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MICROBIOLOGY

Phenotypic chlorhexidine and triclosan susceptibility in clinical *Staphylococcus aureus* isolates in Australia

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Summary

Antiseptics such as chlorhexidine gluconate and triclosan are widely used in healthcare settings for both skin antisepsis and decolonisation of *Staphylococcus aureus*. We determined the minimum inhibitory concentration (MIC) of 198 methicillin susceptible and resistant *Staphylococcus aureus* clinical isolates to both chlorhexidine and triclosan using an agar dilution method. Of these, 10% (19/198) showed a raised MIC to chlorhexidine and 3% (6/198) showed an elevated MIC to triclosan. The multilocus sequence type (MLST) of each isolate was predicted using a binary method, and although ST93-MRSA-IV was the most common, ST22-MRSA-IV was shown to have statistically higher chlorhexidine MIC values compared with non ST22-MRSA-IV isolates ($z = -8.7$, $p < 0.01$). Additionally, isolates from patients known to have failed decolonisation were included and did not demonstrate elevated MIC to the decolonisation antiseptic. Monitoring for non-susceptibility of clinical isolates to biocides is important to determine trends, and may have clinical implications in terms of sub-lethal concentration in residues and concomitant antibiotic resistance.

Key words: Antiseptic resistance; chlorhexidine; triclosan; *Staphylococcus aureus*.

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INTRODUCTION

Staphylococcus aureus infection presents a significant contribution to morbidity and mortality both in hospital and in the community.[1] Measures including disinfection and antisepsis are the cornerstone of infection prevention in hospital, and biocidal agents are increasingly being used in the domestic environment as well.[2–4] Patients who are persistently colonised with *S. aureus* and whom have repeated infections may also undergo decolonisation or pre-procedure staphylococcal load reduction regimens, commonly with nasal mupirocin and antiseptic washes or antiseptic impregnated single-use wipes with agents such as chlorhexidine gluconate or triclosan.

Biocides differ from antibiotics in that they act on a multitude of cellular targets and often have differing effects at low and high concentrations.[3,5] Their in-use concentrations usually greatly exceed the concentration required to kill microorganisms. However, biocide tolerance and its possible cross resistance or co-resistance to antibiotics has been of theoretical concern for some time,[2,6–9] and can be due to either intrinsic or acquired mechanisms.[8]

Chlorhexidine gluconate is part of the biguanide family of biocides, and is commonly used as an antiseptic agent in hand rubs, skin antisepsis and others. Its mechanism of action has multiple targets, including cell membrane damage, collapse of membrane potential, leakage of intracellular contents and at high concentrations, coagulation of cytosol.[3,8,10] Decreased susceptibility to chlorhexidine is mediated by multiple resistance genes including *qacA, qacB, smr, norA, ebr* and others.[5,8,10] The most significant of these in terms of level of biocide resistance and association with antibiotic resistance is *qacA*, which encodes a multi-drug efflux pump.[11] In *S. aureus, qacA* is usually plasmid borne, and significantly is found on the pSK1 family of multi-resistant plasmids,[12] however has also been found on other transmissible plasmids, integrated into the chromosome, as well as on integrons.[3,13,14]

Triclosan belongs to the bisphenol class of biocides used in many indications including antisepsis (hand washes, soaps), and many domestic products such as plastics, pillows, cosmetics, dental products and fabrics.[15] At high concentrations it has multiple cytoplasmic and membrane targets,[16] but at low concentrations its mechanism of action primarily involves blockage of lipid synthesis through inhibition of enoyl-acyl carrier protein (ACP) reductase enzyme.[17] Decreased susceptibility in *S. aureus* is mediated by either overexpression or mutation of the gene *fabI*[17–19] primarily, though other mechanisms have been implicated.[19–21]

Pathology North services the Hunter area region, which includes one 800 bed tertiary hospital, as well as several regional hospitals and private practices. During 2013, methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from any specimen site were stored, as well as methicillin-susceptible *Staphylococcus aureus* (MSSA) isolated from sterile sites (blood culture, joint aspirate, pleural fluid, etc). The local health district uses chlorhexidine in hand washes and surgical scrubs, as well as for skin antisepsis. The district decolonisation regimen recommends triclosan washes with mupirocin nasal ointment. We wanted to detect the prevalence of phenotypic tolerance to both biocides in clinical *S. aureus* isolates, including isolates from patients who have had repeated isolation of MRSA after decolonisation (failed decolonisation). We also compared the prevalence of raised minimum inhibitory concentration (MIC) to chlorhexidine and/or triclosan in MRSA compared with MSSA, and whether raised MIC was related to clonal spread of MRSA or present in multiple different MRSA clones.

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METHODS

Isolates, growth conditions, and antibiotic susceptibility testing

Over a 12 month period in 2013, 1244 clinical isolates of *Staphylococcus aureus* were cultured and stored. Using Microsoft Excel (Microsoft, USA), from this group 188 isolates of *Staphylococcus aureus* (151 MRSA and 37 MSSA) were randomly selected. These isolates represented a wide range of clinical samples including blood cultures, swabs, aspirates, tissues, sputa and urine, and were predominantly inpatient samples from acute wards of one tertiary hospital and eight regional hospitals. Two isolates thought to be MRSA were later found to be other organisms and discarded. In addition to this, 12 MRSA isolates from screening swabs of patients who had undergone previous attempts at decolonisation with mupirocin nasal ointment and triclosan body washes were included. Two non-clinical isolates of *S. aureus* were also sourced from the Antibiotic Resistance and Mobile Elements Group at the University of Western Sydney, both ST239-MRSA-III; one with pSK1 resistance plasmid containing *qacA*, and one isolate with *qacA* integrated into the chromosome. A control strain of *S. aureus* (ATCC 25923) was used as a control on each plate.

These isolates had previously been tested for antibiotic susceptibility to penicillin, erythromycin, flucloxacillin, ciprofloxacin, trimethoprim+sulfamethoxazole, clindamycin, mupirocin and doxycycline. MRSA strains were also tested against rifampicin, fusidic acid and vancomycin. Susceptibility testing was performed at time of initial culture using Clinical and Laboratory Standards Institute (CLSI) methods for disc diffusion on Mueller Hinton agar (interpretation by CLSI M100-S22 January 2012),[22] with the exception of vancomycin which was tested on a vancomycin-containing agar at a concentration of 6 mg/L and reported as susceptible or resistant. These results were not repeated. The isolates were cultured on horse blood agar for 24 h at 35°C aerobically to ensure purity prior to plating onto test agar.

Determination of minimum inhibitory concentration

The MIC of each *S. aureus* isolate to chlorhexidine gluconate and triclosan was determined using an agar dilution method.[23] Chlorhexidine gluconate and triclosan were obtained in their stock solution forms (Whiteley Chemicals, Australia). Agar plates were prepared, incorporating the antiseptics in concentrations from 0.06 mg/L to 256 mg/L in Mueller Hinton agar. Each *Staphylococcus aureus* isolate was adjusted to 0.5 McFarland standard (containing $1-2 \times 10^8$ CFU/mL), and the final inoculum was delivered onto the plates via a multi-point inoculator at a concentration of 10^4 CFU per spot. With 3 mm pins, this was achieved by diluting preparation at 1:10 with sterile saline to obtain a concentration of 10^7 CFU/mL. A growth control plate containing no antiseptic was initially inoculated as a control plate, followed by plates of increasing concentrations of antiseptic agents. A second growth control plate was inoculated at the end to ensure that no contamination or significant antiseptic carryover had occurred. Once dry, the plates were incubated aerobically at 35 °C for 48 h. Plates were examined at 24 and 48 h, and the MIC was taken as the lowest concentration inhibiting visible growth at 48 h. Testing was repeated on 20 isolates to ensure results were reproducible. The MICs for chlorhexidine were considered elevated if ≥4 mg/L.[24,25] The MIC for triclosan is expected to be 0.01 to 0.1 mg/L in a wild-type *S. aureus* population,[26] so reduced susceptibility was considered to occur at MIC values ≥1.0 mg/L.[27]

Comparison of MIC values for MSSA versus MRSA and ST22 MRSA versus non-ST22 MRSA were compared using a two-sample Wilcoxon rank-sum (Mann–Whitney) test as the MIC distributions were not normally distributed (Stata; StataCorp, USA).

RESULTS

Of 198 single isolates, 161 were methicillin-resistant *Staphylococcus aureus* (including 12 decolonisation samples) and 37 were susceptible to methicillin. The distribution of MIC values for chlorhexidine is shown in Fig. 1, and for triclosan in Fig. 2.

With regard to chlorhexidine, the MICs ranged from 0.5 to 4 mg/L for MSSA and from 1.0 to 8 mg/L for MRSA. The median MIC value for MSSA and MRSA was 1 mg/L ($t = 1.987$, $p = 0.48$, df 196). With regard to triclosan, the MICs ranged from 0.06 to 2 mg/L for MSSA and from 0.06 to 16 mg/L for MRSA. The median MIC value was <0.06 mg/L for both MSSA and MRSA ($t = 0.083$, $p = 0.93$, df 196).

As expected, the isolates with known resistance mechanisms (*qacA*) both exhibited raised MIC to chlorhexidine, with the isolate with a pSK1 plasmid (including *qacA*) having an MIC of 4.0 mg/L and the isolate with chromosomal *qacA* having an MIC of 8.0 mg/L. Both isolates had a MIC to triclosan of 0.125 mg/L (susceptible). Given the plasmid contained numerous (seven) copies of the *qacA* gene, and only one copy was present in the chromosome of the other isolate, one may have expected a higher level of phenotypic resistance from the plasmid mediated resistance genes.

Of the 12 decolonisation patient samples, two had raised MICs to chlorhexidine (both 4.0 mg/L), which is not the

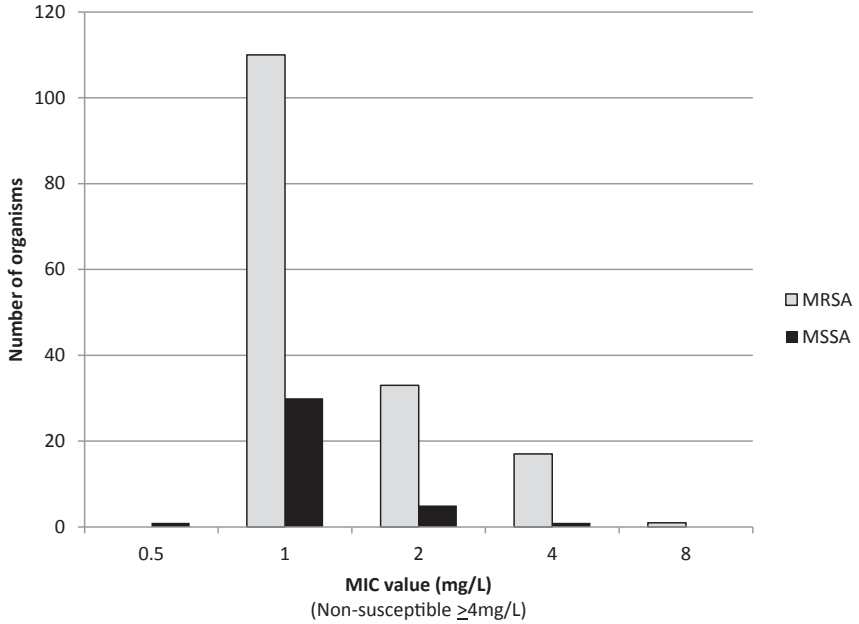

Fig. 1 Number of organisms by minimum inhibitory concentration (MIC) value of chlorhexidine.

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