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In vitro activity of a novel compound, the metal ion chelating agent AQ^+, against clinical isolates of *Staphylococcus aureus*

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***Objectives*: To determine the efficacy of a novel antimicrobial compound, AQ^+, against a genetically heterogeneous collection comprising 213 *Staphylococcus aureus* isolates from global sources. AQ^+ is an aqueous preparation containing 0.5% 8-hydroxyquinoline.**

***Methods*: MICs were found for all the isolates tested using the BSAC microdilution method. Time–kill studies were performed according to NCCLS guidelines. Transmission electron microscopy (TEM) was used to view the ultrastructural effects of AQ^+.**

***Results*: AQ^+ was shown to strongly inhibit the growth of all isolates with a median MIC of 0.25% at a pH optimum of 9.2. Lowering the pH to 7.5 gave an ~4-fold reduction in efficacy and at pH 5.5 there was an ~8-fold reduction in efficacy. Methicillin-resistant *S. aureus* (MRSA) as well as vancomycin-intermediate *S. aureus* were shown to be as equally susceptible to AQ^+ as methicillin-susceptible *S. aureus*. Time–kill curves for AQ^+ were similar to those for gentamicin. TEM showed that AQ^+ actively disrupts the cell wall of *S. aureus* leading to cell lysis.**

***Conclusions*: These results suggest that AQ^+ has strong antimicrobial activity and may be useful in preparations to reduce nasal and skin carriage of MRSA.**

Keywords: 8-hydroxyquinoline, *S. aureus*, MRSA, time–kill, antimicrobial susceptibility

Introduction

The Gram-positive bacterial pathogen *Staphylococcus aureus* is responsible for widespread community-acquired and nosocomial infections worldwide.[1] This versatile pathogen can cause a wide range of disease in humans from boils and furuncles to more serious conditions such as endocarditis, septicaemia and pneumonia.[2]

S. aureus has a proven ability to adapt to different selection pressures and is a particularly acute problem in the hospital environment owing to its ability to rapidly acquire resistance to antibiotics and disinfectants.[3] Resistant isolates have emerged following the introduction of all antibiotic classes. This was the case following the introduction of methicillin (a semi-synthetic penicillin) in 1959 with the first isolation of methicillin-resistant *S. aureus* (MRSA) in 1961.[4] This example has been repeated for various antibiotics and now the emergence of MRSA strains with decreased susceptibility to the antibiotic of last resort, vancomycin, has been reported.[5] The prevalence of antibiotic-resistant *S. aureus* strains has risen dramatically in the last decade and in the UK MRSA bacteraemias (as a percentage of *S. aureus* bacteraemias) rose from <2% in 1990 to 42% in 2000.[6] Coupled to its predominance as a nosocomial infection MRSA is also emerging as a community-acquired pathogen in several countries.[7]

Effective hospital infection control practices including stringent isolation and disinfection guidelines are important in controlling the spread of antibiotic-resistant pathogens in hospitals.[8] Regular handwashing in healthcare workers is often cited as being critical in reducing direct transmission of *S. aureus* and there is some evidence for this.[9] Unfortunately, increasing resistance to commonly used disinfectants reduces the efficacy of regular handwashing[10–12] and dermatological side effects associated with some compounds may lead to poor adherence to handwashing guidelines.[13,14]

AQ^+ is a novel antimicrobial compound based on an aqueous formulation of 8-hydroxyquinoline. 8-Hydroxyquinoline is a potent lipophilic metal chelator whose 8-hydroxyquinoline copper chelate has been used as a fungicide in agriculture and for the

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preservation of textiles, wood and paper. We have determined MICs of AQ^+ for 213 diverse isolates of *S. aureus* including representatives of all major MRSA clones.[3] Time–kill studies were performed with AQ^+, gentamicin, oxacillin and vancomycin to determine the comparative killing effect of the compound. Transmission electron microscopy (TEM) was used to evaluate the effect of AQ^+ on cellular ultrastructure.

Materials and methods

Microorganisms

We examined a collection of 213 genotypically and phenotypically diverse *S. aureus* isolates including 147 isolates from pandemic MRSA lineages[3] and 66 isolates representing community-acquired MRSA and methicillin-susceptible *S. aureus* (MSSA) lineages. This collection contained 110 MSSA and 103 MRSA representing all major MRSA lineages and included six isolates with reduced susceptibility to vancomycin [vancomycin-intermediate *S. aureus* (VISA)]. NCTC strain 6571 was used as a control isolate according to British Society for Antimicrobial Therapy (BSAC) guidelines.[15] Isolates were stored at –80°C in a glycerol broth solution. Fresh subcultures were used for each experiment.

AQ^+

AQ^+ is the trademark name of a patented compound whose active ingredient is 8-hydroxyquinoline. This compound is buffered in a solution containing water, propyl glycol, Synperionic 91/6 and tetra-sodium EDTA. AQ^+ is manufactured by AQ^+ Plc, Angus, UK. It is not possible to test the active ingredient 8-hydroxyquinoline separately from its buffering solution owing to the instability of the compound in a non-aqueous phase. The AQ^+ formulation tested contained 0.5% 8-hydroxyquinoline and MICs were found as the percentage of the complete formulation required to inhibit the visible growth of each strain. For ease of understanding MICs are referred to as $\%AQ^+$ in this study—as an example, an MIC value of 0.5% AQ^+ would represent 25 ppm of the active compound 8-hydroxyquinoline.

Susceptibility testing

Susceptibility to different antibiotics and AQ^+ was assessed using broth microdilution according to the BSAC guidelines for the determination of MICs.[15] Iso-Sensitest broth (ISB; Oxoid, Basingstoke, UK) was used for all AQ^+ susceptibility testing.

The susceptibility of isolates to oxacillin, gentamicin, vancomycin, cefoxitin, ciprofloxacin, clindamycin, erythromycin, linezolid, tetracycline, fusidic acid and penicillin was also measured using the agar dilution method outlined in the BSAC guidelines.[15] These susceptibility data were corroborated using Etest strips (AB Biodisk, Sweden). Iso-Sensitest agar (Oxoid, UK) was also used for all plate dilution susceptibility testing.

Susceptibility of *S. aureus* to AQ^+ was investigated at the optimum pH of 9.2. The effect of lowering the pH to 7.5 and 5.5 on the efficacy of AQ^+ was also examined on a random subset of 50 isolates. The Mann–Whitney *U*-test was used to test for a significant difference in MIC between pH treatments.

Antimicrobial activity of buffer solution

Owing to the nature of 8-hydroxyquinoline it is very difficult to produce in a non-aqueous phase. Thus, all testing was performed on the complete formulation known by its trademark name of AQ^+. This formulation contains two other compounds known to have antimicrobial activity; EDTA and Synperionic 91/6. Both of these compounds are present in very low concentrations but in order to be certain that the antimicrobial activity of AQ^+ is mainly owing to the active compound (8-hydroxyquinoline) the buffer alone was tested against a representative subset of isolates.

Time–kill studies

Time–kill studies were performed on a subset of the strain collection comprising an epidemic MRSA isolate of clone UK EMRSA-7, a carried isolate from Oxford, D137, a laboratory control strain NCTC 6571 and the homo-VISA (homogeneous-VISA) isolate Mu50. Time–kill experiments were performed according to NCCLS guidelines[16] using a protocol based upon a published study that examined the killing effect of tea tree oils.[17] Isolates were grown overnight in ISB at 37°C with shaking. Overnight cultures were diluted to a final concentration of between 1×10^5 and 5×10^5 cfu/mL. Flasks were then shaken at 150 rpm for 90 min at 37°C so that the cultures were in logarithmic growth phase.

Each isolate was inoculated into two flasks, containing ISB and AQ^+, oxacillin, gentamicin or vancomycin and a negative growth control of broth only. After 90 min of incubation at 37°C, 100 µL of the broth was taken to determine the initial inoculum by serial dilution. AQ^+ or an antibiotic was then added to one flask and the buffer solution to the control flask. The final concentration of each antibiotic used was one doubling dilution above the MIC for each particular isolate. AQ^+ was added in various concentrations above the MIC in order to determine how the killing rate of AQ^+ varied with concentration. The MICs for each isolate are shown in Table 1. Each time–kill experiment was performed in triplicate.

Colony counts were performed by making appropriate dilutions in sterile distilled water then plating each onto blood agar (TSA, Oxoid) supplemented with 5% defibrinated sheep blood (TCS, Botolph Claydon, UK). Plates were incubated for 18 h at 37°C and viable counts were calculated to give cfu/mL.

Electron microscopy

TEM was performed on the laboratory strain 6571, and a homo-VISA isolate Mu50. These two isolates were chosen to reflect the diversity of the strain collection. The 6571 strain is an MSSA strain from the 1960s and Mu50 is a multidrug-resistant strain from the late 1990s. Samples were grown overnight in TSB, then inoculated into flasks containing either TSB or TSB supplemented with AQ^+. Final concentrations of bacteria were $1–5 \times 10^8$ cfu/mL with AQ^+ concentrations of 0.25% in treated flasks. Samples were then incubated for 90 min with shaking prior to centrifugation at 10 000 **g** for 10 min to pellet the cells. The supernatant was discarded and the cells were washed three times with PBS (Sigma, UK) prior to treatment for electron microscopy.

Table 1. MICs for four *S. aureus* isolates examined using time–kill studies

	MIC (% or mg/L)				
Strain	AQ^+ pH 9.2	Vancomycin	Oxacillin	Gentamicin	Linezolid
6571	0.25%	1	0.25	0.125	0.5
D137	1%	3	2	2	0.75
EMRSA-7	0.25%	2	>256	0.5	0.75
Mu50	0.25%	8	>256	>256	0.75

Table 2. Summary table showing the MICs of AQ^+ for 213 global isolates of *S. aureus* at pH 9.2

	MIC_{50} (%)	MIC_{90} (%)	MIC range (%)	Median MIC (%)	*n*
MSSA	0.25	1	0.125–10	0.25	114
MRSA	0.25	1	0.25–10	0.25	93
VISA	0.25	0.5	0.25–0.5	0.25	6

Table 3. MIC data for various antibiotics against the strain collection

Antibiotic	Percentage resistance[a]	Median MIC (mg/L)
Vancomycin	2.9	1.5
Ciprofloxacin	26.0	0.38
Cefoxitin	47.1	4
Gentamicin	24.5	0.75
Tetracycline	20.2	0.38
Erythromycin	40.9	0.5
Fusidic acid	5.8	0.094
Clindamycin	15.9	0.125
Oxacillin	48.6	4
Linezolid	0	0.75
Penicillin	84.6	8

[a]Percentage resistance indicates the proportion of isolates with MICs above the breakpoint for each particular antibiotic.

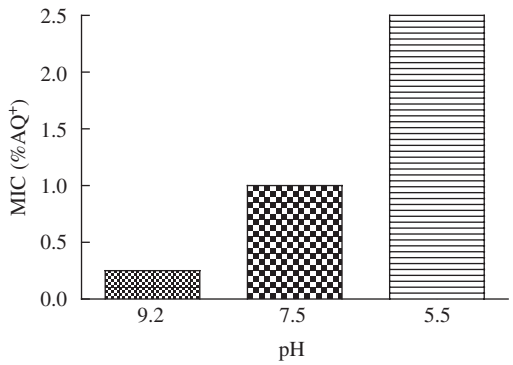

Figure 1. Effect of pH on the efficacy of AQ^+ at inhibiting the growth of isolates of *S. aureus*.

To fix the cells 2% gluteraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate was added and samples were incubated overnight at 4°C. To post-fix the cells 1% osmium tetraoxide was added and the samples were incubated for 1 h. The samples were then dehydrated in graded acetone dilutions of 30, 50, 70, 90 and 100% with three washes at each stage. Infiltration was achieved with various ratios of propylene oxide and Spurr's resin prior to an overnight incubation in 100% Spurr's resin and polymerization in 100% Spurr's resin at 70°C for 8 h.

Sample blocks were then trimmed and ultrathin-sectioned using a Reichert Ultracut Ultramicrotome to produce sections ~100 nm thick. These sections were then viewed on a JEOL1200 TEM and images were taken with a 35 mm camera.

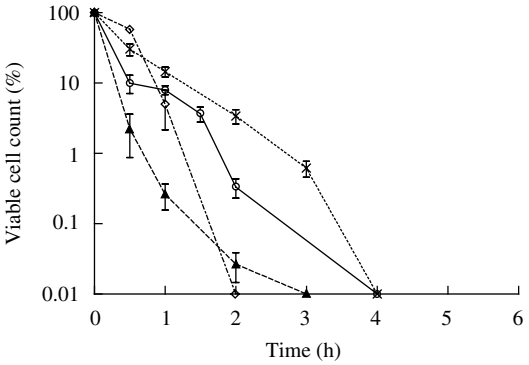

Figure 2. Killing of *S. aureus* by AQ^+. Circles, epidemic MRSA strain EMRSA-7; crosses, nasally-carried MSSA strain D137; diamonds, VISA strain Mu50; and triangles, control MSSA strain 6571.

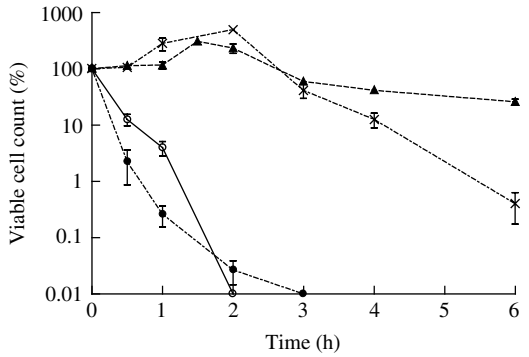

Figure 3. Killing of MSSA strain 6571 by AQ^+, oxacillin, vancomycin and gentamicin. Filled circles, AQ^+; open circles, gentamicin; crosses, oxacillin; and filled triangles, vancomycin.

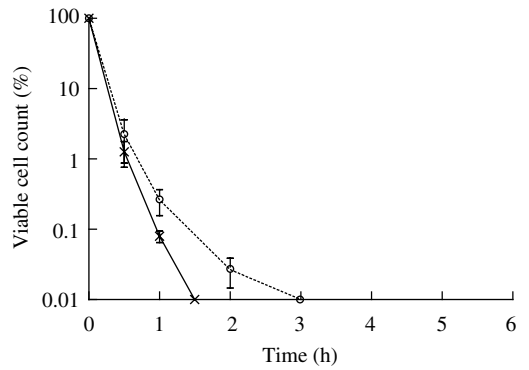

Figure 4. Killing of laboratory MSSA strain 6571 by different concentrations of AQ^+. Circles, 0.5%; crosses, 5%.

Results

Susceptibility testing

All isolates were inhibited by 10% AQ^+, or lower, at an optimum pH of 9.2. The median MIC at this pH was 0.25%. MSSA, MRSA and VISA isolates were shown to be equally susceptible to AQ^+. The susceptibility of the strain collection to AQ^+ is illustrated in Table 2. The isolates were shown to have a considerable range of antibiotic resistance and this is summarized in Table 3.

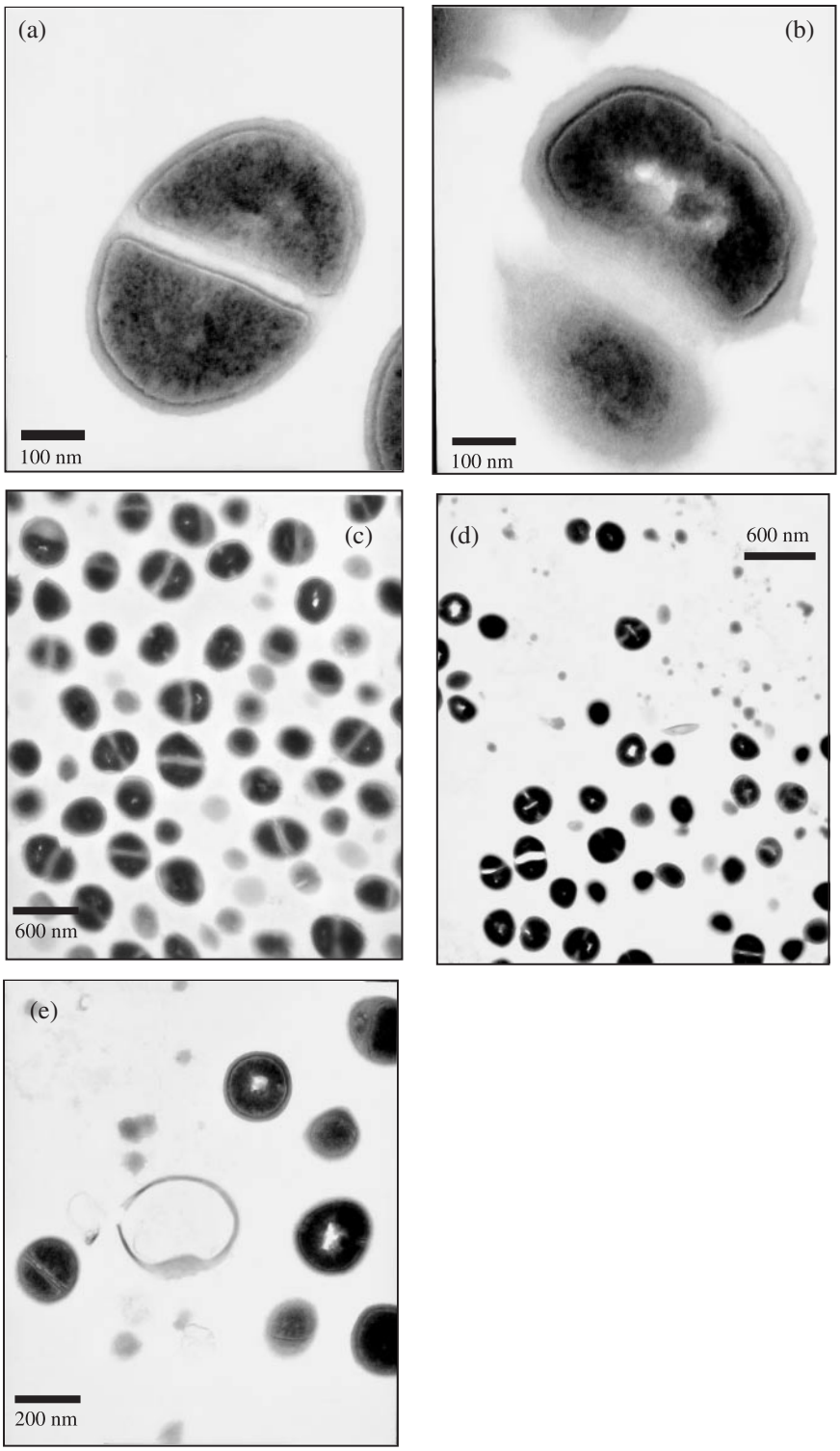

Figure 5. TEM images taken of *S. aureus* 6571 and Mu50 cells treated with AQ^+ at concentrations equal to their MIC. (a and c) Untreated controls. The cell walls of treated cells are darker indicating an increase in electron density, they are also more diffuse and ejection of wall material appears common (b). Ghost cells were also seen in treated samples together with large amounts of cell debris (d and e).

The susceptibility of *S. aureus* to AQ^+ was not apparently influenced by the presence or absence of other antibiotic resistance determinants.

It was found that the buffer without 8-hydroxyquinoline was only inhibitory at concentrations of 20% or above. The median MIC for the strain collection was 0.25%, representing an ~80-fold higher antimicrobial activity with the main active ingredient than without.

The effect of pH on the efficacy of AQ^+ was investigated in a subset of 50 isolates from the collection. At pH 7.5 the median MIC

was significantly increased to 1% ($P = 0.0000$) and at pH 5.5 it was again significantly increased to 2.5% ($P = 0.0004$). Figure 1 shows the effect of changing pH on the efficacy of AQ^+.

Time–kill studies

Measurements of killing for AQ^+, gentamicin, vancomycin and oxacillin were made for each isolate. Antimicrobials were added at a concentration of one doubling dilution above the MIC for each particular isolate unless otherwise stated. Each experiment was performed in triplicate and in all cases the buffer solution-containing controls showed at least a 10^3 increase in cfu/mL by the end of the 6 h test period.

AQ^+ was shown to kill 99.9% of all cells from test isolates within the 6 h test period. The control MSSA strain 6571 was killed within 1 h, and Mu50 within 2 h. It took 6 h for a 99.9% kill of the MRSA strain EMRSA-7 and an MSSA strain D137 (Figure 2).

The time–kill curve for AQ^+ against 6571 was similar in appearance to that of gentamicin. Oxacillin and vancomycin did not reach 99.9% kills over the 6 h study period (Figure 3).

Isolates were killed more rapidly at higher concentrations of AQ^+ (Figure 4). It took 2 h for a 99.9% kill of 6571 at a concentration one dilution above the MIC for that isolate. Increasing the concentration 10-fold to 5% reduced the time required to reach a 99.9% kill to 1 h. The latter concentration is much closer to those in AQ^+-containing products.

Electron microscopy

TEM was used to investigate the nature of the interaction between AQ^+ and *S. aureus*. Two exponentially growing isolates of *S. aureus* were treated with AQ^+ at concentrations equal to their MIC (Table 1) before they were fixed, ultrathin-sectioned and examined using TEM. We examined the laboratory MSSA strain 6571 and the homo-VISA strain Mu50.

The images of cells treated with AQ^+ were greatly different from those left untreated. Untreated cells were uniform in structure with intact cell walls, whereas many of the treated cells had very diffuse cell walls and appeared to have shed cell wall material (Figure 5). In addition, many lysed cells and large amounts of cell debris were present with only empty wall structures remaining. These results suggest that AQ^+ induces lysis and subsequent cell death in *S. aureus*.

Discussion

The number of infections caused by multidrug-resistant bacteria is increasing and this causes major problems in healthcare settings. With increasing antibiotic and disinfectant resistance, effective infection control becomes increasingly important. Our *in vitro* results suggest that AQ^+ might be a useful topical agent for the eradication of *S. aureus* including multidrug-resistant MRSA.

AQ^+ is the trademark name of an aqueous solution containing the lipophilic metal chelator 8-hydroxyquinoline. Here, we demonstrated the bactericidal activity of this novel compound against a collection of MSSA, MRSA and VISA including representatives of all major nosocomial MRSA clones. The median MIC of 0.25% equates to a very low concentration of active ingredient (12.5 ppm) indicating the extremely active nature of this compound.

The median MIC was increased significantly by lowering the pH from 9.2 to 7.5 and further increased at pH 5.5. However, all isolates were inhibited even at these lower pHs and the median MIC at pH 7.5 was 1%, which equates to a much lower concentration of active ingredient than is found in product formulations.

Time–kill studies show that increasing the AQ^+ concentration from 0.5% (one doubling dilution above the MIC of 0.25%) to 5% decreases the 99.9% kill from 2 h down to 1 h. Killing curves also demonstrate the ability of AQ^+ to kill all bacterial populations of MSSA, MRSA and homo-VISA within 6 h.

Cells treated with AQ^+ show altered cell wall structure and were also more electron dense than untreated controls. The presence of ghost cells was common in treated samples indicating cell death and subsequent lysis.

MRSA is preferentially carried in the anterior nares and mupirocin-containing nasal ointment is widely used to eradicate nasal carriage; however, resistance to this antibiotic, although not yet widespread, is increasing.[18] Our results suggest that AQ^+ could be a viable alternative to mupirocin in eradicating nasal carriage of MRSA although the activity of the compound against mupirocin-resistant isolates was not tested here.

MRSA and other drug-resistant pathogens are difficult to eradicate from the skin and direct contact is known to be an important route of transmission.[19] Various studies have highlighted the importance of adhering to a strict hand disinfection regimen[20] with the use of alcohol gels promoted by various bodies.[21,22] However, regular use of alcohol gels may promote dermatological side effects.[23] These may lead healthcare personnel to reduce their compliance with handwashing regimens recommended by Infection Control specialists. The rapid time–kill of AQ^+ against MRSA indicates that it could be an effective active ingredient as part of a hand cleaning product. No dermatological side effects have been observed with heavy use (more than 30 washes per day) of this compound (B. R. D. Short, unpublished results).

We conclude that AQ^+ could be a valuable antimicrobial agent for use in formulations to eradicate carriage and help reduce MRSA transmission.

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Transparency declarations

MCE is a paid consultant of AQ^+ PLC. The funding source had no role in study design, data collection, analysis, interpretation or writing of the report.

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