Comparison of in vitro disc diffusion and time kill-kinetic assays for the evaluation of antimicrobial wound dressing efficacy

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There is a plethora of new silver-containing dressings on the market today. Various manufacturers attempt to show that their dressings are the most efficacious and therefore should be preferentially employed by health care workers based on the results of their in vitro tests. However, there have been no studies that clearly identify which tests are appropriate for comparison purposes. The purpose of this study was to determine which in vitro test is most appropriate for evaluating the antimicrobial efficacy of silver-containing dressings. This was done by testing seven silver-containing dressings and two non-silver-containing topical agents against 17 clinically relevant microorganisms using zone of inhibition assays and time-kill kinetic assays in complex media. The results for the two assays were then correlated to determine whether the methods generated similar results. It was determined that the two methods do not correlate at all. This is most likely a result of the silver interacting with the media in the zone of inhibition test, thus invalidating the results of this test. We therefore conclude that zone of inhibition data generated for silver-containing dressings is of little value when assessing antimicrobial efficacy and that time-kill assays are of greater use. **(WOUND REP REG 2005;13:412–421)**

Because cutaneous wounds are open to the environment and wound beds are such favorable environments for bacterial growth, heavy bacterial colonization is commonly associated with acute and chronic wounds.[1] The presence of bacteria significantly increases the likelihood of overt, clinical wound infection.[2,3] Bacterial colonization and infection can interfere with the wound healing process because many organisms produce a variety of toxins, proteases, and proinflammatory molecules, which in turn may promote an altered host response indicative of an excessive inflammatory response.[4–6] Therefore, prevention and treatment of bacterial colonization or infection is a major concern in wound care. Traditionally, topical antimicrobial agents such as silver sulfadiazine (SSD), silver nitrate ($AgNO_3$), and mafenide acetate have been widely used in a variety of situations, including burn units. More recently, several new dressings with antimicrobial activity have been developed and used clinically.[7,8]

CZOI	Corrected zone of inhibition
MBC	Minimum bacteriocidal concentration
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *S. aureus*
SSD	Silver sulfadiazine
VRE	Vancomycin resistant *Enterococcus*

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Crede, who used 1 percent $AgNO_3$ solutions to treat ophthalmia neonatorum, recognized silver as an antimicrobial agent in 1884.[9] This was perhaps the first formal medical use of silver and came only 7 years after Pasteur had published his germ theory of disease. Three years later, von Behring reported that a 250-mg/L solution of $AgNO_3$ (158.8 mg Ag^+/L) was effective in destroying the typhoid bacillus in 2 hours.[9] Since these first reports, there have been numerous papers published that have examined the efficacy of silver ions in controlling bacteria and other microorganisms using a variety of techniques including zone of inhibition and log reduction assays. There are at present no guidelines available for the selection of antimicrobial efficacy assays for use in the evaluation of topical agents such as silver.

The selection of the appropriate assays is important if medical devices such as dressings are to be critically evaluated for efficacy, as is required to reduce the incidence of resistance to antimicrobial agents such as silver.[10] It is well established that resistance is selected for when the concentrations of active agents are at sublethal levels. Heggers and Robson[11] questioned the use of SSD when 80 percent to 90 percent of the isolates tested were resistant to 200 mg/L (60 mg/L Ag^+) of the active agent. This concentration was about 2 percent of the clinical dose yet caused concern 26 years ago with regard to the potential development of resistance. This concern was well founded, as was shown by Li et al.,[12] who developed silver-resistant (>1000 mg/L Ag^+) strains of bacteria in the laboratory using a multistep exposure protocol with an initial silver concentration near 2.5 parts per million. There is a growing body of evidence that suggests that concentrations at or near the minimal inhibitory concentration (MIC) are optimal for the selection of resistance.[13]

Researchers have routinely evaluated the antimicrobial activity of silver using zone of inhibition tests. Standard zone of inhibition testing should not be mistaken for Kirby-Bauer testing; the Kirby-Bauer or disk sensitivity assay has a large body of clinical data supporting its use. The most important data are those that relate the size of a zone of inhibition to a clinical outcome. Thus, the appearance of a zone is not in itself sufficient to determine efficacy. The zone width must meet certain standards, or the organism will be classed as insensitive or resistant. The data relating zone width to clinical efficacy have not been collected for silver. This missing information makes interpretation of the silver zone of inhibition data difficult. Spadaro et al., who showed that, although the amount of silver released anodically from silver-coated materials is generally proportional to the current, the size of the zone of inhibition is not, highlighted this difficulty.[14] His data showed that zone size reaches a maximum size at low concentrations of silver. He concluded that, because zone size was not proportional to current and therefore silver concentration, additional factors relating to the transport of silver during incubation must determine it. Similarly, Rosenkrantz and Carr[15] found the zone of inhibition test to be unacceptable for the evaluation of SSD efficacy because it did not correlate with MIC test results. This clearly makes the use of zone of inhibition data in efficacy determinations questionable for antimicrobial dressings in general and for silver topicals and dressings specifically.

Log reduction assays are used to determine microbicidal activity of antimicrobial agents. In sterilization assays, the time required to kill 90 percent of the population is referred to as the *D*-value (D10 value or decimal reduction time).[9] In antibiotic sensitivity testing, a compound may be considered cidal if it reduces the population by three orders of magnitude (99.9 percent kill).[16] These assays have been used extensively in assessing antimicrobial efficacy of various compounds, including silver. Of particular importance is the ability to determine the bactericidal properties of an active agent. Spacciapoli et al.[17] have shown that a threshold concentration of silver for clinical use may be the minimum bactericidal concentration. Other authors have suggested that speed of kill is important and that clinical outcomes tend to be better for treatments of infections such as spinal meningitis and endocarditis when the antimicrobial action is very fast. Jackson and Riff[18] showed that clinical outcomes more closely followed the maximal killing rates than MICs. Chandrasekar et al. compared a variety of test techniques (e.g., MICs and time-kill kinetic studies) and found that the latter were the best predictor of therapeutic outcome.[19] Speed of kill can easily be determined using a modified log reduction (time-kill kinetic) assay.

Although both of these techniques are widely reported in the literature as methods for determining silver dressing efficacy, there has been no effort to correlate results from the two techniques and determine whether one is more appropriate than the other for assessing activity. Therefore, it is the purpose of this study to assess the antimicrobial efficacy of nine silver dressings and topical agents against 17 clinically relevant microorganisms using log reduction and zone of inhibition assays in a standardized in vitro testing system and to compare the results of the two tests to determine whether one method is better than the other.

MATERIALS AND METHODS

Topical preparations

$AgNO_3$ (lot #1004) was purchased from Fisher Scientific (Nepean, ON, Canada). Mafenide acetate powder was supplied by the Pharmacy Department,

University of Alberta Hospital (Edmonton, AB, Canada) or purchased from Sigma Chemical Corp. (St. Louis, MO; lot #54H0680). SSD cream (1%) was purchased as Silverzine (Pharmascience Inc., Montreal, Canada) and Flamazine (Smith & Nephew Inc., Lachine, Canada, lot #9776).

Antibacterial wound dressings

Avance Antibacterial Polyurethane Foam Film Dressing (lot #01106943) was made by SSL International Plc. (Oldham, UK). Arglaes Film Dressing (Maersk Medical Ltd, lot #522301) was purchased from Medline Industries, Inc. (Mundelein, IL). Actisorb Silver 220 (lot #9825–04) was produced by Johnson & Johnson Medical Ltd. (Skipton, UK). Silverlon Contact Wound Dressing (lot #32201) was purchased from Argentum Corp. (Lakemont, GA). Kerlix A.M.D. Dressing (lot #JG2733AG) was made by Tyco Healthcare Group LP. (Mansfield, MA). Acticoat nanocrystalline silver-coated dressing (Smith & Nephew Inc., Lachine, Canada, lot #010806 A-05) was supplied by Nucryst Pharmaceuticals Corp. (Fort Saskatchewan, AB, Canada).

Microorganisms used for testing

Seventeen microorganisms were used for the assessments. *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27317) were purchased from the American Type Culture Collection (Manassas, VA). Other microorganisms used were clinical isolates provided by the Biofilm Research Group, University of Calgary, or Calgary Laboratory Services (Calgary, Canada). They included Methicillin-resistant *S. aureus* (MRSA, 8911 A), Vancomycin-resistant *Enterococcus* (VRE, 118271), antibiotic-resistant *P. aeruginosa* (137366), *Burkholderia cepacia* (364), *Staphylococcus epidermidis* (R297190), *Klebsiella pneumoniae* (D-16), *Escherichia coli* (R476), *Alcaligenes* spp. (144218), *Citrobacter koseri* (150938), *Acinetobacter* spp. (150938–2), *Bacteroides fragilis* (ATCC 25285), *Candida albicans* (14053), *Candida glabrata* (14362), *Mucor* spp. (MY744-97/65), and *Aspergillus* spp. (Calgary isolate).

Corrected zone of inhibition test

This test was carried out as reported previously.[20] Briefly, the bacterial or fungal isolates were grown in broth for 4 to 6 hours, and the broth was used to streak Muller-Hinton agar plates in three directions to form a confluent lawn. The various topical preparations (carried on 2.5 cm × 2.5 cm squares of nonwoven rayon-polyester Sontara 8411 (E. I. du Pont de Nemours and Company, Old Hickory, TN)) and dry dressings (2.5 cm × 2.5 cm) were applied to the center of each lawn, and all plates were incubated for 24 hours at 37 °C (bacteria) or 30 °C (fungi). The zone of growth inhibition surrounding the test dressing was then determined. No plate dehydration was observed surrounding the dressings at the time of measurement. All corrected zone of inhibition (CZOI) tests were repeated in triplicate.

Logarithm reduction test protocol

All logarithm reduction tests were performed in triplicate. A sterile nonwoven absorbent material (Sontara 8411, 2.5 cm × 2.5 cm) was used as a control. $AgNO_3$ and mafenide acetate were tested by applying 100 μl of a working solution (1.5% and 15%, respectively) to squares of Sontara. SSD cream was tested by applying 600 μl of the cream to other Sontara squares (approximate depth 4 mm). All other dressings were cut into 2.5 cm × 2.5 cm pieces. The foam dressing Avance was prepared by applying 1.8 mL of sterile distilled water to the dressing, followed by incubation at room temperature for 2 hours with the administration of slight compression. Sterile nonantimicrobial foam dressing (Lyofoam, SSL International, Oldham, UK) was pretreated as described above and served as a control for the Avance.

For aerobic bacteria, colonies from an overnight culture were inoculated in 5 mL of tryptic soy broth and incubated for 4 to 6 hours at 37 °C to reach an ODU 600 of 0.08 to 0.2 (0.5 McFarland turbidity). Volumes of 200 μl of the test bacterial suspensions were transferred onto the topical preparations and dressings. For the $AgNO_3$ and mafenide acetate solutions, this resulted in final concentrations of 0.5% and 5% respectively. After incubation at 37 °C, triplicate samples were taken out at 0.5 and 2 hours. The surviving bacteria were recovered by vortexing the samples in an ice-cold solution containing 0.85% sodium chloride, 1% polysorbate (TWEEN) 20, and 0.4% sodium thioglycollate. Bacterial survival numbers were obtained using a standard plate count procedure using Muller Hinton medium, and the log reduction was calculated as the difference between the log numbers of bacteria surviving on the control dressings and the test dressings.

B. fragilis was tested in a similar fashion except that the bacterium was grown in a brain/heart infusion broth and plated on brucella blood agar with vitamin K and hemin. The incubation was carried out in an anaerobic chamber. *Mucor*, *Aspergillus*, *C. albicans*, and *C. glabrata* were tested as previously described.[21]

Comparison of CZOI and log reduction results

The log reduction data was plotted against the CZOI data, and correlation coefficients were calculated for the 30-minute and 2-hour log reduction assays.

Silver diffusion in solutions

Before determining silver diffusion rates, electrical resistances of solutions containing different concentrations of $AgNO_3$ were measured to determine the relationship between silver concentration and conductivity. The resistance of the solution changed linearly with the $AgNO_3$ concentration and showed a good correlation. Using this information, the following method was used to compare silver diffusion rates in water and normal saline.

To prevent the mixing of components in solution by convection and to allow for collection of multiple samples without perturbing the entire experimental system, all liquid diffusion experiments were performed within a nonreactive foam matrix. Open-celled polyurethane foam pieces (1 cm thick, $n = 3$) were cut to just fit into 10 cm diameter Petri dishes. Holes (0.5 cm diameter) were made at the center of the foam pieces. The Petri dishes containing the foam were filled with ultrapure (18 megaohm) water, and because air inside the foam can prevent the diffusion of $AgNO_3$, the foam pieces were mechanically degassed to remove any trapped air bubbles. The final water level was flush with the top of the foam. Water was removed from the center well immediately before the placement of a rayon polyester nonwoven gauze roll (1 cm $\times$ 0.5 cm) saturated in a solution of 0.03 M $AgNO_3$ to reduce any disturbance of the system. Conductivity probes were placed 3.5 cm from the edge of the central well. When silver and nitrate ions reached the probes, the resistance dropped dramatically. The time was recorded, and 200-µl samples were collected at three different positions (1 cm, 2 cm, and 3.5 cm from the center) in the Petri dishes.

The same experiment was repeated using a 5-g/L sodium chloride (NaCl) solution instead of ultrapure water. Samples (200 µl) were taken at three different positions (1 cm, 2 cm, and 3.5 cm from the center) at the average time obtained in the ultrapure water experiment, because the resistance changes could not be monitored because of the overwhelming presence of non silver ions. All experiments were run in triplicate

Silver concentration was measured in water and saline using flame atomic absorption spectroscopy. Samples were filtered through 0.45-micron filter, to remove particulates, and acidified with nitric acid before analysis. The calibration coefficient of determination (R^2) of the spectrometer was at least 99.9 percent for data acceptance.

RESULTS

Corrected zone of inhibition test

The results for CZOIs generated by antimicrobial agents and dressings are presented in Table 1. The CZOI data showed that, for all silver-based products that release adequate silver, comparable inhibitory zone sizes were generated against each individual microorganism. This is shown with *S. aureus*, where $AgNO_3$, Arglaes, Silverlon, and Acticoat, all dressings that are purported to release silver, generated zones between 8 mm and 10 mm in size. The nonreleasing silver dressing, Actisorb did not produce a zone of inhibition. In addition, it was clear that different organisms produced different-sized zones of inhibition against the same dressing. For instance, zones of inhibition around *S. aureus* were about 10 mm, whereas around *K. pneumoniae*, the zones were 1 mm to 3 mm. Mafenide acetate generated inhibitory zones for most microorganisms but not for *B. cepacia*, VRE, *C. albicans*, *E. coli*, *Mucor*, or *Aspergillus*. Avance and Kerlix did not induce any inhibitory zones on any of the microorganisms tested except for Kerlix on *C. albicans*.

Logarithm reduction test

The logarithmic reduction of viable bacterial counts induced by different antimicrobial agents and dressings is shown in Table 2. For the purpose of this study, low antimicrobial activity was considered to be less than a 1 log reduction, moderate activity between a 1 and 3 log reduction, and high antimicrobial activity as greater than a 3 log reduction.

The topical preparations $AgNO_3$ and SSD each showed low to moderate activity against most bacteria tested, including MRSA and VRE strains. Mafenide acetate, in comparison, showed poor bactericidal activity in general. In addition, none of the topical preparations were particularity effective against the fungal isolates tested. All three topical agents tended to exhibit a much greater efficacy at 2 hours than at 30 minutes, indicating a slow killing of the microbial isolates.

The dressings tested in this study are all commercially available and all are advertised as antimicrobial wound care dressings. Despite these claims, a substantial variation between the various dressings in log reduction activity was observed in terms of spectrum of activity and in their effectiveness over time. The respective activities of the dressings are as follows. Avance, a foam dressing, was not effective at killing any of the microbial isolates tested at 30 minutes or 2 hours. Arglaes and Actisorb 220 were moderately effective at killing most of the Gram-negative bacteria after 2 hours, with the exception of the VRE, the *Acinetobacter* spp., and *B. fragilis*. In addition, Arglaes and Actisorb 220 showed minimal efficacy against all Gram-positive and fungal isolates tested. Silverlon showed a pattern of moderate to high antimicrobial activity, in which *C. glabrata* and all Gram-negative bacteria except VRE showed between a 2.3 and 6.9 log reduction. Nevertheless, Silverlon still

Table 1. Corrected zone of inhibitions (mm) generated by topical antimicrobial agents and dressings

Microorganism	Silver Nitrate	Mafenide Acetate	Silver Sulfadiazine	Avance	Arglaes	Actisorb Silver 220	Silverlon	Kerlix	Acticoat
S. aureus	10	10.3	13	0	9	2	8.3	0	10.3
P. aeruginosa	5.3	17.3	8.3	0	5.5	0	5.3	0	5.3
MRSA	2.3	14	3.7	0	2.3	0	3.7	0	3.3
VRE	6	0	6.3	0	5	0	4	0	7
Antibiotic-resistant *P. aeruginosa*	10	15	10.7	0	11	0	12.3	0	9.7
B. cepacia	2	0	6.3	0	2	0	0	0	2
S. epidermidis	8.7	4.7	9.7	0	2	1	7.7	0	10.7
K. pneumoniae	2.7	6.7	16.7	0	2	0	1	0	2
E. coli	7.3	0	9.3	0	6.7	0	7	0	9.7
Alcaligenes spp.	10	12.7	12.3	0	5.7	2	6	0	8.7
C. Koseri	2.7	6.3	12.3	0	2	0	2	0	3
Acinetobacter spp.	3.1	29.3	18	0	3.5	1.3	3.9	0	3.3
B. fragilis	N/A	26.5	N/A	0	N/A	N/A	N/A	0	N/A
C. albicans	9.3	0	10.3	0	12.7	3.3	8.7	11.3	16.3
C. glabrata	13.3	2	10	0	11.3	0	12.7	0	8.7
Mucor spp.	3.3	0	2.3	0	2.3	0.2	2.1	0	2
Aspergillus spp.	7.3	0	7.5	0	6.3	4.1	6.5	0	5.5

N/A: Data not available because hemolysis of BBA plates was induced.

produced poor antimicrobial activity against all Gram-positive isolates and all other fungi. Kerlix, a non-silver-based dressing, had moderate to high activity against Gram-negative bacteria, Gram-positive bacteria, and yeast alike. Additionally, Kerlix showed similar log reduction values for 30 minutes and 2 hours, indicating that the majority of the microbes tested were killed within a short period of exposure. Even so, Kerlix was limited in its spectrum of activity, showing poor log reduction values for *B. cepacia* and the fungal isolates *Mucor* and *Aspergillus*. Acticoat, a nanocrystalline silver dressing, showed high antimicrobial activity against all 17 isolates tested. Log reductions from 2.9 to 7.9 were observed in the 30-minute test and from 4.2 to 8.6 in the 2-hour test. In most cases, this resulted in apparent 100 percent killing of the tested microbe.

Comparison of CZOI and log reduction results

Figures 1 and 2 show the graphs of CZOI and log reduction data generated at 0.5 and 2 hours. The R^2 values were determined for Gram-positive organisms, Gram-negative organisms, and fungi, for both time points. All groups had an R^2 value of 0.16 or less. When the data for all microorganisms was combined, the R^2 values were 0.0016 and 0.0149 for the 30-minute and 2-hour tests, respectively, indicating no correlation between the CZOI and log reduction data.

Silver diffusion in water and saline

Figure 3 shows the concentration of silver at distances of 1, 2, and 3.5 cm from the source of $AgNO_3$ in water and saline after 5.5 hours of diffusion time. The silver diffusion in the 5 g/L NaCl solution was limited because it was below the limit of detection for the silver analysis technique at all three distances from the source. Measurable silver levels were found at all three distances in the ultrapure water test.

DISCUSSION

This study clearly shows that the zones of growth inhibition produced by silver dressings in a CZOI assay are not reflective of the antimicrobial efficacy of those dressings or topical agents, as measured by direct killing (log reduction) assays. This is of critical importance to wound care practitioners and industry alike, because silver has been recognized as an important topical antimicrobial agent for use on chronic and acute skin wounds. Realization that zone of inhibition assays are not optimal for use in evaluating silver dressings will have a serious effect on the use and development of efficacious silver dressings. This in turn will benefit the wound-healing field, not only in terms of patient quality of life, but also in terms of reduced health care spending on ineffective dressings and topicals and reduced incidence of silver resistance in microorganisms due to use of ineffective or inappropriate dressings. Thus, this study advocates that the standard of use for testing the antimicrobial efficacy of silver-containing dressings and agents should be the log reduction assay and not a zone of inhibition test.

There is no correlation between log reduction and CZOI results for silver in this study (Figures 1 and 2). Similar observations have been made and reported in the literature. Rosenkranz and Carr[15] studied the susceptibility of bacteria to SSD. They found no

Table 2. Log reduction (30 minutes/120 minutes) induced by topical antimicrobial agents and dressings

Microorganism	Silver Nitrate	Mafenide Acetate	Silver Sulfadiazine	Avance	Arglaes	Actisorb Silver 220	Silverlon	Kerlix	Acticoat
S. aureus	1.2/3.1	0.1/0.4	0.2/2.5	0/0.1	0.1/0.2	0.8/1.7	0/0	3.3/5.6	4.0/6.0
P. aeruginosa	1.3/6.4	0/0.8	1.2/6.4	0/0.1	0.1/3.1	0.6/4.3	0.8/5.6	5.0/6.0	4.7/6.2
MRSA	0.9/1.5	0.4/0.9	1.6/<1.2	0/0	0.2/0.2	0.6/1.6	0/0	3.1/5.7	3.7/4.8
VRE	<0.1/2.2	<0.1/0	0.2/1.5	0/0.1	<0.5/<0.3	0.7/0.6	0/0	4.4/4.8	5.0/5.1
Antibiotic-resistant *P. aeruginosa*	0.1/4.2	0.9/2.0	0.3/4.3	0/0	0.4/3.7	0.5/3.4	0.6/4.2	5.0/5.3	5.0/6.5
B. cepacia	1.4/6.3	0.5/1.0	3.1/6.3	0.2/0	<0.6/3.6	0.9/3.0	0.4/4.1	<1.6/<1.8	6.1/6.5
S. epidermidis	1.8/4.2	0.7/2.2	0/3.4	0.2/0.3	0/0.1	0.3/<1.1	0.2/<1.1	5.5/5.6	4.9/4.2
K. pneumoniae	1.5/4.8	0.6/2.5	1.1/3.4	0.2/<1.2	1.1/2.3	0.8/3.0	1.6/2.9	5.9/6.7	5.8/6.8
E. coli	0.9/6.7	0.6/1.4	2.1/6.7	0.2/0.1	0.6/4.7	1.1/3.6	0.9/4.5	6.0/6.3	6.1/6.7
Alcaligenes spp.	0.7/5.9	0/2.2	0.7/5.9	0/0.1	0/3.5	0.4/1.9	2.3/4.0	2.8/4.0	5.6/6.0
C. Koseri	2.4/7.1	1.6/5.7	2.4/7.1	0/0.1	0.7/4.6	2.7/3.6	1.6/6.9	5.0/7.0	5.0/7.0
Acinetobacter spp.	1.8/8.4	1.2/1.9	7.9/8.4	0.1/0.2	0.7/1.7	0.5/1.3	2.8/3.7	7.9/8.4	7.9/8.4
B. fragilis	2.0/8.6	0/1.1	3.4/8.6	1.2/0.3	0.4/0.9	0.5/1.4	3.1/2.3	1.2/8.6	3.1/8.6
C. albicans	1.1/2.4	0.1/0.1	5.1/5.0	0/0	0/0.4	0.7/1.3	0/0.3	4.8/4.9	4.7/4.7
C. glabrata	<0.7/<1.6	<0.7/2.0	<0.7/<1.6	0/0.1	0.1/0.9	0.7/0.7	2.7/3.5	1.0/4.4	5.4/5.4
Mucor spp.	0.1/0	0/0.1	0.3/0.1	0.1/0.1	0.2/0	0.2/0	0.3/0.2	0.2/0.7	6.7/6.6
Aspergillus spp.	0/0	0/0	0.2/0	0.1/0	0/0	0.2/0	0.2/0.2	0.9/1.0	2.9/4.5

FIGURE 1. Plot of corrected zone of inhibition vs. log reduction at 30 minutes for 17 organisms and nine dressings/topical agents with a fitted line and a coefficient of determination of 0.0016.

relationship between zone size and MIC values, and they recommended using only MIC tests to evaluate the efficacy of SSD, as they had done in an earlier study.[22] Wright et al.[20] used zone of inhibition testing to evaluate longevity. In these tests, a piece of dressing was transferred to new bacterial lawns daily. Zones were measured before each transfer, and when zones no longer appeared, it was assumed that the dressing was no longer releasing significant amounts of silver. They found that different silver-releasing dressings had different in-use lifetimes that were not apparent in the first-day results. The differences were magnified when the test organism required a higher dose of silver to inhibit it. They noted that a highly silver-sensitive *P. aeruginosa* was inhibited for 4 and 9 days and a more-silver-resistant *S. aureus* for 1 and 9 days by

FIGURE 2. Plot of corrected zone of inhibition vs. log reduction at 120 minutes for 17 organisms and nine dressings/topical agents with a fitted line and a coefficient of determination of 0.0149.

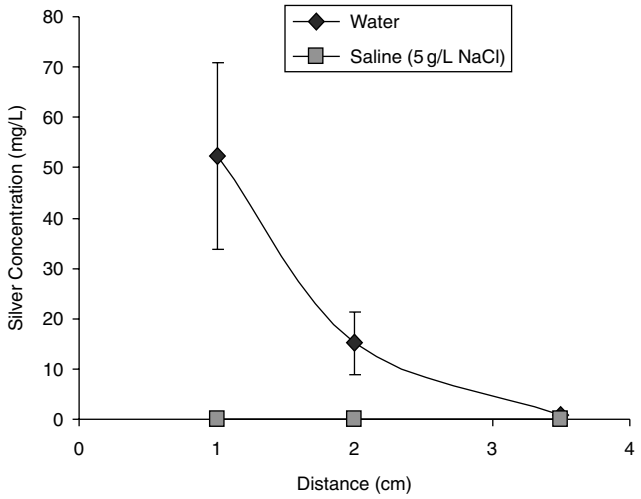

FIGURE 3. The concentrations of silver in solution after 5.5 hours of diffusion in water and 5 g/L saline at 1, 2, and 3.5 cm from the edge of a 0.03-M silver nitrate source. Error bars = standard error of the mean.

Silver calcium phosphate ($AgCaPO_4$) and nanocrystalline silver-coated dressings, respectively. More importantly, they compared bactericidal efficacy in a log reduction test in trypticase soy broth and showed that, with the nanocrystalline silver dressing, there were no recoverable organisms after 30 minutes (>5 log reduction), whereas the $AgCaPO_4$ dressing had log reductions between 0.1 and 0.6 for the same time. When the test solution was saline, there was virtually no change in the outcome of the comparison as measured using log reduction. This shows that, as other authors have noted, the source, species, and concentration of silver are critical factors in efficacy. Thus, in the study by Wright et al.,[20] a single zone of inhibition test would have created the impression that the dressings were equivalent, but the log reduction and silver-release tests confirmed that the dressings were different in terms of silver released, longevity, and bactericidal properties.

Yin et al.[23] compared the efficacy of $AgNO_3$, SSD, nanocrystalline silver, and mafenide acetate using zones of inhibition, MIC, minimum bacteriocidal concentration, and log reduction assays with five different organisms. They found that the silver zones of inhibition were all about the same size and the mafenide acetate zones were generally larger. These findings were consistent with those of Richard et al.,[24] who reported that two different silver dressings generated zones of approximately the same size for numerous organisms. The minimum bacteriocidal concentrations for each of the silver products were similar on a silver basis that corresponds with the zone of inhibition data. However, in log reduction assays, there were significant differences between the various forms of silver delivery. The nanocrystalline silver was clearly superior in terms of bactericidal efficacy, generating 5 log to 6 log reductions in one-half to one-sixth the time it took the other forms of silver. This highlights the problem with silver zone of inhibition testing. It shows that the technique has severe limitations as an assessment tool for determining antimicrobial properties of silver compounds. Richard et al.[24] demonstrated the limitation of zone of inhibition testing in their study comparing silver delivery systems. They found that the zones of inhibition between two dressings were not significantly different but that there were large differences in bactericidal efficacy as measured using a log reduction type assay. They reported that a silver-plated nylon dressing was significantly less efficacious than a nanocrystalline silver dressing in terms of bactericidal activity.

Spadaro et al.[25] have shown that various silver salts generate different-sized zones of inhibition when they are mixed with polymethyl methacrylate cement. They found that all silver salts would eventually create a zone of inhibition if the silver concentration was high enough but that $AgSO_4$ was the best in terms of activity and longevity.

Deitch et al.[26] used a novel diffusion assay to evaluate silver-coated nylon materials. They found that, for every 2 mm of diffusion through agar, a 10-fold decrease in the activity of the silver occurred. This was the first observation that silver diffusion assays do not involve simple linear relationships and was the first indication that silver interacts with the test medium. This suggests that silver does not obey a fundamental rule regarding zone of inhibition tests in that the active agent must not interact with the test medium for a valid result. Others have made similar observations regarding silver. Falcone and Spadaro[27] observed in their work on electrically activated silver nylon materials that the zones of inhibition generated experimentally were complex and appeared to be self-limited in size because of diffusion and competing complexing processes. An example whereby the diffusion (or lack thereof) of an antimicrobial agent may limit the formation of zones of inhibition is shown in the data for Kerlix, presented in Table 1. The active ingredient in this dressing is polyhexamethylene biguanide, a moderately effective antimicrobial agent as indicated by log reduction tests. Nevertheless, polyhexamethylene biguanide is water insoluble. This type of product cannot therefore be expected to generate a zone of inhibition and should not be tested using zone of inhibition techniques. Likewise, Actisorb is a nonreleasing silver dressing that did not produce significant zones of inhibition for most microbes. This further indicates that diffusion-based assays are not appropriate for testing many antimicrobial topical agents and dressings.

Similarly, George et al.[28] evaluated the efficacy of Silvazine against 200 clinical isolates. They were able to show that *S. aureus*, MRSA, *P. aeruginosa*, and

coagulase-negative staphylococci generate consistent-sized zones within the genera. Zone sizes were 19.7 ± 1.6 mm, 16.9 ± 1.6 mm, 15.3 ± 1.1 mm, and 20.8 ± 2.1 mm for *S. aureus*, MRSA, *P. aeruginosa*, and coagulase-negative staphylococci, respectively. These observations are in agreement with the data collected here for zones of inhibition, where all silver products yielded similar-sized zones for any given organism but, in some cases, markedly different log reduction values, suggesting differences in available silver that were not identifiable in the CZOI test.

As several authors have commented on and Spadaro et al.[29,30] have shown graphically, there is an apparent problem using zone of inhibition data to evaluate efficacy of Ag^+ containing solutions. These difficulties have their basis in the reactivity of Ag^+ with Cl^- and other inorganic moieties such as sulfide and organic molecules (e.g., proteins and peptides in serum). The results of the diffusion experiments in water and 5 g/L saline (Figure 3) clearly show the effect of chloride on diffusion of silver. After 5.5 hours of incubation there is no detectable silver at the point closest to the silver source (1 cm) in saline while in the nonchloride test, silver had clearly diffused 3.5 cm. Because the diffusion was limited in the saline environment, it suggests that the silver gradient from the source outward is steep. This would make it difficult to interpret zone of inhibition results from tests in which the active agent is silver. That is, it would not be possible to make any determination regarding the total silver at the source based on the size of the zone of inhibition. Appendix 1 shows how a simple Cl^--containing system will theoretically influence the size of a zone of inhibition and compare it with published experimental data to confirm the model.

The conclusion of this analysis (Appendix 1) is that zone of inhibition is only qualitative and provides no means of assessing quantities of silver that have been released, although this does not mean that the zone of inhibition has no role to play in assessment of other properties of the dressings or topical agents. Wright et al.[20] employed a plate-to-plate transfer zone of inhibition test that generated useful data on in-use longevity in conjunction with log reduction and silver-release analysis. In their studies, these authors showed that *S. aureus* zones of inhibition were the same on Day 1 for two controlled-release silver dressings, but one dressing did not generate any further zones in plate-to-plate transfers, whereas the other produced zones for at least 9 days. The difference between the dressings was clearly shown in log reduction tests, one of which was excellent (>5 log reductions) and the other poor (<0.3 log reductions) at 30 minutes for seven bacteria and fungi species. Silver analysis provided an explanation for the difference between the dressings in that the dressing with poor antimicrobial efficacy and longevity released 100 percent of its silver in 2 hours and reached a concentration in solution of 30 mg/L, whereas the more-efficacious dressing released about 11 percent of its silver and reached a concentration of 65 mg/L, where it equilibrated. Thus, the plate-to-plate tests provided useful data on longevity and total available silver.

Log reduction data presented in the literature are difficult to compare because of the widely differing methodologies and silver concentrations used by various authors. It is clear that, in noncompeting environments, such as buffers, significant log reductions can be achieved with low concentrations of silver ions.[17] It is equally clear that, in chemically competitive environments, the level of kill declines quickly. Ricketts et al.[31] and Spacciapoli et al.[17] have demonstrated this phenomenon.

Ricketts et al.[31] measured the percentage survival of *P. aeruginosa* in a nonbuffered (no Cl^- or organic matter) $AgNO_3$ solution and the supernatant of a AgCl precipitate. In the nonbuffered 0.5 mg/L $AgNO_3$ solution, they reported a 2.0 to 2.5 log reduction in 30 minutes When a 1-mg/L $AgNO_3$ solution was used, they observed a greater than 4.5 log reduction in 15 minutes The supernatant of the AgCl precipitate showed no appreciable bactericidal activity. The Cl^- had a large effect on bactericidal activity of Ag^+ through the formation of AgCl. Spadaro et al.[32–34] reported 2 log drops in 4 hours at 79 mg Ag^+/L for *S. aureus* and 1.5 and 2.5 log drops at 3 and 4 hours with 2.0 mg and 5.8 mg Ag^+/L for *E. coli*. Marino et al.[35] studied log reductions of *S. aureus*, *P. aeruginosa*, and *C. albicans* by electrolytically generated silver. *P. aeruginosa* populations were reduced by 0.4 and 2 logs in solutions containing 27 mg/L, 25 mg/L, and 21 mg/L of silver. They reported similar trends with *S. aureus* and *C. albicans* that had 1.3, 4, and 3 and 0.3, 0, and 0 log reductions, respectively, at silver concentrations of 27 mg/L, 25 mg/L, and 21 mg/L of silver.

Spacciapoli et al.[17] determined log reduction values for several metal-containing compounds including $AgNO_3$ for periodontal pathogens. These organisms displayed a wide range of sensitivities to $AgNO_3$. Three organisms had 3 log reductions when exposed to 0.05 mg $AgNO_3$/L, and nine more had 3 to 4 log reductions at 0.5 mg/L. Two organisms were not affected by 25 mg $AgNO_3$/L exposures and three were not affected at 50 mg $AgNO_3$/L. In their study, the impact of human serum on $AgNO_3$ activity was determined via a log reduction assay using *P. gingivalis*. This organism was sensitive to $AgNO_3$ in phosphate-buffered saline solution (0.05 mg $AgNO_3$/L), but in serum its sensitivity decreased dramatically. As indicated previously, the organism was 125 to 5000 times less sensitive to $AgNO_3$ in serum than it was in phosphate-buffered saline solution. This shows the important effects that

organic matter has on sensitivity and suggests that this must be accounted for in sensitivity tests because medical applications of silver generally result in the exposure of the silver ions to competing environments such as serum. In the present study, the tryptic soy broth in which the final inocula were grown supplied the organic component.

The data presented in Table 2 clearly show that not all of the dressings had the same bactericidal efficacy in 0.5-hour or 2-hour tests. The $AgNO_3$ and SSD dressings had activities that vary from very low to very high in the 30-minute test but were consistently high at 2 hours. These dressings contain about 3200 mg/L and 3000 mg/L of silver, respectively. All of the other silver dressings, with the exception of Acticoat have limited bactericidal activity and are generally thought to release minimal silver. The Kerlix dressing is an effective bactericide, with only six of the test organisms not being highly affected at 0.5 hours. At 2 hours, only three organisms were not killed. The most effective dressing was the nanocrystalline silver dressing which was highly effective at 30 minutes and better at 2 hours. It also releases more silver (70–100 mg/L) than do the other silver dressings and is more effective than the silver topical agents because of the controlled release aspect of the dressings.[36]

Silver dressings and water-insoluble topical agents (e.g., hexamethylene biguanide) should not be tested in single-day zone of inhibition assays to determine their antimicrobial efficacy. Silver reacts with inorganic and organic molecules within most test growth medium, and water insoluble compounds cannot diffuse into the test medium. Both cases result in the generation of unreliable results that have no correlation with the antimicrobial efficacy of the agents to be tested and should not be used to evaluate most of the topical agents and bandages that are used to treat burns or chronic wounds. In addition, zone of inhibition assays do not provide useful information regarding the potential for development of silver resistance of a particular clinical isolate over the duration of treatment. Zone of inhibition assays may be used to provide useful data as to dressing longevity in plate-to-plate transfer assays. Longevity assays, however, must be run in conjunction with other tests such as log reductions or active agent release studies. Log reduction assays, performed as single or multiple time point studies, provide useful data on the efficacy of test agents. As a result, we recommend that log reduction tests be the standard for the assessment of the antimicrobial efficacy of silver-based dressings and other topical agents. Generating data using this type of test will allow clinicians to select dressings that are most effective (well above the MIC for the colonizing or infecting bacterial strains), clinically relevant, and least likely to contribute to the development of silver resistance in the future.

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APPENDIX 1

Due to the extensive nature of this appendix, it is presented only as an online resource at http://www.cphs.wayne.edu/wrr/downloads/V13I4G.html.

REFERENCES

1. Bowler P. The anaerobic and aerobic microbiology of wounds: a review. Wounds 1998;10:170–8.
2. Mertz PM, Patti JM, Marcin JJ, Marshall DA. Model for studying bacterial adherence to skin wounds. J Clin Microbiol 1987;25:1601–4.
3. Mertz PM, Ovington LG. Wound healing microbiology. Dermatol Clin 1993;11:739–47.
4. Robson MC. Wound infection: a failure of wound healing caused by an imbalance of bacteria. Surg Clin N Am 1997;77:637–50.
5. Schraibman IG. The significance of beta hemolytic streptococci in chronic leg ulcers. Ann Royal Coll Surg Eng 1990;72:123–4.
6. Heggers JP, Haydon S, Ko F. *Pseudomonas aeruginosa* exotoxin A: its role in retardation of wound healing. J Burn Care Rehab 1992;13:512–8.
7. Silver S. Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. FEMS Microbiol Rev 2003;27:341–53.
8. Tredget EE, Shankowsky HA, Groenveld A, Burrell R. A matched-pair, randomized study evaluating the efficacy and safety of Acticoat silver-coated dressing for the treatment of burn wounds. J Burn Care Rehabil 1998;19:531–7.
9. Grier N. Silver and its compounds. In: Block SS, editor. Disinfection, sterilization, and preservation, 3rd Ed. Philadelphia: Lea & Febiger, 1983:375–89.
10. Gupta A, Matsui K, Lo J-F, Silver S. Molecular basis for resistance to silver cations in *Salmonella*. Nat Med 1999;5:183–8.
11. Heggers JP, Robson MC. The emergence of silver sulphadiazine-resistant *Pseudomonas aeruginosa*. Burns 1978;5:184–7.
12. Li X-Z, Nikaido H, Williams KE. Silver-resistant mutants of *Escherichia coli* display active efflux of Ag^+ and are deficient in porins. J Bacteriol 1997;179:6127–32.
13. Drlica K. The mutant selection window and antimicrobial resistance. J Antimicrob Chemother 2003;52:11–7.
14. Spadaro JA. Antibacterial effects of silver electrodes. Annual Conference of Electrochemistry—IEEE. Eng Med Biol Society 1981:215–8.
15. Rosenkranz HS, Carr HS. The determination of the susceptibility of bacterial isolates to silver sulfadiazine. Chemotherapy 1978;24:143–5.
16. Hall RE, Bender G, Marquis RE. Inhibitory and cidal antimicrobial actions of electrically generated silver ions. J Oral Maxillofac Surg 1987;45:779–84.
17. Spacciapoli P, Buxton D, Rothstein D, Friden P. Antimicrobial activity of silver nitrate against periodontal pathogens. J Periodont Res 2001;36:108–13.

18. Jackson GG, Riff LJ. *Pseudomonas* bacteremia: pharmacologic and other bases for failure of treatment with gentamicin. J Infect Dis 1971;124:S185–91.
19. Stratton CW, Cooksey RC. Susceptibility tests: special tests. In: Hausler WJ Jr, editor. Manual of clinical microbiology, 5th Ed. Washington: American Society for Microbiology, 1991:1153–65.
20. Wright JB, Hansen DL, Burrell RE. The comparative efficacy of two antimicrobial barrier dressings: In vitro examination of two controlled release of silver dressings. Wounds 1998; 10:179–88.
21. Wright JB, Lam K, Hansen DL, Burrell RE. Efficacy of topical silver against fungal burn wound pathogens. Am J Infect Control 1999;27:344–50.
22. Carr HS, Wlodkowski TJ, Rosenkranz HS. Silver sulfadiazine: In vitro antibacterial activity. Antimicrobial Agents Chemother 1973;4:585–7.
23. Yin HQ, Langford R, Burrell RE. Comparative evaluation of the antimicrobial activity of Acticoat antimicrobial barrier dressing. J Burn Care Rehabil 1999;20:195–200.
24. Richard JW, Spencer BA, McCoy LF, Carino E, Washington J, Edgar P, Rosenblatt J, Goodheart R, Heggers JP. Acticoat versus Silverlon: the truth. J Burns 2002;1:11–9.
25. Spadaro JA, Webster A, Becker RO. Silver polymethyl methacrylate antibacterial bone cement. Clin Orthop Rel Res 1979;143:266–70.
26. Deitch EA, Marino AA, Malakanok V, Albright JA. Silver nylon cloth: in vitro and in vivo evaluation of antimicrobial activity. J Trauma 1987;27:301–4.
27. Falcone AE, Spadaro JA. Inhibitory effects of electrically activated silver material on cutaneous wound bacteria. Plast Reconstr Surg 1986;77:455–9.
28. George N, Faoagali J, Muller M. Silvazine (silver sulfadiazine and chlorhexidine) activity against 200 clinical isolates. Burns 1997;23:493–5.
29. Spadaro JA. Electrical activation of Silver bacteriostasis. In: 7th Annual Meeting of the Society for Biomaterials, Troy, NY: Trans Society Biomater, 1981;4:70.
30. Spadaro JA. Bone formation and bacterial inhibition with silver and other electrodes. Reconstr Surg Traumatol 1985;19:40–50.
31. Ricketts CR, Lowbury EJL, Lawrence JC, Hall M, Wilkins MD. Mechanism of prophylaxis by silver compounds against infection of burns. Br Med J 1970;2:444–6.
32. Spadaro JA, Chase SE, Webster DA. Bacterial inhibition by electrical activation of percutaneous silver implants. J Biomed Mater Res 1986;20:565–77.
33. Spadaro JA, Berger TJ, Barranco SD, Chapin SE, Becker RO. Antibacterial effects of silver electrodes with weak direct current. Antimicrobial Agents Chemother 1974;6:637–42.
34. Spadaro JA, Becker RO. Some specific cellular effects of electrically injected silver and gold ions. Bioelectrochem Bioenerg 1976;3:49–57.
35. Marino AA, Deitch EA, Malakanok V, Albright JA, Specian RD. Electrical augmentation of the antimicrobial activity of silver-nylon fabrics. J Biol Phys 1984;12:93–8.
36. Burrell RE. A scientific perspective on the use of topical silver preparations. Ostomy/ Wound Manage 2003;49:5a(suppl):19–24.