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Susceptibility of *Legionella pneumophila* to Three Cooling Tower Microbicides

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Investigation of epidemic outbreaks of Legionnaires disease by Center for Disease Control personnel has resulted in the isolation of *Legionella pneumophila* from water in the air-conditioning cooling towers or evaporative condensers at the site of the outbreak. It is suspected that improperly maintained open, recirculating water systems may play a role in the growth and dissemination of this pathogen. The objective of this study was to determine the antimicrobial activity of three chemically different, commerically available, cooling tower microbicides against *L. pneumophila*. Using two in vitro test systems, a combination of *N*-alkyl dimethyl benzyl ammonium chloride and bis (tri-*n*-butyltin) oxide was found to kill *L. pneumophila* at a concentration 25 times less than the minimum recommended use concentration, whereas *N*-alkyl 1,3-propanediamine and methylene bis (thiocyanate) were active at concentrations equal to or greater than the concentrations recommended for use by the manufacturer.

During and after the meeting of the American Legion, Department of Pennsylvania, in Philadelphia in July 1976, 182 people developed a severe lower respiratory tract infection and 29 people died (7). Subsequently, a gram-negative bacillus was identified as the etiological agent (8), and the name *Legionella pneumophila* was proposed for this organism (2). By the end of 1978, nine additional epidemic outbreaks had been recorded, resulting in a total of 558 confirmed cases of Legionnaires disease and 70 deaths (3). At three of the outbreak sites, *L. pneumophila* was isolated from water in air-conditioning cooling towers or evaporative condensers. Epidemiological data from two of these outbreaks suggest that the cooling tower or evaporative condenser was the source of dissemination of organisms (6).

Pure chemicals or hospital disinfectant products not formulated for use as cooling tower microbicides (4, 10) have been used in preliminary studies on the activity of chemical microbicides against *L. pneumophila*. The objective of this study was to determine the antimicrobial potential of three cooling tower microbicides, available from a number of sources, against *L. pneumophila* by employing methodology specified by the U.S. Environmental Protection Agency for proof of efficacy (9).

MATERIALS AND METHODS

Microbicides. Aliquots for study were taken from 5-gallon (ca. 18.927 liters) commercial packages of three cooling tower microbicides. The active ingredients of these three microbicides, Ty-Ion A-35, Ty-Ion A-39, and V-709 (Vestal Laboratories, St. Louis, Mo.), are, respectively, *N*-alkyl dimethyl benzyl ammonium chloride (12.5%) and bis (tri-*n*-butyltin) oxide (2.25%), *N*-alkyl 1,3-propanediamine (15%) and methylene bis (thiocyanate) (9.3%). Dilutions recommended for use by the manufacturer for each microbicide were 1:6,667 to 1:20,000, 1:10,000 to 1:20,000, and 1:10,000 to 1:40,000, respectively.

Test organism. *L. pneumophila* (Philadelphia I) was obtained from the Center for Disease Control, Atlanta, Ga. The microorganism was inoculated to an agar-overlay system and maintained by weekly subculturing.

Media. Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with L-cystein hydrochloride and soluble ferric pyrophosphate (F-G agar) was prepared as described by Feeley et al. (5). F-G broth was similarly prepared except that Mueller-Hinton broth (BBL Microbiology Systems or Difco Laboratories, Detroit, Mich.) was used instead of Mueller-Hinton agar.

To prepare the medium, we poured 50 ml of F-G agar into sterile disposable plastic tissue culture flasks (Corning no. 25110, Corning Glass Works, Corning, N.Y.) which were then slanted, and the agar was allowed to solidify. F-G broth (35 ml) was then added to each tissue culture flask containing the solidified F-G agar. The biphasic medium was incubated for 48 h at 35°C to detect contamination and was used to grow the inocula only. In preliminary studies, concentrations of 10^6 to 10^7 colony-forming units of the test organism per ml were consistently achieved after 6 to 7 days of incubation in the biphasic system. Results were neither as consistent, nor were colony counts as high, in F-G broth alone.

F-G broth was used as the diluent in preparing the various test concentrations of the microbicides and for the serial dilutions of test samples.

Minimum inhibitory and minimum lethal concentration tests. Base two dilution series of the three microbicides were prepared in F-G broth to yield 10-ml volumes for minimum inhibitory concentration (MIC) determinations. In addition, an F-G broth tube containing no microbicide was inoculated as a positive growth control. Each tube was then inoculated with 0.1 ml of a 7-day-old culture, late log phase, of *L. pneumophila*. The test solutions were incubated at 35°C in a nonaerated 2.5% CO_2 atmosphere. After incubation for 24 h, 0.1 ml was aseptically transferred from each test solution in the MIC series to 9.9 ml of F-G broth containing no microbicide to overcome inhibition with a 100-fold further dilution. The derivative minimum lethal concentration (MLC) test series was then incubated in parallel with the MIC series for 30 to 45 days. The MIC-MLC experiment was replicated three times.

ASTM E-645-78 standard test method for efficacy of microbicides used in cooling systems. The standard test method (1) was followed with the exception that sterile physiological saline buffered to pH 6.9 was substituted for a naturally contaminated cooling tower water sample as the test substrate. Five concentrations covering an appropriate test range for each microbicide were prepared in saline in addition to an untreated saline control. In the first experiment, a working volume of 49.5 ml was inoculated with 0.5 ml of a 7-day-old culture of *L. pneumophila*. In the second and third experiments, the working volumes were increased to 99 and 1 ml, respectively. The test concentrations and saline control were incubated for 3 h at 35°C. Quantitative plate counts were performed on the saline control immediately after inoculation and at the end of the 3-h exposure. The test concentrations were sampled and plated at the end of the 3-h exposure. From each serial dilution, 0.1 ml was spin plated on the surface of an F-G agar plate with a sterile glass spreader. The plates were incubated at 35°C in a 2.5% CO_2 atmosphere until visible colonies could be counted.

RESULTS

The MICs of the three microbicides against *L. pneumophila* were found at dilutions of 1:4,096,000 for the combination of *N*-alkyl dimethyl benzyl ammonium chloride and bis (tri-*n*-butyltin) oxide, <1:16,000 for *N*-alkyl 1,3-propanediamine, and 1:16,000 for methylene bis (thiocyanate) (Table 1). MLCs for the three microbicides were found at dilutions of 1:512,000, <1:16,000, and 1:16,000, respectively.

Among the replicates of the ASTM E-645 standard method experiments for evalution of cooling tower microbicides, there was a variable drop in titer between the inoculum count and the 0-h time count in the saline controls that cannot be readily explained (Table 2). This is not a critical issue. The important observation was that there was no reduction in the number of organisms in the saline controls during the test period, thus proving the viability of the inoculum in the test procedure. The average $\log_{10}$ reduction of *L. pneumophila* for the combination of *N*-alkyl dimethyl benzyl ammonium chloride and bis (tri-*n*-butyltin) oxide ranged from 4.96 at a dilution of 1:80,000 to 0.19 at a dilution of 1:1,280,000 (Table 3). *N*-Alkyl 1,3-

TABLE 1. *MIC and MLC test results for three cooling tower microbicides against L. pneumophila*[a]

Microbicide	Expt no.[b]	Test	Growth[c] at the following reciprocal of dilution of microbicide (× 10^3):									
			16	32	64	128	256	512	1,024	2,048	4,096	8,192
N-Alkyl dimethyl benzyl ammonium chloride and bis (tri-*n*-butyltin) oxide	1	MIC	−	−	−	−	−	−	−	−	NT	NT
		MLC	−	−	−	−	−	−	+	+	NT	NT
	2	MIC	−	−	−	−	−	−	−	−	−	−
		MLC	−	−	−	−	−	−	−	−	−	−
	3	MIC	−	−	−	−	−	−	−	−	−	+
		MLC	−	−	−	−	−	−	−	+	+	+
N-Alkyl 1,3-propanediamine	1	MIC	−	−	−	−	+	+	+	+	NT	NT
		MLC	−	−	+	+	+	+	+	+	NT	NT
	2	MIC	+	+	+	+	+	+	+	+	+	+
		MLC	−	−	−	−	+	+	+	+	+	+
	3	MIC	−	+	+	+	+	+	+	+	+	+
		MLC	−	−	+	+	+	+	+	+	+	+
Methylene bis (thiocyanate)	1	MIC	−	−	−	−	−	−	+	+	NT	NT
		MLC	−	−	−	−	−	−	−	+	NT	NT
	2	MIC	−	−	+	+	+	+	+	+	+	+
		MLC	−	−	−	−	+	+	+	+	+	+
	3	MIC	−	+	+	+	+	+	+	+	+	+
		MLC	−	−	−	+	+	+	+	+	+	+

[a] In all three experiments, viability controls were positive.
[b] Inocula for experiments 1, 2, and 3 were 2.0×10^7, 3.0×10^6, and 1.8×10^6 colony-forming units per ml, respectively.
[c] −, No growth; +, growth; NT, not tested.

propanediamine produced an average $\log_{10}$ reduction that ranged from 4.27 at a dilution of 1:20,000 to 0.14 at a dilution of 1:320,000. The average $\log_{10}$ reduction for methylene bis (thiocyanate) ranged from 4.30 at a dilution of 1:20,000 to 0.28 at a dilution of 1:320,000. The dilution of the three microbicides that satisfied the test criterion of a 90% reduction in inoculum in 3 h can be interpolated from a $\log_{10}$ normal plot of the average $\log_{10}$ reduction data (Fig. 1). This criterion was met at dilutions of 1:560,00, 1:82,000, and 1:115,000 for the three microbicides, respectively.

DISCUSSION

Current epidemiological data implicate cooling towers and evaporative condensers of air-conditioning systems as possible reservoirs of *L. pneumophila* and point sources for dissemination of the organism in sufficient quantity to produce infection (6). If this causal association is valid, then prophylactic treatment of open recirculating water systems with a microbicide known to be effective in killing *L. pneumophila* would be expected to eliminate this source of the organism.

One microbicide that fulfills the standard criterion for efficacy of cooling tower microbicides (ASTM E-645) at the 2-ppm (0.002 ml/liter) level against *L. pneumophila* is the combination of *N*-alkyl dimethyl benzyl ammonium chloride and bis (tri-*n*-butyltin) oxide, which inhibits the organism at 0.25 ppm (0.00025 ml/liter) and kills it at 2 ppm. These results are consistent with those of Zedler and Beiter (R. J. Zedler and C. B. Beiter, Proc. Annu. Meet. Chem. Specialties Manuf. Assoc. 1961, p. 127-130), who reported that the combination of a quaternary ammonium compound and an organo-tin compound produced synergistic activity against both gram-positive and -negative bacteria as compared with the individual components. In a preliminary study of six chemicals used to formulate cooling tower microbicides, a quaternary ammonium compound was found to be among the three most promising agents to inactivate *L. pneumophila* (4). Wang et al. (10) similarly found that low concentrations of a quaternary ammonium compound were inhibitory to the organism.

TABLE 2. *Viability of L. pneumophila in saline control for the ASTM E-645 method*[a]

Expt no.	Initial inoculum ($\log_{10}$ CFU/ml)	Viability ($\log_{10}$ CFU/ml) at:	
		0 h	3 h
1	7.30	5.24	5.69
2	8.48	5.41	5.47
3	9.94	6.08	6.10

[a] CFU, Colony-forming units.

The other microbicides evaluated in the present study showed a lower order of efficacy but, importantly, tubes became positive in successively higher concentrations during the incubation period until the reported results were observed in the MIC-MLC experiment. This was probably due to the initial survival of very small numbers of organisms that remained viable until adaptation to the particular concentration of microbicide took place. This adaptation phenomenon did not occur with the combination of *N*-alkyl dimethyl benzyl ammonium chloride and bis (tri-*n*-butyltin) oxide.

The variability in dose response observed among the replicates of the MIC-MLC tests is attributed to a combination of events such as run-to-run differences in inoculum titer and organism susceptibility; the prolonged, 30- to 45-

TABLE 3. *ASTM E-645 standard test results for three cooling tower microbicides against L. pneumophila*

Microbicide	Expt no.	$\log_{10}$ change in CFU/ml at reciprocal of dilution of microbicide ($\times 10^3$):						
		20	40	80	160	320	640	1,280
N-Alkyl dimethyl benzyl ammonium chloride and bis (tri-*n*-butyltin) oxide	1	NT[a]	NT	−5.24[b]	−5.24[b]	−5.24[b]	−1.11	−0.34
	2	NT	NT	−5.41[b]	−5.41[b]	−2.02	−0.32	−0.13
	3	NT	NT	−4.23	−2.57	−1.09	−0.17	−0.11
	Mean			−4.96	−4.41	−2.78	−0.53	−0.19
N-Alkyl 1,3-propanediamine	1	NT	NT	−1.32	−0.34	−0.06	+0.12	+0.52
	2	−5.41[b]	−1.64	−0.92	−0.46	−0.07	NT	NT
	3	−3.12	−2.49	−0.69	−0.36	−0.29	NT	NT
	Mean	−4.27	−2.07	−0.98	−0.39	−0.14		
Methylene bis (thiocyanate)	1	NT	NT	−2.62	−0.97	−0.39	−0.23	+0.02
	2	−5.41[b]	−2.37	−1.47	−0.49	−0.34	NT	NT
	3	−3.18	−1.93	−1.15	−0.23	−0.12	NT	NT
	Mean	−4.30	−2.15	−1.75	−0.56	−0.28		

[a] NT, Not tested.
[b] All subcultures were negative.

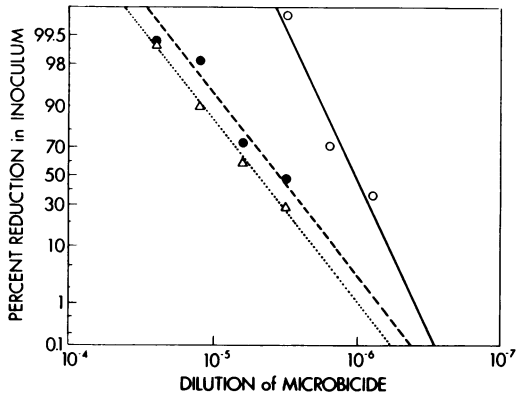

FIG. 1. *Percent reduction in inoculum of L. pneumophila after exposure to a combination of N-alkyl dimethyl benzyl ammonium chloride and bis (tri-n-butyltin) oxide* (—), *N-alkyl 1,3-propanediamine* (····), *and methylene bis (thiocyanate)* (----) *in the ASTM E-645 standard test.*

day recovery incubation period which maximized conditions for repair, survival, and growth of very low numbers of initial survivors; and the inherent variability of any extinction procedure within the endpoint range. Therefore, we interpreted the results conservatively, accepting the poorest dose response of the replicates as the endpoint. In instances where the primary (bacteriostatic) tube produced delayed growth resulting in a positive reading when the secondary (bactericidal) tube remained negative, the apparent bactericidal result was considered to be due to the low sampling probability for a very low number of initial survivors in the primary tube; the bactericidal endpoint was therefore assumed to be at a lower dilution than the bacteriostatic endpoint.

Although this study demonstrates the susceptibility of *L. pneumophila* to cooling tower microbicides in vitro, the fact that a microbicidal endpoint was reached with a combination of a quaternary ammonium compound and an organo-tin compound in two different standard test methods at 4% of the minimum concentration recommended for use suggests that the product would probably exhibit similar efficacy under actual use conditions. Further studies are planned with naturally contaminated cooling towers in order to answer this question.

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