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Susceptibility of Members of the Family *Legionellaceae* to Thermal Stress: Implications for Heat Eradication Methods in Water Distribution Systems

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To ascertain the feasibility of heat inactivation as an eradication method applicable to all members of the family *Legionellaceae*, we tested the heat resistance of 75 isolates which represented 19 members of this family of organisms. The ranges of thermal death times at 60, 70, and 80°C were 1.3 to 10.6, 0.7 to 2.6, and 0.3 to 0.7 min, respectively. These data suggest that the method of heat eradication will be effective against all members of the family *Legionellaceae*.

The epidemiologic reservoir for nosocomial infections caused by *Legionellaceae* has been shown to be the water distribution system (2, 10, 13). These organisms have been shown to be part of the ecologic flora that normally populates water distribution systems (16). Control of *Legionella pneumophila* in hospital water systems has been accomplished by elevating the temperature of the hot water supply to 70 to 80°C (2, 6). Although this eradication method is being used with increasing frequency, the thermal stability of *L. pneumophila* and related species remains unknown.

The objective of this study was to determine the susceptibility of the family *Legionellaceae* to thermal stress. We also assessed in greater detail the heat resistance of *L. pneumophila* serogroup 1, the most common pathogen in the *Legionellaceae* family, under conditions which mimic those of hospital water systems.

Seventy-five isolates representing the family *Legionellaceae* were tested for their ability to withstand thermal stress. Eighteen strains were obtained from the American Type Culture Collection, Bethesda, Md. These were *L. pneumophila* Philadelphia 1 (serogroup 1), Togus 2 (serogroup 2), Bloomington 2 (serogroup 3), Los Angeles 2 (serogroup 4), Dallas 1E (serogroup 5), Chicago 8 (serogroup 7), and Concord 3 (serogroup 8); *L. jordanis* (B1-540); *L. oakridgensis* (OR-10); *L. longbeachae* Longbeach 4 (serogroup 1) and Tucker 1 (serogroup 2); *L. wadsworthii* (Wadsworth 81-716A); *L. sainthelensi* (Mt. St. Helens 4); *Tatlockia micdadei* (Tatlock); *Fluoribacter bozemanae* (WIGA); *F. dumoffii* (TEX-KL and NY-23); and *F. gormanii* (LS-13). Four of the isolates were obtained from the Centers for Disease Control, Atlanta, Ga. They were *L. pneumophila* Atlanta 1 (serogroup 2), Houston 1, and Chicago 2 (serogroup 6) and *F. bozemanae* (MI-15). The remainder were strains isolated from clinical and environmental specimens submitted to the Pittsburgh Veterans Administration Medical Center. *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923) were also included. Stock cultures were maintained in 50% (vol/vol) fetal bovine serum and tryptic soy broth at −20°C. Buffered yeast extract broth (BYEB) was the liquid medium used to prepare suspensions of stationary-phase cells. All isolates and test samples were inoculated onto buffered charcoal yeast extract agar plates (4, 5, 14).

Determination of heat resistance. Stock isolates were plated on buffered charcoal yeast extract agar plates and incubated at 37°C for 72 h. Each bacterial suspension was prepared in 2.0 ml of sterile water and matched to a 1.0 MacFarland standard (approximately 10^8 CFU/ml). BYEB (10 ml) was then inoculated with 0.2 ml of a bacterial suspension and incubated at 37°C for 72 h in a shaker incubator. The stationary-phase cells were harvested by centrifugation at 600 × *g* for 20 min. The supernatant was removed, and the pellet was resuspended in 100 ml of fresh broth. Five milliliters of the broth culture was transferred into sterile tubes. Tubes were placed in covered water baths at 60, 70, and 80°C for specified periods. Tubes were removed from the 60°C water bath at 0, 5, 7, 10, and 20 min; from the 70°C water bath at 0, 1, 1.5, 3, and 10 min; and from the 80°C water bath at 0, 1, 1.5, 2, 2.5, and 3 min. After heating, each tube was rapidly transferred to an ice water bath (approximately 5°C) for a 2-min quench period. Serial dilutions were made in sterile distilled water and surface plated on buffered charcoal yeast extract plates with the spiral plating instrument (Spiral Systems Instruments, Inc., Bethesda, Md.). All plates were allowed to dry before incubation at 37°C in a humidified atmosphere for 3 to 5 days. Heat resistance was determined by plotting the number of survivors versus time of exposure at a particular temperature. This plot is referred to as the thermal death time curve (11). When plotted on a semilogarithmic scale, the thermal death time curve for all *Legionellaceae* was a straight line which indicated progressive death of the population. This straight survivor curve can be described by the D value or decimal reduction time. This is the time required to destroy 90% of the organisms and is numerically equal to the time (in minutes) required to reduce the number of viable organisms by a factor of 10 (1, 7). For example, a D value of 10 min at 60°C (D60°C = 10 min) indicates that an exposure time of 10 min is required to reduce the concentration of an organism suspension by 1 log.

A spiral plating instrument (Spiral Systems) was used to calculate the concentration of bacteria in a given sample. The accuracy of this method has been previously described (8, 9). In addition, we performed parallel experiments using

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TABLE 1. Heat resistance of *Legionellaceae* in BYEB

Organism	No. of strains tested	Range of D[a] values (min) at a temp (°C) of:		
		60	70	80
L. pneumophila serogroup:				
1	21	2.3–4.8	1.2–1.4	0.4–0.6
2	3	3.6–5.0	1.3–2.6	0.5
3	3	2.8–3.4	1.1–2.4	0.4
4	3	2.8–4.0	1.2–2.2	0.4–0.5
5	3	2.3–3.4	1.2	0.4
6	5	2.6–3.7	1.3	0.4–0.5
7	1	3.7	1.2	0.4
8	1	2.5	1.3	0.4
L. jordanis	1	4.8	1.6	0.6
L. oakridgensis	1	2.4	1.3	0.4
L. longbeachae serogroup:				
1	1	2.8	1.3	0.4
2	1	1.3	0.7	0.5
L. wadsworthii		2.6	1.4	0.5
L. sainthelensi	1	1.4	0.7	0.5
T. micdadei	24	4.5–10.6	1.1–2.5	0.5–0.7
F. bozemanae	2	1.4–3.0	1.3–2.2	0.3–0.5
F. dumoffii	2	1.4–2.8	1.2	0.4
F. gormanii	1	1.8	1.1	0.5

[a] Decimal reduction time.

the standard tube dilution and plate count methods (J. E. Stout, unpublished data).

Comparison of the heat resistance of *L. pneumophila* in broth, tap water, and concentrated hot-water tank water. The thermal death times for two environmental isolates of *L. pneumophila* serogroup 1 were determined in broth, sterile tap water, and concentrated hot-water tank water (sediment). A 500-ml water sample collected from the bottom of a hot-water storage tank was concentrated by centrifugation at 2,800 × *g* for 30 min. The supernatant was removed, and the concentrate (sediment) was resuspended in 1/10 of the original volume of supernatant and autoclaved. Viability of *L. pneumophila* within these suspensions was determined by the spread plate method; 0.1 ml of the appropriate sample dilution was spread over the surface of a buffered charcoal yeast extract plate using a sterile bent-glass rod. The organism for inoculation of sediment suspensions was prepared as previously described except that after centrifugation the stationary-phase cells were suspended in 10 ml of sterile water. One-half-milliliter samples of this suspension were added to several tubes which contained 4.5 ml of the sterile sediment suspension. Thermal death times were determined at 60, 70, and 80°C.

Statistical analysis. The D60°C values of all *L. pneumophila* serogroup 1 isolates and all *T. micdadei* isolates were compared. Comparisons were made to determine whether a significant difference in D60°C could be attributed to the origin of the isolates, i.e., clinical versus environmental, and whether the D60°C of *T. micdadei* differed significantly from that of *L. pneumophila*. Data were analyzed by the *t* test for unpaired data (Prophet System, Division of Research Resources, National Institutes of Health, Bethesda, Md.).

Table 1 summarizes the heat resistance of 19 members of the family *Legionellaceae*. When only one serogroup or strain of an organism was tested, the D values that appear in Table 1 represent an average of replicate experiments. Figure 1 illustrates survivor curves for *L. pneumophila* serogroup 1 in BYEB at 60, 70, and 80°C. The D values of *S. aureus* and *P. aeruginosa* at 60, 70, and 80°C were 4.4, 1.3, and 0.5 and 2.6, 1.3, and 0.7 min, respectively.

The thermal stabilities of two environmental strains of *L. pneumophila* serogroup 1 were virtually unaffected when heat resistance was determined in broth, water, or water which contained particulates. The D60°C values for these organisms in broth, tap water, and tap water plus sediment were 3.2 and 2.6, 3.1 and 3.4, and 4.0 and 3.1 min, respectively. The D70°C values were 1.3, 1.2, and 1.5 and 1.3 min, respectively. The D80°C values were 0.5 and 0.4, 0.4, and 0.4 min, respectively.

We found no differences for D60°C values of clinical versus environmental isolates of *L. pneumophila*. The ranges of D60°C values for 11 clinical and 10 environmental isolates were 2.4 to 4.8 (mean, 3.4) and 2.4 to 3.8 (mean, 3.2) min, respectively. We did find that environmental strains of *T. micdadei* had a significantly higher D60°C than clinical strains ($P < 0.01$; *t* test for unpaired data). The ranges of D60°C values for 12 clinical and 12 environmental isolates were 3.4 to 6.4 (mean, 5.1) and 4.5 to 10.6 (mean, 7.1) min, respectively. Also, the D60°C values for all isolates of *T. micdadei* were significantly higher than those of clinical or environmental isolates of *L. pneumophila* isolates ($P < 0.01$; *t* test).

Although *L. pneumophila* and *T. micdadei* account for most cases of pneumonia caused by *Legionellaceae*, other members of this family are being implicated as etiologic agents of pneumonia. We expect that nosocomial pneumonia due to these organisms will, as with *L. pneumophila* and *T. micdadei*, be found to result from dissemination of organisms via large-volume water systems. A study of the susceptibility of all *Legionellaceae* to heat inactivation (an eradication methodology successfully used to control *L. pneumophila* and *T. micdadei* in hospital water systems) would provide the foundation for the utility of heat inactivation as a general eradication method applicable to all *Legionellaceae*.

All members of the family *Legionellaceae* experienced

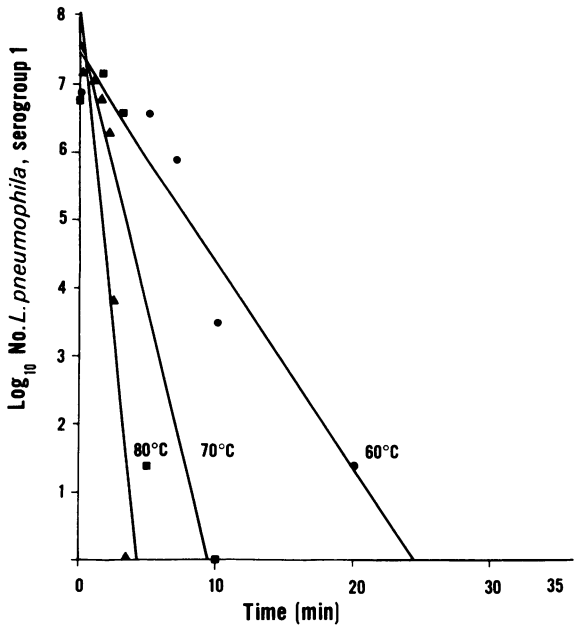

FIG. 1. Thermal death time curves for *L. pneumophila* serogroup 1 in BYEB at three temperatures.

rapid and precipitous loss of viability when exposed to temperatures exceeding 50°C. Only slight differences in D values obtained at 60, 70, and 80°C were observed for *L. pneumophila* even under conditions more representative of a water distribution system, i.e., when suspended in water or water plus sediment. The D60°C value determined in water plus sediment was slightly higher for one of two environmental organisms than that in tap water (3.1 versus 4.0 min). We did not, however, interpret this difference to represent significant resistance. Our system for evaluating the effect of sediment on the heat resistance of *Legionella* spp. may not be totally reflective of the situation in hot-water systems. However, the successful use of elevated water temperature to eradicate *L. pneumophila* from sediment-laden water systems suggests that heat resistance under these conditions is not a significant problem.

The D60°C values for *T. micdadei* were significantly higher than those for *L. pneumophila* ($P < 0.01$). This difference does not, however, suggest that *T. micdadei* would survive a heat eradication protocol. Given that the mean D60°C values for environmental isolates of *T. micdadei* and *L. pneumophila* were 7.1 and 3.2 min, respectively, the recommended 72-h duration of the heating procedure with a 30-min flushing of distal outlets with 70°C water should be sufficient to eliminate both organisms.

Although it has been documented that elevating hot water temperatures to 70°C with systematic flushing of distal outlets can be successful in reducing the concentration of *Legionella* spp. (2, 6), complete *Legionella* eradication may be difficult to achieve. This study supports the finding that complete elimination of *L. pneumophila* may not be achieved unless careful attention is directed to flush temperatures and duration of exposure. Flush temperatures must exceed 60°C, and the duration of each flush should be approximately 30 min or longer. Approximately 25 min at 60°C was required to sterilize a suspension of *L. pneumophila* which contained approximately 10^8 CFU/ml (Fig. 1).

We also suggest that, following implementation of the eradication protocol, the temperature of hot-water tanks should be maintained at 60°C because thermal death of *L. pneumophila* at temperatures of 50°C or less decreases dramatically (3, 12). It has also been reported that hot-water storage tanks which are maintained at approximately 60°C are less likely to contain *L. pneumophila* (15, 17). Temperature stratification within hot-water tanks may require that the thermostat set point be 65 to 70°C to achieve a temperature of 60°C at the bottom of the tank (where *L. pneumophila* proliferates).

These data also explain our empiric observations regarding the efficacy of instantaneous steam-heating units (Constantemp; Leslie Co., Parsippany, N.J.) in maintaining *Legionella*-free water distribution systems (R. M. Vickers, N. Carmen, V. L. Yu, and S. Hanna, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 163, 1984). This system heats the incoming cold water to 90 to 100°C using steam and high pressure. Heated water is subsequently blended with cold water to achieve the desired temperature.

In conclusion, the results of this study provide a scientific basis for the use of the heat eradication method. We project that eradication measures which have proven to be effective against *L. pneumophila* and *T. micdadei* will also be effective against other members of the family *Legionellaceae*.

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