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ABSTRACT

Water samples from air conditioning cooling towers receiving different treatment protocols on five large municipal buildings in San Juan, Puerto Rico were assayed for various species and serogroups of Legionella spp. using direct immunofluorescence. Several water quality parameters were also measured with each sample. Guinea pigs were inoculated with water samples to confirm pathogenicity and recover viable organisms. Legionella pneumophila (1-6), 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, reaching 10^5 cells/ml, 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 (AODC), were inversely correlated with bacterial density. This study demonstrates that Legionella spp. are present in tropical air-conditioning cooling systems, and without continuous biocide treatment may reach densities that present a health risk.

INTRODUCTION

Legionellosis accounts for almost 4% of all patients with 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 a year occur in the United States. Reports of legionellosis from the tropics were rare until twenty four people that 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 the patients were vacationing [11]. Recently, studies in Puerto Rico have demonstrated the 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 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 fifteen stories high in San Juan, Puerto Rico.

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MATERIALS AND METHODS

Sampling procedures. Samples for the detection of Legionella were taken from the air-conditioning cooling systems of buildings over 15 stories high in the banking area in Hato Rey (San Juan), Puerto Rico. The cooling towers were examined for fecal coliforms, Legionella spp., the existence 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 sodium thiosulfate bags (Nasco), if the water source was chlorinated. 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** using a Hydrolab surveyor (digital model 4041, Hydrolab Corp., Austin, Tex.). Alkalinity and hardness were also measured **in situ** by standard methods [1] using Spectrokits (Bausch and Lomb, Rochester, N.Y.). Other samples were collected in 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 using Standard Methods for Water and Waste Water Analysis [1].

In order to have an index of biological contamination, fecal coliform densities were done for every sample. Determination of fecal coliform densities was performed by membrane filtration of triplicate

samples, plating on m-FC media, and incubation at 44.5 ± 0.1°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 cell ability to reduce INT to INT-formazan during respiration as described by Zimmermann et al. [14]. All methods are as described previously [10].

Direct enumeration of Legionella spp. Ten liters of water were collected in 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 x g for 15 min at 4°C. The pellet and residual water was filtered onto a 0.2 µm pore size, 47-mm diameter membrane (Nuclepore Corp., Pleasanton, Calif.). The filter was eluted by shaking with sample water and 10 µl aliquots placed into the 8 wells of a toxoplasmosis slide (Cell Line Associates, Newfield, N.J.). The the aliquots were fixed with formalin and the slide subsequently stained with fluorescent antibody to L. pneumophila (serogroup 1-6), 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 U.S. Dept. of Health and Human Services, Center for Disease Control, Atlanta, Georgia. Stained slides were examined with an epifluorescence microscope (Model 16 + IV FL Vertical illuminator, Carl Ziess Inc., N.Y.). The percentage of respiring Legionella spp. was determined using the FAINT technique as described by 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 1 x 10^3 Legionella-like cells/ml, 2 ml was inoculated intraperitoneally; if at least 1 x 10^2 cells/ml but less than 1 x 10^3 cells/ml were present, 3 ml were inoculated intraperitoneally; if less than 1 x 10^2 cells/ml were present, the sample was concentrated by centrifuging the sample at 2,900 x g for 30 min, discarding the supernatant, resuspending the sediments in 6 ml sucrose phosphate glutamate buffer, and inoculating 3 ml intraperitoneally, as described by Morris et al. [8].

Five guinea pigs were used in each sampling. One guinea pig was used as a positive control, inoculating it directly with Legionella pneumophila (ATCC 33152), and another guinea pig as a negative control, inoculating it with sample water filtered through a 0.2 µm-pore size membrane filter. Before inoculation, each animal's mean baseline temperature was established from 5 daily measurements. After inoculation the guinea pig's temperature was measured at a predetermined time each day for 7 days. A rise of 0.6°C over the baseline temperature for 2 consecutive days was considered a fever, and febrile animals were sacrificed immediately as well as the other 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 and inoculated onto media as described below [2].

Legionella viable counts and isolation. Four liter samples were collected in sterile polycarbonate containers and transported on ice to the laboratory. All samples were than pretreated with acid to reduce background organisms as described by Cherry et al. [2]. Treated samples were than plated on 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 [5]. 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 than subjected to immunofluorescent staining for confirmation.

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

RESULTS AND DISCUSSION

Previous studies by our laboratory [10] demonstrated that Legionella bozemanii, L. dumofii, L. micdadei, L. gormanii, L. longbeachae, and L. pneumophila were found widely distributed in natural waters of Puerto Rico. The present study has shown that air-conditioning cooling towers in the tropics can also harbour Legionella spp. Legionella spp. were found at all five sites with densities from 10^4 to 10^5 cells/ml (Table 1). Densities of 10^5 to 10^6 cells/ml, are believed to be potentially pathogenic [4]. The densities reported in this study were similar, though slightly lower than those reported for cooling tower waters in temperate areas [5, 9]. L. bozemanii, L. micdadei, L. pneumophila, L. gormanii, and L. dumoffii were isolated from the cooling towers (Table 2). L. longbeachae and L. oakridgesis were not detected in the cooling towers, but were observed in natural waters of Puerto Rico [10]. Only L. pneumophila was found in all 5 cooling towers. L. dumofii and L. gormanii were found in four of the 5 cooling towers, whereas L. micdadei was only found in 2 cooling towers, and L. bozemanii was only found at 1 site (Table 2). The most abundant species was L. pneumophila (40.75%). L. pneumophila serogroups 1 and 3 were the most abundant serogroups found, each accounting for 39.4% and 29.6%, respectively (Table 3). The most abundant species found in the potable water system linked to an outbreak of legionellosis on the adjacent island of St. Croix were also L. pneumophila serogroups 1 and 3 [11]. Natural waters of Puerto Rico were also shown to be dominated by

serogroups 1 - 3 [10] as were cisterns on the adjacent island of St. Thomas [Hazen, unpublished data). This suggests that in the Caribbean and perhaps in other tropical areas L. pneumophila is the dominant species of Legionella spp. and that serogroups 1-3 are the dominant serotypes.

The pathogenicity of the Legionella spp. from each cooling tower was established through guinea pig inoculation and recovery from homogenized tissues of moribund animals. Though 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 Legionella recovery. Isolation using media is very difficult due to high levels of contamination [9]. Indeed, in the present study Legionella spp. could not be isolated directly from cooling tower water using media due to over-growth by yeasts, similar results were obtained previously for natural waters in Puerto Rico [10].

The cooling tower which was not being treated by antimicrobial compounds (site B), had the highest densities of Legionella (Table 2). Biocidal treatment of sites A, C, D, and E helped control to some extent Legionella, even though the organisms in site C had a high level of activity (Table 2). Fliermans et al. [4] reported 5-36% respiration for Legionella spp. in water samples taken from freshwater lakes and ponds. In this 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 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 if a more active population of Legionella is also more pathogenic.

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

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Table 1. Cooling tower water quality by site.

SITES	WTEMP	DO	pH	HARD	NO_{2+3}	PO_4	TP	CHLA	%R	%A	FC
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

*All values are mean ± one standard error, WTEMP = water temperature (°C), D O = dissolved oxygen (mg/L), HARD = Hardness (mg/L $CaCO_3$), $NO_{2\pm3}$ = nitrites plus nitrates (mg/L), PO_4 = orthophosphate (mg/L), TP = total phosphorus (mg/L), CHLA=chlorophyll *a* (mg/L), %A = percentage of total bacteria active (AODC), %R = percentage of total bacteria respiring (INT), FC = fecal coliforms (CFU/ml).

Table 2. Density, activity and pathogenicity of Legionella by site.

SITES	TL	LG	LD	LB	LM	LL	LO	LP	FAINT	GP
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)

*All densities are mean ± one standard error x 10^3 cells/ml (n = 4), TL = total Legionella, LG = L. gormanii, LD = L. dumoffii, LB = L. bozemanii, LM = L. micdadei, LL = L. longbeachae, LO = L. oakridgensis, LP = L. pneumophila (serogroup 1-6), FAINT = percentage of LP that were respiring as measured by INT reduction, GP = guinea pig recovery of Legionella spp. number of positive recoveries / number tested (number of fatal infections).

Table 3. Densities of Legionella pneumophila serotypes by site.

	SEROTYPES					
SITE	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
Percent of total	39.4	10.4	29.6	7.6	6.5	5.9

*All densities in cells/ml by DFA