mixed with either Naegleria or Acanthamoeba in CGVS or CGV, respectively, there was an initial decrease in recoverable, viable LDB (Table 12). However, some, but not all, cultures of amoebae that were fed LDB and held for several weeks were found to be heavily inundated by LDB. Microscopic examination of LDB/amoeba mixtures using

Table 4. Characteristics of Oak Ridge isolates of *Legionella*

Characteristics	Isolates				
	OR4	OR6	OR23	OR24	OR30
Growth on:					
CYE	+	+	+	+	-
BHI	-	-	-	-	-
Blood agar	-	-	-	-	-
Gram stain	Gr.-[a]	Gr.-	Gr.-	Gr.-	Gr.-
Catalase[b]	+	+	+	+	-
Reaction with conjugates against					
L. pneumophila (1-6)	-	-	-	-	-
L. bozemanii	-	-	-	-	-
L. gormanii	-	-	-	-	-
L. micdadei	-	-	-	-	-
L. dumoffii	-	-	-	-	-
L. longbeachae	-	-	-	-	-
L. OR	+	+	+	+	-

[a]Gr.- = gram-negative rods with morphology suggestive of *Legionella*.

[b]The presence of the enzyme catalase was detected by reacting bacterial suspensions with hydrogen peroxide.

Table 5. Distribution and concentration of L. pneumophila and the Oak Ridge strain of Legionella in artificially heated and control source waters[a]

	Approximate Legionella concentrations per liter of water			
	L. pneumophila[b]		L. OR	
Site	Heated	Unheated	Heated	Unheated
A	7.6×10^5	4.0×10^4	3.2×10^5	2.8×10^4
B	3.0×10^5	$<1.3 \times 10^4$	8.0×10^4	1.1×10^5
D	8.0×10^5	4.0×10^5	8.0×10^4	8.0×10^4
F	1.0×10^5	4.1×10^5	1.3×10^5	4.0×10^4
G[c]	5.7×10^5	5.4×10^6	3.2×10^6	1.5×10^5
H	1.0×10^5	1.7×10^5	4.0×10^4	5.3×10^5
I	8.0×10^4	2.6×10^5	2.0×10^5	2.6×10^5
J	4.8×10^5	1.6×10^5	2.8×10^5	4.0×10^4

[a]Samples collected in spring and summer of 1981.

[b]Serogroups 1-4.

[c]Site of isolation of L. oakridgensis.

Table 6. Presence of thermophilic Naegleria in cooling tower water

Site	Sample type/vol.	Amoebic outgrowth at 45°C	Morphology	Flagella	Pathogenicity
A	H_2O-100 mL	+	NN[a]	NT[b]	NA[c]
A	H_2O-100 mL	+	NP[d]	+	Neg
A	H_2O-100 mL	+	NN	NT	NA
A	H_2O-100 mL	+	NN	NT	NA
B	H_2O-400 mL	+	NN	NT	NA
B	H_2O-400 ml	+	NN	NT	NA
C	H_2O-100 ml	+	NP	+	Neg
C	H_2O-100 mL	-	NA	NA	NA
D	H_2O-100 mL	+	NN	NT	NA
D	H_2O-100 ml	+	P[e]	+	Pos
F	H_2O-100 mL	-	NA	NA	NA
F	H_2O-100 mL	+	NP	+	Neg
F	H_2O-100 mL	+	NP	+	Neg
F	H_2O-100 mL	-	NA	NA	NA
H	H_2O-100 mL	-	NA	NA	NA
H	H_2O-100 mL	-	NA	NA	NA
H	H_2O-100 mL	-	NA	NA	NA
H	H_2O-100 mL	-	NA	NA	NA
I	H_2O-400 mL	+	NN	NT	NA
I	H_2O-400 mL	+	NP	+	Neg
J	H_2O-250 ml	+	NN	NT	NA
J	H_2O-250 mL	+	NN	NT	NA
J	Sediment	+	NN	NT	NA
J	Sediment	+	NP	+	Neg
J	Sediment	+	NN	NT	NA
J	Sediment	+	NN	NT	NA
J	Sediment	+	NN	NT	NA
J	Sediment	+	NN	NT	NA
K	H_2O-400 mL	+	NN	NT	NA
K	H_2O-400 mL	+	NN	NT	NA
K	H_2O-100 mL	-	NA	NA	NA
K	H_2O-100 mL	+	NN	NT	NA

[a]NN = not Naegleria.

[b]NT - not tested.

[c]NA = not applicable.

[d]NP = nonpathogenic Naegleria.

[e]P = indicative of pathogenic Naegleria.

Table 7. Presence of thermophilic Naegleria in cooling tower source water

Site	Sample type/vol.	Amoebic outgrowth at 45°C	Morphology	Flagella	Pathogenicity
A	H_2O-250 mL	+	NN[a]	NT[b]	NA[c]
A	H_2O-100 mL	-	NA	NA	NA
A	H_2O-100 mL	-	NA	NA	NA
A	H_2O-100 mL	-	NA	NA	NA
C	H_2O-100 mL	-	NA	NA	NA
C	H_2O-100 mL	-	NA	NA	NA
D	H_2O-100 mL	-	NA	NA	NA
D	H_2O-100 mL	-	NA	NA	NA
F	H_2O-250 mL	+	NN	NT	NA
F	H_2O-100 mL	-	NA	NA	NA
F	H_2O-100 mL	+	NN	NT	NA
H	H_2O-100 mL	+	NN	NT	NA
H	H_2O-100 mL	+	NP[d]	+	Neg
I	H_2O-400 mL	+	NN	NT	NA
I	H_2O-100 mL	-	NA	NA	NA
J	H_2O-250 mL	-	NA	NA	NA
J	Grass	-	NA	NA	NA
J	Grass	-	NA	NA	NA
J	Grass	-	NA	NA	NA
J	Sediment	-	NA	NA	NA
J	Sediment	-	NA	NA	NA
K	H_2O-400 mL	-	NA	NA	NA
K	H_2O-400 mL	-	NA	NA	NA

[a]NN = not Naegleria.

[b]NT = not tested.

[c]NA = not applicable.

[d]NP = indicative of nonpathogenic Naegleria.

Table 8. Water quality characteristics of power plant and source waters, summer 1980

Site	Temp. (°C)	pH	Conductivity (µS)	Chemicals (µg/mL)						Total bacteria (No./mL)
				NO_3-N	NO_2-N	P	SO_4	Cl	TOC[a]	
A-test	23	8.3	700	0.47	0.012	0.108	196	27	4.4	10^4
A-control	19	8.0	150	0.08	0.004	0.006	11.5	5	5.3	10^4
C-test	30	7.5	3000	0.30	0.021	0.034	633	603	9.2	10^4
C-control	20	8.2	800	0.071	0.007	0.004	71	148	11	10^5
D-test	12	8.3	5000	3.11	0.013	0.010	12.5	36	12	10^3
D-control	4	8.3	2900	1.21	0.007	0.008	27	13	7.3	$<10^3$
F-test	28	8.1	1100	10.7	0.034	0.82	429	38	15	10^4
F-control	22	8.4	550	5.9	0.043	0.16	34	20	4.3	10^4
G-test	28	8.4	450	0.70	0.10	0.138	43	16	11	10^3
G-control	24	8.5	450	0.66	0.10	0.145	45	22	10	10^4
H-test	16	8.1	1700	8.1	0.005	1.0	542	115	5.8	10^3
H-control	18	83	1150	4.2	0.003	0.5	353	66	2.7	10^3
I-test	32	8.0	390	0.67	0.095	0.016	93	21	9.9	10^5
I-control	22	7.9	390	0.68	0.096	0.001	92	22	12	10^5
J-test	30	7.6	1200	2.10	0.003	0.007	74.0	7.2	11.0	10^4
J-control	22	7.0	1200	2.03	0.003	0.009	11.4	7.4	8.9	$<10^3$
K-test	32	8.2	420	0.44	0.027	<0.001	89	13	13	$<10^3$
K-control	16	8.2	280	0.25	0.006	<0.001	23.3	8.93	9.1	$<10^3$

[a]Total organic carbon.

Table 9. Water quality characteristics of power plant and source waters, spring/summer of 1981

Site	Temp. (°C)	pH	Conductivity (µS)	Chemicals (µg/mL)						Total bacteria (No./mL)
				NO_3-N	NO_2-N	P	SO_4	Cl	TOC[a]	
A-test	44	7.2	800	0.40	0.021	0.079	365	25	17	$<10^3$
A-control	9	7.4	129	0.10	0.003	0.009	10	3.3	8.0	$<10^3$
B-test	27	7.24	1070	2.19	0.011	0.071	514	38	20	10^4
B-control	26	7.33	62.5	0.04	0.003	0.006	3.3	1.8	8.5	10^3
C-test	40.6	7.92	3630	0.32	0.006	0.104	379	507	18	10^4
C-control	29.5	7.92	830	0.14	0.036	0.021	57	158	15	10^4
D-test	21	8.09	519	3.36	0.063	0.001	72	32	11	$<10^3$
D-control	11	7.84	352	2.00	0.060	0.011	41	19	9.3	10^4
E-test	26	7.45	455	1.18	0.047	0.016	139	27	7.1	$<10^3$
E-control	18.5	7.34	275	0.87	0.060	0.062	81	18	9.5	10^3
F-test	NT	6.92	1750	11.6	0.069	0.78	853	89	20	10^5
F-control	NT	7.89	530	3.47	0.048	0.010	48	21	8.0	10^4
G-test	27.6	7.74	324	2.07	0.003	0.133	33	17	16	10^4
G-control	25.2	7.68	428	1.84	0.040	0.093	32	17	16	10^4
H-test	NT	7.39	1060	2.48	0.037	2.9	309	107	19	10^3
H-control	NT	8.04	1190	2.92	0.013	0.62	383	59	14	$<10^3$
I-test	25	7.39	2010	0.77	0.005	0.040	45	9.9	10	$<10^3$
I-control	24	7.33	2010	0.87	0.006	0.040	43	11	10	$<10^3$
J-test	26.6	6.86	223	0.45	0.041	0.025	71	12	12	10^4
J-control	11.6	7.32	67.8	0.24	0.014	0.003	10	5.5	9.0	$<10^3$
K-test[b]	NT	8.25	292	0.37	0.001	0.017	24	9.3	12	NT
K-control	NT	8.03	333	0.39	0.003	0.023	57	10.4	14	NT

[a]Total organic carbon.

[b]Sampled January 1982.

Table 10. Results of stepwise regression analysis[a] for the dependent variable $\log_{10}$ (LDB cell density)

Step number	Percent of variance explained (R^2 x 100)	Mallow's Cp[b]	Variables	Coefficient	Prob > F[c]
1	24.5	19.1	Total organic carbon	0.086	0.003
2	38.1	12.3	Total organic carbon	0.082	0.002
			Nitrite	8.22	0.014
3	54.6	3.6	Total organic carbon	0.061	0.011
			Nitrite	9.71	0.002
			Sulfate	0.002	0.003

[a]The "stepwise" variable selection method was used in this case.

[b]Mallow's Cp provides an estimate of the efficiency of the model. The "perfect" model should have the value of the Cp statistic equal to the number of variables used plus one.

[c]The probability of finding this result by chance.

Table 11. Relative ability of E. coli and Legionella pneumophila (Los Angeles serotype) to support the migration of Naegleria lovaniensis and Acanthamoeba royreba

Amoeba	Bacterium	Day[a]	Mean radius of outgrowth[b]	Mean increase in radius[b]
N. lovaniensis	E. coli	0	7.8 ± 0.9	NA[c]
		1	12.4 ± 0.6	4.7 ± 0.9
		2	19.3 ± 1.0	6.8 ± 0.4
		3	32.0 ± 0.8	12.8 ± 0.6
	Legionella pneumophila	0	8.3 ± 2.1	NA[c]
		1	12.4 ± 1.2	4.1 ± 1.0
		2	17.7 ± 1.6	5.3 ± 0.4
		3	24.1 ± 0.9	6.7 ± 0.8
A. royreba	E. coli	0	8.3 ± 1.1	NA[c]
		1	11.6 ± 0.4	3.3 ± 0.5
		2	16.2 ± 0.6	4.6 ± 0.6
		3	23.2 ± 1.2	7.1 ± 0.6
		4	32.3 ± 0.8	9.1 ± 1.1
	Legionella pneumophila	0	7.6 ± 1.0	NA[c]
		1	11.2 ± 1.2	3.5 ± 0.3
		2	14.6 ± 1.8	4.4 ± 0.1
		3	19.5 ± 2.4	5.5 ± 0.6
		4	24.5 ± 2.4	5.5 ± 0.6

[a]Days after plating.

[b]In millimeters, mean of three experiments ± standard error.

[c]NA = not applicable.

Table 12. Destruction and/or growth of *Legionella pneumophila* in axenized cultures of *Naegleria lovaniensis* and *Acanthamoeba royreba*

Test cultures[a]	Time (in days) between exposure of LDB to amoeba and titration of LDB	Dilution of LDB inoculum relative to media change	Approximate no. of viable LDB/mL[b]	Serotype of LDB as determined by fluorescent antibody
A. royreba + LDB (LA)	0.25	NA[c]	1×10^6	NT[d]
	1.0	NA	1×10^2	NT
	250.0	10^{30}	1×10^{10}	LA
LDB (LA)	0.25	NA	1×10^6	NA
	1.0	NA	1×10^6	NA
N. lovaniensis + LDB (LA)	0.25	NA	1×10^6	NT
	1.0	NA	1×10^1	NT
	150.0	10^{30}	1×10^9	LA
LDB (LA)	0.25	NA	1×10^6	NA
	1.0	NA	1×10^6	NA
N. lovaniensis + LDB (Knox.)	115.0	10^{23}	1×10^9	Knox.
	115.0	10^{23}	$<10^1$	NA
	115.0	10^{23}	$<10^1$	NA
	115.0	10^{23}	1×10^9	Knox.

[a]Amoeba-LDB cultured in CGV or CGVS media; media replaced every 5 d with YE broth; LA = Los Angeles serotype of LDB; Knox. = Knoxville serotype of LDB.

[b]As determined by endpoint titration in YE broth and/or CYE plates unless otherwise indicated. Cultures were frozen at -70°C and thawed twice prior to titration. When titrated in CGVS, the LDB did not grow.

[c]NA = Not applicable.

[d]NT = Not tested.

combined transmitted light and epifluorescent bright-field microscopy showed amoebae filled or covered with fluorescent LDB debris, and in many cases with accumulation of intact bacteria and antigen localized at the cell membrane. When held for additional weeks, microscopic examination showed a continual presence of LDB concomitant with the continuing propagation of amoebae. When titrated in CGV, CGVS, or YE media, the LDB growing in conjunction with the amoebae was unable to grow in the casitone media but showed titers of 10^{10} LDB/mL in YE broth (Table 12). Some of these cultures had more than thirty media changes, representing a 10^{30} dilution of the original LDB inoculum, and still contained 10^{9} to 10^{10} LDB/mL of culture fluid (Table 12).

That the LDB propagated in the amoebae cultures was serotypically identical to the LDB originally inoculated into the amoebae was indicated by fluorescent antibody (FA) analysis (Table 12). Similarly, no marked increase in virulence was seen on inoculation of guinea pigs (Table 13). Guinea pigs inoculated with LA-LDB, cultured in YE broth or in CGV or CGVS in conjunction with amoebae, showed an initial increase in temperature which subsided 3 to 4 d after inoculation. The LA-LDB could be detected in the spleen tissue by FA analysis and could be recovered from some pigs 3 to 5 d after inoculation, but the animals did not succumb to their infections (Table 13).

Intranasal inoculation of mice with amoebae containing associated LDB or LDB antigens did not elicit any obviously altered pathogenic sequelae relative to that elicited by either LDB or amoebae inoculated alone (Table 12). Amoebae were isolatable from lung and/or brain tissue of mice inoculated with A. royreba or A. royreba + LDB. The propensity for amoebic localization in lung and brain tissue was not markedly altered by the associated LDB (Table 12). Similarly, the gross pathology evident in A. royreba-infected mice was not obviously altered in mice infected with amoeba-fed LDB. Mice infected with LDB had slight lung pathology 5 d after inoculation. Viable LDB could be isolated from lung tissue of all five mice 5 d after the inoculation of only LDB. Conversely, LDB could not be isolated from mice inoculated with the Naegleria + LDB mixture. Some mice inoculated with the A. royreba + LDB mixture yielded viable LDB from infected lung tissue.

DISCUSSION

The results of the LDB analysis of power plant cooling waters show generally low levels of LDB. In most cases where increases in heated water were observed, the concentrations of LDB in cooling waters were increased by approximately tenfold or less relative to source water (Table 1). In some cases, levels of LDB were greater in source water than in cooling water (Table 1).

Although concentrations of the four major serogroups of L. pneumophila were not particularly high in either source or heated waters, infectious LDB was demonstrable in many of the samples. Four of ten samples of cooling waters taken in the spring and/or summer of

Table 13. Results of intraperitoneal inoculation of guinea pigs with Legionella pneumophila (Los Angeles serotype) grown in yeast extract media or cocultivated with amoebae in casitone media

Guinea pig	Source of LDB inoculation[a]	Viable LDB inoculated[b]	Duration of fever[c]	Day of sacrifice	Presence of LDB in tissues[d]	
					FA	Culture
1	N. lovaniensis	10^9	1-2	NS	NT	NT
2	A. royreba	10^9	2-3	NS	NT	NT
3	YE (stock)	10^{10}	2-4	5	+	+
4	N. lovaniensis	10^8	1-3	3	+	-
5	A. royreba	10^0	1-2	5	+	-
6	N. lovaniensis	10^{10}	3-4	NS	NT	NT
7	N. lovaniensis	10^{10}	1-5	6	NT	+
8	A. royreba	10^9	2-5	NS	NT	NT
9	A. royreba	10^9	2-5	6	NT	-
10	A. royreba	10^8	1-4	NS	NT	NT
11	A. royreba	10^8	1-3	NS	NT	NT
12	N. lovaniensis	10^{10}	1-6	NS	NT	NT
13	N. lovaniensis	10^{10}	1-4	4	+	+
14	N. lovaniensis (cell assoc.)[e]	10^7	1-6	NS	NT	NT
15	N. lovaniensis (cell assoc.)[e]	10^7	1-4	4	+	+
16	YE (stock)	10^{10}	1-5	NS	NT	NT
17	YE (stock)	10^{10}	1-5	6	NT	-

[a]YE = stock LDB grown in yeast extract media; N. lovaniensis = LDB cocultivated with Naegleria lovaniensis in CGVS media; A. royreba = LDB cocultivated with Acanthamoeba royreba in CGV media.

[b]As determined by endpoint titration in YE broth and on CYE agar plates.

[c]Days after inoculation when rectal temperature was <40.0°C.

[d]Days after inoculation when animals were necropsied and spleen, liver, and peritoneal fluid tested by FA or culture on CYE agar for presence of LA-LDB.

[e]Amoebae washed with CGVS and amoebae with associated LDB was titrated and then inoculated into guinea pigs.

NT = not tested.

NS = not sacrified.

1981 were shown to be infectious for guinea pigs, with Knoxville, Los Angeles, and Chicago serotypes of L. pneumophila being the resultant isolates. In addition, one apparently new species of Legionella (OR) was isolated from site G (Orrison et al., submitted; Tyndall et al., submitted). Interestingly, source water from three of the four positive test sites were also positive for infectious LDB and yielded Knoxville, Bloomington, Los Angeles, and Chicago isolates. The presence of infectious LDB in the cooling waters at these sites is not surprising, considering their concomitant isolation from the source water.

Five samples of cooling water from the fall samples also contained infectious LDB, as shown by guinea pig inoculation. These samples yielded Knoxville, Los Angeles, and Chicago isolates (Table 3). The OR species of Legionella was again isolated from heated water at site G. Only two of eleven fall samples of source water were positive for infectious LDB. The Knoxville and Los Angeles serotypes of L. pneumophila were isolated from these samples, as they were from the corresponding samples of heated water (Table 3). Thus, in the samples taken in 1981, seven of eleven test sites were shown to contain infectious LDB and five of these seven sites had infectious LDB in the source water. While the source water contained infectious L. pneumophila, only thermally altered water yielded the infectious Oak Ridge isolates. Whether thermal discharges enhance or select for the infectious form of these Legionella will require additional study.

That the infected guinea pigs were injected with only 10^4 to 10^5 (Tables 2 and 3) LDB indicates a rather high degree of virulence in these populations because the observations of Berendt et al. (1980) indicated that the LD_{50} of virulent L. pneumophila (serogroup 1) is 3.0×10^6 when injected intraperitoneally.

In addition to the isolation of four of the six known serotypes of L. pneumophila in these studies, one site yielded the new species of Legionella tentatively named Legionella oakridgensis (OR). Two unusual characteristics were apparent on the initial isolation of these Legionella-like bacteria. First, the colonies did not appear until three or more days after plating of the tissue on CYE agar. Second, the bacteria, although presumably Legionella, did not cross react with antisera prepared against known Legionella species. The poor growth on CYE agar with these isolates has also been noted by W. Cherry (personal communication), as has the lack of serologic identity with known species of Legionella. Orrison et al. (1982) showed that the isolates are a new species of Legionella (i.e., Legionella oakridgensis) and that DNA relatedness and the fatty acid profiles are unique, as are the serologic characteristics.

That L. oakridgensis is widely distributed was initially indicated by its isolation from two sites 2400 km (1500 miles) apart (Tyndall et al., submitted). Subsequently, fluorescein-tagged antisera prepared against L. oakridgensis was used to survey microscopically for the presence of this Legionella species in other disparate locales. These studies indicated a wide distribution for this species (Table 5).

Some guinea pigs from which L. oakridgensis were isolated also yielded L. pneumophila. Thus the elevated fever and other signs of illness (i.e., lethargy, ruffled fur, etc.) in these animals could have been due to infection with L. pneumophila as opposed to L. oakridgensis. Both guinea pigs inoculated with water concentrates from a spring sample at site G, however, yielded only L. oakridgensis. These pigs were febrile and showed overt signs of illness. This indicates that L. oakridgensis is pathogenic. It will be of interest to determine whether evidence of human infection with L. oakridgensis can be found. This is of particular interest considering the wide distribution and relatively high concentrations of this Legionella species.

While the goal of this ongoing study is to assess the presence of infectious LDB in cooling waters of electric power plants, the isolation of the new species of Legionella illustrates the value of such survey information as input to clinical studies. For example, many clinical specimens are placed in fixative for histological analysis, obliterating any possibility of isolating new species of pathogens. Thus environmental material serves not only as a source of clinical infection but as a reservoir from which the isolation of clinically important pathogens is possible.

As was previously reported for other sites (Tyndall et al. 1980), cooling water from most of the present sites showed the presence of thermophilic free-living amoebae. Twenty percent of the present sites (2 of 11) were also positive for pathogenic Naegleria. One of these two sites was previously shown to contain relatively high levels of pathogenic Naegleria and was not reported as part of this study. The pathogenic isolates from both sites produced the typical fulminating encephalitis when inoculated intranasally into weanling ICR mice. Gross observation of the brain tissue revealed obvious swelling and areas of hemorrhage. As expected, the Naegleria were readily reisolated from infected brain tissue.

Pathogenicity of thermophilic free-living amoebae, other than Naegleria, was not tested. All sites except site H yielded thermophilic free-living amoebae.

The observation that Naegleria and Acanthamoeba can interact with LDB either destructively or supportively may be important, particularly in regard to the prevalence of such amoebae in many cooling towers. A better understanding of the interaction may be important in understanding the prevalence or absence of LDB in cooling waters.

While the migration of amoebae on nonnutrient agar plates seeded with a lawn of LDB was slower than that on plates seeded with E. coli, the LDB did serve as a sole food source. That LDB was ingested or concentrated by the amoebae was obvious from the FA analysis of the amoeba-LDB mixtures. We did not see deleterious effects of LDB on amoebae as reported by Rowbotham (1980). However, we were using low-virulence LDB cultures as opposed to more virulent strains shown to have adverse effects on free-living amoebae.

The animal studies also reflect the prolonged survival of Acanthamoeba-associated LDB relative to Naegleria-associated LDB. The only animals given amoeba-associated LDB from which viable LDB could be isolated were those inoculated with Acanthamoeba + LDB. Viable LDB could not be recovered from mice inoculated with Naegleria + LDB.

Naegleria lovaniensis is a nonpathogenic thermophilic amoeba (Stevens et al. 1980). We and others have not seen pathologic changes in mice inoculated with this amoeba (unpublished observations). Similarly, there was no indication in this study suggesting pathogenicity by the amoebae following ingestion of LDB (Table 3). Similarly, the results of this study indicate little or no alteration in the pathogenic potential of A. royreba on ingestion of LDB. The results, however, confirm the previously observed persistence of the amoebae in tissue. A. royreba is known to produce pulmonary lesions on intranasal inoculation (Willaert et al. 1978). On occasion the amoebae can be isolated from the brain, although fatal encephalitis is not generally associated with exposure to this amoeba.

The observation that viable LDB was propagated symbiotically with Naegleria and Acanthamoeba has various ramifications. The fact that the LDB in these cultures was not adapted to growth in CGV or CGVS indicates that the amoebae are providing either an intracellular niche or extracellular factors that support the growth of LDB. Tison et al. (1980) previously showed that blue-green algae can provide extracellular growth factors that promote the growth of LDB. Similarly, it has been shown that LDB can grow intracellularly in monocytes, and in doing so the virulence is enhanced (Wong et al. 1980, 1981). In the case of the amoebae in this study, however, it was surprising to see that propagation of LDB was preceded by a rapid destruction of the bacterium. While destruction followed by growth promotion suggests a selection for, or an enhancement of, a subpopulation from the original stock culture, immunologic or pathogenic differences between the stock LDB and that grown in conjunction with the amoebae have not, as yet, been demonstrated. The results thus far indicate that free-living amoebae might provide a necessary niche for maintaining viable LDB where conditions might not otherwise support the survival or growth of the microorganism.

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