

Legionella spp. in Puerto Rico Cooling Towers

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Received 18 April 1988/Accepted 13 July 1988

Water samples from air conditioning cooling towers receiving different treatment protocols on five large municipal buildings in San Juan, P.R., were assayed for various *Legionella* spp. and serogroups by using direct immunofluorescence. Several water quality parameters were also measured for each sample. Guinea pigs were inoculated with water samples to confirm pathogenicity and recover viable organisms. *Legionella pneumophila* serogroups 1 to 6, *L. bozemanii*, *L. micdadei*, *L. dumoffii*, and *L. gormanii* were observed in at least one of the cooling towers. *L. pneumophila* was the most abundant species; its density reached 10^5 cells per ml, which is within the range that is considered potentially pathogenic to humans. A significantly higher density of *L. pneumophila* was observed in the cooling tower water that was not being treated with biocides. Percent respiration (INT) and total cell activity (acridine orange direct count) were inversely correlated with bacterial density. This study demonstrates that *Legionella* spp. are present in tropical air-conditioning cooling systems and that, without continuous biocide treatment, they may reach densities that present a health risk.

Legionellosis accounts for almost 4% of all cases of atypical pneumonia (7). The disease has been reported in many parts of the United States and Europe. Fliermans (3) has estimated that over 200,000 cases per year occur in the United States. Reports of legionellosis from the tropics were rare until 24 people who visited St. Croix, U.S. Virgin Islands, acquired legionellosis (11). *Legionella pneumophila* serogroups 1 and 3 and several new species were isolated from the potable water system in the resort where these people had been vacationing (11). Recently, studies in Puerto Rico have demonstrated that *Legionella* spp. are widely distributed in natural environments and may reach potentially pathogenic densities (10). Ortiz-Roque and Hazen (10) also demonstrated, from autopsy analysis, that legionellosis in Puerto Rico has an overall mortality rate of 25% and that at least 52 cases should be diagnosed every year, yet only 4 retrospective cases have ever been reported. The present study was undertaken to determine the incidence, density, and pathogenicity of *Legionella* spp. in cooling towers for air-conditioning systems in buildings over 15 stories high in San Juan, P.R.

(This study was part of the M.S. thesis of A. Negrón-Alvíra at the University of Puerto Rico, Río Piedras, P.R., 1987.)

MATERIALS AND METHODS

Sampling procedures. Samples for the detection of *Legionella* spp. were taken from the air-conditioning cooling systems of buildings over 15 stories high in the banking area in Hato Rey (San Juan), P.R. The cooling towers were examined for fecal coliforms, *Legionella* spp., the presence of algae in the tanks, the state of maintenance of the cooling units, and fill material. Samples for bacteriological analysis were collected by grab sampling and placed into sterile Whirl-Pak Bags (Nasco International, Fort Wilkinson, Wis.) or, if the water source was chlorinated, sodium thiosulfate bags (Nasco). Standard fixation and storage techniques were

performed (1). Time from collection to analysis never exceeded 6 h.

Water quality. Conductivity, pH, temperature, and dissolved oxygen were measured in situ by using a Hydrolab surveyor (digital model 4041; Hydrolab Corp., Austin, Tex.). Alkalinity and hardness were also measured in situ by standard methods (1) by using Spectrokits (Bausch & Lomb, Inc., Rochester, N.Y.). Other samples were collected, placed into Nalgene bottles, fixed, and transported to the laboratory for further analysis. These fixed samples were tested for nitrites plus nitrates, sulfates, phosphates, total phosphorus, and chlorophyll *a* trichromatic as recommended in *Standard Methods for the Examination of Water and Wastewater* (1).

To obtain an index of biological contamination, fecal coliform densities were determined for every sample. Fecal coliform densities were determined by membrane filtration of triplicate samples, plating on m-FC medium, and incubation at $44.5 \pm 0.1^\circ\text{C}$ for 24 h in a block-type incubator (1).

Total bacteria cell counts were determined by acridine orange staining (AODC) as described by Singleton et al. (12). At the same time, total bacterial activity was measured in terms of the ability of the cells to reduce INT to INT-formazan during respiration by the method of Zimmermann et al. (14). All methods used were described previously (10).

Direct enumeration of *Legionella* spp. Ten liters of water was collected and placed into sterile polycarbonate containers at each sampling site, incubated with INT for 30 min (14), fixed with Formalin, and transported on ice to the laboratory. These samples were centrifuged at $5,000 \times g$ for 15 min at 4°C . The pellet and residual water were passed through a 0.2- μm -pore-size, 47-mm-diameter membrane filter (Nucleopore Corp., Pleasanton, Calif.). The filter was eluted by shaking it with sample water, and 10- μl aliquots were placed into the eight wells of a toxoplasmosis slide (Cell Line Associates, Newfield, N.J.). The aliquots were fixed with Formalin, and the slide was subsequently stained with fluorescent antibody to *L. pneumophila* (serogroups 1 to 6), *L. gormanii* (serogroup 1), *L. dumoffii* (serogroup 1), *L. bozemanii*, *L. micdadei*, *L. longbeachae*, and *L. oakridgensis*. All sera and antigens were supplied by the Centers for Disease Control, U.S. Department of Health and Human

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TABLE 1. Cooling tower water quality by site^a

Site	WTEMP (°C)	DO concn (mg/liter)	pH	HARD (mg of CaCO ₃ /liter)	NO ₂₊₃ (mg/liter)	TP (mg/liter)	PO ₄ (mg/liter)	ChlA (mg/liter)	%R	%A	FC (CFU/ml)
A	27 ± 0.5	6.6 ± 0.2	7.1 ± 0.2	76 ± 6.3	1.7 ± 0.2	1.7 ± 0.2	0.6 ± 0.1	8.1 ± 0.2	16.7 ± 1.1	38.9 ± 2.0	11 ± 1.0
B	29 ± 0.3	8.8 ± 0.2	7.9 ± 0.3	40 ± 6.0	1.4 ± 0.1	3.4 ± 0.1	1.7 ± 0.2	6.8 ± 0.2	16.6 ± 1.5	32.4 ± 1.3	66 ± 4.0
C	28 ± 0.6	5.0 ± 0.3	7.1 ± 0.1	43 ± 3.3	5.2 ± 0.1	4.8 ± 0.1	4.1 ± 0.1	8.4 ± 0.2	30.1 ± 3.7	39.7 ± 1.1	10 ± 4.8
D	27 ± 0.1	4.0 ± 0.2	7.2 ± 0.2	30 ± 5.8	4.4 ± 0.1	4.4 ± 0.1	0.5 ± 0.1	8.1 ± 0.2	14.1 ± 2.6	58.9 ± 4.0	7.4 ± 1.0
E	28 ± 0.3	4.3 ± 0.6	7.2 ± 0.6	37 ± 5.8	6.9 ± 0.1	3.9 ± 0.1	0.4 ± 0.1	7.5 ± 0.3	14.0 ± 2.0	37.7 ± 7.0	7.4 ± 1.5

^a All values are means ± 1 standard error. Abbreviations: WTEMP, water temperature; DO, dissolved oxygen; HARD, hardness; NO₂₊₃, nitrites plus nitrates; PO₄, orthophosphate; TP, total phosphorus; ChlA, chlorophyll a; %A, percentage of total bacteria active (AODC); %R, percentage of total bacteria respiring (INT); FC, fecal coliforms.

Services, Atlanta, Ga. Stained slides were examined with an epifluorescence microscope (model 16, with an IV FL vertical illuminator; Carl Zeiss, Inc., New York, N.Y.). The percentage of respiring *Legionella* spp. was determined by using the FAINT technique of Fliermans et al. (6).

Inoculation of guinea pigs. Sample processing and inoculum dosages varied with the total number of organisms (DFA) found. Unfixed water samples were prepared for inoculation into guinea pigs as follows. If the sample contained more than 10³ *Legionella* sp.-like cells per ml, 2 ml was inoculated intraperitoneally; if at least 10² cells per ml but less than 10³ cells per ml were present, 3 ml was inoculated intraperitoneally; if less than 10² cells per ml were present, the sample was concentrated by centrifuging it at 2,900 × g for 30 min, discarding in supernatant, suspending the sediments in 6 ml of sucrose-phosphate-glutamate buffer, and inoculating 3 ml intraperitoneally by the method of Morris et al. (8).

Five guinea pigs were used in each sampling. One guinea pig was used as a positive control; it was inoculated directly with *L. pneumophila* (ATCC 33152). Another guinea pig was used as a negative control; it was inoculated with sample water filtered through a 0.2-μm-pore-size membrane filter. Before inoculation, the mean base-line temperature of each animal was established from five daily measurements. After inoculation, the temperature of each guinea pig was measured at a predetermined time each day for 7 days. A rise of 0.6°C over the base-line temperature for 2 consecutive days was considered a fever, and febrile animals were sacrificed immediately, as well as were any animals with other signs of illness (ruffled fur, watery eyes, prostration, and hypothermia). All guinea pigs were sacrificed at 7 days. The tissue homogenates were examined by fluorescent antibody assay and inoculated onto media as described below (2).

***Legionella* viable counts and isolation.** Four-liter samples were collected, placed into sterile polycarbonate containers, and transported on ice to the laboratory. All samples were

then pretreated with acid to reduce background organisms by the method of Cherry et al. (2). Treated samples were then plated onto *Legionella* Agar Base and *Legionella* Agar Enrichment (Difco Laboratories, Detroit, Mich.). After 2 to 5 days of incubation in an aerobic and humid chamber containing 2.5% carbon dioxide at 35°C, colonies that appeared light blue to blue-gray in color were considered positive (4). Isolates were then Gram-stained and subcultured to a fresh agar plate and to a blood agar plate that did not contain L-cysteine. Typical isolates were then subjected to immunofluorescence staining for confirmation.

Data analysis. Statistical analyses were done with programs developed for the Apple IIe and Macintosh computers. Heteroscedastic data were made more homoscedastic by using the appropriate transformation prior to analysis. Any statistical probability equal to or less than 0.05 was considered significant (13).

RESULTS AND DISCUSSION

Representative water quality data are given in Table 1. Previous studies by our laboratory (10) demonstrated that *L. bozemanii*, *L. dumoffii*, *L. micdadei*, *L. gormanii*, *L. longbeachae*, and *L. pneumophila* were widely distributed in natural waters of Puerto Rico. The present study has shown that air-conditioning cooling towers in the tropics can also harbor *Legionella* spp. *Legionella* spp. were found at all five sites, with densities of 10⁴ to 10⁵ cells per ml (Table 2). Densities of 10⁵ to 10⁶ cells per ml are believed to be potentially pathogenic (5). The densities reported in this study were similar to, although slightly lower than, those reported for cooling tower waters in temperature areas (4, 9). *L. bozemanii*, *L. micdadei*, *L. pneumophila*, *L. gormanii*, and *L. dumoffii* were isolated from the cooling towers (Table 2). *L. longbeachae* and *L. oakridgensis* were not detected in the cooling towers but have been observed in natural waters of Puerto Rico (10). Only *L. pneumophila* was found in all

TABLE 2. Density, activity, and pathogenicity of *Legionella* spp. by site

Site	Density (10 ³ cells/ml) of the following species ^a :								Faint ^b	GP ^c
	TL	LG	LD	LB	LM	LL	LO	LP		
A	25 ± 5.1	29 ± 9.0	25 ± 7.0	ND	ND	ND	ND	13 ± 3.5	15 ± 1.5	19/20 (2)
B	290 ± 37	ND	27 ± 5.3	ND	11 ± 4.1	ND	ND	110 ± 37	14 ± 2.4	20/20 (1)
C	20 ± 3.9	25 ± 6.8	ND	8.2 ± 5.3	ND	ND	ND	13 ± 3.2	22 ± 5.5	15/16 (0)
D	22 ± 6.4	28 ± 8.0	20 ± 5.8	ND	ND	ND	ND	14 ± 8.8	11 ± 1.9	15/16 (0)
E	19 ± 1.8	15 ± 3.1	12 ± 4.5	ND	12 ± 4.2	ND	ND	1.4 ± 0.9	9.7 ± 1.8	16/16 (0)

^a All densities are means ± 1 standard error (n = 4). Abbreviations: TL, total *Legionella* spp.; LG, *L. gormanii*; LD, *L. dumoffii*; LB, *L. bozemanii*; LM, *L. micdadei*; LL, *L. longbeachae*; LO, *L. oakridgensis*; LP, *L. pneumophila* (serogroups 1 to 6).

^b FAINT, Percentage of *L. pneumophila* that were respiring, as measured by INT reduction.

^c GP, Number of guinea pigs with isolatable *Legionella* spp. per number tested (number of fatal infections).

TABLE 3. Densities of *L. pneumophila* serotypes by site^a

Site	Density (cells/ml) of serotype:					
	1	2	3	4	5	6
A	1.6×10^4	2.6×10^3	1.1×10^4	3.0×10^3	1.1×10^3	2.4×10^3
B	2.3×10^4	8.5×10^3	3.7×10^4	2.4×10^3	4.5×10^3	2.2×10^3
C	9.4×10^3	2.0×10^3	7.9×10^3	2.8×10^3	2.7×10^3	3.4×10^3
D	6.3×10^3	2.4×10^3	6.4×10^3	3.9×10^3	0	7.0×10^2
E	9.0×10^3	9.2×10^3	0	8.1×10^2	8.9×10^2	0
% of total	39.4	10.4	29.6	7.6	6.5	5.9

^a Determined by DFA.

five cooling towers. *L. dumoffii* and *L. gormanii* were found in four of the five cooling towers, whereas *L. micdadei* was found in only two cooling towers and *L. bozemanii* was found at only one site (Table 2). The most abundant species was *L. pneumophila* (40.75%). *L. pneumophila* serogroups 1 and 3 were the most abundant serogroups found, accounting for 39.4 and 29.6%, respectively (Table 3). The most abundant species found in the potable water system which was linked to an outbreak of legionellosis on the adjacent island of St. Croix was also *L. pneumophila*, serogroups 1 and 3 (11). Natural waters of Puerto Rico were also shown to be dominated by serogroups 1 and 3 (10), as were cisterns on the adjacent island of St. Thomas (T.C.H., unpublished data). This suggests that in the Caribbean and perhaps in other tropical areas *L. pneumophila* is the dominant *Legionella* sp. and that serogroups 1 and 3 are the dominant serotypes.

The pathogenicity of the *Legionella* spp. for each cooling tower was established through inoculation of guinea pigs and recovery of organisms from homogenized tissues of moribund animals. Although all animals that became ill after inoculation had isolatable *Legionella* spp. in their tissues, not all of them died (Table 2). This could indicate that the *Legionella* strains present were less virulent. Guinea pig inoculation is still the most appropriate method for recovery of *Legionella* organisms. Isolation by using media is very difficult owing to high levels of contamination (9). Indeed, in the present study *Legionella* spp. could not be isolated directly from cooling tower water by using media owing to overgrowth by yeasts; these results were similar to those obtained previously for natural waters in Puerto Rico (10).

The cooling tower which was not being treated with antimicrobial compounds (site B) had the highest densities of *Legionella* spp. (Table 2). Biocidal treatment of sites A, C, D, and E helped to control *Legionella* spp. to some extent, even though the organisms in site C had a high level of activity (Table 2). Fliermans et al. (5) reported 5 to 36% respiration for *Legionella* spp. in water samples taken from freshwater lakes and ponds. In the present study, the percentage of respiration ranged from 10 to 35% for the total bacterial community and from 5 to 30% for *L. pneumophila*. At site B, where the highest cell densities of *L. pneumophila* were observed, the lowest percentage of respiring cells was observed; conversely, at site C, where the lowest cell densities were observed, the highest proportion of respiring cells was observed. The total bacterial population was also more active, as indicated by AODC, in the cooling towers which were receiving biocides (Table 2). This suggests that biocides reduce the density of *Legionella* spp. and other bacteria in the cooling tower water but that the remaining population is more active since there is less competition and

more resources. It remains to be seen whether a more active *Legionella* population is also more pathogenic.

It is interesting that densities of *Legionella* organisms in Puerto Rico cooling tower waters were quite high considering the low nutrient levels of the water and the fastidious nature of the organism. Although previous studies have shown relationships between *Legionella* spp. and protozoa and between *Legionella* spp. and cyanobacteria, such relationships were not observed in these cooling towers. However, densities of fecal coliforms and the activity of the total bacterial population (Table 1) may indicate poorer-quality source water and higher bacterial turnover rates than were previously reported for cooling towers (4). These conditions could also reduce the effectiveness of biocides in the cooling towers.

The presence of pathogenic *Legionella* spp. in air-conditioning cooling towers in the tropics at concentrations high enough to cause disease, especially in immunocompromised or elderly people, suggests that legionellosis may be underdiagnosed in the tropics. Considering the year-round use of these cooling towers and the large proportion of the population that may be exposed, monitoring and treatment of these systems are essential for prevention of legionellosis.

ACKNOWLEDGMENTS

We are especially grateful to Carl B. Fliermans, who made many helpful suggestions regarding the manuscript.

This work was supported by the Water Resources Institute of the University of Puerto Rico at Mayagüez and in part by Sea Grant R/LR-08-87-THA1 and by Public Health Service grants RR-2657 and RR-8102 from the National Institutes of Health. In addition, portions of the information contained in this article were developed during the course of work under contract no. DE-AC09-76SR00001 with the U.S. Department of Energy.

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