High-Level Resistance to Quaternary Ammonium Compounds in Clinical MRSA Isolates

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Abstract

Background: Quaternary ammonium compounds (QACs) are microbicides commonly used in hard surface disinfectants and in alcohol-free hand sanitizers. There is an extensive body of research regarding resistance of microorganisms to QACs. Resistance to QACs typically occurs through efflux pumps, which are found in both gram-positive and gram-negative bacteria. At least 5 different QAC efflux (*qac*) genes have been described in *Staphylococcus aureus;* which may be located together with antibiotic resistance genes on transferable elements such as plasmids and transposons. The majority of studies to date have focused on minimum inhibitory concentration (MIC) data and the current dogma is that the resistance conferred by *qac* genes is small and well below in-use concentrations.

Objective: The objective of this study was to determine whether clinical MRSA strains exhibit resistance to killing by the QAC benzethonium chloride (BZE) in short contact times relevant to hand sanitizers. A second objective was to determine whether resistance to killing by BZE correlates with the presence of *qac* genes.

Methods: Eleven strains of *Staphylococcus aureus* (8 MRSA and 3 MSSA) were used. An isogenic pair (RN4420 and RN4220/pGO1) was included to examine the influence of the *qacC* gene on QAC resistance. Standard PCR was used to detect the presence of *qac* genes (*qacA/B, qacC, qacF, qacG, qacH*). MICs were performed by the microdilution method. *In vitro* Time-Kill experiments were carried out according to ASTM E 2315 using a 15-second contact time.

Results: BZE MICs for S. *aureus* strains ranged from 2 to 8 µg/ml. Higher MICs were associated with the presence of a *qac* gene. The concentrations of BZE required to achieve complete kill in 15-second Time-Kill experiments were much higher and ranged from 150 to >4800 µg/ml (0.015% to >0.48%). Notably, USA400 was not killed at the highest concentration of BZE tested (0.48%), which is above the typical concentrations used in QAC-based hand sanitizers (0.1%-0.2%). We did not find a strong correlation between the presence of a *qac* gene and the concentration of BZE required to kill *S. aureus*.

Conclusions: These results demonstrate that MICs are not appropriate for determining resistance to QACs. Time-Kill experiments revealed that the concentrations of BZE required to kill MRSA in short contact times can approach or exceed those used in alcohol-free hand sanitizers. Because no correlation was found between BZE resistance and the presence of *qac* genes, a novel resistance mechanism may be involved. Further research is needed to determine whether MRSA strains are resistant to BZE-based products in actual use.

Introduction

There is a large body of research pertaining to resistance of microorganisms to QACs.[1-3] Previous studies have demonstrated that QACs are substrates for non-specific efflux pumps in both gram-negative and gram-positive bacteria. At least 5 different QAC efflux (*qac*) genes have been described in *Staphylococcus aureus* (designated *qacA, qacB, qacC, qacG and qacH*).[1-4] These genes have been shown to confer small increases in MICs (minimum inhibitory concentration) and MBCs (minimum biocidal concentration) to QACs. The *qac* genes can be carried on mobile genetic elements (plasmids, integrons, and transposons), which may be transferred vertically or horizontally. They are often linked genetically to resistance genes for antibiotics such as aminoglycosides or ß-lactams. It remains controversial whether exposure to QACs can select directly for maintenance or transfer of these mobile elements.[1-3] Because MICs and MBCs in strains harboring *qac* genes remain well below typical in use concentrations for hand sanitizers and hard surface disinfectants, others have concluded that resistance to QACs in *S. aureus* is not clinically relevant.[1] However, MIC and MBC measurements, which are typically employed for antibiotics, measure growth inhibition and/or kill after a 24-hour exposure to the antimicrobial. In contrast, hand sanitizers and skin disinfectants are required to produce reductions of at least 4- to 5-log steps in very short contact times. In the case of hand sanitizers, contact times are typically between 15 and 30 seconds. MICs and MBCs may therefore be inappropriate means to measure "resistance" of bacteria to QACs. The *in vitro* Time-Kill methodology is commonly used to evaluate the rate of kill and spectrum of activity of biocidal actives and products and is highly useful for identifying differences in the sensitivities of bacterial strains to killing by biocides. Recent studies have begun to demonstrate that bacteria can develop resistance to killing by quats at levels approaching the in use concentration when short contact times are used.[5]
The objective of this study was to evaluate the level of BZE required to kill MRSA strains in short contact times typical of hand sanitizers. A second objective was to determine whether resistance to killing by BZE correlates with the presence of *qac* genes or with elevated BZE MICs.

Methods

Test Strains: Eleven strains of *Staphylococcus aureus* were used in the study. Seven MRSA Isolates were obtained from the Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA): NRS123 (USA 400), NRS382 (USA 100), NRS383, (USA 200), NRS384 (USA 300), NRS385 (USA 500), NRS483 (USA 1000), NRS484 (USA 1100). To evaluate the influence of *qacC* on QAC sensitivity an *S. aureus* isogenic pair NRS144 (RN4220) and NRS106 (RN4220 / pGO1 were employed.
An additional MSSA strain (ATCC 6538) and MRSA strain (ATCC 33591) were used as controls.

Minimum Inhibitory Concentrations: Minimum Inhibitory concentrations (MICs) were determined by the microdilution method (M7-A5) using cation-adjusted Mueller Hinton broth.[6] The lowest concentration of an antimicrobial agent that totally inhibited bacterial growth after 24 h of incubation at 37°C was considered the MIC.

PCR of *qac* genes, *aac-aphA*, and *mecA*: Primers used to amplify *qacA/B, qacC, qacG* and *qacH, aacA-aphD,* and *mecA* have been previously described.[4] Reaction mixtures were subjected to 30 cycles of amplification. The conditions for each cycle were denaturation for 10 s at 98°C, annealing for 10 s at 61°C, and primer extension for 40 s at 72°C. Finally, reaction mixtures were incubated at 72°C for 1 min. PCR products were separated by electrophoresis in agarose gels, stained with ethidium bromide, and visualized under UV light.

Time-Kill Studies: *In vitro* Time-Kill suspension tests were performed following the ASTM E 2315 Standard Guide.[7] The test products were evaluated at a 99% (v/v) concentration, where an aliquot of the challenge suspension was transferred to a tube containing test product and exposed for 15 or 30 seconds. Immediately following exposure, the test product / challenge suspension mixture was diluted ten-fold in BBP++ to neutralize the antimicrobial active. Additional ten-fold dilutions were prepared in BBP++ as appropriate. From these dilutions aliquots were pour-plated in duplicate using TSA+ and plates were incubated at 35°C for 48 to 72 hours. Following incubation, colonies were counted, and log reductions were calculated by subtracting post-exposure recovery values from the pre-exposure culture titer. Curves were generated using a sigmoidal dose response (variable slope) model.

Summary and Conclusions:

- The presence of *qacC* in *S. aureus* conferred only a slight increase (2-4-fold) in QAC MICs and did not provide protection against the rapid killing of *S. aureus* by BZE.
- The concentration of BZE required for rapid killing of *S. aureus* by *in vitro* Time-Kill was approximately 100-fold greater than the MIC.
- Clinical MRSA isolates displayed varied sensitivities to rapid killing by BZE. In particular USA 400 and USA 500 resisted killing by marketed BZE-based hand sanitizers after a contact time (15 s) reflective of in use conditions.
- The concentration of BZE required to kill USA 400 in 15 seconds was above the typical level used in alcohol-free hand sanitizers. Extending the contact time to 30-s shifted this concentration by 10-fold indicating that contact time plays a critical role in killing by BZE.
- Neither USA 400 or USA 500 was found to contain known *qac* resistant determinants suggesting that a novel mechanism is responsible for the high-level BZE resistance in these strains.
- These results demonstrate that MICs alone are not appropriate for determining resistance to QACs.
- Further studies are needed to determine whether QAC-based products would be ineffective against QAC-resistant strains under actual use conditions.

Additional Information

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Results

Table 1: Correlation of MICs to the Presence of Antimicrobial Resistance Genes in *S. aureus*

Strain	MIC (µg/ml)[a]				Resistance Gene (PCR Result)		
	BZE	BZK	Kan	Mec	*qac*[b]	*aacA-aphD*	*mecA*
RN4220	2	2	1.56	0.78	-	-	-
RN4220/pGO1	8	4	50	1.56	*qacC*	+	-
ATCC 6538	2	2	n.t.	0.78	-	-	-
ATCC 33591	8	4	50	25	*qacC*	-	+
USA 100	4	2	50	25	-	-	+
USA 200	8	4	50	25	-	+	+
USA 300	4	2	3.13	25	-	-	+
USA 400	2	2	3.13	25	-	-	+
USA 500	8	4	50	125	-	+	+
USA 1000	4	4	3.13	50	-	-	+
USA 1100	4	2	3.13	25	-	-	+

Table 1: [a]BZE, benzethonium chloride; BZK, benzalkonium chloride; Kan, kanamycin; Mec, methicillin.
[b] Strains were screened for presence of *qacA/B, qacC, qacF, qacG, qacH.*

Table 2: Rapid Biocidal Activity of Hand Sanitizers Against MRSA Strains

Strain	Log_{10} Reduction (15-sec exposure)		
	Product A (62% Ethanol)	Product B (0.10% BZE)	Product C (0.15% BZE)
ATCC 6538	≥6.149	5.848	≥6.149
ATCC 33591	≥6.297	4.964	≥6.297
USA 100	≥6.134	4.256	≥6.134
USA 200	≥6.929	≥6.929	≥6.929
USA300	≥6.234	≥6.234	≥6.234
USA 400	≥6.152	1.367	5.851
USA 500	≥6.250	1.462	≥6.250
USA 1000	≥7.045	6.2	≥7.045
USA 1100	≥7.135	5.918	≥7.135

TABLE 2 shows the log_{10} reductions achieved by 3 commercial hand sanitizers against 8 MRSA and 1 MSSA strain in a 15-s Time-Kill experiment. A ≥ symbol denotes that no viable organisms were recovered after product contact.

Figure 1 : Influence of *qacC* on Sensitivity to Killing of *S. aureus* by BZE

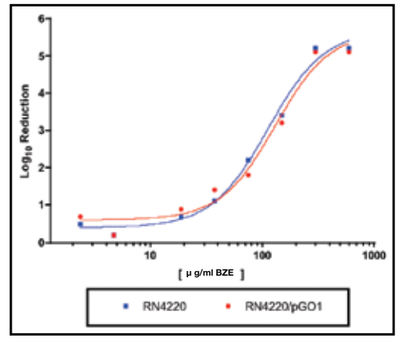

FIGURE 1 shows the log_{10} reductions achieved by increasing concentrations of BZE in 15-s Time-Kill experiments against 2 *S. aureus* strains. RN4220 and RN4220/pGO1 are an isogenic pair where the latter strain contains *qacC*. The presence of *qacC* did not affect sensitivity to killing by BZE.

Figure 2: Concentration Dependent Killing of MRSA by BZE in a 15-s Exposure

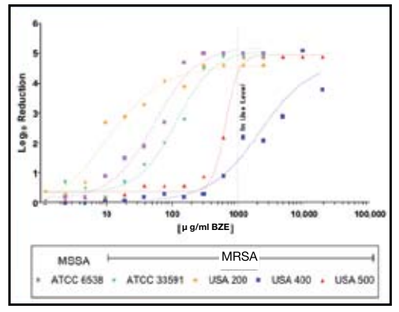

FIGURE 2 shows the log_{10} reductions achieved by increasing concentrations of BZE in 15-s Time-Kill experiments against 1 MSSA and 4 MRSA strains. The concentration of BZE required for effective kill (i.e. ≥ 4 log reduction) ranged from approximately 100 µg/ml to 10,000 µg/ml depending on the test strain. The concentration required for effective kill of USA 500 was near the in-use level (1000 µg/ml or 0.1%) and was 10-fold above the in-use concentration for USA 400 .

Figure 3: Influence of Exposure Time on Killing of USA 400 by BZE

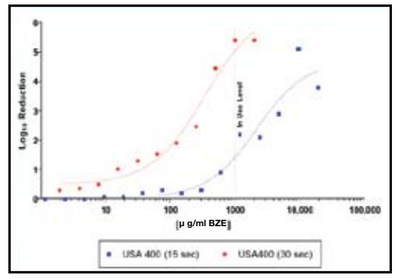

FIGURE 3 shows the log_{10} reductions achieved by increasing concentrations of BZE in 15-s and 30-s Time-Kill experiments against USA 400. The concentration required to kill USA 400 was decreased by approximately 10-fold by extending the contact time to 30 s.