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Inactivation of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Vancomycin-Resistant *Enterococcus faecium* (VRE) on Various Environmental Surfaces by Mist Application of a Stabilized Chlorine Dioxide and Quaternary Ammonium Compound-Based Disinfectant

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Contaminated environmental surfaces are important reservoirs in the transmission of many human pathogens. Although several options exist for disinfecting contaminated environmental surfaces, few are compatible with use on both hard smooth non-porous (hard) and soft porous surfaces (soft) while still offering significant disinfection of the contaminating organisms. This study evaluated the efficacy of mist application of a stabilized chlorine dioxide and quaternary ammonium compound-based disinfectant (Cryocide20) for inactivation of methicillin-resistant Staphylococcus aureus *(MRSA) and vancomycin-resistant* Enterococcus faecium *(VRE) on various environmental surfaces. MRSA and VRE were applied to hard and soft surfaces (glass, steel, tile, carpet, and cotton fabric), allowed to dry, and exposed to a uniform mist application of the disinfectant solution. After 1 hr of contact time, the residual disinfectant was neutralized, and the bacteria were recovered and enumerated on brain heart infusion (BHI) agar. Reduction of both test bacteria was observed on most of the hard and soft surfaces tested. Log_{10} reduction of the organisms tended to be higher on steel, tile, and carpet than glass or cotton. Overall, these results suggest that mist application of Cryocide20 disinfectant may be an effective option for reduction of low levels of infectious bacterial pathogens from contaminated environmental surfaces.*

Keywords disinfection, environmental surface, mist application, MRSA, VRE

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INTRODUCTION

Environmental surfaces have been implicated as important reservoirs in the transmission of many human pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE),[1–3] which can persist and remain viable for days to months on various environmental surfaces, such as countertops, floors, equipment, and worker clothing.[4–11] MRSA and VRE are strains of common bacterial pathogens that have developed resistance to traditional antimicrobials. Although still considered emerging infectious agents in some countries, these drug-resistant pathogens have become endemic in the United States, Europe, and Australia and are associated with increased morbidity and mortality in these countries. These pathogens are commonly found in hospitals and clinics and are increasingly implicated in community-acquired illness.[3,12–19] They are typically spread by direct contact with other infected individuals or indirectly from fomites.[9,20–21]

Several factors should be considered in selecting disinfectants for environmental surfaces, in particular, the type and amount of organic soil present, necessary contact time, their effectiveness against various microorganisms, compatibility with various environmental surfaces, and method of application.[10] Although many classes of compounds have been used in disinfecting contaminated environmental surfaces, none of them have proved to have all the ideal properties.

Chlorine dioxide (ClO_2) has many of the characteristics desired in a surface disinfectant (e.g., broad efficacy against different organisms, compatible with many surfaces). However, it is poorly stable in solution. When stabilized with carbonate and maintained at a high pH (>8.5), chlorine dioxide is present primarily as chlorite, a more stable form.[22] Although chlorite has nearly the same oxidative capacity as chlorine dioxide, the germicidal potential of chlorite is not well known. If stabilized chlorine dioxide is acidified, however, ClO_2 gas will be regenerated. This is an important

consideration for application, since chlorite is a respiratory irritant but has no established exposure limit, while ClO_2 has an occupational exposure limit of 0.1 ppm. Stabilized chlorine dioxide products that have been activated are reported to be efficient bactericides, with previous studies showing as little as 0.75 mg/L of available chlorine dioxide capable of at least a $\log_{10}$ reduction = 4 for vegetative bacteria.[23–24]

Quaternary ammonium compounds (QACs) are another promising surface disinfectant. QACs are a class of surface active agents that have varying degrees of antimicrobial activity. QACs may have good germicidal properties against bacteria, fungi, and lipophilic viruses at levels as low as 10 to 50 mg/L and may inhibit bacterial growth at lower levels.[25,26] However, some QACs can be inhibited by environmental factors, such as protein load and water hardness, although as a class, twin chain QACs are considered to have a high tolerance for such inhibiting factors.[25]

In addition to the choice of disinfectants in terms of their effectiveness against various microorganisms and compatibility with various environmental surfaces, method of application is another important factor in surface disinfection. There are two methods commonly used to evaluate the efficacy of disinfectants on hard surfaces: (1) the (AOAC) use-dilution method and (2) the germicidal spray products test.[27] Unfortunately, neither of these standardized methods is adequate to replicate delivery of disinfectants by mist- or fog-based applications. In this study, a novel mist-chamber method was developed for the evaluation of fine-mist application of a stabilized chlorine dioxide and QAC-based disinfectant (Cryocide20; R.P. Adam Ltd., Selkirk, Scotland) for disinfection of MRSA and VRE on various environmental surfaces.

METHODS

Test Bacteria

Type strains of MRSA (ATCC #700698) and VRE (ATCC #700221) were obtained from American Type Culture Collection (Manassas, Va.). Stock cultures were prepared from the rehydrated strains according to ATCC protocol and stored at –80°C with 20% glycerol until use. Prior to each disinfection trial, frozen MRSA and VRE stocks were inoculated into BHI broth and incubated overnight at 37°C.

Carrier Materials

Five carrier types were examined in disinfectant trials, including three hard surfaces and two soft surfaces. Hard surfaces examined were borosilicate glass, #316 stainless steel (Biosurface Technologies Corp., Bozeman, Mont.), and vinyl composition floor tile (Armstrong World Industries, Lancaster, Pa.). For glass and stainless steel, independent 2 cm diameter disc carriers were used. For the floor tile surface, 30.5-cm^2 floor tiles were divided into discretely delineated 6 cm diameter circles (diameter of contact plates). Soft surfaces examined were synthetic-fiber carpet and cotton fabric. Carpet carriers were fashioned from a 2-cm^2 region of a used nylon/olefin blend carpet remnant with 6.4 mm pile, 9.5 mm gauge and stitch rate, and a latex backing. Cotton fabric carriers were 2 cm diameter pieces punched from bleached cotton muslin with a thread count of 717 threads per cm. All carriers were autoclaved (121°C for 15 min) prior to use and their sterility verified by sampling unseeded carriers during each disinfection trial.

Disinfectant

A commercial disinfectant product, Cryocide20, was used in this study. It contains stabilized chlorine dioxide and a twin-chain quaternary ammonium compound (dimethyl-didecyl-ammonium chloride) at 7500 mg/L and 2500 mg/L (as listed by the manufacturers), respectively. The intended pH of Cryocide20 (from the material safety data sheet) is 9.0. For mist disinfection experiments, 5 mL of liquid Cryocide20 (or sterile deionized water) was applied as an atomized fine mist. To maximize the consistency of disinfectant dose, only freshly opened bottles of Cryocide20 were used in each reported experimental trial.

Disinfection Chamber and Application

For surface disinfection experiments, a 0.5 m^3 chamber (127 cm long, 77 cm wide, and 52 cm high) was designed and fabricated from 6.4-mm plexiglass (Figure 1). Supplemental diffused air was supplied to maintain an air exchange rate of 4.8 to 6 air changes per hour (ACH) and provide mixing of the air within the chamber. A Devilbiss model DV151 atomizer (Sunrise Medical HHG, Inc., Somerset, Pa.), driven by house air, was used for delivery of the Cryocide20 disinfectant (or sterile deionized water for controls) to the test chamber from the tapered terminus of the chamber. The atomizer provided a conical dispersion pattern and had a forward spray distance

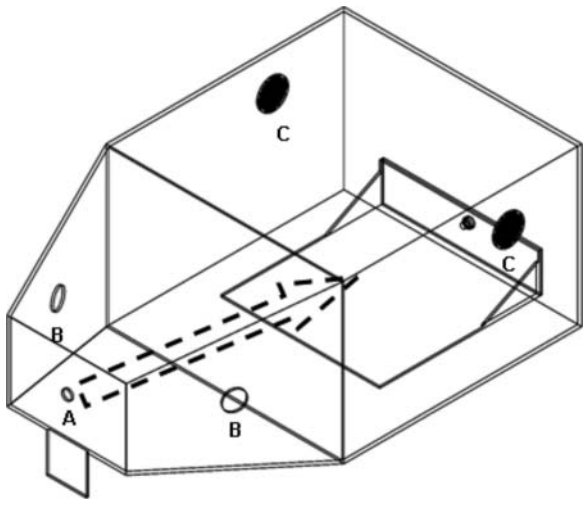

FIGURE 1. Disinfection chamber. The dotted arrow represents the direction of disinfectant (or control) flow, following introduction through the A port. Additional airflow was introduced through B ports to provide turbulent mixing of the disinfectant plume. C ports were filtered vents.

of roughly 1.2 m, under the air pressure applied. The bulk of the atomized liquid settled evenly within a triangular pattern on the floor of the chamber. Application was calibrated prior to each trial to verify even and adequate coverage.

Surface Disinfection Test Procedure

Overnight cultures of MRSA and VRE were serially diluted in sterile deionized water, and $\sim 10^6$ colony forming units per mL (cfu/mL) were applied to carrier surfaces. All surfaces received the inocula in a liquid volume of 10 μL, except carpet and tile, which received 20 μL (due to absorptive capacity) and 100 μL (due to size), respectively. Suspending liquid was allowed to dry completely on the surfaces in a Class II B2 biological safety cabinet for approximately 30 to 90 min, depending on surface type. Once dried, carriers were transferred to the disinfection chamber and immediately exposed to 5 mL ($\pm$ 0.5 ml) of either sterile, deionized water (control) or Cryocide20 disinfectant (test solution).

After a contact time of 1 hr at room temperature, carriers were removed from the chamber, and immediately neutralized with a neutralizing agar (BHI agar with 0.6% lecithin and 0.7% sodium thiosulfate) or a neutralizing eluant (PBS supplemented with 0.6% lecithin and 0.7% sodium thiosulfate to neutralize quaternary ammonium compounds and chlorine compounds, respectively), depending on recovery and assay method. For all surface disinfection trials, carrier surfaces were sampled in triplicate, with the exception of tile (sampled in duplicate). Three to eight independent disinfection trials were conducted for each carrier type.

Sample Recovery and Viability Assay

For tile surfaces, bacteria were recovered and assayed directly by pressing contact plates of D/E neutralizing agar onto the appropriate delineated circle for approximately 60 sec. For all other surfaces, bacteria were recovered via rinse and elute method. Briefly, carriers were placed in individual 8-mL vials with 1 mL (2 mL for carpet carriers) of neutralizing solution and vortexed vigorously for 60 sec. Recovered bacteria were diluted serially in PBS, spotted (as replicate 10 μL spots) on BHI agar, then incubated for 24 hr at 37°C and the resulting colonies in each spot quantified. Inoculum titer was confirmed for each trial by assay of initial serial dilution by spot-titer method on BHI agar.

Liquid Disinfection Test Procedure

Batch liquid suspension tests were conducted for comparison with surface disinfection tests. Overnight cultures of test bacteria were serially diluted 10-fold in PBS, and $\sim 10^6$ cfu in an inoculum volume of 10 μL was transferred to each 1.5 mL polypropylene tube. Disinfectant solution was then added at a volume to volume ratio of 1:1, 1:5, or 1:10 (inoculum volume to disinfectant volume). After a contact time of 1 hr, the reaction suspensions were diluted to 1 mL with PBS supplemented with 0.6% lecithin and 0.7% sodium thiosulfate. The neutralized suspensions were then serially diluted in PBS and plated on BHI agar. The serial dilution of the inoculum was concurrently plated on BHI agar to determine initial titer. For liquid disinfection trials, each suspension was sampled in at least triplicate, with each organism replicated six to nine times. All tests were conducted at ambient room temperature (22 $\pm$ 2°C).

Data Analysis

The $\log_{10}$ reduction factor for bacteria was calculated for each disinfectant trial according to the following formulas:

$$\text{Log}_{10}\,\text{Reduction} = -\text{Log}_{10}\,(T_T \div T_C)$$

where T_T is the average titer of bacteria recovered from Cryocide20-treated samples, and T_C is the average titer of bacteria recovered from the deionized water-treated controls. For surface disinfection trials, the median $\log_{10}$ reduction factor is reported for each bacterium on each carrier type. For the liquid suspension disinfection trials, the median $\log_{10}$ reduction factor is reported for each seeding density and each volumetric ratio. Maximum and minimum $\log_{10}$ reduction factors are also reported. Significance of differences in the levels of disinfection between MRSA and VRE were determined by the Mann-Whitney U-test. Differences in the reductions for a particular organism between carrier types were evaluated using the Kruskal-Wallis test. All statistical analysis was performed using Statistica, v. 6.1 (Statsoft, Inc., Tulsa, Okla.).

RESULTS

Table I summarizes the $\log_{10}$ reduction of MRSA on different environmental surfaces by mist application of Cryocide20 disinfectant. Maximum values reported as ">" indicate that T_T fell below the lower limit of detection. Median values reported as ">" indicate that in at least half the trials, T_T fell below the lower limit of detection. Although the reduction of MRSA was somewhat variable, there was reduction of MRSA on most of the hard and soft surfaces tested. Table II summarizes the $\log_{10}$ reduction of VRE on different environmental surfaces by mist application of Cryocide20 disinfectant. Although the reduction of VRE was somewhat lower than MRSA, there was a $\log_{10}$ reduction $\geq$2 for VRE on most of the hard and soft surfaces tested.

Table III summarizes the $\log_{10}$ reduction of MRSA and VRE, respectively, in liquid disinfection trials. Liquid disinfection experiments were conducted to determine the

TABLE I. Log_{10} Reduction of MRSA on Surfaces by Mist Application of Cryocide20 Disinfectant

Surface	Glass	Steel	Rug	Cotton	Tile
Median	1.9	3.2	3.1	2.7	>4.0
Max	3.7	>4.0	>4.0	>4.0	>4.0
Min	0.4	2.0	2.4	0.5	2.6
Number of trials	4	4	6	8	3

TABLE II. Log_{10} Reduction of VRE on Surfaces by Mist Application of Cryocide20 Disinfectant

Surface	Glass	Steel	Rug	Cotton	Tile
Median	1.9	2.4	2.0	0.6	2.6
Max	2.9	>4.0	2.2	>4.0	>4.0
Min	0.9	0.5	1.6	0.4	2.1
Number of trials	6	4	6	6	3

disinfection potential of the test disinfectant (Cryocide20) without the variability introduced during the surface disinfection trials, such as organism desiccation during surface drying and poor recovery from surfaces. As expected, median reductions of both test bacteria were greater than observed for the mist-based applications. Higher levels of inactivation were observed with higher concentrations of the disinfectant.

DISCUSSION

It is difficult to compare the results of this study with the ones in previous studies because of the differences in disinfectants, carrier materials, and application methods. However, the maximum reductions of MRSA and VRE on glass and steel in the current study are similar to the average reduction values demonstrated for other vegetative bacteria in previous studies with use-dilution application of chlorine dioxide or QAC-based disinfectants.[24,28]

Previous surface disinfection studies have typically been concerned with the efficacy of disinfectants to inactivate microorganisms on glass and stainless steel carrier surfaces, consistent with the standard AOAC International methods.[27] However, glass and stainless steel may not adequately represent the range of surfaces in the real world that may require disinfection. In this study, disinfection of MRSA and VRE was evaluated on a range of hard (glass, stainless steel, and floor tiles) and soft surfaces (carpet and cotton fabric). No significant difference was found in the level of disinfection observed between different surface types ($p > 0.05$). Surface type differences were potentially occluded by the variability between inter-trial results for particular surfaces. The log_{10} reduction factor for MRSA was generally greater than or equal to that of VRE, regardless of surface type. However, only the log_{10} reduction difference on carpet was significant ($p = 0.004$).

TABLE III. Log_{10} Reduction of MRSA and VRE by Liquid Application of Cryocide20 Disinfectant

Disinfectant	1:1 Strength		1:5 Strength		1:10 Strength	
Ratio (v/v)	MRSA	VRE	MRSA	VRE	MRSA	VRE
Median	3.1	3.8	3.0	>4.0	>4.0	>4.0
Max	>4.0	>4.0	>4.0	4.1	>4.0	>4.0
Min	2.5	2.4	2.5	3.3	2.6	3.8
Number of trials	6	9	6	9	6	9

Another difference between this study and others is the methodology typically used to test the efficacy of the disinfectants. Previous studies have been limited to the use-dilution method or the germicidal spray test, which do not necessarily adequately represent all disinfectant application methods because of differences in volume and distribution of disinfectant delivered to the test surface.[27] Cryocide20 can be easily applied by several application methods, including fine mist- and fog-based applications. These application strategies have shown to be an efficient means for delivery of disinfectants to a broad range of environmental surfaces.[29] Unfortunately, current disinfectant testing methods do not allow adequate evaluation of the efficacy of the disinfectants when delivered by these methods.[27]

In the use-dilution method, the entire carrier surface is immersed in excess disinfectant, whereas in the germicidal spray test the carrier surface is applied in a coarse spray. These application methods should be contrasted with fog- and mist-based application methods in which smaller volumes of disinfectant are applied on a real basis and in which the distribution of the disinfectant may not be uniform.

To address this issue, a novel mist chamber method was developed in this study to mimic fine-mist and fog-based delivery of disinfectant. The mist application method resulted in delivery of only a few microliters of disinfectant to carrier surfaces. This reduced volume of disinfectant and possible unevenness in the application also undoubtedly contributed to the variability in disinfection between trials. Despite this variation, this method is more likely to represent the natural variability in disinfection efficacy by the spray-mist or fog-based application than the use-dilution or germicidal spray test methods.

Although there were significant reductions of MRSA and VRE in terms of maximum log_{10} reduction factors, median log_{10} reduction factors for both test bacteria on different carrier surfaces demonstrated considerable variability. There could be several factors to explain this variability, but the most important factor would be the recovery rates of the test bacteria between different surface types. That is, recovery rates between surface types were highly variable (data not shown), with recovery from hard surfaces being the highest and most consistent. As a result, disinfection was normalized based on the average recovery from control carriers on each surface type for an individual trial.

Despite normalization of disinfection results, it is likely that intra-trial variability in the recovery from carriers contributed to the overall variability observed in the log_{10} reduction factors. Differences in the characteristics of the surface materials (e.g., hydrophobic nature of synthetic carpet fibers vs. the absorptive nature of the cotton) also may have contributed to differences in disinfection between carriers by affecting delivery of disinfectant or recovery of organisms.

Liquid disinfection experiments were conducted to determine the efficacy of the test disinfectant without the artifacts introduced during the surface disinfection trials. Assuming a conical spray pattern with even coverage of the disinfectant on the chamber floor during mist application, it is estimated that each carrier received 5–8 μL of disinfectant (2-cm carriers) and 40–70 μL (6-cm carriers). Based on the upper estimate (8 μL) of the disinfectant dose for a 2-cm carrier for spray-based studies, 10 μL was chosen as the volume of disinfectant used for the 1:1 volume ratio liquid application experiments. In the liquid disinfection experiments, the least inactivation occurred at the lowest disinfectant volume ratio, and the greatest inactivation occurred at the highest disinfectant volume ratio for both bacteria.

Comparing mist application with liquid application with the 1:1 volume ratio, median reductions of the test bacteria following liquid application were always greater, except in one instance when the limit of detectable reduction for liquid was exceeded. In addition to differences in disinfectant dose applied between the two application methods, desiccation during the drying period and losses due to poor recovery from surfaces might play an important role in differences in reduction observed in the two application methods.

Previous studies have indicated a need for activation of the stabilized ClO_2 prior to disinfection, typically by acidification.[24,30] The ClO_2 and QAC-based disinfectant (Cryocide20) is marketed as a product requiring no activation prior to use. The bulk of the ClO_2 in Cryocide20 is stabilized as chlorite, thus, little dissolved ClO_2 gas should be available in the solution for disinfection. The manufacturer estimates only 0.5 to 1 mg/L of ClO_2 is available in fresh solution. Previous studies with the use-dilution method have shown that this concentration is adequate for several orders of magnitude reduction of most vegetative bacteria.[24,27,30] Further, a recent study examining the disinfection of *S. aureus* in liquid suspension by the QAC contained in Cryocide20, found high levels of inactivation (log_{10} reduction $\geq$4) within a few minutes over contact.

Centers for Disease Control and Prevention (CDC) recommendations for disinfection and sterilization of health care facilities do not recommend disinfectant fogging for routine purposes,[31] nor is the practice common. However, the guidelines do recognize that the technique has been used in hospital rooms. Under routine circumstances, disinfectant fogging would be unlikely to significantly reduce transmission of nosocomial agents and may pose a chemical concern to patients. However, in circumstances where contamination is significant or for instances in which direct patient contact risks are limited, disinfectant fogging may be an appropriate supplement to routine cleaning or disinfectant processes for non-critical surfaces. Furthermore, as community-acquired MRSA is a growing concern, the use of disinfectant fogs outside the health care setting may become more common. Disinfectant fogging is also used more frequently in agricultural and veterinary settings where MRSA may be an issue.

CONCLUSION

Mist application of Cryocide20 with delivery of only a few microliters of disinfectant per square centimeter of carrier surface resulted in log_{10} reduction $\geq$4 for MRSA and VRE on most hard and soft surfaces tested. Furthermore, evidence from liquid disinfection experiments suggests that using greater volumes of disinfectant during mist application would likely lead to even greater reduction of the test organisms. Overall, these results suggest that mist application of Cryocide20 disinfectant may be an effective option for reduction of infectious bacterial pathogens from contaminated environmental surfaces.

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