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Legionella Incidence and Density in Potable Drinking Water Supplies†

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The incidence and density of *Legionella* spp. in raw water, water at various stages of treatment, and in potable distribution water were determined by direct immunofluorescence. The number of cells reacting with *Legionella*-specific fluorescent antibody conjugates in raw waters ranged from about 10^4 to 10^5 cells/liter, whereas the concentrations of fluorescent antibody-positive cells in the distribution waters were generally 10- to 100-fold lower than in the raw source waters. No viable or virulent *Legionella* strains were isolated from either the source or distribution waters. However, *Legionella* spp. are infrequently isolated from water at temperatures below 15°C as was the case in the systems surveyed in this study.

The etiological agents of Legionnaires disease and Pontiac fever, bacteria of the genus *Legionella*, have been isolated from aquatic habitats associated with outbreaks of illness (3, 6, 15) and also from lakes and streams in areas where no documented cases of these diseases had occurred (9, 10, 18). There have also been a number of reports of the isolation of *L. pneumophila* from water storage tanks, hot and cold water taps, and shower heads, where potable waters were implicated as the sources of these bacteria (7, 11, 19, 20). The isolation of *L. micdadei* from a hospital shower has recently been reported (4). These reports implicating water distribution systems as sources of *Legionella* spp. prompted us to initiate a survey to determine the effectiveness of water treatment procedures in eliminating *Legionella* spp. from these systems.

Water treatment systems sampled between April and August of 1981 were of four basic types: (i) water receiving only chlorination and stored in closed reservoirs; (ii) water receiving chlorination and stored in open reservoirs; (iii) slow sand filtration with chlorination; and (iv) water receiving flocculation, mixed media filtration, and chlorination. Samples of raw water (8 to 20 liters) were collected in sterile polypropylene carboys. Treated water was collected in sterile 20-liter polypropylene carboys containing sodium thiosulfate to neutralize residual chlorine (1). Microbial biomass accumulations consisting of algae and cyanobacteria, identified by fluorescence microscopy (22), and associated bacteria were collected from the walls of flocculation and filter tanks and open reservoir walls by scraping with an ethanol-sterilized knife blade and stored in sterile 50-ml polyethylene containers. Samples were transported to the laboratory at ambient temperature and processed within 24 h. Temperature was determined by using a thermistor (model 43TD; YSI, Yellow Springs, Ohio) calibrated to a mercury thermometer, and free and total residual chlorine were determined by using a model CN-66 HACH DPD colorimetric analysis field kit. Standard plate count bacteria were enumerated by the membrane filter procedure with incubation at 35°C for 48 h (17). Total coliform bacteria were enumerated by the membrane filter method with m-Endo agar (Difco Laboratories, Detroit, Mich.) incubated at 35°C for 24 h (1).

Raw water samples and samples from various stages in the treatment process containing visible turbidity were centrifuged at 3,000 × *g* for 30 min (10, 14). The supernatant fluids were decanted and concentrated by membrane filtration (16). The pellet and membrane filter were suspended in 40 ml of sample water, and the concentrated sample was then homogenized to suspend cells trapped on the filter (16). Treated water samples with no apparent turbidity were concentrated by membrane filtration, and the filters were homogenized in 40 ml of sample water (16). Biomass samples were homogenized in 40 to 50 ml of water from the sample site in a blender for 15 s. Concentrated sample (10 to 25 μl) was spotted onto multiwelled slides (Cell-Line Associates, Minolta, N.J.), air dried, gent-

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TABLE 1. Numbers of *Legionella* spp. determined by direct immunofluorescence and water temperature and free residual chlorine concentrations in raw waters and distribution waters treated by different procedures

Sample type	No. of direct immunofluorescence-positive cells per liter	Temperature (°C)	Free residual chlorine (ppm)
Raw water (n = 5)	1.4×10^4 to 1.7×10^5	10.1 to 12.0[a]	—[a]
Distribution water treated by chlorination only (n = 7)	$<8 \times 10^3$ to 1.4×10^4	10.5 to 13.0	0.2 to 0.6
Distribution water treated by slow sand filtration and chlorination (n = 6)	$<5.4 \times 10^3$ to 4.6×10^4	12 to 20	0.0 to 0.4
Distribution water treated by flocculation, mixed-media filtration, and chlorination (n = 6)	$<8 \times 10^3$ to 2.2×10^4	12	0.5 to 2.0

[a] Not applicable

ly heat fixed, and then stained with a fluorescent antibody (FA) conjugate to *L. pneumophila* serogroups 1 through 6, *L. micdadei*, *L. gormanii*, *L. dumoffii*, and *L. bozemanii*, and preimmune serum conjugated with fluorescein isothiocyanate as a negative control (5). Each conjugate was quality assurance tested against its specific antigen as a positive control (5). FA conjugates were supplied by the Biological Products Section, Centers for Disease Control, Atlanta, Ga. The number of cells reacting with a specific FA conjugate was quantitated microscopically (5).

Isolation of *Legionella* spp. from samples with FA-positive cells was attempted by inoculation of guinea pigs with 3 to 5 ml of concentrated sample (12) and by direct isolation with buffered charcoal-yeast extract agar (8) as modified by Bopp et al. (2). A 0.1-ml sample was spread directly onto modified buffered charcoal-yeast extract agar plates, and samples were also spread after 5, 15, and 30 min in pH 2.2 buffer (2). Plates were incubated at 37°C for 7 to 10 days. *L. pneumophila* serogroup 1 (ATCC 33152) was used as a quality assurance control for each batch of medium.

The raw water sources and the distribution systems sampled were generally low in standard plate count and total coliform bacteria. Concentrations of standard plate count bacteria in the raw water ranged from about 50 to 650 per ml, whereas concentrations in the treated waters were 73 to <1 per ml. Total coliform bacteria were detected in only two distribution water samples, although the concentrations were less than 10 per 100 ml in both cases. The number of cells reacting with *Legionella* spp. FA conjugates are summarized in Table 1.

Concentrations of *Legionella* in the raw water sources were comparable to those observed in a survey of aquatic habitats in the southeastern United States, in which numbers of *Legionella* ranged from 10^3 to 10^6 per liter (15). After treatment, the concentrations of *Legionella* were at least one order of magnitude less than those in the raw waters. A statistical evaluation of the data (t test) showed that there was a significant difference ($P < 0.001$) in the mean number of cells reacting with fluorescent antibody conjugates in distribution waters ($\bar{x}$, 5.5×10^3 cells/liter) when compared with raw waters ($\bar{x}$, 7.8×10^4 cells/liter). The concentrations of *Legionella* in the biomass samples collected from the walls of a flocculation tank and filter bed (4×10^3 cells/ml) and from the wall of an open reservoir (8×10^4 cells/ml) were greater than in the surrounding water. These results indicate that the physical nature of these biomass accumulations and the potentially high concentration of dissolved organics in these niches may protect the resident bacterial community from the bactericidal effect of chlorine in the surrounding water and allow for regrowth of *Legionella* and other bacteria.

Microscopic detection of cells by direct immunofluorescence does not distinguish between viable and nonviable cells. Therefore, it is necessary to isolate *Legionella* to show that viable cells are present, especially in the case of waters which have been chlorinated. Both the animal inoculation and the direct isolation procedure, with and without the low-pH treatment, were used in attempts to isolate *Legionella*. No *Legionella* or other bacteria which cross-reacted with the FA conjugates were isolated by either of these procedures, indicating that (i) those cells observed by direct immunofluorescence were not viable; (ii) viable *Legionella* were not virulent in the test animals (13); or (iii) viable cells were injured by the treatment processes and were unable to survive the isolation procedures. It should be noted that in a recent survey of rivers and lakes in the southeastern United States, *Legionella* were isolated from 15% (47 of 318) of FA-positive samples, and only 4% (2 of

47) of the waters at temperatures below 13.5°C yielded viable *Legionella* (15). Temperatures in this range (<13.5°C) were recorded for all but three of the samples collected in this study.

In summary, an examination of six water treatment facilities and water distribution systems for *Legionella* spp. showed that all stages of the treatment processes were positive for *Legionella* by direct immunofluorescence, but no viable or virulent isolates were obtained. A total of 38 samples consisting of raw waters, water after various stages of treatment, samples of accumulated biomass, and distribution water were examined. These water treatment processes were apparently successful in reducing the numbers of *Legionella* from those observed in raw waters. The potential does exist, however, for *Legionella* to enter distribution waters through breakdowns in the treatment process (19) and to survive in distribution lines that are not frequently used or where low flow rates prevail (18, 20, 21). The lack of *Legionella* isolations from the cool water specimens collected during this survey does not preclude occurrences of viable virulent cells in drinking water of higher temperatures.

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