Major Article

Demonstrating the persistent antibacterial efficacy of a hand sanitizer containing benzalkonium chloride on human skin at 1, 2, and 4 hours after application

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Background: Use of hand sanitizers has become a cornerstone in clinical practice for the prevention of disease transmission between practitioners and patients. Traditionally, these preparations have relied on ethanol (60%-70%) for bactericidal action.

Methods: This study was conducted to measure the persistence of antibacterial activity of 2 preparations. One was a non-alcohol-based formulation using benzalkonium chloride (BK) (0.12%) and the other was an ethanol-based formulation (63%) (comparator product). The persistence of antibacterial activity was measured against *Staphylococcus aureus* using a technique modification prescribed in American Society for Testing and Materials protocol E2752-10 at up to 4 hours after application.

Results: The test product (BK) produced a marked reduction in colony-forming units at each of the 3 time points tested (3.75-4.16-$\log_{10}$ reductions), whereas the comparator produced less than 1-$\log_{10}$ reduction over the same time. The differences were highly significant.

Discussion: In the course of patient care or examination, there are instances where opportunities exist for the practitioner's hands to become contaminated (eg, key boards and tables). Persistent antibacterial activity would reduce the chances of transfer to the patient.

Conclusions: These results show a major improvement in persistent antibacterial activity for the BK formulation compared to the comparator ethanol-based formulation.

The prevention of nosocomial infections has been a goal for the medical community since the elucidation of the germ theory of disease. Modern approaches include extensive facilities sanitation programs and multiple personal hygiene practices.[1] Of the latter, regular hand washing and the use of hand sanitizer products are now routine.[2] Hand sanitizer formulations have traditionally contained ethanol or other short-chained alcohols (60%-70%) as the active ingredient responsible for the antibacterial action. Ethanol provides its antimicrobial action through desiccation of the target organisms. Applied to the skin, the ethanol-based sanitizers are effective in reducing the bioburden of many types of microbes.[3] However, alcohols are volatile and can evaporate from the skin's surface, so the residual antibacterial activity may be limited.[4] The importance of persistent antimicrobial activity has been increasingly recognized in the medical/surgical setting.[2,5] Recent reports have also shown that certain pathogen populations are becoming more tolerant to ethanol exposure.[6] These data suggest that the use of alternative antibacterial actives might be a benefit in the clinical setting.

Alcohol-free formulations have been developed, with the surfactant benzalkonium chloride (BK) as the active antibacterial agent. This active ingredient acts by disrupting the cell membranes of the target organisms and is active at relatively low concentrations (0.12%-0.13%).[7] Since this surfactant is not volatile, it is expected to remain on the skin as the product dries. Although this report focuses only on the antibacterial action of BK against *Staphylococcus aureus*, this surfactant has also been studied for virucidal activity against influenza, Newcastle disease, and avian infectious bronchitis viruses.[8]

This study was performed to measure the residual antibacterial activity of 2 hand sanitizer products using the standard method prescribed in the American Society for Testing and Materials protocol E2752-10.[9] The test product was a surfactant-based product using BK (0.12%) as its active antibacterial agent, and the second product was a standard commercial ethanol-based formulation (with 63% ethanol but no other antibacterial actives), which served as the comparator product. The comparator product's ethanol concentration falls within the recognized effective concentration range for effective immediate contact antimicrobial activity.[3] Persistence of antibacterial activity was measured as a function of $\log_{10}$ kill of reference bacteria versus time after application of the hand sanitizer. The antibacterial activity was measured from 1-4 hours after application of the products. The test product was evaluated at 1, 2, and 4 hours after application, whereas the comparator product was evaluated at 1 and 4 hours after application.

METHODS

For this study of residual antibacterial activity on the skin, 2 products were compared. The commercial brand DAB hand sanitizer (active ingredient 0.12% BK) and a comparator hand sanitizer, containing 63% ethyl alcohol), were provided by Best Sanitizers (Walton, KY) to the testing laboratory, Biosciences Laboratories, Inc. (Bozeman, MN).[10] The DAB brand is produced by Best Sanitizer under contract to Three Kings Inc. (Corinth, MS). The study was conducted in compliance with good laboratory practices for nonclinical studies (21CFR58). As stated in the study protocol, "The purpose of this study was to evaluate the residual antibacterial efficacy of 1 test product verses a comparator ethanol-based product, as determined by the difference between the number of challenge bacteria species recovered following exposure to the test materials and the number recovered from the untreated (negative control) test sites."

Panelists and skin preparation

The study was performed on 24 subjects (19-63 years old) with healthy skin (16 men and 8 women). The study protocol and informed consent form were approved by the Gallatin Internal Review Board. The volar forearms were used, and the test sites were marked for the test product, comparator product, and negative control. The volar forearm was chosen to provide multiple replicate test sites on each arm, which would not be possible using the hands. The sites and arms were randomized among the treatment groups to prevent anatomical bias. The arms were washed with nonmedicated soap to remove surface dirt and oil, dried, and finally decontaminated with 70% isopropyl alcohol and allowed to air dry. The test sites and control sites were marked with a surgical marker as rectangles (2 × 6 inch [5.08 × 15.24 cm]) for the test product on 1 arm and as rectangles (2 × 4 inch [5.08 × 10.16 cm]) for the comparator product on the other arm. An area for the untreated control skin (no further treatment) was also marked. The areas for the test and comparator products were randomized between arms across the test panel. Within the test sites, 3 circles (2 cm in diameter) were marked with a surgical marker. Only 2 circles were marked in the 2 × 4-inch box for the comparator product, as only 2 time points were to be assessed. These were the sites to which the bacteria were to be applied.

Challenge bacteria

The challenge bacterial strain for this study was *S aureus* (ATCC 6538). *S aureus* is a common skin contaminant and therefore provides an appropriate test organism.[11] Fresh, active stocks were prepared in broth medium daily. The day before testing, a sample of the broth culture was applied to and spread over the surface of a tryptic soy agar plate and incubated for 24 hours. Just before beginning the study, a portion of the bacteria on the surface of the agar plate was transferred to phosphate buffered saline. After mixing the bacteria into the saline to form a uniform suspension, the turbidity of the suspension was measured and the sample diluted to approximately 1.0×10^8 colony-forming units (CFU) per mL of suspension. Ten microliters of this suspension (approximately 10^6 CFU) were applied to and spread over the 2-cm circles at the appropriate times.

Product neutralizer

It is essential that once the bacteria are removed from the treated skin that residual skin sanitizer not continue to act on the bacteria as they are being prepared (diluted and plated). To this end, a product neutralizer was prepared and added to the dilution liquids. For this study, the same product neutralizer was selected for both the test and comparator products. Before the study began, the effectiveness of the product neutralizer was confirmed using American Society for Testing and Materials E1054 (2013), Standard Test Method for Evaluation of Inactivators of Antibacterial Agents.[12] Four replicate samples for each of the 2 exposure periods (1 and 30 minutes) were tested for each treatment condition: untreated control, test product, comparator product, Butterfield's Phosphate Buffer (BPB++), and Stripping Suspension Fluid (SSF++). The "++" refers to the presence of the product neutralizer. In addition, the antibacterial efficacy of the test and comparator products without neutralization were verified.

Evaluation of antibacterial efficacy

Application of the test and comparator products

Each product was applied to the skin at a rate of 0.25 mL per square inch (0.039 mL/cm^2) (3 mL for the 2 × 6-inch test rectangle and 2 mL for the 2 × 4-inch comparator product rectangle). In both cases, the liquid was applied in stages, spread over the whole area, and allowed to dry for 1-2 minutes between each application. Once all of the applications were made, the subjects were sequestered and monitored at the test facility to ensure test site integrity.

The persistent efficacy of the test product was evaluated at 1, 2, and 4 hours after application of the product to the skin. The comparator product was evaluated at only 1 and 4 hours after application. At each time point, 10 μL of the bacterial suspension were applied to 1 of the 2-cm circles in the test product treatment area and spread over the surface with a sterile glass rod. The procedure was repeated on the comparator product treatment area (except for the 2-hour time point) and on the negative control area. Each inoculation was allowed to dry in place for at least 20 but not for more than 25 minutes. At the end of this exposure period, a 2-step procedure known as the cup scrub technique was used to remove the bacteria for determination of viability. A sterile stainless steel cylinder with an interior area of 3.46 cm^2 was held against the skin within the 2-cm circle. A volume of 2.5 mL of sterile SSF was dispensed into the cylinder. The fluid contained the specific product neutralizer (SFF++) to stop the action of the test and comparator products. A sterile rod was used to massage the skin for 1 minute to lift the bacteria from the skin into the fluid. This fluid was transferred to a sterile tube, and a second 2.5 mL volume of SSF++ was dispensed into the cylinder. Again, the skin was massaged for 1 minute, and the second fluid sample was combined with the first. This process was repeated for each exposure condition at that time point. For example, at the 1-hour postexposure time point, 3 bacterial suspensions were collected from each of the 24 subjects; 1 from the test product-treated skin, 1 from the comparator product-treated skin, and 1 from the negative control-treated skin. To determine the number of viable bacteria (number of CFU) in each sample, serial 10-fold dilutions of each bacterial suspension sample were prepared in BPB solution again containing the product neutralizer (BPB++). Samples from each dilution were spread onto 2

Table 1
Mean $\log_{10}$ microbial recoveries and reductions from the untreated control of *Staphylococcus aureus* (ATCC 6538), 1 hour following application of the test product or comparator product

Measure	Untreated $\log_{10}$ microbial recovery	Test product 1 h after application		Comparator product 1 h after application	
		Treated $\log_{10}$ microbial recovery	$\log_{10}$ difference	Treated $\log_{10}$ microbial recovery	$\log_{10}$ difference
Median	5.23	0.86	4.22	4.81	0.51
Mean	5.20	1.08	4.12	4.50	0.70
SD	0.189	0.395	0.359	0.727	0.703
		P value (1 tailed)	*P* <.001		

individual mannitol salt agar plates, which were incubated at $35\pm2°C$ for 48 hours. On mannitol salt agar, *S aureus* produce golden-yellow colonies, and only those colonies were counted.

Calculation of the recovery of viable CFU of bacteria

By definition, a CFU is 1 bacterium that is capable of continued replication to produce a large number of bacteria to form a colony. Each inoculum to the skin contained approximately 10^6 CFU. Each sample from the skin was serially diluted and samples plated. Knowing the area of the skin sampled (3.46 cm^2), the volume of SSF (5 mL), the dilution of the sample producing the counted plate, and volume of the sample added to the plate, the number of CFU per unit area on the skin could be calculated.

The number of CFU from each site at each postapplication time was converted to a $\log_{10}$ value. The residual antibacterial activity was calculated by comparing the $\log_{10}$ value from the negative control site (time matched) to the $\log_{10}$ value from the test and comparator product-treated sites to determine the $\log_{10}$ difference (antibacterial effectiveness) for each treatment. The relative values were internally controlled for each subject. For the 1- and 4-hour postexposure times, the statistical significance between the $\log_{10}$ difference for the test and comparator values for the 24 subjects was evaluated using a paired Student t test (Excel).

RESULTS

The results of the product neutralizer testing showed the efficacy of the neutralization formulation. In all cases, there was no significant difference between the mean untreated control $\log_{10}$ colony counts (n = 4) and the mean treated $\log_{10}$ colony counts (n = 4), indicating that there was no significant residual antibacterial activity.

The results of the study are expressed as $\log_{10}$ mean recovery of CFU of *S aureus* from the untreated control site, the test product, and the comparator product sites for each postapplication time point. The mean values from the individual postapplication time point values for the test and the comparator products are provided (Tables 1-3).

DISCUSSION

This study was performed to measure the antibacterial efficacy of a benzalkonium-based test product in comparison with a comparator

Table 2
Mean $\log_{10}$ microbial recoveries and reductions from the untreated control of *Staphylococcus aureus* (ATCC 6538), 2 hours following application of the test product

Sample	Sample size	Mean ($\log_{10}$)	SD
Untreated $\log_{10}$ microbial recovery (2 h)	23*	5.17	0.20
Treated $\log_{10}$ microbial recovery (2 h)	24	1.01	0.37
$\log_{10}$ difference (2 h)	23	4.16	0.35

*One untreated control sample lost.

product containing 63% ethanol as a function of time after application of the individual products to human skin. *S aureus* was used as the test organism since it is a known skin pathogen.[11] The test and comparator products were applied to defined areas of opposing forearms at 0.039 mL/cm^2. Within those areas, 2-cm diameter circles were marked, to which the bacterial suspension would be applied at the specific times after application of the products. For the test product treatment, bacteria were applied at 1, 2, and 4 hours after product application and for the comparator product treatment, bacteria were applied at 1 and 4 hours after product application. Bacteria were applied to untreated skin at each time point to provide the baseline bacterial recovery. The difference in the recovery between the test and comparator products was striking. Although the test product reduced bacterial viability by 3-4 $\log_{10}$ at each time point, the comparator product did not reduce bacterial viability by even 1 $\log_{10}$. The differences in efficacy were statistically significant at $P < .001$. These data suggest that the active ingredient BK (0.12%) can provide a marked improvement in persistent antibacterial activity over the 63% ethanol-based product.

The effectiveness of BK as an antibacterial agent on skin has been evaluated in the past. Dyer et al (1998) compared the efficacy of 3 hand sanitizer preparations containing either ethanol (63% or 70%) or BK (0.13%) against *Serratia marcescens* applied to the hands.[7] In this study, the hands were contaminated with 5 mL of *S marcescens*, spread over the hands, and allowed to dry for 45 seconds. Five grams of test product were used to "wash" the hands, and then the remaining bacteria were recovered using the "glove juice sampling method." Polyethylene gloves with 50 mL of recovery fluid were placed, and the hands and the fluid massaged for 1 minute to recover the bacteria. The bacterial suspension was diluted and plated to obtain the number of CFU recovered. This process was

Table 3
Mean $\log_{10}$ microbial recoveries and reductions from the untreated control of *Staphylococcus aureus* (ATCC 6538), 4 hours following application of the test product or the comparator product

Measure	Untreated $\log_{10}$ microbial recovery	Test product 4 h after application		Comparator product 4 h after application	
		Treated $\log_{10}$ microbial recovery	$\log_{10}$ difference	Treated $\log_{10}$ microbial recovery	$\log_{10}$ difference
Median	5.08	0.86	3.96	4.58	0.17
Mean	4.92	1.17	3.75	4.59	0.32
SD	0.420	0.503	0.602	0.649	0.597
		P value (1-tailed)	*P* <.001		

repeated 10 times for each treatment condition, and the reduction factors were calculated. The process took approximately 10 minutes per cycle. Only the BK formulation produced a progressive increase in effectiveness (increased reduction factor) over the 10 cycles. The ethanol formulations showed declines in effectiveness relative to the first cycle for each.

The concentration of ethanol in the hand sanitizer formulation can have a marked impact on antibacterial activity. Kampf (2008) compared 4 ethanol-based formulations (85%, 62%, 61%, and 60%) and 2 application volumes of 2.4 and 3.6 mL (total both hands) were evaluated.[13] Again, *S marcescens* was used as the test bacterium. Approximately 5 mL of bacterial suspension were rubbed over the hands and allowed to dry. The viable bacteria were recovered using the glove juice sampling method described in the preceding text. The bacterial suspension was diluted and plated to obtain the number of CFU recovered. The untreated recovery values were compared to the treated conditions where either 2.4 or 3.6 mL were provided to rub over the hands (covering all skin). Both volumes were sufficient to cover the hands of most of the 16 subjects in each test group. The mean $\log_{10}$ reductions for each treatment were statistically compared by an analysis of variance analysis. Although all of the preparations reduced the number of viable bacteria, the larger volume was more effective at all ethanol concentrations and the 85% ethanol formulation was statistically more effective than the other 3 concentrations. For the 3.6 mL application volume, the mean $\log_{10}$ reduction for the treatment groups were 3.04 ± 0.81 (85%), 2.85 ± 0.51 (62%), 2.63 ± 0.59 (61%), and 2.53 ± 0.60 (60%). However, 85% ethanol is much higher than what is normally contained in current commercial hand sanitizer formulations.

Although *S aureus* accounts for a large fraction of the hospital-acquired infections, other bacteria are a concern. *Enterococcus faecium* is a gram-positive bacterium, which has become a leading antibiotic-resistant pathogen (bloodstream, urinary tract, and surgical wounds).[14] Hospital strains can be resistant to multiple antibiotics, which make them particularly difficult to treat once the infection is established.[15] The rise in incidents of nosocomial infections has raised concerns that preventive measures, such as the use of ethanol-based hand sanitizers, have applied selection pressure on the populations to select for more tolerant strains. Pidot et al (2018) have examined the resistance to isopropyl alcohol in 139 strains of hospital-associated *E faecium* isolated from 2 major Australian hospitals over 17 years.[6] These hospitals have active hand sanitation programs based on alcohol-based hand disinfectants. To measure resistance, bacterial suspensions were exposed to 23% isopropyl alcohol for 5 minutes and the number of remaining CFU determined. The concentration of isopropanol and time of exposure were selected to maximize resolution among the strains. Breaking the isolates into groups by date of isolation (1997-2003, 2004-2009, and 2010-2015), there was a high statistically significant decrease in mean sensitivity (based on mean $\log_{10}$ reduction) for the 2010-2015 isolates compared to the 1997-2003 and to the 2004-2009 isolates. These data suggest that there has been a population selection, which has reduced the overall sensitivity to the alcohol-based infection control measures.

Selection for increased tolerance to other disinfectants as a function of repeated use/exposure has been examined under various environmental exposure conditions. Holah et al (2002)[16] compared *Listeria monocytogenes* and *Escherichia coli* populations found in cannery processing lines where quaternary anomia disinfectants were routinely used. These isolates were compared to isolates from sites not routinely subjected to disinfectant use. They concluded that the persistent populations on the cannery lines were not inherently more tolerant to the disinfectant but that other factors (ie, surface attachment, biofilm formation, and growth rate) were likely responsible for their ability to persist in the disinfectant-treated environment. Kim et al (2018)[17] examined the impact of continuous exposure to BK on

bacterial populations isolated from contaminated river sludge. The sediment samples were maintained for extended periods (3 years) in bioreactors containing nutrient medium and increasing concentrations of BK or nutrient medium alone. Changes in benzalkonium tolerance were measured using the minimal inhibitory concentration assay on nutrient agar. Certain species (ie, *Pseudomonas aeruginosa*) showed increased tolerance to BK (200 vs 50 mg/L), whereas others did not (ie, *Klebsiella michiganensis*). The basis for the difference in the selected strains with increased tolerance was a small change in the antibiotic efflux gene sequence.

It is not surprising that disinfectants can provide some selective pressure on bacterial populations. This pressure is most effective at sublethal concentrations of the disinfectant, which allow the more tolerant subpopulations to thrive and predominate. Lethal concentrations are less likely to select for tolerant clones where the surviving fraction of the population is very low.[18,19] The current study was not designed to measure selection pressure on the *S aureus* population. It was designed to measure persistence of antibacterial efficacy. The persistence of high antibacterial efficacy from the BK-containing test product may reduce the chances for selection of more tolerant clones.

Normal clinical infection control protocols specify use of hand sanitizers between patients to prevent patient-to-patient microbial transfer. That is not expected to change with the use of a persistent antimicrobial agent. However, in the course of patient care or examination, there are instances where there are opportunities for the practitioner's hands to become contaminated. Various surfaces such as key boards, tables, chairs, bed frames and other fixtures will need to be touched or handled. Use of a persistent antimicrobial hand sanitizer would be expected to reduce the opportunity for microbial transfer to the patient.

This study was undertaken to measure the absolute and relative persistence of antibacterial activity under very controlled test conditions. Having demonstrated persistent activity, the logical next step would be a clinical use study. As a first evaluation, a study is planned that will compare a 70% ethanol product and the test product from this study. Subjects will be medical clinic personnel, who will use both products in a cross-over study design.

In the United States, hand sanitizers (both medical professional and consumer) fall under the purview of the U.S. Food and Drug Administration, the 1994 tentative final monograph or proposed rule (the 1994 TFM) for over-the-counter antiseptic drug products (Federal Register of June 17, 1994 [59 FR 31402]). These rules are in the process of being revised to separate the professional and consumer products, and the agency is seeking additional data on active ingredients, including ethanol and BK. One factor to consider is the persistence of the antibacterial activity on the skin. This study provides quantitative data on the persistence of BK-induced antibacterial action, which could be a marked benefit in the prevention of nosocomial infections.

CONCLUSIONS

These results show a major improvement in persistent antibacterial activity for the BK formulation compared to the comparator ethanol-based formulation. Persistent antibacterial activity may be beneficial in the patient care setting to reduce the chances of incidental contamination of the hands and subsequent transfer to the patient.

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