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Chlorhexidine Resistance in Methicillin-Resistant *Staphylococcus aureus* or Just an Elevated MIC? An In Vitro and In Vivo Assessment

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Chlorhexidine (Hibiscrub; ICI) is generally accepted to be effective as an antiseptic hand wash for methicillin-susceptible *Staphylococcus aureus* (MSSA), but there is dispute whether the chlorhexidine MIC for methicillin-resistant *S. aureus* (MRSA) strains is higher than that for MSSA strains and, indeed, whether it is relevant. In addition, the link between resistances to chlorhexidine, gentamicin, and "nucleic acid-binding" compounds (NAB; which code, in particular, for propamidine isethionate and ethidium bromide) requires clarification. We performed chlorhexidine MIC and rate of kill tests on a number of MSSA and MRSA isolates. Two gentamicin-resistant MRSA isolates without NAB plasmids were more susceptible (0.25 and 0.5 μg/ml) than four of eight MSSA that we tested (range, 0.25 to 2 μg/ml). Chlorhexidine MICs were higher (4 to 8 μg/ml) for seven distinct MRSA isolates with plasmids conveying resistance to gentamicin and NAB (GNAB). Curing of the GNAB plasmid from MRSA strains resulted in a fall in the MIC (1 to 3.3 μg/ml), but no consistent fall in killing by chlorhexidine was observed. No effect on the chlorhexidine MIC or killing was observed when we cured strains of methicillin resistance. GNAB plasmid transfer resulted in a rise in the chlorhexidine MIC for the strains but no consistent fall in killing by chlorhexidine. Ethical approval was granted for 10 volunteers to each have a methicillin-susceptible, GNAB-resistant, derived transcipient and its GNAB-susceptible isogenic parent applied to separate sites in an in vivo skin test; no significant difference was seen in survival rates after the application of chlorhexidine. These results suggest that chlorhexidine appears to be as effective as a hand-washing agent for MRSA isolates with or without NAB plasmids as it is for MSSA isolates.

Nosocomial infection with methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major problem worldwide, and certain MRSA strains may possess an epidemic potential (EMRSA), although it is unclear what factors are involved (7). Particular EMRSA isolates from the southeast of England (in this report they are termed EMRSA) and Australia (7, 21) appear to be very similar if not identical and possess plasmids with genes coding for resistance to gentamicin, propamidine isethionate, and nucleic-acid binding (NAB) compounds (e.g., ethidium bromide) and, hence, are often termed GNAB plasmids (10).

Guidelines (3) published previously to control MRSA have recommended the use of antiseptics to reduce the MRSA load on superficial colonized sites. Chlorhexidine, in particular, has become a controversial choice because of reports of elevated MICs for MRSA strains compared with those for methicillin-susceptible *S. aureus* (MSSA) strains. Brumfitt et al. (5) found that the chlorhexidine MIC for EMRSA (mean MIC, 4.19 μg/ml) is higher than that for nonisogenic MSSA (0.94 μg/ml), and Mycock (14) reported similar findings, while this was not observed by Al-Masaudi et al. (1). Haley et al. (12) failed to show a difference between three MSSA and three MRSA, all local American strains, in a test of the rate of killing by chlorhexidine, but they did not state whether their strains were resistant to GNAB.

Others have preferred to examine isogenic isolates. Coleman et al. (6), in a study of MRSA isolates in Dublin, Ireland, found that transfer of plasmids conveying gentamicin and ethidium bromide resistance resulted in a threefold rise in the chlorhexidine MIC. We observed a similar fall in the MIC when we cured the GNAB plasmid from strains of EMRSA (8), but previously (7), we failed to show a consistent rise in the chlorhexidine MIC for derived transcipients with these same GNAB plasmids (7).

Platt and Bucknall (15) have criticized the evaluation of the clinical efficacy of antiseptics as hand washes from their MICs and suggested an in vivo skin test (the ring test of Story [18]) as an alternative. However, MRSA isolates cannot ethically be applied to human skin for such an in vivo test. In this study, we performed chlorhexidine MIC and rate of kill tests on a number of MSSA, MRSA, and derived strains. We also performed the Story (18) ring test on an MSSA transcipient strain with the GNAB plasmid transferred from an isolate of EMRSA.

MATERIALS AND METHODS

Isolates used. The MRSA (methicillin MICs [see below] for all these isolates were 16 μg/ml or greater), MSSA (methicillin MICs for all these isolates were less than 2 μg/ml), transcipient, and recipient isolates used in this study are listed in Table 1. In addition, three fresh EMRSA isolates from two different outbreaks were examined. They were isolated from two patients who had each received at least 10 days of chlorhexidine therapy. One was an isolate from the throat of a patient receiving chlorhexidine gargles, and the other two were from the perineum and urine of another patient receiving chlorhexidine as an antiseptic and bladder washout.

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TABLE 1. Strains used in the study

S. aureus category	Isolate codes	Chlorhexidine MIC (μg/ml)
MRSA isolates with GNAB resistance[a]		
Typical EMRSA	STH M	8 (2)[b]
	STH H	6.66 (6.66[c]) (2[d])
EMRSA with 83A/84/85 phage reactions	STH N	8 (2)
EMRSA with a cryptic plasmid	STH C	8 (3.3)
	STH W	8 (3.3)
EMRSA with biotype changes	STH G	6.66 (4[c]) (1)
EMRSA with chloramphenicol resistance	STH F	8 (2)
MRSA with GNAB resistance (nonepidemic)	GH 19	4 (4[c]) (1)
Recipient strains		
RN 450 lysogenized with phage 95	BDC 822	2
RN 450 lysogenized with J phage	WG 3358	1
GNAB trancipient strains derived by mixed culture		
STH H transferred to BDC 822	BDC 2HG	8
STH N transferred to BDC 822	BDC GU2N	4
STH C transferred to BDC 822	BDC 2CG	8
STH G transferred to WG 3358	BDC 3GG	8
GNAB transductant strain STH F with J phage to WG 3358	BDC F01	8
Gentamicin resistant MRSA without NAB resistance		
STH 696/82		0.25 (0.25[c])
STH 4682/82		0.50
MSSA strains		
NCTC 8532		0.75
NCTC 4163		0.36
ATCC 6538		0.75
ATCC 9144		0.50
A8601-008[e]		0.50
A8601-011[e]		0.38

[a] See reference 8 for descriptions of these strains.
[b] Values in parentheses are chlorhexidine MICs for isolates cured of plasmid GNAB.
[c] Cured of methicillin resistance.
[d] Cured of methicillin and GNAB resistance.
[e] Clinical isolates from Withington Hospital, Manchester, England.

Derivation of isolates. Strains were cured of their plasmids by growth at 42°C (8). The loss of GNAB plasmids was confirmed by testing susceptibility by a standard comparative disk diffusion method with disks containing gentamicin (10 μg), propamidine isethionate (25 μg), and ethidium bromide (25 μg). Our resistant isolates grew to within 5 mm of these disks. Plasmid loss was also confirmed by running plasmid extracts in 0.8% agarose gels (8). The strains were also cured of methicillin resistance by growing them at 42°C or by long-term storage at room temperature (13).

Transfer experiments. Mixed-culture plasmid transfer was performed with the MSSA recipient isolates listed in Table 1, as described previously (7). In addition, one strain was constructed by the propagation of J phage derived by mitomycin C induction from strain WG 3358 (strain RN 450 lysogenized with J phage [9]) on a strain of EMRSA (STH F) by the soft agar method of Swanstrom and Adams (19) and by the addition of phage lysate, at a multiplicity of infection of 1, to a broth culture of strain WG 3358. The culture was incubated for 30 min at 37°C and washed once with 5 ml of 0.01 M sodium citrate, and then 1 ml was spread onto selective medium as described above for the mixed-culture experiments.

Serial passaging of resistance. EMRSA isolates STH M, STH N, and STH G; transcipients BDC 2HG and BDC 2CG; and the transductant BDC F01 (see Table 1) were subcultured serially from the highest point of growth on gradient plates containing up to 32 μg of chlorhexidine per ml or 320 μg of gentamicin per ml. This was done by direct inoculation of the colonies and by flooding the plates with 3 ml of an inoculated Oxoid no. 2 nutrient broth, which was incubated for 18 h; and then the excess fluid was removed, and the plates were dried. The MICs of these agents were measured when growth to the top of the gradient had occurred or after 25 gradient passages.

MIC test. MICs of chlorhexidine, propamidine isethionate, and gentamicin were determined in duplicate on plates as described previously (8). Briefly, the inoculum used was an overnight broth culture diluted so that approximately 10^4 or 10^6 CFU could be applied with a multipoint inoculator; the inocula were checked with a laser colony counter (Spiral System Instruments, Bethesda, Md.). Plates were incubated at 37°C and examined at 24 and 48 h (30°C for methicillin); the lowest concentration that produced 10 or fewer CFU was read as the MIC.

Rate of kill test. The chlorhexidine rate of kill test (chlorhexidine killing) was based on British standard 3286 (4). One milliliter of bacterial suspension diluted 1:10 in sterile dis-

tilled water was added to 9 ml of test solution in a sterile MacCartney bottle (United Glass Containers St. Albans, United Kingdom). Samples of 1 ml were removed, usually after 1, 3, and 10 min (but, on occasion, after 20 s instead of or in addition to 10 min), and placed into 9 ml of 3% asolectin (Fluka, Buchs, Switzerland)–20% polysorbate 80 (ICI Pharmaceuticals, Cheshire, United Kingdom) in tryptone soy broth (TSB; Oxoid, Basingstoke, United Kingdom) to neutralize any chlorhexidine carried over from the test solution (17). Pour plates of 1 ml were prepared from this.

Further dilutions were made in 0.75% asolectin–5% polysorbate 80 in TSB to allow survivor counts to be made with the spiral plate system (Don Whitley Scientific, Shipley, United Kingdom). Water controls were included for each test organism by adding 1 ml of bacterial test suspension to 9 ml of sterile distilled water. Samples of 1 ml were withdrawn from this after 3 min and diluted accordingly for viable counting. All plates were prepared by using tryptone soy agar (TSA; Oxoid) containing 0.3% asolectin and 2% polysorbate 80. For chlorhexidine, pour plates were additionally neutralized with 1% sodium suramin (Mobay Chemical Co., Pittsburgh, Pa.). The plates were incubated for 48 h, and the survivor counts were then determined. Log_{10} reductions in viable counts per milliliter were determined by subtracting the numbers of survivors at each contact time from the count of the water control.

Story ring test. The ring test of Story (18), which has also been described elsewhere (15), was performed on the forearms and hands of five female and five male volunteers with intact healthy skin, no history of sensitivity to any of the formulations or active ingredients to be used in the study, and no skin allergy. Written informed consent was obtained, as demanded by the ethical committee.

The MSSA isolates tested were the parent strain WG 3358 and strain BDC 3GG, a transcipient in which GNAB resistance but not β-lactamase resistance was transferred (7). These were applied to four areas (each strain to two areas) on the right inner forearm of each of 10 volunteers in a statistically randomized manner. All test organisms were grown overnight on 50 ml of TSA in a 250-ml flask; the agar for BDC 3GG contained 10 μg of gentamicin per ml, to maintain the GNAB plasmid. The organisms were thoroughly resuspended with the aid of sterile glass beads in 10 ml of sterile distilled water. This suspension was then clarified by centrifugation, and the pellet was resuspended in 10 ml of sterile distilled water, diluted 1:10 in sterile distilled water, and stored in 1-ml aliquots in liquid nitrogen. The stored suspension was thawed as required and further diluted 1:10 in sterile distilled water before use.

The volunteers first washed their hands and forearms with nonmedicated soap for 30 s; they then rinsed their hands and forearms well with water and dried them on paper towels. The inner right forearm was then swabbed with 70% alcohol, and four 3.5-cm-diameter circles were marked on the skin. Each marked area was contaminated with 20 μl of the appropriate test inoculum (approximately 10^7 organisms) with a micropipette by the randomized format. The bacterial suspension was spread over the area with a glass rod and left to dry for 1 min. Areas were then treated with 0.1 ml of 4% chlorhexidine gluconate detergent solution (Hibiscrub; ICI Pharmaceuticals PLC) or water by the randomized format. After the 1-min contact time, the number of viable organisms recoverable was determined by enclosing the marked area with a sterile glass cylinder pressed firmly against the skin. Five milliliters of 0.75% asolectin–5% polysorbate 80 in TSB was poured into the ring, and the skin was swabbed in a standard manner with a sterile swab for 15 s. The sample fluid was pipetted off, and viable counts were determined on TSA containing 0.3% asolectin and 2% polysorbate 80 neutralizers by a technician blinded to which test organism or treatment had been used. All organisms recovered were saved for subsequent retesting of chlorhexidine, propamidine isethionate, and gentamicin susceptibility and the presence of the GNAB plasmid.

On completion of each test, the hands and forearms of the volunteers were swabbed thoroughly with 70% alcohol and washed with plain bar soap to eliminate surviving organisms and to remove traces of the formulation. A peptone-moistened swab was applied vigorously to the test areas and cultured for residual gentamicin-resistant organisms on plates containing 2 μg of gentamicin per ml. Volunteers were asked to report any untoward sensations or change in skin appearance at the inoculum sites.

Statistical analyses. All analyses were performed with the SAS system (16). For chlorhexidine killing tests, the numerical differences between the pairs of MRSA with and without GNAB plasmids were calculated and a 95% (two-sided) confidence interval for the average difference was estimated. MRSA and MSSA data were not paired, and so a 95% (two-sided) confidence interval for the difference in the group means was estimated. A pooled estimate for the variance from the two groups was used to estimate the interval.

Data from the Story ring test (18) were examined by analysis of variance.

RESULTS

Table 1 lists the chlorhexidine MIC results for MRSA, MSSA, and the various cured and transcipient isolates. In the initial experiments, there were problems with the reproducibility of chlorhexidine MICs for EMRSA isolates and the transcipients with the GNAB resistance plasmid. However, when the maintenance of GNAB plasmids in those organisms grown on the MIC plate with the highest chlorhexidine concentration was examined by comparative diffusion tests by comparing growth on gentamicin, ethidium bromide, and propamidine isethionate plates (and, later, confirmational MICs and plasmid analyses), it was found that if there had been a two- to fourfold increase in the chlorhexidine MIC, compared with the relevant MRSA cured of GNAB plasmids, or with the recipient in the case of a transcipient (Table 1), then the organism was always resistant to these three agents and the GNAB plasmid was present. However, if the chlorhexidine MIC was not elevated, then the converse was true and the plasmid was lost. The mean chlorhexidine MIC (Table 1) for the eight MRSA isolates with GNAB plasmids (seven EMRSA isolates and strain GH19) was higher (4 to 8 μg/ml) than it was for isolates of MSSA (0.37 to 2 μg/ml) or the MRSA isolates without NAB (0.25 and 0.50 μg/ml). Cure of methicillin resistance from an MIC of ≥16 to 2 or 4 μg/ml did not change significantly the chlorhexidine MICs for three GNAB-resistant MRSA isolates (two isolates of EMRSA and GH19) or strain STH 696/82, which was not resistant to NAB. However, when one derived methicillin-susceptible EMRSA strain (STH H) was subsequently cured of GNAB plasmids, the MIC did fall (Table 1).

It proved to be impossible to increase resistance to chlorhexidine after repeated exposure in vivo or serial passage in vitro. For the three fresh EMRSA isolates isolated from treated sites, the chlorhexidine MIC was not higher (4 μg/ml)

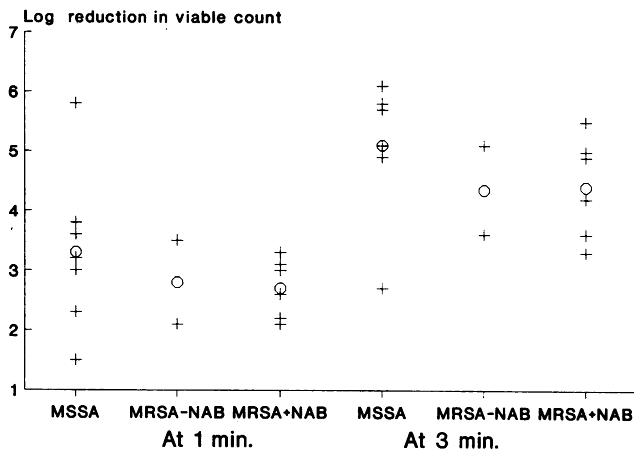

FIG. 1. Chlorhexidine killing at 1 and 3 min for MSSA and gentamicin-resistant MRSA with and without NAB. +, datum points; O, mean datum points.

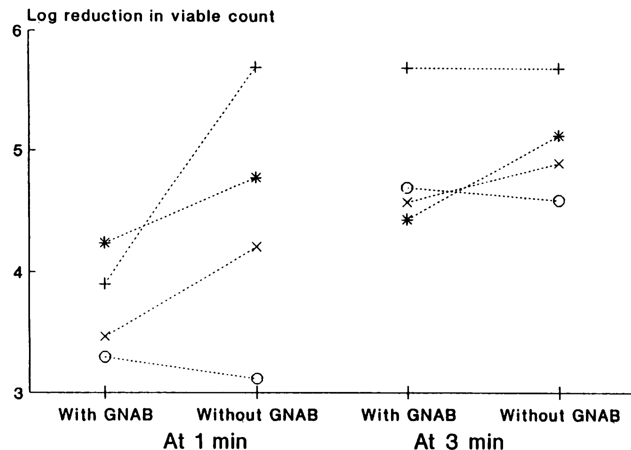

FIG. 2. Chlorhexidine killing at 1 and 3 min for MRSA and their isogenic pairs cured of GNAB. ×, STH N; +, STH M; *, STH W; O, GH 19. Isolates STH N, STH M, and STH W were EMRSA; isolate GH 19 was non-EMRSA.

than those for other isolates of EMRSA tested. Neither was it possible to raise the chlorhexidine MIC for the three EMRSA isolates, two transcipients, and the transductant after 25 passages on chlorhexidine gradients. The isolates grew readily after two passages up to the top of the 320-μg/ml gentamicin gradients, but, although the gentamicin MIC was appropriately raised, there was no increase in the chlorhexidine MICs for these isolates.

Preliminary data showed that an operator could carry out reproducible chlorhexidine killing tests for the same organisms when run on the same day. Experiments were thus performed on the same day by this same operator on the groups of isolates which were to be compared, i.e., MRSA and MSSA, recipients and transcipients, parents, and derivatives cured of antibiotic resistance.

There was no significant difference in chlorhexidine killing (Fig. 1) between MRSA with GNAB and MSSA, or between MSSA and the two MRSA without NAB. However, the range of chlorhexidine killing for MSSA was greater, and the chlorhexidine killing rate for one MSSA isolate (A8601-011) was the poorest, yet the chlorhexidine MIC for this isolate was the lowest (0.25 μg/ml). When the three EMRSA isolates were cured of their GNAB plasmids, the chlorhexidine killing for these isolates increased at 1 min (it was particularly marked for isolate STH M), and for two isolates it was also increased at 3 min (Fig. 2). For isolate GH 19, a nonepidemic MRSA isolate with GNAB, there was a slight decrease in chlorhexidine killing at both times. However, none of these changes analyzed in toto achieved a level of statistical significance ($P > 0.05$) at either time. For the freshly isolated EMRSA isolates from chlorhexidine-treated patients, chlorhexidine killing was not lower compared with those for the EMRSA isolates from untreated subjects from the same outbreak (STH M and STH H; data not shown). Neither was there a decrease in chlorhexidine killing for the GNAB transcipients compared with the killing for their appropriate controls (isogenic recipients), and this was also true for the transcipients and EMRSA isolates serially passaged on up to 320 μg of gentamicin per ml and the isolates cured of methicillin resistance when they were tested in the same run (data not shown).

Results of the Story ring test (18) showed no significant difference ($P > 0.05$) between the mean logarithmic reductions achieved with chlorhexidine against the recipient strain WG 3358 (3.040) and transcipient strain BDC 3GG (3.165) containing the GNAB plasmid (Fig. 3). Isolates of BDC 3GG taken from each volunteer at the end of the test had unchanged gentamicin, propamidine isethionate, and chlorhexidine susceptibilities, and their GNAB plasmids were still present. Volunteers for the Story ring test (18) suffered no adverse effects, and no EMRSA isolates were isolated from the forearms of the volunteers after the final alcohol rinse.

DISCUSSION

In this study, we found, as before (8), that when EMRSA strains were cured of GNAB plasmids there was a fall in the chlorhexidine MICs for these strains. We failed previously to show a consistent rise in the chlorhexidine MIC for our GNAB plasmid transcipients (7), but in this study, we found that this was probably due to the inherent instability of the GNAB plasmid. Instability was observed on occasion in the donor EMRSA strains during an MIC run, and this may also

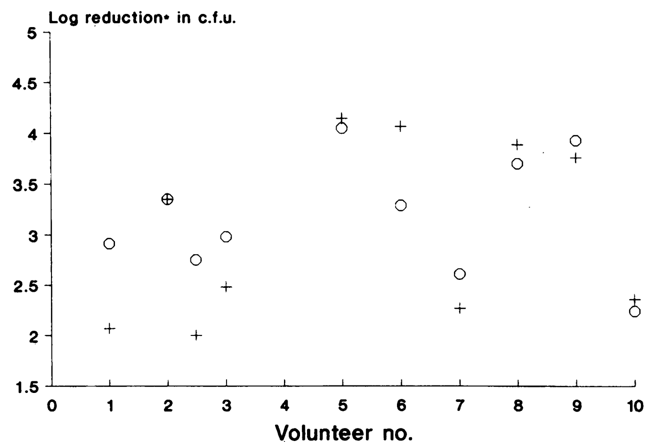

FIG. 3. Story ring test (18) results for 10 volunteers. *, results were compared with those for water-treated controls; O, recipient WG 3358 for which the chlorhexidine MIC was 1 μg/ml; +, transcipient BDC 3GG for which the chlorhexidine MIC was 8 μg/ml.

explain why other workers have failed to detect elevated chlorhexidine MICs (1).

A similar rise in the chlorhexidine MICs for transcipients has been reported by Coleman and coworkers (6) in their Irish MRSA strains, although those strains were distinct from EMRSA (21).

The hypothesis of linked NAB and chlorhexidine "resistance" was supported by the increased chlorhexidine MIC for MRSA isolates with NAB compared with those for MSSA isolates. In turn, the MICs for two gentamicin-resistant, NAB-susceptible MRSA isolates were comparable to those for the MSSA isolates. Curing of methicillin resistance did not alter the chlorhexidine MICs for MRSA isolates with or without NAB. Thus, our results of the examination of nonisogenic MRSA isolates confirm and extend those of Brumfitt et al. (5) and Mycock (14).

Haley et al. (12) and Gordon and McClure (11) could not detect a difference between MRSA and MSSA isolates in their chlorhexidine killing tests. However, it is impossible to compare the results of our chlorhexidine killing with the results of either of those groups of investigators because of the different methods used and because of the fact that neither group determined the MICs of chlorhexidine or NAB compounds for their isolates.

When tests were performed on the same day it was possible to compare chlorhexidine killing results, but further work is required to examine the reproducibility of chlorhexidine killing testing so that results may be compared between operators and between experiments performed on different days and at different centers. No consistent relationship between a raised chlorhexidine MIC and an increased chlorhexidine killing was observed in the isolates or transcipients examined in this study, nor could this be related to gentamicin or methicillin resistance per se. We have no explanation for the poor chlorhexidine killing of the MSSA isolate (A8601-011); other, as yet undescribed factors may await elucidation.

The molecular basis of the elevated chlorhexidine MIC is under investigation. Tennent et al. (20) described preliminary experiments which implicate the *qacA* determinant and a common efflux system for ethidium bromide, propamidine isethionate, and other compounds, including chlorhexidine. Those workers did not refer to the work of Yamamoto et al. (22), who had already cloned a 3.5-kb fragment from plasmid pSAJ1 isolated from a Japanese MRSA isolate into *Escherichia coli*. That fragment coded for resistance to NAB compounds and chlorhexidine (two- to fourfold increase in the MIC). They pointed out that this plasmid differed from the plasmid pSK1 used by Tennent and coworkers (20) only in that it did not encode resistance to trimethoprim, and so it perhaps resembled the GNAB plasmid of STH M (the index case of outbreak with strain S1a [8], called pBDC1 here). It is impossible to compare the two cloned fragments of Tennent et al. (20) and Yamamoto et al. (22), because the restriction enzymes used to map them were quite different. Furthermore, unlike pBDC1 and pSK1, pSAJ1 was described as a conjugative plasmid, although it transferred at a very low frequency and no attempt was made to transfer it in mixed culture. Thus, there is the possibility that it may closely resemble pSK1 and pBDC1.

Ethical considerations prevent the testing of MRSA isolates on human volunteers, and so for our Story ring test (18), we chose a transcipient strain for which the elevated chlorhexidine MIC was the same as that for MRSA but which could be treated effectively with antibiotics such as penicillin or erythromycin if it caused problems in the volunteers.

In this test, which closely mimics the in-use application of the antiseptic, there was no significant difference between the rate of killing of the GNAB transcipient and that of the susceptible recipient. It will be interesting to see how these isolates compare in other models which might be proposed to mimic the prolonged use of antiseptics in the clearance of MRSA.

In conclusion, we confirmed that for isolates of MRSA there is a GNAB-related rise in the chlorhexidine MICs. However, the results of the in vitro rate of killing tests and volunteer skin tests used in this study suggest that MRSA isolates are as susceptible as MSSA isolates to the bactericidal action of chlorhexidine.

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