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Negative Enrichment Procedure for Isolation of *Legionella pneumophila* from Seeded Cooling Tower Water

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A negative enrichment procedure was developed which was capable of isolating *Legionella pneumophila* directly from seeded air-conditioning cooling tower water onto laboratory media. This procedure was based on an 8-h incubation under conditions that were bactericidal to the indigenous water microflora but merely bacteriostatic to *L. pneumophila*.

The epidemiological findings from recorded epidemics of Legionnaires disease suggest that this disease is spread by the airborne route from a common environmental source (3). However, the natural habitat of *Legionella pneumophila* and the mechanism by which it becomes airborne are more speculative. Several epidemics of legionellosis have been associated with contaminated air-conditioning cooling towers (1, 3). A recent study by Fliermans et al. (4) reported that this bacterium is also found in many nonepidemic-related aquatic habitats. Identification of *L. pneumophila* in samples from the environment usually involves screening of concentrated water samples by direct immunofluorescence microscopy, with confirmation by inoculation into guinea pigs (4). The direct culture of this bacterium onto laboratory media from environmental samples has been unsuccessful to date, due in part to overgrowth and interference by the normal resident microflora. This note describes a negative enrichment procedure that is capable of recovering *L. pneumophila* directly on laboratory media from seeded cooling tower water.

L. pneumophila strains Knoxville 1, Togus 1, Bloomington 2, and Los Angeles 1, representing the four serogroups, were obtained from the Center for Disease Control, Atlanta, Ga. GC-FC agar (5) containing GC medium base (Difco) supplemented with L-cysteine (0.4 g/liter) and soluble ferric pyrophosphate (0.25 g/liter) was used for routine maintenance of the cultures. Cultures were maintained at 37°C on GC-FC agar slants in a moist atmosphere containing 5% CO_2 and were transferred weekly. All cultures were routinely monitored for purity based on characteristic growth, pigment production, and microscopic morphology. Stock cultures of all strains were also stored at −70°C in tryptic soy broth (Difco) containing 20% (vol/vol) glycerol.

Inocula of *L. pneumophila* for addition to cooling tower water were prepared from cultures grown for 72 h on GC-FC agar plates. Cells were removed with a sterile cotton swab and suspended in 5 ml of sterile tap water to a turbidity of approximately 25 Klett units (ca. 10^7 colony forming units [CFU]/ml). Portions of this suspension were then added to samples of cooling tower water or sterile tap water to achieve a cell concentration of approximately 10^4 or 10^2 CFU/ml.

Water samples were obtained from the basins of operating cooling towers in the Louisville metropolitan area during the months of September through December. The temperature of each sample was recorded at the time of collection. The pH was measured in the laboratory with a Corning model 7 pH meter. Levels of indigenous microflora were determined by routine plate count procedures with sterile distilled water as a diluent. Samples were plated on the surface of GC-FC agar plates. Plates were incubated at 37°C with 5% CO_2 and read after 48 to 72 h. Colonial color and morphology along with Gram-stain characteristics were used to classify individual isolates. No attempt was made to further identify these organisms.

The negative enrichment procedure involved mixing a sample (2.5 ml) of cooling tower water (or seeded sterile tap water) with an equal volume of negative enrichment broth. This latter solution was prepared by reconstituting the contents of one 10-ml vial of CNV antibiotics (Difco) in 50 ml of double strength tryptic soy broth (Difco). When mixed with an equal volume of cooling tower water as described above, the final antibiotic concentrations of colistimethate, nystatin, and vancomycin were 75 μg/ml, 125 U/ml, and 30 μg/ml, respectively. Samples were then incubated without shaking at room temperature (22°C). At 1-h intervals samples (0.01 and 0.1 ml) were removed and plated directly on GC-FC agar plates. Viable cell counts were also per-

formed as described above. After incubation, these plates were examined for the presence of indigenous microflora and for growth of characteristic *L. pneumophila* colonies.

The effect of the negative enrichment procedure on the survival of the indigenous microflora in the tower water, compared with *L. pneumophila* strain Togus 1 (in sterile tap water) is shown in Fig. 1. The indigenous microflora decreased from 2.2×10^4 CFU/ml to undetectable levels ($<10^1$ CFU/ml) within 6 h. In contrast, *L. pneumophila* was unaffected by the antibiotics during incubation for 8 h. Preliminary experiments showed the Togus strain to be the most sensitive of the four isolates to these antibiotics. Incubation beyond 8 h, however, eventually resulted in decreased viability of *L. pneumophila*. The culture was still unaffected at 15 h, but by 24 h the viability had dropped to undetectable levels (data not shown). Resistant populations of indigenous microflora also routinely appeared by 72 h. Based on these results, the negative enrichment procedure was standardized to an incubation for 8 h.

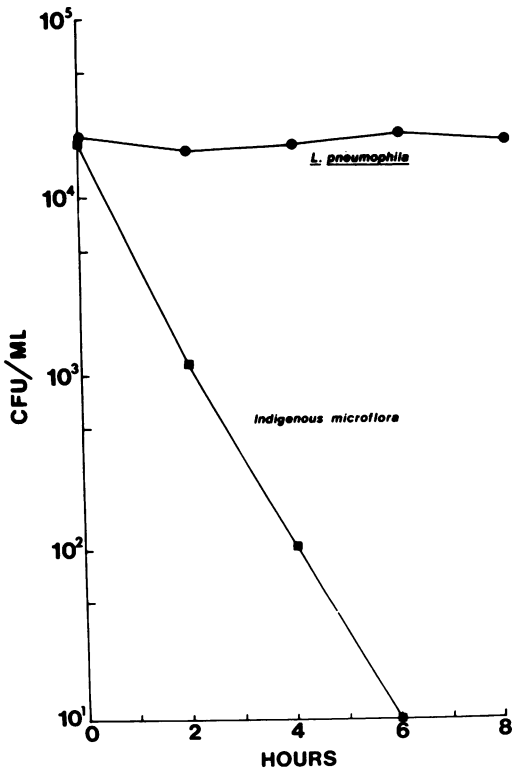

FIG. 1. *Effect of the negative enrichment procedure on the survival of the indigenous microflora in the tower water compared with the survival of L. pneumophila strain Togus 1 in sterile tap water. Tower water sample G was used for this experiment (see Table 1).*

A total of eight cooling tower samples were processed through the negative enrichment procedure (Table 1). These water samples varied with respect to sampling temperature (24 to 32°C), pH (5.5 to 8.3), prior chemical treatment, and levels of indigenous microflora (1.5×10^4 to 1.4×10^5 CFU/ml). Although the bacteria in these samples were not identified, they were, for the most part, gram-negative bacilli usually representing at least four different species based on colonial description and microscopic morphology. An occasional gram-positive bacillus or fungus colony was also encountered. The microflora of all eight water samples was reduced to $\leq 6 \times 10^1$ CFU/ml after 8 h in the antibiotic mixture. This level of reduction was acceptable for direct plating of 0.01-ml samples (<1 colony per plate). In several cases the microflora was reduced to levels undetectable when plating samples of 0.1 ml.

Four different isolates of *L. pneumophila* were tested for survival in the negative enrichment procedure. Each isolate was suspended to an initial concentration of approximately 10^4 CFU/ml in either cooling tower water or sterile tap water and then processed through the negative enrichment procedure. Each of the four isolates demonstrated complete survival after 8 h in the antibiotic mixture (Table 2). No difference in survival was observed between *L. pneumophila* in tap water compared with that in cooling tower water. The best results were obtained by direct plating of 0.01-ml samples from the negative enrichment. This sample size gave excellent recovery of *L. pneumophila* (Table 2) with only an occasional colony of indigenous microflora. Plating of larger samples (i.e., 0.1 ml) occasionally resulted in a somewhat reduced recovery of *L. pneumophila* plus an increased number of potential colonies of indigenous microflora on each plate (data not shown).

The potential sensitivity of the negative enrichment procedure for detecting *L. pneumophila* would be ca. 2×10^2 CFU/ml based on plating 0.01-ml samples. To determine whether this sensitivity could be realized in practice, we inoculated *L. pneumophila* strain Togus 1 into a cooling tower water sample at an initial concentration of 6×10^2 CFU/ml. After 8 h of incubation with the antibiotics the concentration of *L. pneumophila* was 5.7×10^2 CFU/ml.

The results of this study have demonstrated the effectiveness of our negative enrichment procedure for isolation of *L. pneumophila* from cooling tower water seeded with this organism. This procedure is based on the observation that

TABLE 1. *Effect of the negative enrichment procedure on the viability of indigenous microflora in samples of cooling tower water*

Water sample	Temp (°C)[a]	pH	Chemical additives[b]	Viability (CFU/ml)	
				Initial (0 h)	After treatment (8 h)
A	30	8.0	Chromate	4.6×10^4	6.0×10^1
B	30	8.2	Quat, phos	6.2×10^4	3.0×10^1
C	24	8.2	Quat, phos	2.4×10^4	4.0×10^1
D	30	7.8	None	4.4×10^4	1.0×10^1
E	24	8.3	Quat, phos	1.4×10^5	$<10^1$
F	30	8.1	Quat, phos	1.5×10^4	2.0×10^1
G	29	8.3	Quat, phos	2.2×10^4	$<10^1$
H	32	5.5	Quat, phos	4.0×10^4	$<10^1$

[a] Temperature of the water sample at the time of collection from the cooling tower.

[b] Chemical additives with which the tower was being treated at the time of collection. Information on the exact concentration of these compounds in the tower water at the time of collection was not available. Chromate is a corrosion-inhibiting additive; quat, quaternary ammonium biocide; phos, phosphonate anti-scale and dispersant additive.

TABLE 2. *Effect of the negative enrichment procedure on the viability of L. pneumophila in cooling tower water or sterile tap water*[a]

Strain	Sterile tap water (CFU/ml)		Cooling tower water (CFU/ml)[b]	
	Initial (0 h)	After treatment (8 h)	Initial (0 h)	After treatment (8 h)
Knoxville 1	5.0×10^4	5.3×10^4	ND[c]	5.2×10^4
Togus 1	2.2×10^4	2.0×10^4	ND	2.2×10^4
Bloomington 2	4.8×10^4	5.1×10^4	ND	5.0×10^4
Los Angeles 1	2.0×10^4	2.2×10^4	ND	2.0×10^4

[a] The data in this table were based on plating of 0.01-ml samples.

[b] Cooling tower water sample G was used in these experiments.

[c] ND, Not determined. Initial levels of indigenous flora present precluded determination of exact numbers of *L. pneumophila*. However, since the cooling tower water and sterile tap water were inoculated from the same stock inoculum, the initial concentration in the tower water should, in each case, be reasonably close to the initial values for the sterile tap water.

the antibiotics and incubation conditions used in this study were bactericidal to the indigenous microflora but were merely bacteriostatic to *L. pneumophila*. The resistance of *L. pneumophila* to these antibiotics (vancomycin and polymyxin B) has been reported recently by Edelstein and Finegold (2), who described a semiselective medium for isolation of *L. pneumophila* from clinical specimens. We were unsuccessful in using this medium or similar selective media for direct isolation of *L. pneumophila* from environmental samples, due to similarities in the minimal inhibitory concentrations of these antibiotics for *L. pneumophila* and the environmental microorganisms. The success of our negative enrichment may be based on the incubation in tryptic soy broth at 22°C. These conditions are unfavorable for growth of *L. pneumophila* and may result in antibiotic-induced bacteriostasis rather than lethality.

The applicability of the negative enrichment procedure for isolation of naturally occurring *L. pneumophila* from environmental samples was not addressed in this study. However, if the antibiotic resistance of the naturally occurring organisms is similar to that of the laboratory-maintained cultures used in this study, then the negative enrichment procedure or modifications of it should be successful. This procedure would be a valuable addition to the laboratory procedures currently available for detection of *L. pneumophila* in the environment, and studies designed to ascertain its effectiveness in these areas are in progress.

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