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INACTIVATION OF *MYCOBACTERIUM AVIUM* BY COPPER AND SILVER IONS

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Abstract—*Mycobacterium avium* and *Legionella pneumophila* are opportunistic pathogens that are found in hot water systems of hospitals. Hospital water supplies have also been suggested as sources for *M. avium* in AIDS patients. Copper/silver ionization has been shown to be an effective method for controlling *Legionella* in hospital hot water systems. The effect of copper/silver ions on *M. avium* is unknown. The susceptibility of *M. avium* to copper/silver ions was tested by performing kill-curve experiments in 100 ml buffered water at the following concentrations of copper/silver ions: (1) 0.1/0.01; (2) 0.2/0.02; (3) 0.4/0.04; (4) 0.8/0.08 mg/l. Initial *M. avium* concentration was *ca.* 3×10^6 CFU/ml. Viability was assessed daily for 1 week by plate count on Middlebrook 7H10 agar, and by the BACTEC system. Culture plates and BACTEC bottles were incubated at 37°C for two weeks. Contact times (days) required for 99% and 99.9% inactivation of *M. avium* by copper/silver ion concentrations of 0.1/0.01; 0.2/0.02; 0.4/0.04; 0.8/0.08 were 3 d and 5 d, 3 d and 5 d, 2 d and 4 d, 1 d and 2 d, respectively. The data indicate that *M. avium* is susceptible to these metal ions. However, *M. avium* are more resistant to the bactericidal effects of copper/silver ions than *Legionella*, requiring 100 times longer exposure to achieve comparable killing *in vitro*. Nevertheless, this suggests that the copper/silver ionization system may rid hospital hot water systems of both of these potential pathogens. Published by Elsevier Science Ltd

Key words—copper, disinfection, kinetics, *Mycobacterium*, silver

INTRODUCTION

Among mycobacterial species, *Mycobacterium avium* complex (MAC) is second only to *M. tuberculosis* as a human pathogen (Iseman *et al.*, 1985). Disseminated MAC infections have become a major problem in AIDS patients (Keihn *et al.*, 1985; Young *et al.*, 1986). *M. avium* has been found in hot water systems of hospitals and linked to hospital-acquired disease (du Moulin *et al.*, 1988; Singh and Yu, 1994; von Reyn *et al.*, 1994). It has been suggested that MAC infection could be prevented by avoidance of non-sterile potable water by high-risk patients as well as disinfection of the water distribution systems (von Reyn *et al.*, 1994).

Copper/silver ionization is a new technology that has been used in more than 30 hospitals in the United States to control *Legionella* in hot water systems. Copper and silver ions attach to the negatively charged bacterial cell wall and disrupt cell wall permeability. This action coupled with protein denaturation induces cell lysis and death (Friedman and Dugan, 1968; Bitton and Freihofer, 1978; Slawson *et al.*, 1990). The advantages of copper/silver ionization compared to other disinfection techniques include straight-forward installation, easy maintenance, and the presence of the residual disinfection throughout the system.

The efficacy of copper and silver ions in eradicating MAC has never been investigated. Thus, the objective of this study was to determine the *in vitro* susceptibility of MAC to copper and silver ions. The bactericidal effects of copper and silver ions against MAC were determined by batch disinfection studies at various concentrations including those that have been found to be effective for eradicating *Legionella* from hospital hot water systems.

MATERIALS AND METHODS

Organisms

Strains of *Mycobacterium* tested included patient and environmental strains of *M. avium*, patient and environmental strains of *M. fortuitum*, and a patient strain of *M. intracellulare*. Organisms were maintained at −70°C in broth before being subcultured to a Middlebrook and Cohn 7H10 agar plate and incubated for 7 d. The culture was removed and suspended in *ca.* 30 ml of sterile deionized water. Two milliliters of suspension was removed and standardized by comparison with the turbidity of McFarland No. 1 standard (approximate density of

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3×10^8 colony forming unit (CFU)/ml). One milliliter of the standardized suspension was transferred to the test solution to achieve the initial concentration of 3×10^6 CFU/ml for each experiment.

Copper and silver ions

Copper and silver ion solutions were prepared by dissolving $CuCl_2$(s) and AgCl(s) (Aldrich, Milwaukee, WI) in deionized water. Stock solutions of Cu^{2+} and Ag^+ containing 10 and 1 mg/l, respectively, were prepared in advance and transferred to test solutions using a proper dilution scheme. Actual ion concentrations were confirmed at the beginning of each experiment by atomic adsorption spectrophotometry (AAS). Flame AAS and HGA graphite furnace AAS (Model 4000, Perkin Elmer, Norwalk, CT) were used to measure high and low concentrations of metallic ions, respectively. Teflon flasks (250 ml) were used for all batch experiments to prevent loss of Cu^{2+} and Ag^+ from solution due to the adsorption onto the walls of the container. All test solutions were sterilized by steam sterilization and/or membrane filtration.

Batch disinfection study

Batch disinfection experiments were used to evaluate the effectiveness of copper and silver ions in killing *M. avium*. Approximately 3×10^6 CFU/ml of *M. avium* were introduced in deionized water buffered at pH 7.0 by sodium bicarbonate solution which was adjusted to 7.0 with 12 N HCl(l). Actual bacterial concentration was determined using the plate count of the sample withdrawn at time zero. Teflon flasks were placed on a shaker and temperature was controlled at 37°C. Upon the addition of disinfectant solution containing copper/silver ions at 10:1 ratio, *M. avium* concentration was monitored daily for 7 d.

One milliliter of sample withdrawn from the batch reactor was mixed with 10 μL neutralizer solution immediately, serially diluted, and 0.1 ml of sample was plated in duplicate onto Middlebrook and Cohn 7H10 agar plates. The neutralizer solution contained 14.6% sodium thiosulfate and 10% sodium thioglycolate and was used to prevent any further disinfection of *M. avium* during incubation and enumeration (Landeen *et al.*, 1989). The culture plates were sealed and incubated for 14 d at 37°C and colonies were enumerated (detection limit = 10 CFU/ml). Each disinfection experiment was performed in triplicate to improve statistical precision of the results. Each data point depicted on time-kill curves represented an average from three experiments performed at different days and each sample was analyzed in duplicate.

After 7 d of exposure to the copper and silver ion test solution, viability of *M. avium* was also assessed in a liquid medium for rapid detection of *M. avium*. 0.5 ml of the test solution was injected into a BACTEC 12B standard Middlebrook radiometric 7H12 broth medium (BACTEC 12B; Becton Dickinson Diagnostic Instrument Systems, Sparks, MD). The 7H12 medium contains a ^{14}C-labeled substrate. Following incubation at 37°C, the amount of $^{14}CO_2$ detected in the vial reflects the amount of growth, and is expressed as the "Growth Index" (GI). A GI of greater than 10 indicated growth of *M. avium* in the vial. The 12B vials were tested on a BACTEC 460 instrument for the radiometric determination of the Growth Index ($^{14}CO_2$) (Becton Dickinson Microbiology Systems, Cockysville, MD). Vials were tested after 1, 3, 6, 10 and 12 d.

Disinfection kinetics

The inactivation rate of *M. avium* observed in batch studies was modeled using a Gard model (Montgomery Engineers, 1985). According to this model, the inactivation of organisms follows a declining rate as expressed by the following equation:

$$-\frac{dN}{dt} = \frac{kN}{[1 + ax(Ct)]}$$

where, N is the concentration of viable organisms at time t ($N = N_0$ when $t = t_0$), C is the disinfectant concentration held constant over time, k is the first-order rate of deactivation effected at time zero, a is the rate coefficient, t is the contact time.

Coefficients k and a were determined using a non-linear regression analysis. The Ct value calculated for each data point represented a product of disinfectant concentration, C, (as $[Cu^{2+}]$ in mg/l) and the contact time, t, (in h).

RESULTS

The susceptibility of *M. avium* to copper/silver ions was tested at concentrations of 0.1/0.01, 0.2/0.02, 0.4/0.04, and 0.8/0.08 mg/l (Table 1 and Fig. 1). The lowest copper/silver ion concentration (0.1/0.01 mg/l) required 72 and 120 h to achieve 99% and 99.9% reduction, respectively. However, the highest ion concentration tested (0.8/0.08 mg/l) required only 24 and 48 h to achieve the same reduction. No *M. avium* were recovered from the culture plates after 7 d of contact with copper and silver ions at all concentrations. The Ct value at 99.9% kill was 82 mg/l × h for *M. avium* by copper and silver ions (Fig. 2).

Four additional strains of *Mycobacteria* were tested for susceptibility to copper/silver ions at concentrations of 0.2/0.02 and 0.8/0.08 mg/l. There was no significant difference in susceptibility of *M. avium* (patient isolate), *M. fortuitum* (patient and environmental isolates), or *M. intracellulare* (patient isolate) compared to *M. avium* (environmental isolate) (data not shown).

The Growth Index reading using the BACTEC system demonstrated the presence of viable *M. avium*. All readings were positive (> 999) even after 7 d of exposure to all copper/silver ion concentrations evaluated in this study (data not shown). Thus, despite contact with copper/silver ions for 7 d, some viable *M. avium* grew in the liquid media.

DISCUSSION

Mycobacterium has been found in hot water systems of hospitals (du Moulin *et al.*, 1988). *Mycobacterium* produces cell walls of unusually low permeability, which contribute to their resistance to

Table 1. Susceptibility of *M. avium* to copper and silver ions

Cu^{2+}/Ag^+ (mg/l)	Time required for 99% kill (h)	Time required for 99.9% kill (h)
0.1/0.01[a]	72	120
0.2/0.02[b]	72	120
0.4/0.04[c]	48	96
0.8/0.08[d]	24	48

The error range of ion concentration in solution verified by atomic absorption: [a]±0.05/0.01; [b]±0.08/0.01; [c]±0.1/0.01; [d]±0.12/0.02 (mg/l).

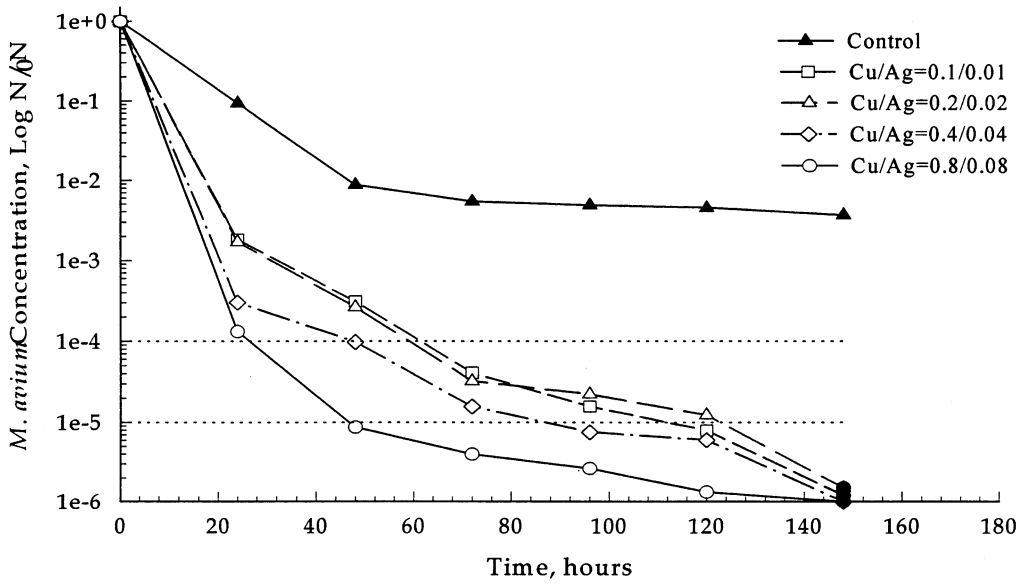

Fig. 1. *M. avium* was susceptible to copper and silver ions at concentrations from 0.1/0.01 mg/l (99.9% kill in 120 h) to 0.8/0.08 mg/l (99.9% kill in 48 h).

bactericidal agents (Brennan and Nikaido, 1995). *Mycobacterium* has been found to be resistant to chlorine in water supply (Pelletier *et al.*, 1988) and in swimming pools (Martin Delgado *et al.*, 1992). Thus, residual chlorine from the water treatment plant cannot effectively eradicate *Mycobacterium*.

Copper and silver ions can kill many waterborne pathogens *in vitro* including *Legionella* (Landeen *et al.*, 1989; Lin *et al.*, 1996), *Naegleria fowleri* (Yahya *et al.*, 1994), Coliphage MS-2, poliovirus (Yahya *et al.*, 1992) and *Pseudomonas cepacia* (Pyle *et al.*, 1992). This study assessed the *in vitro* efficacy of copper and silver ions against *M. avium*. The copper/silver ion concentrations tested in this study ranged between 0.2/0.02–0.8/0.08 mg/l, concentrations which have been found to be effective for eradicating *Legionella* from hospital hot water systems (Liu *et al.*, 1994).

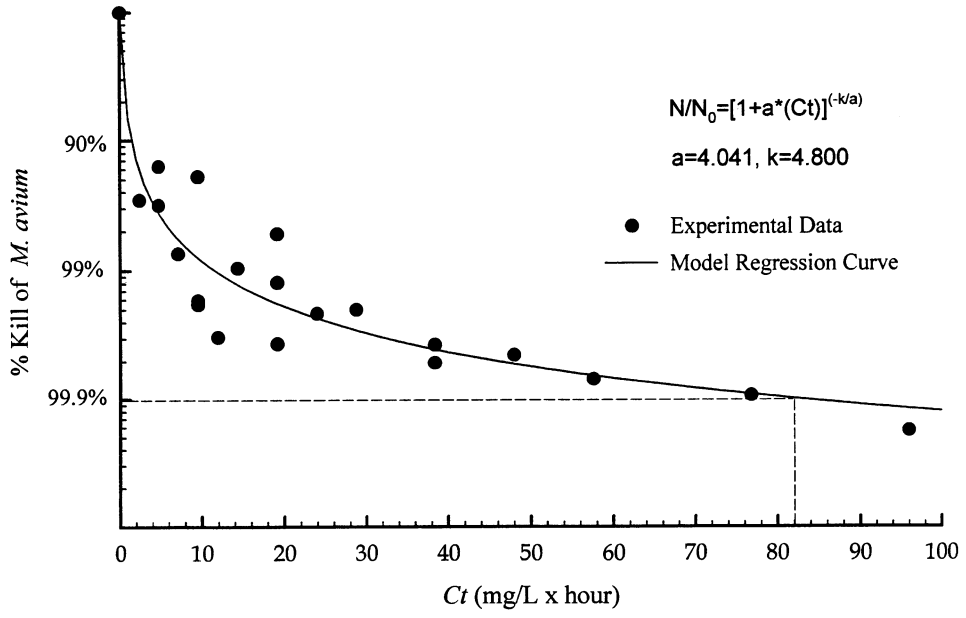

Fig. 2. *Ct* of copper/silver ions for *M. avium* was 82 mg/l × hour for 99.9% kill determined by Gard Model.

The bactericidal effects of copper and silver ions on *M. avium* were determined by kill curve experiments at four different ion concentrations. All combinations of copper and silver ion concentrations tested in this study were able to reduce *M. avium* viability by 99.9% (Fig. 1 and Table 1). The 99.9% (3-log) kill has been the primary standard for drinking water treatment for several pathogens mandated by the Environmental Protection Agency. Thus, copper/silver ions may have potential for eradicating *M. avium* in drinking water.

The *Ct* value for 99.9% reduction (3-log kill) in *M. avium* viability in this study was 82 mg/l × h (Fig. 2). We previously demonstrated that the *Ct* values at 99.9% kill for copper and silver ions on *L. pneumophila* inactivation were 0.08 and 0.35 mg/l × h, respectively (Lin *et al.*, 1996). Thus, *M. avium* is more resistant to copper/silver ions than *Legionella*.

Viable *M. avium* was detected in the liquid media by the BACTEC system, but not recovered from the culture plates after 7 d of contact with copper and silver ions at all concentrations tested. Growth in the liquid medium may represent injured cells that are viable but non-culturable on the solid media (7H10 plate). The significance of this finding is unknown, however, this phenomena has also been seen with chlorine-injured cells (Byrd *et al.*, 1991).

In summary, copper/silver ion concentrations of 0.1/0.01–0.8/0.08 mg/l achieved 99.9% kill of *M. avium*. However, *M. avium* was more resistant to the bactericidal effects of copper/silver ions than *Legionella*, requiring 100 times greater exposure time to achieve comparable kill *in vitro*. *In situ* evaluation of copper/silver ionization for *M. avium* inactivation in a real water distribution system is necessary to confirm our findings. Nevertheless, copper/silver ionization may be an attractive alternative for *M. avium* inactivation. These *in vitro* findings can be useful in establishing optimal copper/silver ion concentrations for eradication of *M. avium* in water distribution systems.

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