Susceptibility to chlorhexidine in multidrug resistant clinical isolates of *Staphylococcus epidermidis* from bloodstream infections.

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ABSTRACT

Emergence of *Staphylococcus* isolates with reduced susceptibility to chlorhexidine is being increasingly reported. We present an update to a previous report showing continuing efficacy of chlorhexidine-based infection control measures against *Staphylococcus aureus* over six years. We screened *qacA/B* genes in *Staphylococcus* isolates collected over another six years in the same intensive therapy unit in Scotland where chlorhexidine baths form an essential component of long-term control of nosocomial infections. Consistent with our previous study we report minimal presence of *qacA/B* in *S. aureus* strains from screening samples and bacteraemia patients but the new finding of a high proportion of *qacA/B* carriage in *Staphylococcus epidermidis* associated to reduced susceptibility to chlorhexidine. *S. epidermidis* isolates positive for *qacA/B* were clonally diverse although 65% of isolates belonged to the multidrug resistant clone ST-2. These findings raise concerns in relation to selection of multidrug resistant strains by chlorhexidine and are important in the context of recent evidence emphasising the benefits of targeting bloodstream infections associated with coagulase-negative staphylococci.

KEYWORDS: chlorhexidine baths, intensive therapy unit, *Staphylococcus aureus, Staphylococcus epidermidis, qac* genes, multidrug resistance, ST-2.

1. INTRODUCTION

For over 50 years, chlorhexidine-based preparations have been used with remarkable success for control of healthcare-associated infections, for example use of chlorhexidine baths for the prevention and control of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals (1). It has been recently shown that universal decolonization of patients in intensive care settings was more effective than targeted strategies in reducing MRSA-

positive clinical cultures and bacteraemias from any pathogen (2, 3). However, the debate around use of targeted versus universal decolonisation approaches is still ongoing (4). Indeed, a number of reports have suggested the emergence of *Staphylococcus* clinical isolates with decreased susceptibility to chlorhexidine *in vitro* (5-8), although the clinical significance of these findings is still controversial (9-11). Phenotypic susceptibility to chlorhexidine is mostly based on assays which measure MICs and MBCs. Measurement of chlorhexidine MIC and MBC relates to bacteria tested against much lower concentrations of chlorhexidine compared to those achieved in clinical practice. The lack of agreed breakpoint values for biocide susceptibility testing along with other limitations inherent to phenotypic measurement of susceptibility to disinfectants has hampered the development of standardised assays (9) and encouraged screening of clinical isolates for genetic markers potentially associated with resistance. *Qac* genes encode for proton-dependent efflux pumps which are known to bind a variety of lipophilic cations including quaternary ammonium compounds such as chlorhexidine. Of the genes known to be associated with biocide resistance, *qacA* has been more strongly associated with decreased susceptibility to chlorhexidine in *Staphylococcus* (8, 12). The QacB efflux pump carries amino acid differences compared with QacA including a substitution from Asp to Ala which determines inability to bind divalent cations (13). *QacA/B* genes are located on mobile genetic elements and their co-presence on plasmids with antibiotic resistance genes has pointed to the possibility of cross-resistance between biocides and antibiotics in *Staphylococcus* (10, 14). However, it is yet unclear whether or not the presence of *qac* genes selects for the presence of antibiotic resistance genes.

Another medically important *Staphylococcus* species is *Staphylococcus epidermidis.* While previously considered to be a non-pathogenic skin commensal, it is now recognized a key opportunistic pathogen associated with nosocomial infections including bacteraemias. *Qac* genes have been identified in *S. epidermidis* (11, 15). Some studies have suggested horizontal transfer of plasmids carrying *qac* genes among strains of *S. aureus* and other

staphylococci (16). The study here presented was designed as a follow-up to a previous report showing continuing efficacy of MRSA infection control measures in intensive care settings and the absence of emergence of resistance over a period of six years (17). We screened *qacA/B* genes in *Staphylococcus* isolates collected over another 6 years in the same intensive therapy unit (ITU) of a hospital in the North East of Scotland where use of chlorhexidine baths forms an essential component of long-term control of nosocomial infections (1). Isolates included MRSA strains from clinical samples obtained from screening upon admission to the ITU as well as *S. aureus* and *S. epidermidis* strains from patients with bacteraemia.

2. METHODS

2.1. Setting, intervention and sample collection: This study took place between November 2007 and February 2014 in the ITU of Aberdeen Royal Infirmary and involved analysis of eighty-one *Staphylococcus* isolates. Forty strains of MRSA were randomly selected from patients screened at multiple body sites on admission over the whole period of the study. Forty-one strains were obtained from blood cultures and comprised sixteen strains of *S. aureus* and twenty-five strains of *S. epidermidis*. This was a random collection of strains representing 12% and 32% of the total number of bacteraemias related to *S. aureus* and *S. epidermidis,* respectively, that occurred over the study time period. For both screening samples and blood cultures one isolate per patient was included in the study. The MRSA infection control measures following screening have already been described (17). Bacterial isolation and characterisation was carried out as previously described (17).

2.2 DNA extraction: Genomic DNA was extracted from overnight pure cultures of the *Staphylococcus* isolates using the High Pure PCR template preparation kit (Roche Diagnostics, Germany) following the manufacturer's instructions. Samples were pre-treated with 5µl of Lysozyme (Sigma, 10mg/ml) and 4µl of Lysostaphin (Sigma, 10mg/ml) and incubated for 30 minutes at 37°C for complete lysis of the cell pellet prior to the extraction.

Genomic DNA was quantified on a Qubit Fluorimeter (Thermo Fisher Scientific, Waltham, MO) and quality-assessed on a Tapestation (Agilent Technologies, Santa Clara, CA) with genomic DNA screentapes.

2.3 *qacA/B* PCR: Bacterial 16S rRNA gene was amplified using the universal eubacterial primers 27F and 1492R to confirm DNA suitability for further analysis. The 16S rRNA PCR yielded a product of approximately 1500bp. *Qac* genes (*qacA* and *qacB*) were identified using previously described specific primers (8) which yielded a product of approximately 800bp.

2.4 Whole genome sequencing: Dual indexed TruSeq libraries were prepared from 200ng of genomic DNA using the TruSeq Nano DNA library preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions using a Bioruptor Pico (Diagenode, Seraing, Belgium) for fragmentation to 550bp. Libraries were quantified by qPCR, pooled at equimolar concentrations and 14pM of the pool was sequenced on a MiSeq using version 3 chemistry and 300bp paired end reads (Illumina, San Diego, CA), with 29.3 million pass filter reads generated.

2.5 Sequence analyses: Sequences were trimmed using Trimmomatic (18), assembled using SPAdes (19) and quality-assessed with QUAST (20). The contig files were then uploaded to the Center for Genomic Epidemiology server (https://cge.cbs.dtu.dk/services/MLST/) for MLST analysis (21).

2.6 Susceptibility testing to antimicrobials: Susceptibility to chlorhexidine was tested using an agar dilution technique according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Briefly, chlorhexidine digluconate (Sigma-Aldrich, Dorset, UK) was incorporated into Mueller Hinton agar (Oxoid, Hants, UK) at two-fold dilutions. The range of concentrations tested was 0.125 to 64 mg/L and inoculation delivered at 10^4 CFU/spot using a multipoint replicating device. Incubation was carried out in ambient air at 35 °C for 20 hours. ATCC 29213 and 25923 were used for control strains. The MIC was determined as the lowest concentration of chlorhexidine that completely inhibited growth. Ethidium bromide was used as positive control of QacA pump activity and was tested at 4-1024 mg/L. Susceptibility

testing to 22 antibiotics was carried out using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) until 2010. Subsequently, susceptibility testing to antibiotics was carried out using a Vitek instrument (bioMerieux, Basingstoke, UK) and the EUCAST guidelines. Antibiotic susceptibility data of selected isolates are reported in Supplemental table 1.

3. RESULTS

3.1 Qac typing. Genomic DNA was obtained from all 81 *Staphylococcus* isolates investigated in this study, as confirmed by positive detection of the 16S rRNA gene (data not shown). Of the bacteraemia strains that were found positive for the *qacA/B* gene, 20 strains were *S. epidermis* (Table 1) and 2 strains were *S. aureus* and accounted for 80% and 13% respectively of the total *S. epidermidis* and *S. aureus* strains isolated from blood samples. Only 1 out of 40 (2%) MRSA strains isolated from screening samples was found positive for the *qacA/B* gene (STAPH12, data not shown). This strain was isolated from a throat swab and showed 100% homology to *qac*A (GenBank accession no. HE579074.1) along with the 2 *qacA/B* positive *S. aureus* strains from blood cultures.

Sixteen out of 20 (80%) *qacA/B* positive *S. epidermidis* isolates from bacteraemia samples showed 99-100% homology to the full length *qac*A, while 3 strains (STAPH51, STAPH53, STAPH59) showed 5-6 nucleotide differences compared to *qacA* (Table 1). Three of these single nucleotide polymorphisms result in single amino acid substitutions present in *qacB* (C455T, G871A, T1139C) and have been named *qacAB* in Table 1. Polymorphic sites of *qac* nucleotide sequences relative to *qacA* and respective amino acid substitutions are summarised in Table 2. STAPH77 appeared to carry only a partial *qacA* sequence (3' 450bp fragment) although a contig break in the middle of the gene and the absence of upstream sequences hindered detailed analysis of the strain.

3.2 Susceptibility to chlorhexidine. *S. epidermidis* strains positive for the full length *qacA/B* gene showed minimal but consistent increase of MIC values (2-4 fold) compared to *S. epidermidis* negative for qacA/B with the exception of strains STAPH59 (Fig. 1, supplemental file 2). For all *S. epidermidis* strains chlorhexidine MICs never exceeded 4 mg/L and there was no evidence of decreasing susceptibility levels to chlorhexidine over the study period (Fig.1, supplemental file 2). Fig. 1 shows chlorhexidine MICs of *S. epidermidis* in relation to ethidium bromide MICs which was used as control in view of the strong correlation of *Staphylococcus* resistance to this compound and positivity for the QacAB efflux pump (8, 22). Indeed, all *qacA/B* strains investigated in this study returned ethidium bromide MIC values ≥256 mg/L except for STAPH77 carrying a deleted *qacA* (Fig.1, supplemental file 2). Chlorhexidine MIC values for *S. aureus* isolates fluctuated between 1 mg/L and 4 mg/L and showed no relationship with *qac* carriage (Supplemental file 2).

3.3 Clonality of *qac* positive *S. epidermidis* strains. *S. epidermidis* isolates positive for *qacA/B* were clonally diverse with 13 out of 20 (65%) of isolates belonging to the multidrug resistant clone ST-2 found prevalent in hospital-acquired infections (23) (Table 1). Multidrug resistance of *qac* positive ST-2 *S. epidermidis* strains was confirmed by sensitivity testing to a broad range of antimicrobials (Supplemental table 1). Both STAPH51 and STAPH59 harbouring the highest number of *qacA* polymorphisms belonged to ST-83 (Table 1). The sequence types accounting for the other 5 *qac* positive *S. epidermidis* strains were ST-5, ST-559, ST-59, and ST-48 (Table 1). As observed for ST-2 these 4 types were also associated with multidrug resistance (Supplemental table 1) and were distinct from the 5 different sequence types (ST-19, ST-210, ST-54, ST-204 and a new ST) that accounted for the 5 *qac* negative *S. epidermidis* strains (Table 1) which were sensitive to most, if not all, antimicrobials (Supplemental table 1).

3.4 Genetic determinants for resistance to triclosan and mupirocin. Whole genome sequencing of *S. epidermidis* strains revealed genetic determinants for resistance to mupirocin in 10 of the *qac* positive strains. A mutation (V588F) of the iso-leucyl tRNA

transferase gene (*ileS*) conferring resistance to mupirocin (24) was observed in 5 of these strains (Table 1). The other 5 strains harboured the *ileS2* gene associated with resistance to mupirocin (25) (Table 1). Sensitivity testing showed high level resistance to mupirocin in *ileS2* isolates tested and low level resistance to mupirocin in 4 out of 5 of the *ileS* (V588F) isolates (Supplemental table 1). The genetic determinants for resistance to triclosan *sh-fabI* (enoyl-acyl-carrier protein reductase) (26) and the F204L mutation in gene *fabI* were identified in 8 and 2 strains respectively with STAPH51 carrying both determinants (Table 1). Five of these strains were also mupirocin resistant (Table 1). As previously reported *sh-fabI* was co-present on the plasmid harbouring *qacA* (26), but also observed in *qac* negative STAPH67 (Table 1).

4. DISCUSSION

This study will inform the current debate around use of universal decolonisation versus approaches to target high-risk pathogens or patient populations that are susceptible to infection from many pathogens.

We report a very low presence of the *qacA* gene in *S. aureus* strains from both screening samples and bacteraemia patients but a higher proportion (74%) of *qacA/B* carriage in *S. epidermidis*. *Qac* carriage in *S. epidermidis* coincided with consistently reduced susceptibility to chlorhexidine compared to *qac* negative *S. epidermidis.* However, chlorhexidine MICs were stable with no evidence of steady decrease of susceptibility to chlorhexidine over time, not withstanding that chlorhexidine had already been in widespread use for 6 years before the start of the present study. *Qac* positive *S. epidermidis* strains STAPH59 and STAPH77 did not show reduced susceptibility to chlorhexidine. The latter appeared to carry only the 3' 450bp portion of the *qacA* sequence and was sensitive to the control compound ethidium bromide, suggesting a defective QacA pump in this isolate. In contrast, there was no obvious correlation between *qac* carriage in *S. aureus* isolates and reduced sensitivity to

196 chlorhexidine as previously reported (8). Most *qac* positive *S. epidermidis* isolates carried
197 genes nearly identical to *qacA* reference genes. *S. epidermidis* isolate STAPH59 as well as
198 STAPH51 and STAPH53 showed 5-6 nucleotide differences with respect to *qacA* although
199 only 3 of these single nucleotide polymorphisms result in single amino acid substitutions
200 present in QacB (Table 2). All three strains lacked the three other substitutions which
201 distinguish QacA from QacB, including the Asp to Ala substitution (D322A) shown to
202 determine substrate specificity (13). While STAPH59 showed lower chlorhexidine MIC
203 compared to all other *qacA/B S. epidermidis* strains, there was no consistent relationship
204 between carriage of such polymorphic *qacA* sequences and susceptibility to chlorhexidine.

205 The widely prevalent multidrug resistant clone ST-2 (23) accounted for the majority of *qac*
206 positive *S. epidermidis*. As previously observed (14) the higher frequency of antibiotic
207 resistance among *qac*-carrying strains suggests that chlorhexidine may select for antibiotic
208 resistance. Nonetheless, presence of five other types amongst *qac* positive *S. epidermidis*
209 isolates was evidence of clonal diversity. ST-83 and ST-5 comprised the strains displaying
210 the most highly polymorphic *qacA* sequences. Strains with identical polymorphic *qacA* gene
211 sequences have been previously detected in both ST-83 and ST-5 sequence types (27, 28).
212
213 Of interest, whole genome sequencing identified genetic determinants of 2 other biocides
214 widely used for MRSA decolonisation, albeit not in ITU settings: mupirocin and triclosan. The
215 V588F *ileS* mutation or the resistance gene *ileS2* conferring mupirocin resistance (24, 25)
216 were identified in 50% of *qac* positive isolates. The triclosan resistance determinants *sh-fabI*
217 and/or the F204L mutation in *fabI* (11, 26) were identified in 9 isolates. Notably, in strain
218 STAPH48 the *sh-fabI* gene was inserted downstream *qacA*, suggesting the potential for
219 horizontal gene transfer of multiple genes associated with reduced susceptibility to biocides
220 by the same plasmid. This may be particularly true of *sh-fabI* which was present within a
221 composite transposon containing the *Staphylococcus haemolyticus*-derived insertion

sequence IS1272 (26). Consistently, a previously observed conserved gene coding Sin recombinase flanking *qacR* (14) was present on some of the *qac*-carrying plasmids.

This study provides no indication of decreased efficacy of chlorhexidine-based infection control measures against *S. aureus* infections in the ITU setting described here, or hospital wide, as already reported (29)**.** Findings are in keeping with our previous report showing no evidence of decreased susceptibility to chlorhexidine with long-term chlorhexidine bathing in intensive care over a 6 year period (17). More recent evidence also shows a lack of association between extended chlorhexidine use and the prevalence of chlorhexidine-resistant MRSA isolates in outpatient settings (30). Clonal spread, the type of population under study and differences in infection control policies are likely to account for the higher prevalence of chlorhexidine resistance genes in *S. aureus* reported in other studies (5, 6, 31).

The higher proportion of *qac* gene carriage observed in *S. epidermidis*, possibly due to long term chlorhexidine exposure, is concerning. Findings are consistent with a recent study according to which *qac* resistance genes were prevalent among *S. epidermidis* isolates associated with deep surgical site infections (15). Future larger scale prospective studies will determine the clinical implications of the high prevalence of *qac* positive *S. epidermidis* strains in this ITU setting. In this context horizontal transfer of resistance genes between *S. epidermidis* and *S. aureus* can be postulated in view of recombinase and IS sequences flanking determinants for reduced susceptibility to chlorhexidine, triclosan and mupirocin. In addition the high prevalence of *qac* in multidrug resistant strains is a concern for selection of multidrug resistant strains by chlorhexidine as previously reported for *S. aureus* (10).

5. CONCLUSIONS

In conjunction with our previous study, we report a negligible presence of *qacA* in *S. aureus* strains from screening samples and bacteraemia patients over an exceptionally long period of time. The new finding of a high proportion of *qac* gene carriage in *S. epidermidis* could however be of concern to all intensive care settings where chlorhexidine is used for universal decolonisation and prevention of bacteraemia. While levels of decreased susceptibility to chlorhexidine associated with *qac* gene carriage in *S. epidermidis* were stable with no evidence of increasing resistance levels over the study period, most *qac* positive *S. epidermidis* strains belonged to a single multidrug resistance sequence type. This raises concerns in relation to potential multidrug resistant strain selection by chlorhexidine. Larger scale prospective studies will determine the clinical relevance of these findings.

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CONFLICTS OF INTERESTS

None to declare

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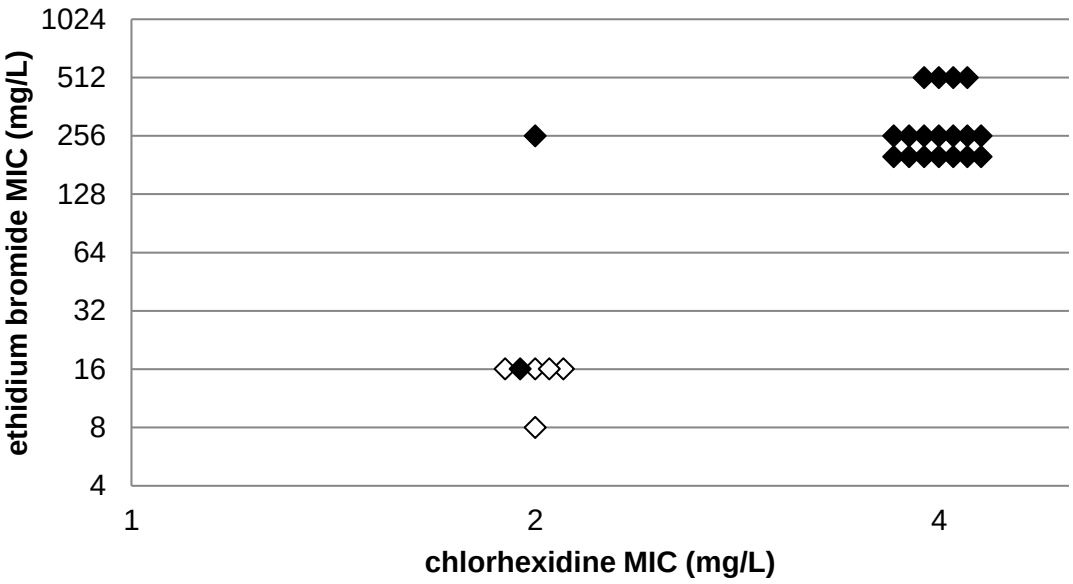
FIGURE LEGEND

Figure 1: Susceptibility of *S. epidermidis* isolates to chlorhexidine. Chlorhexidine MICs are shown in relation to the MICs for ethidium bromide. Each of the twenty-five strains tested is represented by a symbol. Solid symbols indicate strains carrying *qac* genes and open symbols indicate *qac* negative strains. The MIC values for strain STAPH77 carrying a truncated *qac* are also represented by a solid symbol (chlorhexidine MIC = 2 mg/L, ethidium bromide MIC = 16 mg/L).

377 **FIGURE 1**



378

Table 1: Genetic determinants for reduced biocide susceptibility in *S. epidermidis* isolates.

Isolate ID	Date	Sequence type (ST)	Biocide susceptibility genes		
			Chlorhexidine[a]	Mupirocin[b]	Triclosan[c]
STAPH48	Jul-09	ST-559	*qacA*	*ileS* (V588F)	*sh-fabI*
STAPH49	Jul-09	ST-2	*qacA*	*ileS* (V588F)	
STAPH51	May-10	ST-83	*qacAB*	*ileS2*	*fabI* (F204L), *sh-fabI*
STAPH53	May-10	ST-5	*qacAB*	*ileS2*	*sh-fabI*
STAPH54	Jul-10	ST-5	*qacA*		
STAPH56	Aug-10	ST-2	*qacA*	*ileS2*	*sh-fabI*
STAPH58	Mar-11	ST-2	*qacA*		*sh-fabI*
STAPH59	Apr-11	ST-83	*qacAB*	*ileS2*	*fabI* (F204L)
STAPH60	Aug-11	ST-2	*qacA*		
STAPH61	Sep-11	ST-2	*qacA*		
STAPH62	Sep-11	ST-2	*qacA*		
STAPH63	Sep-11	ST-2	*qacA*	*ileS* (V588F)	
STAPH64	Oct-11	ST-2	*qacA*	*ileS* (V588F)	
STAPH66	Apr-12	ST-19			
STAPH67	Jul-12	ST-210			*sh-fabI*
STAPH68	Jul-12	ST-54			
STAPH69	Sep-12	ST-2	*qacA*	*ileS2*	
STAPH70	Dec-12	ST-2	*qacA*		*sh-fabI*
STAPH73	Jan-13	ST-204			
STAPH74	Jan-13	new			
STAPH75	Jan-13	ST-2	*qacA*	*ileS* (V588F)	
STAPH77	Jun-13	ST-59	*qacA* (fragment)[d]		*sh-fabI*
STAPH78	Jul-13	ST-2	*qacA*		
STAPH79	Sep-13	ST-48	*qacA*		
STAPH83	Feb-14	ST-2	*qacA*		

[a] *qac* gene sequences containing 3 nucleotide changes found in *qacB* are indicated as *qacAB*

[b] *ileS* gene mutation or presence of the added gene *ileS2* conferring reduced susceptibility to mupirocin.

[c] *fabI* gene mutation or presence of the added gene *sh-fabI* conferring reduced susceptibility to triclosan.

[d] Truncated *qacA* sequence (only 3' 450bp fragment).

Table 2: Polymorphic sites of the *qac* gene nucleotide sequences and respective amino acid substitutions.

	Isolates	* 11 3444558913 78579567636 64509121890	Sequence type (ST)	Amino acid substitutions
qacA	GenBank GU565967.1	GCCCTCGGATT		
qacA	48,54,56,58,69,70,71,75,78,79,83	.T.........	ST-2, ST-5, ST-48, ST-559	
qacA	49,60,61,62,63,64	.T.G.......	ST-2	A157G
qacAB	51,59	.TT..TAA.C.	ST-83	A151V, A184V, V188I, A290T, M379T
qacAB	53	.TT..T.A.C.	ST-5	A151V, A184V, A290T, M379T
qacB	GenBank AF053772.1	A.T.A..ACC.		V025I, A151V, L166I, A290T, D322A, M379T

* positions of nucleotide polymorphisms with respect to *qacA* are written vertically