

## Efficacy of Ozone in Eradication of *Legionella pneumophila* from Hospital Plumbing Fixtures

PAUL H. EDELSTEIN,<sup>1,2\*</sup> R. E. WHITTAKER,<sup>3</sup> R. L. KREILING,<sup>3</sup> AND CLIFFORD L. HOWELL<sup>1,2</sup>

*Infectious Disease Section, Research and Medical Service, Veterans Administration Wadsworth Medical Center, Los Angeles, California 90073*<sup>1</sup>; *Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024*<sup>2</sup>; and *Aqueonics Division, ARCO Environmental Inc., Subsidiary of Atlantic Richfield Co., Dublin, California 94566*<sup>3</sup>

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The effect of ozonation of supply water for one wing of an unoccupied hospital building which had positive cultures for *Legionella pneumophila* from multiple potable water fixtures was studied in a prospective, controlled fashion. Mean ozone residual concentrations of 0.79 mg/liter eradicated *L. pneumophila* from the fixtures, but so did nonozonated water in the control wing fixtures. The efficacy of the nonozonated water was most probably due to a mechanical flushing effect and to an unexpected rise in the chlorine content of the supply water. Determination of the in vitro activity of ozone against *L. pneumophila* did not predict the efficacy of its eradication from water fixtures treated with ozone.

Contamination of potable water or plumbing fixtures with *Legionella pneumophila* has been found to be a source of outbreaks of Legionnaires disease in the United States and Great Britain (6, 9; K. N. Shands, J. L. Ho, G. W. Gorman, R. D. Meyer, P. H. Edelstein, S. M. Finegold, and D. W. Fraser, Clin. Res. 29:260A, 1981). The methods currently used to decontaminate such sources include replacement or sterilization of plumbing fixtures, raising water temperature to 55 to 65°C, and hyperchlorination (2 to 6 mg of free residual chlorine per liter) (6). No controlled trial of any of these methods has been attempted, primarily because it is considered unwise to leave the potential source of a disease outbreak untreated to serve as a negative control.

Ozone is a very potent oxidizing agent with greater biocidal activity than chlorine (5). It is used in many European countries for water supply and swimming pool disinfection at concentrations of 0.1 to 0.5 mg/liter and is currently used in this country for air-conditioner cooling-tower decontamination.

This study was designed to determine in a controlled fashion whether continuous ozonation of the water supply and, consequently, the fixtures of a building would eradicate *L. pneumophila* from the fixtures.

### MATERIALS AND METHODS

The susceptibility to ozone of two *L. pneumophila* strains (E221ADP and E102A<sub>3</sub>DP) isolated from plumbing fixtures of the building studied was tested first to determine the feasibility of eradicating the

bacterium from the plumbing system. This was performed by using an adaptation of the methods of Skaliy et al. (8). The strains had been isolated by direct plating techniques; one was a serogroup 1 strain, and the other was a serogroup 4 strain. They were passaged twice on buffered charcoal-yeast extract medium supplemented with 0.1%  $\alpha$ -ketoglutarate (BYCE $\alpha$  medium) (2) and harvested from plates to sterile distilled water to approximate the turbidity of a 1.0 McFarland barium sulfate standard diluted 1:2 with 1% H<sub>2</sub>SO<sub>4</sub>. The bacterial suspension was diluted 1:100 in sterile distilled water, and samples (1 ml each) of this were placed in duplicate flasks containing the following concentrations of ozone in 100 ml of sterile, glass-distilled water: <0.01 (nonozonated control), 0.32, 0.47, and 0.63 mg/liter. The ozone was generated in the water by an electrical corona discharge method (Aqueonics model 1T; Aqueonics, Dublin, Calif.), and ozone concentrations were measured by an iodometric method (7). The actual number of organisms placed into the flasks was determined by dilution plating on BCYE $\alpha$  medium in duplicate. Bacteria were allowed to react with the water solutions for 20 min at room temperature (24°C) in the uncovered flasks. The bacterial counts were then determined by using dilution plating in duplicate on BCYE $\alpha$  medium. Also, 1.0-ml samples of each dilution (undiluted, 10<sup>-1</sup>, 10<sup>-2</sup>, and 10<sup>-3</sup>) from each flask were inoculated into two 7-day, antibiotic-free embryonated hen eggs to determine bactericidal activity. Controls in the egg study were ozonated water without bacteria and the two *L. pneumophila* strains in sterile distilled water (10<sup>2</sup> and 10<sup>3</sup> colony-forming units [CFU] per ml).

The building studied is a 14,600-ft<sup>2</sup> single-story building on the hospital grounds. It was unoccupied until 1980, when it was renovated for use as nurses' quarters. At that time, cultures of sink faucets, shower heads, and hot-water heaters in the building revealed heavy growth of *L. pneumophila*; simple flushing of all fixtures for a 1-week period did not eradicate the

organism. The water supply of the building contains free residual chlorine levels of less than 0.1 mg/liter and is culture negative for *L. pneumophila*. For the purposes of this study, the water supply for the building was split into two wings so that identical numbers of fixtures and water heaters were supplied. This enabled the water supply of one side of the building to be treated with ozone while the other side was untreated. There were no pipe connections between the two wings. Water meters were placed in the system so that usage rates in the two wings could be determined.

Supply water for the treated wing was first piped into a 500-gallon (ca. 1,893-liter) plastic reservoir, in which the ozonation process occurred. The ozone gas was generated by an electrical corona discharge method (Aqueonics model 36T), which used bottled medical-grade oxygen as the oxygen source. Water in the reservoir tank was ozonated so that water sampled at the test site faucet in the treatment wing contained the desired amount of ozone.

Since the water supply to the building was not a recirculating system, a faucet water tap in each wing was left open so that when other fixtures were operated, ozone levels quickly rose to the desired steady-state concentrations.

To simulate normal usage patterns in both wings, all sink faucets, toilets, and shower faucets were operated eight times per day for 2 min, at 1000, 1200, 1400, 1600, and 2400 h.

Ozone levels at the continuously flowing faucets on both wings were monitored 10 to 24 times daily, using an iodometric titration method (7). This technique measures all oxidizing substances and is not specific for ozone. Free residual chlorine levels were also measured at the times of bacteriological sampling as well as at other times; these were measured by amperometric means with phenylarsine reagent (7) (Fisher Titrimeter; Fisher Scientific Co., Pittsburgh, Pa.). This method of measuring chlorine is affected by ozone, which causes falsely low chlorine measurements.

The study was carried out in several phases. The pretreatment phase was a 1.5-day period used to determine bacterial counts and base-line ozone and chlorine levels. This was done with all ozonation equipment in place but not functioning. The next phase was a 3-day period during which all fixtures were operated and in which an attempt was made to maintain ozone levels of  $1.0 \pm 0.3$  mg/liter at the monitored tap. Hot-water heaters were turned off so that ozone levels within the heaters were not adversely affected by high temperature. The next phase was a low-level, 3-week maintenance phase, during which fixtures were operated intermittently to simulate normal usage (except for the continuously running faucet on each wing needed to maintain a steady state); ozone levels at the monitored tap were adjusted to  $0.5 \pm 0.2$  mg/liter. The water heaters were turned on during this phase and operated in the range of 42 to 44°C. The next phase was designed to determine whether *L. pneumophila* regrew after the cessation of water treatment (11). The flushing schedule was adhered to in this stage, which lasted for 3 weeks. Then, to determine the effect of water stagnation, bacterial counts were determined for 3 weeks after all fixtures were turned off.

Water sampling was performed in the morning, approximately 8 h after the last flush of the evening before. The same three sites in each wing were sampled each time. These were water heater drainage taken from a valve at the bottom of each water heater, a shower head, and a sink faucet. All samples were collected in 100-ml volumes in sterile plastic tubes containing sodium thiosulfate; the final concentration of sodium thiosulfate after sample collection was 200 µg/ml. This concentration of sodium thiosulfate did not inhibit the growth of *L. pneumophila* in a study of three environmental and two clinical strains (6; unpublished data). The shower heads were disconnected from the wall gooseneck pipe, and the interior surfaces of the gooseneck and shower head were swabbed with a sterile cotton swab moistened in the standing water from the shower head. The first 100 ml was then collected in the tube, and the swab was broken aseptically into it. Material from the interior of the sink faucet was also collected in a similar fashion. The contents of the tubes were blended in a Vortex mixer, and the swab was discarded. Specimens were plated within 2 to 4 h of collection onto 5% sheep blood agar (Cal Labs, Los Angeles, Calif.), BCYE $\alpha$  medium, and BCYE $\alpha$  medium supplemented with antimicrobials (2). Specimens plated for *L. pneumophila* were treated with and without an acid wash as previously described (2). These methods of growing *L. pneumophila* from potable water are more sensitive than guinea pig inoculation, but are not necessarily effective in growing other species of *Legionella* (3). All specimens were plated in 0.1-ml volumes in unconcentrated form and in 10-fold-concentrated form obtained by centrifugation. Also, samples plated for non-*Legionella* bacteria were plated with 0.1 ml of the 10-fold-concentrated form, the undiluted form, and the 1:100 dilution (in sterile distilled water). All plates were incubated at 35°C in humidified air.

Non-*Legionella* plates were held for 4 days, and plates for *L. pneumophila* were held for 7 days. *L. pneumophila* was identified on the basis of colonial morphology, Gram stain, biochemical and growth characteristics, and serological characteristics as defined by direct fluorescent antibody staining (10).

The total non-*Legionella* bacterial count was determined by taking the highest count of non-*Legionella* bacteria on any plate. The *L. pneumophila* count was determined by the highest count of *L. pneumophila* on any plate.

## RESULTS

The results of in vitro susceptibility testing are shown in Table 1. The stated log reductions in bacterial counts are based on a sensitivity of  $\approx 10^3$  CFU per egg for lethality as determined by the use of positive controls and a sensitivity of  $\approx 10$  CFU/ml by plating onto BCYE $\alpha$  medium as determined in unpublished studies performed in this laboratory. All concentrations of ozone (except blank) tested killed the test strains as assayed by egg inoculation, and all but the lowest concentration tested inhibited the strains as determined by plating methods. The killing of the embryonated hen eggs was due exclusively to *L. pneumophila*, as ozonated water without added

TABLE 1. Reduction in counts of *L. pneumophila* exposed to ozone in distilled water for 20 min

Ozone concn (mg/liter)	Run	Log reduction with the following strain and assay method:			
		E221ADP <sup>a</sup>		E102A <sub>3</sub> DP <sup>b</sup>	
		BCYE $\alpha$ <sup>c</sup>	Eggs <sup>d</sup>	BCYE $\alpha$	Eggs
<0.01	1	0	0	0	0
	2	0	0	0	0
0.32	1	4.0	>3.0	>5.2	>3.0
	2	>5.2	>3.0	>5.2	>3.0
0.47	1	>5.2	>3.0	>5.2	>3.0
	2	>5.2	>3.0	>5.2	>3.0
0.63	1	>5.2	>3.0	>5.2	>3.0
	2	>5.2	>3.0	>5.2	>3.0

<sup>a</sup> Initial inoculum size,  $1.5 \times 10^6$  CFU/ml; serogroup 4 environmental strain.

<sup>b</sup> Initial inoculum size,  $1.65 \times 10^6$  CFU/ml; serogroup 1 environmental strain.

<sup>c</sup> BCYE $\alpha$  medium used to count *L. pneumophila*. Sensitivity,  $\approx 10$  CFU/ml.

<sup>d</sup> Embryonated hen egg inoculations used to determine the presence or absence of *L. pneumophila*. Sensitivity,  $\approx 10^3$  CFU/ml.

organisms had no effect on the eggs.

The results of bacterial counts of the showers are shown in Fig. 1, as are schematic representations of ozone levels. Only serogroups 1 and 4 of *L. pneumophila* were detected, in approximately equal numbers. No other serogroup of *L. pneumophila* was detected, nor was any other species of *Legionella*. No change in the propor-

tion of serogroup 1 to serogroup 4 was noted during the study period.

The total bacterial counts decreased initially, but then rose and remained more or less constant with occasional sporadic fluctuations. The counts of *L. pneumophila* initially fell to undetectable levels at all three study sites in both the treatment and control wings. During the maintenance phase, 8 of 12 control wing and 3 of 12 treatment wing cultures had detectable *L. pneumophila* ( $p = 0.10$  by chi-square test, power  $[1 - \beta] = 0.38$ ). Except for one sample, the treatment wing shower remained culture negative for *L. pneumophila* during the maintenance period, whereas the control wing cultures became and remained culture positive. A similar but not identical pattern was observed for the sink sites; in these sites the treatment wing cultures for *L. pneumophila* became positive toward the end of the maintenance phase.

The pretreatment culture for *L. pneumophila* from a water sample from the control wing hot-water heater was negative, whereas the treatment wing water contained 1 CFU/ml. The treatment wing cultures became negative 2 days after ozonation was started; both sites became culture positive at the same rate after the cessation of ozonation.

Water usage rates in the two wings were unexpectedly unequal during all water-use phases of the study. Treatment wing water use during the 3-day high-level phase was 1,127 gallons (ca. 4,266 liters) per h, compared with 2,480 gallons (ca. 9,387 liters) per h for the control wing. During the next phase, usage rates were 222 and 296 gallons (ca. 840 and 1,120

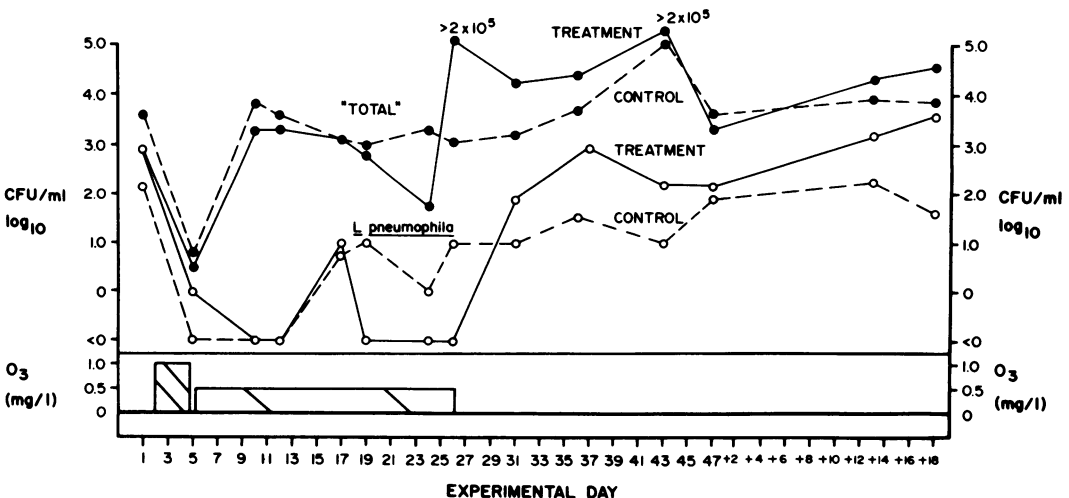


FIG. 1. Viable bacterial counts of *L. pneumophila* (O) and of total bacteria growing on 5% sheep blood agar (●) for treatment (—) and control (---) showers. Ozone (O<sub>3</sub>) concentration is shown schematically. Experimental days shown in small numbers represent days of no water use (stagnation phase).

liters) per h for the control and treatment wings, respectively. Usage rates during the 3-week postozonation phase were 138 and 246 gallons (ca. 522 and 931 liters) per h for the control and treatment wings, respectively.

Pre- and posttreatment oxidant levels measured as ozone were 0.05 to 0.12 mg/liter in both wings. Mean levels during the high-level phase were 0.24 and 0.79 mg/liter for the control and treatment wings, respectively. During the maintenance ozonation phase, mean oxidant levels measured as ozone were 0.12 and 0.58 mg/liter for the control and treatment wings, respectively. During the high-level ozonation phase, the control wing water oxidant level, measured as ozone, rose unexpectedly from the base-line level to an average of 0.24 mg/liter. We found that incoming water to the building, sampled at a site proximal to where the plumbing branched to the two wings, also had high oxidant levels, which correlated closely with those found on the control wing. Determinations of free residual chlorine levels of the control wing and incoming water corresponded closely with the total level of oxidants measured as ozone. No free residual chlorine was detectable (<0.01 mg/liter) in the treatment wing water, presumably due to interference by ozone.

During both ozonation phases (high level and maintenance), the acrid odor of ozone was present in the water and also permeated throughout the building. No one developed any respiratory or gastrointestinal complaints while in the building or afterwards.

## DISCUSSION

The in vitro susceptibility study showed that the test strains of *L. pneumophila* were significantly inhibited by 0.36 mg of ozone per liter. If the usual criterion for bacterial killing, that is, a 3-log reduction in count after broth subculture, is applied, then the minimal bactericidal concentration of ozone for both of the strains is less than or equal to 0.36 mg/liter (1).

*L. pneumophila* could still be recovered from water with ozone concentrations greater than the in vitro-determined minimal bactericidal concentration. Thus, these in vitro values can only be relied upon in a general sense and are not necessarily predictive of the interaction between steady-state ozone and bacteria in a complex environmental situation. A recent retrospective study has suggested that this holds for other biocides active in vitro against *L. pneumophila* (4).

Conclusions regarding the role of ozone in the eradication of *L. pneumophila* from the water system of the treatment wing are difficult to make because of the simultaneous eradication of *L. pneumophila* from the control wing. Several

unanticipated events occurred which may explain the reason(s) for the disinfection of the control wing. Water flow rates through the fixtures were very high and may have had a mechanical cleansing effect. This high water usage rate is compounded by the fact that the water usage rates in the control wing were twice as high as those in the treatment wing during the eradication phase. In conjunction with this, the rise in oxidants in the control wing water, which was probably due to chlorine, may also have contributed to the eradication of *L. pneumophila*. Since both wings were exposed to elevated oxidant levels in incoming water, and since both had very high water usage rates, it is impossible to determine whether ozone had any effect at all during this phase.

An attempt to eradicate *L. pneumophila* from the building by the use of water flushing alone failed several months before this study. Thus, flushing alone should not explain the persistence of negative cultures during the maintenance phase; events occurring during this phase may perhaps be more easily explained by ozonation alone. The level of measured oxidants in the control wing returned to base-line values, in concert with a marked reduction in water usage rates. The trend observed for the showers is at least suggestive that ozonation was effective in keeping the *L. pneumophila* counts low, whereas that observed for the sinks is less convincing.

Whether or not ozone can be considered to be an effective biocide to use for the eradication and continued suppression of *L. pneumophila* in a potable water system unfortunately has not been definitely answered by this study. The results obtained suggest that ozonation of such a system may be efficacious, but do not definitely prove it.

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