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Tolerance of MRSA ST239-TW to chlorhexidine-based decolonization: Evidence for keratinocyte invasion as a mechanism of biocide evasion

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SUMMARY

Objectives: Information on genetic determinants of chlorhexidine tolerance (*qacA* carriage and MIC) *in vitro* is available, although evidence of the clinical impact and mechanisms remain poorly understood. We investigated why, following chlorhexidine intervention, prevalent epidemic MRSA ST22 and ST36 clones declined at an ICU, whilst an ST239-TW clone did not. The chlorhexidine tolerant ST239-TW phenotypes were assessed for their protein binding, cell adhesion and intracellular uptake potential.

Methods: Six ST22, ST36 and ST239-TW bloodstream infection isolates with comparable chlorhexidine MICs were selected from a 2-year outbreak in an ICU at Guy's and St. Thomas' Hospital. Isolates were tested for fibrinogen and fibronectin binding, and adhesion/internalization into human keratinocytes with and without biocide.

Results: Binding to fibrinogen and fibronectin, adhesion and intracellular uptake within keratinocytes ($P < 0.001$) and intracellular survival in keratinocytes under chlorhexidine pressure (ST22 3.18%, ST36 4.57% vs ST239-TW 12.79%; $P < 0.0001$) was consistently higher for ST239-TW.

Conclusions: We present evidence that MRSA clones with similarly low *in vitro* tolerance to chlorhexidine exhibit different *in vivo* susceptibilities. The phenomenon of *S. aureus* adhesion and intracellular uptake into keratinocytes could therefore be regarded as an additional mechanism of chlorhexidine tolerance, enabling MRSA to evade infection control measures.

Introduction

Over the past 20 years there have been two dominant pandemic healthcare-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) clones in the UK, EMRSA–15 (ST22) and EMRSA-16 (ST36), with lower prevalence of other clones (ST8, ST239, ST5 and ST247). HA-MRSA rates have decreased significantly since the mid-2000s[1–4] following the introduction of a comprehensive UK-wide infection control program comprising re-enforcement of basic infection control practices and targeted measures of screening, contact precautions, patient isolation and decolonization.[4–7] We previously reported an approximate 70% reduction in transmission of endemic MRSA clones following introduction of chlorhexidine-based universal decolonization into the intensive care unit (ICU) at Guy's and Thomas' Hospital (GSTT), London in 2004.[4] This is consistent with a number of studies reporting reductions in MRSA transmission, bacteremia and colonization following introduction of universal chlorhexidine skin washes.[8–10]

Introduction of heightened infection control measures also uncovered a hidden two-year outbreak due to an ST239 clone (ST239-TW) which probably originated from Singapore and is one of five major ST239 clades (the Thai clade).[11] While transmission of ST22 and ST36 clones decreased following the introduction of decolonization with chlorhexidine on the GSTT ICU, the transmission of ST239-TW did not.[4,8,12–14]

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We previously reported that ST239-TW had an increase in chlorhexidine MBC compared with non-TW MRSA isolates (ST22 and ST36), based on analysis of five skin isolates identified during the outbreak.[8] MRSA blood stream infection (BSI) isolates from the same outbreak were analyzed for their CHG (chlorhexidine digluconate) MIC in 2013, reportedly clustering into MICs groups of < 2 mg/l and ≥ 2 mg/l.[12] ST239-TWs isolated during that outbreak all carry the plasmid borne *qacA* gene, which encodes a major facilitator superfamily efflux-pump that expels quaternary ammonium compounds and biguanides such as chlorhexidine, and has been associated with biocide tolerance. Carriage of *qacA* currently represents the accepted explanation for the lack of clinical effect of chlorhexidine on Staphylococci.[15–17] However, small increases in staphylococcal MICs or MBCs have been reported elsewhere, for which a correlation with *qacA/B* carriage was found to be equivocal, or otherwise associated with a modest one dilution increase in MIC, but with no effect on the MBC.[18–22] Given that there is no internationally endorsed standard for assessing biocide MICs and MBCs, except for the CLSI guideline (biocide activity measurement)[21] and no epidemiological breakpoints for the resistance of the test organism (BSAC, EUCAST or CLSI), the significance of these increases is unclear; particularly since surface acting biocides are typically applied in much higher concentrations than their *in vitro* MIC or MBC.[23–25] Following the 2009 report on Antibiotic Resistance Effects of Biocides by the Scientific Committee on Emerging and Newly Identified Health Risks[26] Morrissey et al. established in 2014 the first epidemiological breakpoints characterizing *S. aureus* chlorhexidine resistance, setting the chlorhexidine MIC ECOFF at 8 mg/ml and the MBC ECOFF at > 64 mg/ml.[27] Despite working on a similar size *S. aureus* strain collection, Furi et al. were not able to set such breakpoints.[21]

One potential alternative survival mechanism to antimicrobials in bacteria is that of evading topical biocide action by invading human epithelial cells and hiding in an intracellular location. Indeed, staphylococcal adhesion, invasion and persistence has been observed in multiple human cell types (by professional and non-professional phagocytosis) and has been demonstrated as a means of evading the host innate immunity, also fostering deeper tissue and systemic infections, in addition to being postulated as a mode of surviving antibiotic action.[28–33]

S. aureus adhesion and internalization to human endothelial and epithelial cells via binding to fibrinogen and fibronectin has been widely studied,[34–38] and has to be included when investigating bacterial invasion into host cells. The fact that blood stream infection (BSI) isolates of ST239-TW with moderate chlorhexidine susceptibilities can persist after topical decolonization and thus outlive other clones requires detailed examination.[4,8,13,38] We therefore sought to investigate whether ST239-TW isolates present with a biocide evasion strategy, based on a dominant keratinocyte adhesion and internalization phenotype and if this a plausible alternative mechanism of decolonization tolerance over other MRSA clones.

Methods

Bacterial strains and growth conditions

A representative collection of six ST36 (three *qacA*$^+$and three *qacA*$^-$), six ST22 (three *qacA*$^+$and three *qacA*$^-$) and six ST239-TW isolates (all *qacA*$^+$; no *qacA*$^-$ strain initially isolated) were selected from a previously published strain set of 136 MRSA bloodstream infection isolates (BSI), from GSTT ICU patients between 2001 and 2009 (Supplementary Material Tables S1-3).[12] The strains were chosen based on their previously established, comparable and highly susceptible CHG MIC result (all < 2 mg/l; Supplementary Material Table S4).

BSI strain characteristics are stated in the Supplementary Material (Tables S1–3).

S. aureus NCTC 10788, a standard strain for chemical disinfection testing, was obtained from Public Health England and used as a quality control strain in CHG susceptibility testing.

In vitro *chlorhexidine digluconate susceptibility testing*

MICs were determined using a modified Clinical and Laboratory Standard Institute (CLSI) micro-dilution method,[40] with adaptions described by Longtin et al. (2011).[41] A 2-fold serial dilution was prepared from a 20% (w/v) chlorhexidine digluconate stock solution (CHG) (Sigma-Aldrich, Ltd. UK) and final concentrations of 64–0.125 mg/l were tested against 5×10^5 CFU/ml bacteria suspensions.

Bacterial binding to human immobilized fibrinogen and fibronectin

The adherence assay was performed using 10 µg/ml human fibrinogen or fibronectin (Merck Chemicals Ltd, UK). Aliquots of 100 µL stationary or exponential phase bacterial cultures were added to protein coated wells and incubated for 2 h at 37°C. Bound bacteria were fixed with 25% formaldehyde and then stained with 0.1% crystal violet. Absorbance was measured at 570 nm (SpectraMax 190 Absorbance Microplate Reader, Molecular Devices) and readings corrected for background measurement with a bacteria-free control. *S. aureus* 8325-4 (fibronectin binding) or *S. aureus* Newman (fibrinogen binding) were included as binding controls.[42]

Cell culture adhesion and internalization assay

HaCaT cell line maintenance: The immortalized human keratinocyte cell line (HaCaT) was maintained at 37 °C (5% CO_2 atmospheric pressure) in T75 or T175 flasks with DMEM medium (Lonza Ltd., Belgium), supplemented with 10% foetal calf serum (FCS) (First Link Ltd., UK) and 1% penicillin-streptomycin, 50 IU/ml (Life Technologies Inc., UK).

Combined bacterial adhesion and invasion assay

Bacterial adhesion and invasion of human epithelial cells was assayed as previously described[34,37,43] with the following modifications: Briefly, HaCaT cells (4.5×10^4 cells/ml) were seeded onto circular glass coverslips (13 mm diameter, VWR 632-0150) in 24-well tissue culture plates containing DMEM/10% FCS and 1% penicillin-streptomycin and grown to 80–90% confluency. Bacterial cultures were grown at 37 °C in Brain Heart Infusion (BHI) broth to stationary phase, centrifuged, washed and re-suspended in DMEM/10% FCS without antibiotics. A 1×10^6 CFU/ml bacterial aliquot was added to each well of confluent HaCaT cells, incubated for 90 min at 37 °C (5% CO_2), after which cells were carefully washed three times with PBS to remove non-adherend bacteria. The bacterial input CFU was checked for accuracy by serial dilution and plating onto BHI agar and colony counting after overnight incubation. For the combined adhesion and internalization assays, 0.5% trypsin (Worthington Biochemical Corp.) was added for 10 min at 37 °C, to detach cells, and then neutralized with 1 ml DMEM/10% FCS. Cell suspensions were centrifuged (SIGMA 1-14 Microfuge, SciQuip Ltd.) at 14.800 rpm for 20 min and the mixed HaCaT/bacteria pellet resuspended in de-ionized water for 30 min to lyse the eukaryotic cells. Released bacteria were enumerated after overnight growth at 37 °C on BHI agar to establish the proportion of both bound and internalized bacteria combined as CFU/ml.

Bacterial invasion assay: The internalization assays were performed as described for the combined adhesion and uptake assay,

only that after the 90 minute co-incubation of cells and bacteria and the three PBS washes, extracellular adhered and non-adhered bacteria were killed by adding 50 µg/ml lysostaphin in DMEM/10% FCS for 1 h at 37 °C. Externally killed bacteria were washed off using PBS. Detachment of HaCaT cells from the coverslip, cell lysis and bacterial enumeration of internalized bacteria was performed as described above. The HaCat cell viability, when incubated with 50 µg/ml lysostaphin for 1 h and also the bacterial killing efficiency with 50 µg/ml lysostaphin was confirmed using the non-toxic *alamarBlue*® assay (Supplementary Material Fig. S3 and S4).

Bacterial adhesion assay: Bacterial adhesion was calculated by subtracting CFUs/ml obtained from the internalization assay from CFUs/ml obtained from the combined adhesion and internalization assay. The final percentage of adhesion and uptake was calculated in relation to the total CFU input for each bacterial strain.

Chlorhexidine (CHG) killing efficiency

The combined adhesion and internalization assay was performed as described above, to test for superficial CHG efficacy on a keratinocyte-MRSA co-culture. No differentiation between internalized and adhered bacteria was made. After washing off extracellular non-adhered bacteria, CHG was added to the Staphylococci/HaCaT cell co-culture at a final concentration of 6 mg/l and incubated at 37 °C for 1 h, followed by a final PBS wash to remove the CHG solution and non-adhered bacteria. Intracellular bacteria killed by CHG could not be eliminated at this point. Lysostaphin was not applied in this assay, as the CHG activity was investigated on adhered and internalized bacteria combined. The HaCat cell viability, when incubated with 6 mg/ml CHG, was confirmed using the non-toxic *alamarBlue*® assay (Supplementary Material, Fig. S2).

Detachment of HaCaT cells from the coverslip, cell lysis and bacterial enumeration of internalized and adhered bacteria was performed as described above. The final percentage of both, adhered and internalized bacteria surviving the CHG treatment was assessed in relation to the internalization and adhesion CFU result where no CHG was added, thus for each individual bacterial strain. The results for all strains belonging to the same clone were averaged and shown as the percentage CHG survival, representing the CHG efficacy.

Electron microscopy – visualization of adhered and internalized ST239-TW

Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) were performed on 4.5×10^4 HaCaT cells/ml grown to 80–90% confluency on Nunc™ Thermanox™ coverslips (Thermo Fisher Scientific Inc.) in a 24-well tissue culture plate at 37 °C in 5% CO_2. Eukaryotic cells were subsequently incubated with stationary phase grown ST239-TW (1×10^6 CFU/ml) for 90 min under the same growth conditions. HaCaT cell monolayers were washed three times with PBS to remove non-adhered bacteria, not followed by lysostaphin treatment, to allow visualization of adhered as well as internalized bacteria.

For TEM, the epithelial monolayers were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C for 4 h. After rinsing thoroughly with cacodylate buffer, samples were post-fixed with 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) for 1 h at 4°C. Samples were then dehydrated through a graded ethanol series, equilibrated with propylene oxide before infiltration with TAAB epoxy resin and polymerised at 70°C for 24 h. Ultrathin sections (50–70 nm) were prepared using a Reichert-Jung Ultracut E ultramicrotome, mounted on 150 mesh copper grids and contrasted using uranyl acetate and lead citrate. Samples were examined on an FEI Tecnai 12 transmission microscope operated at 120 kV and images were acquired with an AMT 16000M camera.

For SEM, the HaCaT cell monolayers were fixed with 4% (v/v) paraformaldehyde. Before further processing, the samples were placed into 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) and incubated for 24 h. Finally, the samples were dehydrated for 10 minutes each in 20, 50, 70 and 90% ethanol, followed by 30 min incubation in 100% ethanol and a final drying step using hexamethyldisilazane. The samples were mounted onto stubs with a conducting carbon cement applied between, coated with gold and the images assessed with an FEI Quanta 3D FEG scanning electron microscope.

Statistical methods

All experiments were performed on six ST36, six ST22 and six ST239-TW isolates, where each isolate was tested in triplicates on at least two independent occasions and the results shown as the average derived from all strains per clonal group. Statistical analysis was performed using GraphPad Prism 6.0 software. The two tailed non-parametric *t* test (Mann–Whitney ranked sum test) was applied to find differences between ST239-TW to ST22 and ST36 and the control strains, as well as *qacA*$^+$and *qacA*$^-$ strains (MICs). Minimum statistical significance was set to $P < 0.05$.

Results

Chlorhexidine (CHG) MICs

Six *qacA*$^+$ ST239-TW, three *qacA*$^+$ and three *qacA*$^-$ ST36 and ST22 MRSA BSI isolates with comparable low MICs (Supplementary Material Table S4) were randomly selected from an initial 136 isolate cohort[12] and multiple replicate CHG testing performed to verify their initially established MICs (Table 1). Re-testing showed only minor differences in the clones' CHG susceptibilities compared to 2013 (Supplementary Material Table S4). The highest change in MIC was found for the ST22 clones, showing an increase of 45% compared to 2013. MICs of *qacA*$^+$ and *qacA*$^-$ ST36 and ST22 clones (1.75 mg/l ± 0.05 vs 1.47 mg/l ± 0.08, $P = 0.29$) were similar. The fact that all ST239-TW isolates from the initial cohort were *qacA*$^+$ needs to be noted. These results confirm the 2013 established low MICs and imply that *qacA* carriage alone cannot explain the *in vivo* chlorhexidine survival of ST239-TW at the intensive care unit (ICU) of Guy's and Thomas' Hospital. So, we investigated cell adhesion and uptake as an additional explanation for their survival under topically applied chlorhexidine pressure in a clinical setting.

Protein binding assay

Binding of MRSA clones to fibrinogen and fibronectin at stationary and exponential growth phases was first investigated (Fig. 1). In general, protein binding was either equal or higher at stationary than at exponential bacterial growth phase. There were however clear differences between protein binding of the clonal groups in each growth phase. ST239-TW binding to both fibrinogen (OD570nm absorption: stationary 0.192; exponential 0.165) and fibronectin (stationary 0.350; exponential 0.172) was significantly higher than for ST22, ST36 and the control strains ($P < 0.001$) at exponential and stationary growth phases, with the exception of ST36 at exponential growth (fibronectin 0.077, $P = 0.195$; fibrinogen 0.089, 0.175, $P = 0.211$).

Adhesion to and internalization into human keratinocytes

Adherence and internalization of MRSA clones was assessed *in vitro* using an immortalized keratinocyte cell line (HaCaT) (Fig. 2).

Table 1
Chlorhexidine (CHG) MICs for selected representative EMRSA BSI isolates.

Clone	Isolates (n)	MIC mean [mg/l] ± SEM	MIC range [mg/l]
ST239-TW	6	1.29 ± 0.18	1.0–1.5
ST22	6	2.68 ± 0.53	1.9–3.3
ST36	6	1.61 ± 0.18	1.4–1.8
S. aureus 8325-4	1	0.75 ± 0.25	0.1–1.0
S. aureus Newman	1	1.13 ± 0.13	1.0–2.0
S. aureus NCTC 10788	1	0.97 ± 0.18	0.3–1.0

Abbreviations: MIC, minimum inhibitory concentration; EMRSA, epidemic methicillin-resistant *Staphylococcus aureus*; SEM, standard error of the mean.
Note: Results are the means of ≥ 3 independent experiments of each individual isolate, tested in triplicate.

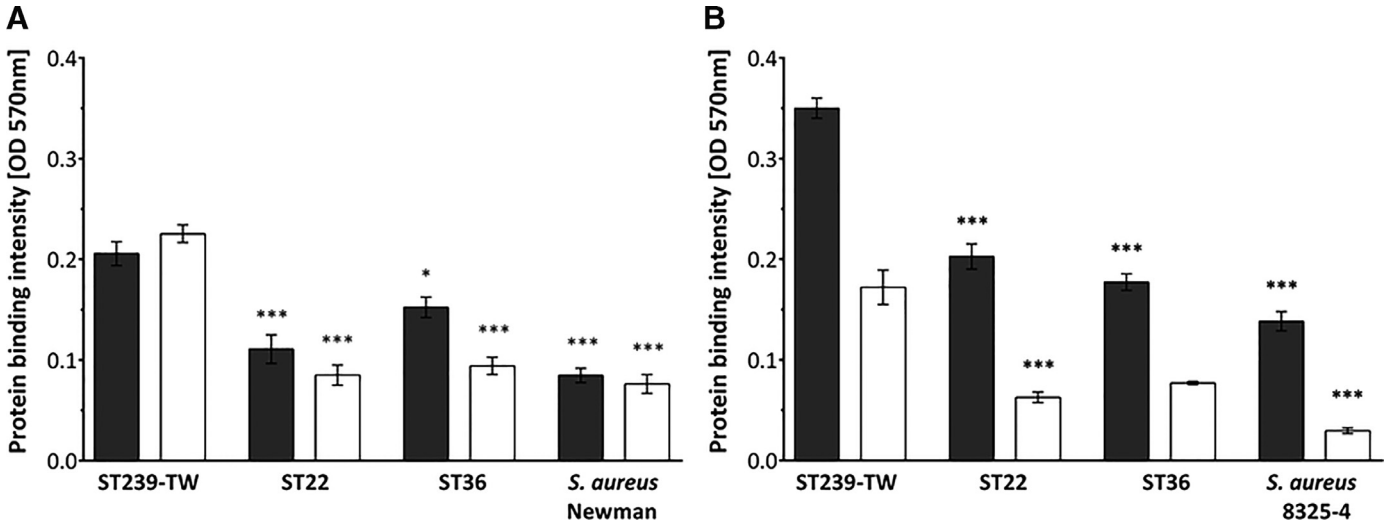

Fig. 1. Bacterial binding to immobilized human fibrinogen (A) and fibronectin (B) at stationary (filled bars) exponential (open bars) growth phases. Bars show the results for ≥ 3 independent experiments performed in triplicate (*S. aureus* Newman, *S. aureus* 8325-4, six ST36, six ST22 and six ST239-TW isolates) with the standard error of the mean for each clonal group. $^{*}P < 0.05$, $^{***}P < 0.001$; [Non-parametric two tailed Mann Whitney U test compared to ST239-TW].

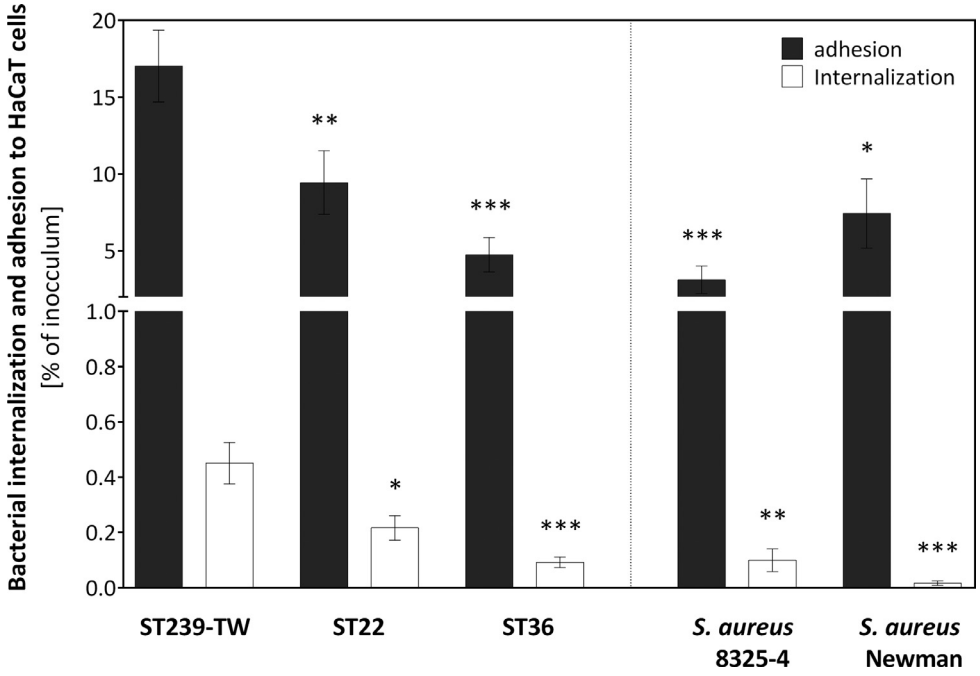

Fig. 2. Adherence (filled bars) and internalization (open bars) of MRSA clones to HaCaT cells. Results are shown for ≥ 3 independent experiments performed in triplicate (*S. aureus* Newman, *S. aureus* 8325-4, six ST36, six ST22 and six ST239-TW isolates) with the standard error of the mean for each clonal group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; [Non-parametric two tailed Mann Whitney U test compared to ST239-TW].

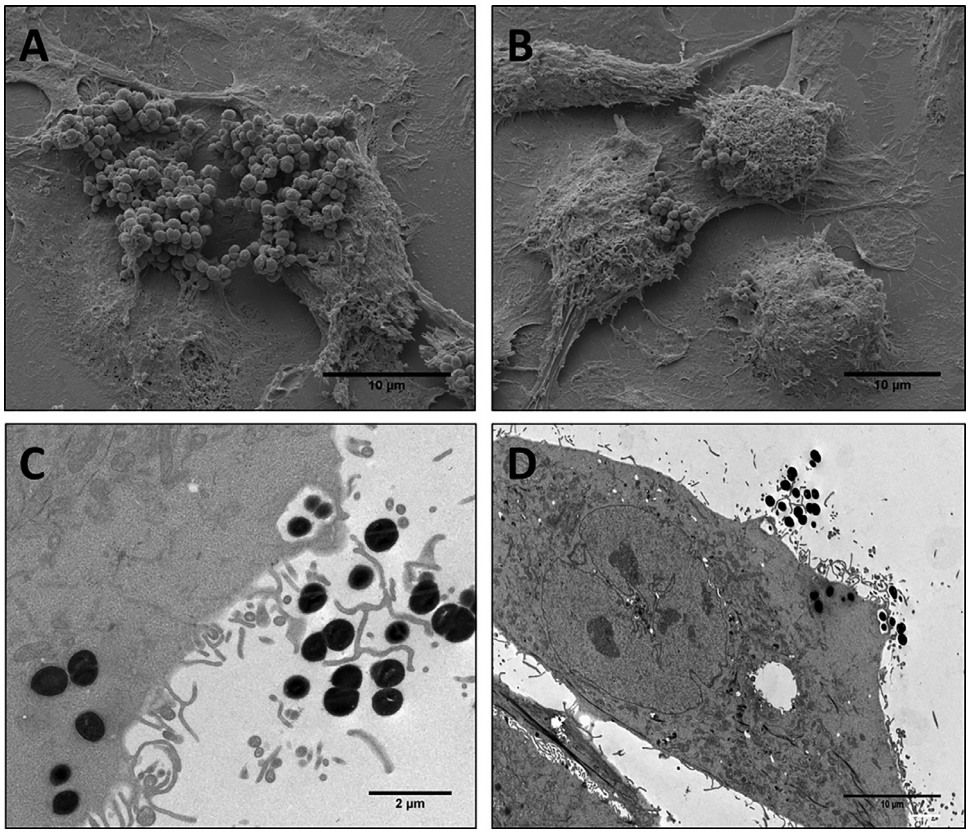

Fig. 3. Electron micrographs of HaCaT cells infected with MRSA ST239-TW strain (TW20)[59]. SEM (A, B) of adhered ST239-TW and TEM (C, D) of surrounding, and internalized ST239-TW, either presenting in vacuoles or embedded in the cell cytoplasm. SEM shows the top view and TEM a cross section of co-incubated HaCaT cells with ST239-TW (TW20) bacteria for one hour and a three time wash with PBS.

Here, only the adhesion and invasion propensities between the three clones was investigated, and not their survival or persistence, to provide preliminary data for further CHG effectiveness testing on *S. aureus* bound and internalized to HaCaT cells. The co-culture incubation time was set to 90 min only, to minimize HaCaT cell degradation and apoptotic processes.

Electron micrographs of HaCaT cells inoculated with the representative isolate ST239-TW (TW20), confirmed bacterial adhesion and internalization (Fig. 3) to HaCaT cells. TW20 bacteria were found on the keratinocyte surface (SEM) and during different stages of internalization (TEM – cross section of co-cultured bacteria with HaCaT cells), either in the process of binding, inside the epithelial cells enclosed within vacuoles, or appearing in the cell cytosol. No quantitative assessment was made.

The percentage of inoculated ST239-TW, then binding to HaCaT cells (16.39%) was significantly greater than for ST22 (9.44%; $P = 0.0021$) and ST36 (4.73%; $P < 0.0001$). Also, with 0.41%, a greater proportion of inoculated ST239-TW bacteria internalized into keratinocytes than ST22 (0.22%; $P = 0.0126$) and ST36 (0.09%; $P < 0.0001$).

Chlorhexidine (CHG) efficacy on co-adhered and internalized MRSA to keratinocytes

CHG at 6 mg/l, which is more than twice the MIC of all tested MRSA clones (Table 1), resulted in 100% killing of bacteria without HaCaT cells (data not shown) and had only minimal cytotoxic effect on the HaCaT cell viability over 4 h of incubation when tested without bacteria (Supplementary Material Fig. S2).

CHG treatment, of infected (bound and internalized bacteria) monolayers showed about a three-fold greater recovery of ST239-TW (12.79% survival; $P < 0.001$) compared with ST22 (3.18%) and ST36 (4.57%), but all three clinical clones were less likely to be killed by CHG than the control strains *S. aureus* Newman and *S. aureus* 8325-4 (< 0.7%; $P < 0.0001$) (Table 2).

Table 2
Killing effect of chlorhexidine (CHG) on adherent and internalized MRSA clones co-cultures with HaCaT cells.

Clone	% bacterial survival	*P*	95% CI
ST239-TW	12.79 ± 1.08		10.34–14.02
ST22	3.18 ± 0.76	***	1.44–4.15
ST36	4.57 ± 1.68	***	1.19–5.20
S. aureus Newman	0.13 ± 0.03	***	0.07–0.19
S. aureus 8325-4	0.66 ± 0.26	***	0.06–1.25

Note: Results of six ST36, six ST22 and six ST239-TW isolates are for ≥ 2 independent experiments performed in triplicate and are presented as the mean and standard error of the mean percentage recovery for each clone; ***$P < 0.001$; [non-parametric two tailed Mann Whitney U test compared to ST239-TW].

Discussion

The widespread use of biocides for decolonization has become an important infection control intervention to decrease transmission of nosocomial pathogens, but has also raised concerns about the development of bacterial biocide tolerance.[13,16,39,44,45] Here we show evidence that bacterial uptake into skin cells together with extracellular adherence can protect some MRSA clones against biocide action. This provides a putative mechanism whereby ST239-TW was able to resist chlorhexidine when introduced on the ICU of GSTT, in the absence of a measurable difference in MIC. Although it had previously been suggested that *qacA* and *B* carriage

confers chlorhexidine tolerance,[15–17] our results can only partially support this. However, any effects of subtle increases of CHG MIC on ST239-TW internalized and adhered to keratinocytes were not evaluated and further, no distinction between *qacA*$^+$ *and qacA*$^-$ isolates was possible. An alternative mechanism suggested by our data is that ST239-TW has a selective advantage compared to ST22 and ST36 clones during topical chlorhexidine treatment due to its enhanced ability to bind and invade skin cells. A further possibility, though not investigated here, is the correlation of *qacA/B* carriage with an *agr* (quorum-sensing operon) dysfunction, recently described by Cho *et al.*, suggesting a selection advantage of ST239 clones over others in an ICU with common chlorhexidine usage.[46]

Staphylococcal adhesion, invasion and persistence has been observed in multiple human cell types and has been postulated as a means of evading the host innate immunity and antibiotic action, leading to either recurrent or chronic infections.[28–32] However, the preliminary findings presented here support an additional hypothesis that by invading and adhering to skin cells, *S. aureus* (ST239-TW) is also protected against the important infection control intervention of chlorhexidine decolonization in healthcare settings. Binding to fibrinogen and fibronectin are key steps in staphylococcal adhesion and invasion, and thus are of relevance in disease pathogenesis.[34–37] We found that compared with ST22 and ST36, ST239-TW showed increased binding to fibrinogen and fibronectin, and increased adherence and internalization into keratinocyte monolayers. This supports the clinical phenomenon of *in vivo* chlorhexidine survival. However, we must note that common experimental investigations for *S. aureus* and eukaryotic cell interactions *in vitro* perform co-incubation for around one hour,[34,37,43] which does not fully represent a long-term clinical exposure to MRSA, but certainly allows for the identification of differences between MRSA clones, whilst minimizing cell apoptotic side effects.[43,47] The previous finding that some ST239 clones exhibit increased colonization potential to human nasal epithelial cells due to carriage of *sasX*, a mobile genetic element–encoded gene[48] also carried by ST239-TW[49] but not tested in this study, provides supporting evidence that host cell linked CHG survival, as also shown elsewhere[28–31] is an important virulence mechanism of this clone.

Persistent skin colonization during antiseptic decolonization is a concern not only for patients on ICUs. It could enable colonization of catheters leading to bacteremia[50] and colonization of healthcare workers' hands facilitating transmission to other patients.[14] Chlorhexidine is used extensively in hospitals for skin cleansing prior to invasive procedures and to remove antimicrobial resistant bacteria to prevent transmission or infection.[51–53] However, skin traumas which are often present in ICU patients due to surgery, intubation or intravenous catheter insertion, allow the exposure of extracellular matrix proteins on the epithelial surface, thus presenting excellent initial binding conditions for MRSA isolates. Indeed, the ST239-TW MRSA strains tested in this study showed elevated survival to CHG *in vitro* when adhered and internalized to keratinocyte monolayers. Similarly Lehar *et al.* showed that a minority of MRSA can actually survive in macrophages, even under antibiotic pressure, and retain their ability to establish infections.[32]

Recent clinical studies have observed high rates of *qacA* carriage by Staphylococci including those isolated from healthcare workers, and in some studies that has been linked with extensive chlorhexidine use.[54,55] Connecting multi-drug efflux pumps such as the one encoded by *qacA* to a decreased staphylococcal susceptibility against cationic antiseptic agents (e.g. chlorhexidine) seems to be reasonable, but remains controversial.[15,16,20,21,45] In contrast with previous findings, showing a two- to four-fold increase in chlorhexidine MBC of five tested *qacA*$^+$ ST239-TW skin isolates,[13] no conclusion could be drawn from our ST239-TW BSI isolates, since all strains specifically selected, had a moderate MIC below 2 mg/l, despite being *qacA*$^+$. It is possible that the elevated MBC phenotype from skin isolates analyzed shortly after the outbreak at GSTT may have been lost after storage. Conversely, serial passaging of six ST239-TW isolates whilst exposed to sub-inhibitory CHG concentrations *in vitro* did not increase their MICs (Supplementary Material Fig. S1). Consistent with other studies, we showed that biocide activity is not, or only partially affected by the presence of a *S. aureus* efflux pump.[18,19,21] Nevertheless, we cannot exclude that *qacA* carriage plays a role in the *in vivo* ST239-TW chlorhexidine tolerance phenotype.[13,50] It remains possible that unknown factors are required to demonstrate the chlorhexidine resistance phenotype of *qacA*$^+$ strains in the laboratory.

The findings presented here provide evidence to support the hypothesis that the clinical chlorhexidine phenotype (*in vivo* decolonization tolerance) of some MRSA clones may be due to adhesion and intracellular invasion of keratinocytes. However, further experiments will be required, with prolonged chlorhexidine exposure and antibiotic co-treatment in primary cell cultures and skin models[56,57] to examine the effects of keratinocyte antimicrobial peptides,[58] and to extend our findings by identifying the bacterial factors involved in protection against chlorhexidine and additionally investigate the consequences of intracellular survival on the host cells. Also, the relative importance of adhesion versus internalization and whether this phenomenon is associated with other clones with a similar *in vivo* phenotype needs to be established. Although our findings need further investigation, we feel that there is compelling evidence that the host cell invasion mechanism employed by MRSA strains to evade innate immunity also confers tolerance to infection control interventions, which is of high clinical importance.

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Conflicts of interest

Jonathan A. Otter is a consultant to Gama Healthcare Ltd., and Pfizer Ltd. All other authors have no conflicts to declare.

Supplementary materials

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