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Isolation of *Legionella pneumophila* from Cooling Tower Water by Filtration

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Methods are described for detection of *Legionella pneumophila* in cooling tower water or other water sources by direct fluorescent-antibody staining. A procedure for isolation of *Legionella* bacteria from water samples by guinea pig inoculation is described. Two different serogroups of *L. pneumophila* were isolated repeatedly from one of the cooling towers.

Cooling towers serving as heat exchangers for industrial and institutional buildings and operations have been implicated as the disseminators of *Legionella pneumophila* in several outbreaks of legionellosis (3, 4, 9). The turbulence generated by transfer of heat from the cooling water to the air produces mists which may drift for several hundred meters (3, 4, 9, 14). If these droplets of water contain viable Legionnaires disease bacteria (LDB), they are potentially infectious when inhaled by a susceptible host (3, 14).

Bettinger, Fynsk, and Fliermans surveyed the water from 12 cooling towers and 6 chilled water systems in six states for the presence of *L. pneumophila* (G. E. Bettinger, A. W. Fynsk, and C. B. Fliermans, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C81, p. 288). By direct fluorescent-antibody (DFA) procedures, 15 of the 18 samples were positive, with serogroups 1 and 4 being most common. Three samples could not be read because of high background fluorescence. None of these air-conditioning units was associated with a disease outbreak.

Procedures for maintaining and efficiently operating cooling towers vary greatly depending upon the design, construction material, water source, operational schedules, and temperature. Most towers are treated on a regular or irregular schedule with one or more biocides and with anticorrosive chemicals. Chlorination is frequently used. No clear consensus has arisen as to the most effective way to prevent the emergence of LDB as a potential health hazard.

We report a filtration method for examining cooling tower water for LDB. Water samples from a number of cooling towers were examined by this method.

MATERIALS AND METHODS

Efficiency of recovery of LDB. Experiments designed to estimate the efficiency of recovery of the LDB from inoculated water samples were performed by the following method. A pure culture of *L. pneumophila* of serogroup 1 (Knoxville) was grown for 24 h on charcoal-yeast extract agar (6) at 36°C. A cell suspension was made in sterile tap water to approximate a cell density of 10^9/ml, and appropriate dilutions (sterile tap water) were made and plated in triplicate on charcoal-yeast extract agar to obtain the colony-forming units in the suspension. The cultures were incubated at 36°C. A total of 2 ml of the original suspension was added per liter of sterile tap water (4 liters).

Possible LDB contamination of the tap water used for rinsing the filtration equipment was tested by filtering and concentrating four liters of water and examining the sediment by DFA staining. No LDB were found.

The inoculated tap water was pressure filtered (N_2) through a membrane filter (porosity, 0.45 or 0.65 μm) of 140-mm diameter placed in a steam-sterilized, stainless steel filter holder (Millipore Corp., Bedford, Mass.). After the test sample was filtered, the membrane with its collected residue was aseptically removed and placed in a 250-ml sterile polycarbonate blending jar to which approximately 50 ml of the sample filtrate was added. The sample was blended for 15 s with an Oster blender at the highest speed setting (Oster Corp., Milwaukee, Wis.).

The blended sample was transferred to a 50-ml sterile polycarbonate tube and centrifuged lightly (650 × *g* in an International PR-2 centrifuge for 10 min) to pellet the membrane particles. The supernatant was decanted and centrifuged at 5,500 × *g* in a Sorvall SS-1 centrifuge for 1.5 h. (We now use 9,000 × *g* for 1 h) The supernatant was discarded except for 5 ml, which was used to resuspend the pellet by blending in a Vortex mixer. The resuspended cells were diluted appropriately, plated in triplicate on charcoal-yeast extract agar, and incubated as described above.

Collection of the bacteria from cooling tower water. Cooling tower water samples varying from 1 to 2 liters in volume were pressure filtered and blended as described above. Membranes of 0.65-μm porosity were used for filtration of all samples except for the first nine, which were filtered through 0.45-μm membranes. After a sample was processed, both the filtra-

tion and blending equipment were thoroughly flushed with tap and then with distilled water before proceeding to the next sample.

DFA studies. Smears of the bacteria concentrated in the 5-ml volume were prepared on multiwell slides (Cel-Line Associates, Minotola, N.J.) for examination by DFA staining. The smears were heat fixed and stained with three pools of conjugates for *L. pneumophila* of serogroups 1 through 6 (5, 12, 13), for *Legionella bozemanii* (1), for *Legionella dumoffii* (1, 11), and for *Legionella micdadei* (10) and *Legionella gormanii* (15). The specific composition of the conjugate pools is given in Table 1. Smears for each water sample were processed individually to avoid any possible carry-over of bacteria from a positive to a negative slide.

Animal inoculation. The concentrated water samples were not cultured directly because the associated contaminants invariably overwhelm the LDB. When bacteria morphologically and serologically resembling the LDB were estimated by the microscopic DFA examination to equal or exceed 10^5 bacteria per ml of concentrate, a guinea pig was injected intraperitoneally with approximately 3.0 ml of the sample. Subsequently, the animals were observed for fever and for clinical signs of infection. If illness developed, they were sacrificed and smears of the peritoneal fluid, spleen, and liver were examined for *Legionella* bacteria by DFA tests. These tissues also were cultured on charcoal-yeast extract agar plates, and the plates were incubated at 36°C for 7 to 10 days. Colonies morphologically typical of *L. pneumophila* were fished from the plates and repurified by two successive platings. All isolates were characterized morphologically, tinctorily, serologically, and biochemically as described previously (2, 8). Gas-liquid chromatographic profiles also were obtained (16).

RESULTS

Experiments on efficiency of recovering LDB. The recovery of *L. pneumophila* from 4 liters of inoculated tap water as measured by the colony-forming units obtained on charcoal-yeast extract agar was tested in each of five experiments. In experiments 1 and 5, Millipore membrane filters of 0.45-μm porosity were used; in the other three experiments, membranes of 0.65-μm porosity were used to enhance flow rates. Within the limits of detectability (50 colony-forming units per liter), *L. pneumophila* was not cultured from the filtrates passed through the membranes of 0.45-μm porosity. To enhance flow rates, membranes of 0.65-μm porosity were tested for ability to retain the bacteria. The loss (0, 1.5, and 0.0074%) of *Legionella* bacteria in the filtrates from three experiments in which the 0.65-μm membrane was used was variable but relatively small when compared with the percentage of viable cells recovered from the filter. The size of *L. pneumophila* cells is about 0.5 by 1 to 2 μm. Under the experimental conditions used, the theoretical limit of detectability was approximately 50 colony-forming units per liter. The maximum loss of colony-forming units inoculated was approximately 1 $\log_{10}$. The recoveries of 78, 20, 9, 19, and 19% in the five experi-

TABLE 1. *Results of tests of 34 water samples from industrial cooling towers*

No. of samples	Water temp (°C)		Treatment received	No. of samples	Results of guinea pig inoculation
	Range	Mean			
18[a]	24–35	28	Chlorine and phosphates	8	ND[b]
			Betz J-12[c]	1	ND
			Unknown	3	ND
			None	6	ND
16[d]	22–24	29	None	3	−(1); +2 (Serogroup 1 and 4)
			Phosphates	1	−1
			Chlorine	2	−(1); +1 (Serogroup 1 and 4)
			Chlorine and Chromate	7	−(1); ND(6)
			Betz 508[e]	1	ND
			Betz J-12	1	ND
			Unknown	1	ND

[a] Samples contained no or $<10^5$ LDB-like cells per ml stained by pool A, B, or C conjugates. Pool A contained serogroups 1, 2, 3, and 4 of *L. pneumophila*; pool B contained serogroups 5 and 6 of *L. pneumophila*, *L. bozemanii*, and *L. dumoffii*; pool C contained *L. micdadei* and *L. gormanii* (15).

[b] ND, Not done.

[c] Betz J-12 is *N*-alkyl [C-12, 5%; C-14, 60%; C-16, 30%, C-18, 5%]-dimethyl benzyl ammonium chloride (quarternary ammonium chloride).

[d] Samples contained $\geq 10^5$ LDB-like cells per ml stained by pool A, B, or C conjugates.

[e] Betz 508 is 2,2-dibromo-3-nitrilopropionamide.

ments appear to be satisfactory, in view of the many manipulations involved and the lack of information on the viability of the LDB under stress.

Recovery of LDB from cooling tower water. Table 1 summarizes the test results of 34 water samples from industrial cooling towers. These samples were divided into two groups on the basis of a quantitative microscopic estimate of the number of cells morphologically typical of *Legionella* cells and fluorescing strongly with one, two, three or more of the polyvalent conjugates or giving a negative test. Of the 34 samples, 18 (group 1) were either negative or had less than 10^5 LDB per ml. The remaining 16 samples (group 2) had more than 10^5 LDB per ml. The water temperature and water treatment of the two groups were similar. None of the group 1 samples was injected into guinea pigs because of the relatively small number of LDB observed by DFA tests. Three pairs (serogroups 1 and 4) of isolates of *L. pneumophila* were obtained from samples in group 2, and all came from the same cooling tower. All six isolates were typical LDB by the criteria outlined above. Two pairs of isolates were obtained before the water received any treatment (Table 1). The third pair was obtained after the tower was hand-fed daily for 6 days with high test hypochlorite tablets [65% $Ca(OCl_2)$; Olin Corp., Stamford, Conn.] at an undetermined level. After chlorination, the tower was treated with 100 ml of Betz 508 (2,2-dibromo-3-nitrilopropionamide) daily for 5 days (Betz Labs, Inc., Trevose, Penn.). It was then treated on day 1, 3, 5, and 7 with 250 ml of Betz J-12 (*N*-alkyl [C-12, 5%; C-14, 60%; C-16, 30%; C-18, 5%]-dimethylbenzyl ammonium chloride). The volume of water in this one-celled cooling tower is quite variable (375 to 750 liters), so accurate concentrations of the added chemicals were not determined; however, the concentration of Betz 508 ranged from 0.13 to 0.26 g/liter; that of Betz J-12 ranged from 0.33 to 0.66 g/liter. DFA tests with polyvalent A conjugate (Table 1) indicated that LDB were still present (at concentrations of 3×10^5, 3×10^4, 1.4×10^5/ml) on day 5, 10, and 12, respectively, after Betz 508 treatment was started. After treatment with the two Betz Chemicals, the level of LDB in the tower was, however, considerably reduced compared with that obtained after chlorination (8×10^6 bacteria per ml).

Water samples taken from the tower after treatment with the Betz chemicals were not injected into the guinea pigs for attempted recovery of the LDB because the microscopic DFA examination indicated insufficient or borderline numbers of organisms.

DISCUSSION

Most laboratories have equipment suitable for filtration of 1 to 4 liters of water and for concentrating the bacteria from the membrane filters. With the 140-mm diameter membrane filters, the choice of pore size (0.45 μm or 0.65 μm) is based on both the appearance and quantity of the water sample to be filtered. A small, turbid sample of a few hundred milliliters volume may be filtered through either of the membranes. Samples of 1 to 4 liters, unless exceedingly clear, should be filtered through 0.65-μm membranes to prevent clogging or reduced flow. It is preferable to collect bacteria from water samples of 100 to 200 ml by centrifugation because this method minimizes the loss of cells.

The variability in recovery of pure cultures of LDB from inoculated 4-liter samples of water was not surprising (i) because of the number of manipulations involved and (ii) because the experiments were done over a period of time to assess the range of conditions that might be encountered. *L. pneumophila* cells may lose viability (i) by inoculation into tap water, (ii) by impaction on the filter membrane or because of toxicity of the membrane itself, (iii) by the trauma of the blending operation, (iv) by centrifugation, and (v) by resuspension and dilution before plating. In addition, physical loss of bacteria probably occurs by adherence of the cells to the membrane particles removed during low-speed centrifugation and by retention of some cells in the supernatant after high-speed centrifugation.

The described method is practical for examining cooling tower as well as other water samples for viable LDB that are infectious for guinea pigs. We have used these methods to isolate LDB from water samples from a variety of natural sources. Some isolates have been obtained from samples as small as 50 ml in volume. Pending the development of selective enrichment media, we are not aware of any procedure that is as good as or better than that described here for collecting and isolating the LDB from water samples up to 4 liters in volume.

Isolates were not obtained from four cooling tower water concentrates that were injected into guinea pigs (Table 1). One sample was untreated, and one was treated with chlorine, one with phosphates, and one with chlorine and chromate. Three of four samples had DFA microscopic counts of greater than 10^5 of the LDB-like cells per ml. The fourth was a sludge sample from the tower that yielded the three pairs of isolates. This sample was rapidly lethal to guinea pigs, presumably because of chemicals concen-

trated in the sludge, and therefore could not be validly examined for the LDB. The surface water sample taken from the above-described tower at the same time as the sludge sample yielded one of the pairs of LDB isolates. Attempted isolation of *L. pneumophila* from large-volume lake and river water samples has shown that the yield of isolates is small (15% of samples tested), even when large numbers of LDB-like bacteria are seen by DFA tests (7). In those studies, as in this one, the isolation of two serogroups of LDB from the same guinea pig was not unusual. There are several possible explanations for the failure to isolate the LDB-like bacteria observed by DFA tests: (i) the viability of the fluorescing LDB-like cells is unknown; (ii) some animals are lost to overwhelming infections by the contaminants associated with the samples; (iii) media may be suboptimum for cultural recovery, (iv) some LDB may be nonpathogenic for guinea pigs, and (v) some of the fluorescing bacteria may belong to other species that are serologically and morphologically similar to *L. pneumophila* (2). Only the last category indicates false-positive DFA tests. If cross-reactivity by DFA staining is a frequent occurrence, it has not been documented, in spite of intensive investigation.

Available data are inadequate for recommending treatment of cooling towers and evaporative condensers to eliminate *L. pneumophila*. Because these systems are proven sources for dissemination of the LDB, methods should be developed to prevent the growth and dispersal of this pathogen from these sources. It is emphasized that water samples failed to show a substantial decrease in LDB counts after routine treatment with chlorine at an undetermined level. Water samples that were analyzed after treatment with bacterial slime inhibitors, however, exhibited substantial decreases in LDB microscopic counts.

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