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Impact of exposure of methicillin-resistant *Staphylococcus aureus* to polyhexanide *in vitro* and *in vivo*

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ABSTRACT

Staphylococcus aureus (MRSA) resistant to decolonization agents such as mupirocin and chlorhexidine increase the need to develop alternative decolonization molecules. The absence of reported adverse reactions and bacterial resistance to polyhexanide makes it an excellent choice as topical antiseptic. In the present study we evaluated the *in vitro* and *in vivo* capacity to generate strains with reduced polyhexanide susceptibility and cross-resistance with chlorhexidine and/or antibiotics currently used in clinic. Here we report the *in vitro* emergence of reduced-susceptibility to polyhexanide by prolonged-stepwise exposure to low concentrations in broth culture. Reduced susceptibility to polyhexanide was associated with genomic changes in the *mprF* and *purR* genes, and with concomitant decreased susceptibility to daptomycin and other cell-wall active antibiotics. However, the *in vitro* emergence of reduced-susceptibility to polyhexanide did not result in cross-resistance to chlorhexidine antiseptic. During *in vivo* polyhexanide clinical decolonization treatment, neither polyhexanide reduced-susceptibility nor chlorhexidine cross-resistance were observed. Together, these observations suggest that polyhexanide could be used safely for decolonisation of carriers of chlorhexidine-resistant *S. aureus* strains but highlight the need for careful use of polyhexanide at low antiseptic concentrations.

INTRODUCTION

Prevention of healthcare-associated infections includes the use of antiseptic agents. Chlorhexidine antiseptic solution is one of the most widely used antiseptics since the 1950s and is administered for hand and skin disinfection prior to surgical intervention, bathing patients in intensive care units, decolonization of carriers of methicillin-resistant *Staphylococcus aureus* (MRSA) and prevention of vascular catheter infections (1). Broad range and long residual activity, safety and good tolerance are key advantages of this antiseptic agent. However, chlorhexidine reduced-susceptibility associated with biocide-efflux pumps (1, 2) has shown to impact clinical outcomes (3, 4).

Increasing chlorhexidine reduced-susceptibility due to its intensive clinical use (5) has led to the development of new antiseptics such as polyhexanide (polyhexamethylene biguanide). This antiseptic was originally developed as a surface disinfectant but, in the early 1990s, was introduced in medicine for local antiseptic treatment (6) and is currently used in the United States for wound disinfection. Polyhexanide shows good safety, tissue compatibility and reduction of bacterial load and infection rate of chronic and burn wounds, and is proposed as an alternative to topical antibiotic treatment (7, 8).

Polyhexanide is a cationic polymer attaching primarily to negatively charged membrane phospholipids, interfering with its stability and leading to membrane permeability. Lipopolysaccharides and teichoic acids, from Gram-negative and Gram-positive bacteria respectively, and peptidoglycan components of the cell wall were also identified as polyhexanide targets (9-11). Accordingly, polyhexanide was shown to have potent antimicrobial activity against both Gram-positive and Gram-negative bacteria (8, 12). In contrast to other antiseptics, reduced susceptibility of polyhexanide and its association with antibiotic resistance has not yet been detected (12-16). Another exceptional characteristic recently identified for polyhexanide is its intracellular

70 bactericidal activity recognized as an important property to potentially treat skin

71 infections caused by intracellular bacteria (17).

72   The lack of reported adverse reactions, selection of bacterial resistance,

73 antagonisms with antibiotic activities and its potential use as an intracellular bactericidal

74 agent makes polyhexanide an excellent choice as topical antiseptic to prevent and treat

75 bacterial infections. To test polyhexanide, we previously assessed its efficacy to

76 eradicate MRSA carriage *in vivo* by a randomized placebo-controlled clinical trial (18),

77 which showed that single polyhexanide decolonization course was not sufficient to

78 significantly eradicate MRSA carriage (18).

79   In the current microbiological study we analyzed the potential reasons that could

80 explain low polyhexanide decolonization rates *in vivo*. We tested the *in vitro* emergence

81 of polyhexanide resistance, the potential cross-resistance with chlorhexidine antiseptic

82 and identified the genetic mutations potentially leading to reduced susceptibility to

83 polyhexanide.

84

85 **RESULTS**

86   ***In vitro* emergence of polyhexanide reduced-susceptibility and cross-**

87 **resistance with chlorhexidine.** To analyze the potential emergence of polyhexanide

88 reduced-susceptibility *in vitro*, we selected three different clinical MRSA strains (COL,

89 134947 and 128822) that were subjected to a stepwise training method in polyhexanide

90 broth cultures (see materials and methods). As shown in Table 1, after several passages

91 of 2 days each on increasing concentrations of polyhexanide, two out of three MRSA

92 strains (with an initial polyhexanide MIC of 0.5 and 1 μg/ml) ultimately grew at

93 polyhexanide concentrations of 2, 4 and 8 μg/ml (Table 1). Prolonged exposure did not

94 increase further the levels of polyhexanide MIC.

95   To further analyze cross-resistance development, we assessed the emergence of

96   chlorhexidine reduced-susceptibility *in vitro*, using the same methodology as described

97   above for polyhexanide. After several passages of 2 days each on chlorhexidine, again

98   two out of three MRSA strains (with an initial chlorhexidine MIC of 2 and 4 µg/ml)

99   ultimately grew at a chlorhexidine concentration of 8 µg/ml (Table 1). Emergence of

100   chlorhexidine reduced-susceptibility was not accompanied by changes in polyhexanide

101   MIC (Table 1). Similarly, emergence of polyhexanide reduced-susceptibility was not

102   accompanied by changes in chlorhexidine MIC, suggesting an absence of cross-

103   resistance between these antiseptics.

104

105   **Antibiotic susceptibility profiles and genomic sequencing of MRSA strains**

106   **with reduced susceptibility to antiseptics.** Previous studies have shown an association

107   of chlorhexidine resistance with resistance to antibiotics (19). To determine whether *in*

108   *vitro* emergence of reduced susceptibility to polyhexanide or chlorhexidine in our

109   strains is associated with emergence to antibiotic resistance, we analyzed the antibiotic

110   susceptibility pattern of each parental isolate and its cognate *in vitro* derived

111   polyhexanide - or chlorhexidine-exposed derivative showing altered susceptibility

112   (COL/COLP3/COLP5, 134947/134947P6, 128822/128822P6, COL/COLP7/COLP10,

113   134947/134947P10, 128822/128822P6). Antibiotic disc diffusion and Etest assays

114   showed that reduced-susceptibility to polyhexanide was accompanied by changes in

115   antibiotic susceptibility profiles (vancomycin, teicoplanin, daptomycin) as compared to

116   the parental strains (Table 2). Interestingly, strains showing reduced susceptibility to

117   polyhexanide showed reduced susceptibility to daptomycin with or without concomitant

118   alteration susceptibility to vancomycin or teicoplanin. In contrast to polyhexanide, no

119   consistent association between reduced susceptibility to chlorhexidine and antibiotic

120   resistance was observed; of three strains with reduced chlorhexidine susceptibility, only

one showed an association with reduced ciprofloxacin susceptibility, in agreement with previous observations (20). In a single background (MRSA128822) showing high initial MIC level against chlorhexidine (MIC = 8µg/ml), we observed reduced susceptibility to daptomycin (Table 2).

To identify genomic changes associated with antiseptic/antibiotic reduced-susceptibility, we performed *de novo* whole-genome sequencing (see materials and methods). Using Illumina-Solexa technology, we obtained between 4'107'708 and 3'452'730 of 300-bp paired-end reads for all strains leading to between 140X and 287X of coverage depth after quality filtering. Genome assembly results in 2'821'361-bp for the *S. aureus* COL strain, 2'794'034-bp for *S. aureus* SA134947 and 2'922'225-bp for MRSA128822. After quality assessment, filtering and genome assembly, single nucleotide polymorphisms, insertions and deletions were identified between antiseptic-selected mutants and their cognate parents. As shown in Table 2, antiseptic-selected mutants showing changes in polyhexanide or chlorhexidine MICs possessed mutations in *mprF purR*, *mepA*, *pldB*, *glpD* genes and in some intergenic regions near *norA*, *ndrL* or other hypothetical genes. Interestingly, these genes affect lipid metabolism (*mprF*, *pldB* and *glpD*) or are already known to affect resistance to chlorhexidine (MepA efflux pump) (20) or to daptomycin (21) and nisin cationic antimicrobial peptide antibiotics (22) (MprF protein and PurR transcriptional activator, respectively). To establish the contribution of the observed *purR* mutation to polyhexanide resistance, the MIC of polyhexanide was determined against *S. aureus* SH1000:*purR* T686G, the construction of which is described elsewhere (22). No change in polyhexanide susceptibility was observed when compared with parental SH1000 or COL, suggesting that this mutation does not contribute to the observed resistance phenotype. Given that nonsynonymous *mprF* mutations were identified (Table 1) in all strains displaying polyhexanide resistance, it seems likely that these mutations are responsible for resistance. MIC

determination was also assessed in strains harboring *mprF*C884T identified in a different experimental context (23). This mutation leading to daptomycin resistance was responsible for a 2-fold factor increase in polihexanide (from 4 to 8 µg/ml). Note that we tried several times but we failed to transfer individual mutation into the parental strain COL, by transduction using several staphylococcal phages.

Polyhexanide and chlorhexidine susceptibility profiles of MRSA isolates before and after polyhexanide decolonization. Our previous published study suggested a limited efficacy of a single polyhexanide decolonization course in eradicating MRSA carriage (18). Despite several possible limitations of our study, one possible explanation was the emergence of resistance to polyhexanide or cross-resistance between chlorhexidine and polyhexanide antiseptics. Indeed, we previously reported that resistance to chlorhexidine in our hospital was associated with the dominant clone, the South German SCC*mec*I ST type 228 MRSA (3). To monitor potential polyhexanide and chlorhexidine reduced-susceptibility in our strain collection, we selected nasal MRSA strains isolated before (D0) and after active polyhexanide decolonization treatment (D28) (Tables 3 and 4). MLVA analysis was performed to confirm the clonal relationship between D0 and D28 bacterial strains isolated from the same patient and to deduce the ST-type of our strain collection (not shown). All selected pairs of strains isolated from the same patients were indeed clonally related and showed ST 228 (n=20), ST5 (n=2), ST8 (n=2), ST105 or ST22 (n=1).

Reduced susceptibility to polyhexanide and chlorhexidine was further measured using macrodilution minimal inhibitory concentration (MIC) method. Our strain collection shows polyhexanide and chlorhexidine MIC ranging between 0.25-1 µg/ml and 0.5-4 µg/ml, respectively, with a modal polyhexanide MIC of 0.5 µg/ml and of chlorhexidine MIC of 4 µg/ml (Table 3). According to the epidemiological cut-off value proposed by Fabry *et al*, our *S. aureus* collection is considered susceptible to

173 polyhexanide and 50% resistant to chlorhexidine (15). However, no correlation between

174 chlorhexidine and polihexanide susceptible profiles or cross-resistance was observed.

175 Moreover, the majority of our D28 isolates showed neither polihexanide nor

176 chlorhexidine MIC changes compared to isolates at D0. Altogether, our results suggest

177 that the limited MRSA decolonization rate previously observed (18, 17) is not related to

178 the presence or selection of strains with reduced-susceptibility to polyhexanide nor with

179 cross-resistance towards chlorhexidine.

180

181

182 **DISCUSSION**

183 This study focused on the development of polyhexanide reduced-susceptibility

184 and emergence of cross-resistance with other antiseptics or antibiotics in various MRSA

185 strains. We previously found that a single polyhexanide decolonization course was not

186 fully effective in eradicating MRSA carriage (18). This study performed in a population

187 mainly composed of MRSA harboring *qac* genes excludes the possibility that the

188 moderate decolonization rate of MRSA relies on the emergence of isolates showing

189 polyhexanide reduced-susceptibility or on potential cross-resistance between

190 polyhexanide and chlorhexidine in MRSA.

191 In the present study, we provide evidence that prolonged *in vitro* exposure to low

192 levels of polyhexanide results in the emergence of polyhexanide reduced-susceptibility

193 in MRSA without cross-resistance to chlorhexidine. Moreover, we repeatedly observed

194 concomitant changes in resistant profiles of daptomycin and glycopeptides, antibiotics

195 used for *S. aureus* clinical treatment. This observation should encouraged further *in vivo*

196 studies, as various local and low disinfectant concentrations can potentially be found

197 after topical administration of this substance or also found at residual levels on surfaces

198 (15, 24).

199   *In vitro*, we detected polyhexanide reduced-susceptibility (MIC changes from

200   0.5 to 4 µg/ml) following step-wise and prolonged (2 days) passages in low

201   concentrations of polyhexanide (< 2 µg/ml of polyhexanide). The occurrence of

202   polyhexanide reduced-susceptibility under low concentrations *in vitro* does not argue

203   against the general use of polyhexanide for decolonization, because the high therapeutic

204   concentration used, will highly exceed the low concentrations that permit resistance

205   development and will rapidly eradicate bacteria. However, it suggests careful follow-up

206   of resistance profiles during topical administration.

207   To understand the molecular pathways leading to polyhexanide reduced-

208   susceptibility, we performed whole genome sequencing and identified genetic changes

209   in strains selected *in vitro* under polyhexanide exposition compared to wild-type strains.

210   Mutations were found in *mprF* genes that can be correlated with polyhexanide reduced-

211   susceptibility. Indeed, polyhexanide is a cationic polymer attaching to negatively

212   charged molecules and acting on bacterial membrane phospholipids,

213   lipopolysaccharides, teichoic acids and peptidoglycan components of cell wall (9, 10).

214   The integral membrane protein MprF, lysinylate membrane lipid phosphatidyl glycerol

215   (PG) and subsequently flips lysyl phosphatidyl glycerol (L-PG) to the outer leaflet of

216   the plasma membrane (21). The *mprF* mutations detected in our strains may potentially

217   increase L-PG synthesis and flipping leading to increase of membrane positive surface

218   charge and consequently charge repulsion for cationic molecules, such as polyhexanide.

219   Interestingly, our mutants showing *mprF* mutations and reduced susceptibility to

220   polyhexanide also show resistance to the cationic antibiotic daptomycin. The identified

221   *mprF* mutation L337S is located in the so-called bifunctional domain of *mprF* known to

222   be a hot spot of *mprF* mutations leading to daptomycin resistance (25). Further studies

223   are underway to highlight the association of *mprF* mutations and antiseptic resistance, a

224   mechanism that to our knowledge has not been previously identified. Regarding *purR*

225 mutations, further studies are needed to understand the mechanistic link between *purR*

226 mutations and reduced-susceptibility to polyhexanide. In addition to containing the

227 *mprF* mutation described above, a polyhexanide-resistant mutant of 134947 (P6) was

228 found to contain a non-synonymous mutation in *purR*. This mutation has been

229 encountered elsewhere when selecting for resistance to the lantibiotic nisin (22), and

230 other *purR* mutations were discovered in mutants displaying resistance to vancomycin

231 (26). However, the observed *purR* mutations had no apparent role in nisin or

232 vancomycin resistance (22, 26). This also seems to be the case in polyhexanide

233 resistance, as an SH1000 strain containing PurR($V_{229}G$) was no more resistant to

234 polyhexanide as its parent. It is not clear why mutations in *purR* emerge when selecting

235 for resistance to antibiotics or antiseptics, however it is apparent that they are not

236 required to confer resistance to these agents.

237   An important observation of this study is the potential emergence of cross-

238 resistance between antiseptics and antibiotics used in clinical routine. This has been

239 observed for antiseptics such as chlorhexidine or triclosan in other bacterial species (27-

240 29). In *S. aureus,* cross-resistance between antiseptics and antibiotics was previously

241 observed after chlorhexidine exposure selecting for resistance to several β-lactam

242 antibiotics (30). To date, a single study assessed and found no correlation between

243 polyhexanide and antibiotic resistant profiles. However, the analyzed collection lacks

244 polyhexanide reduced-susceptible isolates, which prevents any conclusion on cross-

245 resistance between these molecules (15). Our results showed that development of

246 reduced-susceptibility to polyhexanide can be accompanied by changes in resistance to

247 not only daptomycin but cell-wall active antibiotics such as vancomycin and

248 teicoplanin. This can be expected if taking into account the mode of action of

249 polyhexanide. Any molecular change leading to alteration in cell wall could potentially

250 affect the net charge of cell wall and indirectly affect polyhexanide binding. This link

251 was reliably observed in independent experiments and in different bacterial genetic

252 backgrounds. However, we did not observe the development of polyhexanide reduced-

253 susceptibility accompanied always by an identical antibiotic resistant pattern, even

254 though identical genetic mutations were identified. Studies related to whole

255 transcriptomic would probably contribute to clarify the mechanisms leading to

256 alteration of susceptibility.

257 Our experiments were performed *in vitro* which appears as the main limitation. In

258 a recent clinical trials dedicated to assess the decolonisation efficacy of polyhexanide

259 (18), we were able to collect 27 pairs of MRSA resulting from cases of decolonization

260 failure. No significant MIC alterations for antibiotics were observed between pairs of

261 isolates in this small collection following polyhexanide exposition. Note however that

262 *in vivo*, bacteria are probably exposed to the concentrations used in our report at specific

263 body sites and that our design mimicking potential prolonged or repeated exposition to

264 antimicrobial solutions may reflect the *in vivo* situation. In these conditions, we reliably

265 obtained alteration of susceptibility to the polyhexanide as well as alteration in the

266 MRSA antibiotic susceptibility profiles, which is a potential risk of emergence of

267 antibiotic resistances, particularly in area showing generalized and extensive utilization

268 of antiseptic solutions.

269

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study are listed in Tables 3 and 4. Strains COL, 134947 and 128822 are MRSA strains belonging to different ST types and used to analyze phenotypic and genetic alterations following exposure to antiseptic solutions. The other MRSA isolates were collected from a previously published randomized, placebo-controlled trial, assessing the clinical efficacy of polyhexanide in eradicating MRSA carriage at day 28 (D28) after decolonization (18). Briefly, selected MRSA-colonized patients fulfilling inclusion criteria were randomized to receive either active treatment or placebo for 10 days. Active treatment (Prontoderm®Gel light solution containing polyhexanide 20% in a base of Glycerine 99% and Hydroxyethylcellulose; B. Braun Medical AG, Sempach, Switzerland) was applied intranasally three times a day to the anterior nares. At D28, swabs were taken from nares and identification of MRSA was performed as previously described (18). MRSA strains before (D0) and after treatment (D28) were saved frozen in skimmed milk for further determinations.

Molecular MRSA typing. MRSA isolates were subjected to a rapid genotyping assay using Multiple-Locus Variable Number of Tandem Repeats Analysis (MLVA) assay. Briefly, this assay is based on a multiplex PCR using ten primer pairs targeting 9 genes showing variable numbers of tandem repeats and an additional pair of primers allowing amplification of the *mecA* gene as internal control. This method shows discriminatory power that is at least similar to that of pulsed-field gel electrophoresis (31). The analysis was performed on isolated colonies grown on Mueller–Hinton agar disrupted by vortex agitation. PCR amplification was then evaluated using a micro capillary electrophoresis system (2100 Bioanalyzer, Agilent Technologies) and automatically analyzed using specifically developed software (31). The genotype of

295 each strain was deduced by comparison with profiles obtained with characterized

296 control isolates (31).

297 **Polyhexanide susceptibility testing.** Polyhexanide 20% solution was obtained

298 and prepared as recommended by the manufacturer. The stock solution was diluted in

299 the test broth to final polyhexanide concentrations of 0.25, 0.5, 1, 2 and 4 µg/ml.

300 polyhexanide minimum inhibitory concentration (MIC) was determined as previously

301 described (8) but using a macrodilution method. Briefly, one bacterial colony growing

302 on Mueller-Hinton agar was used to inoculate 1 ml of Mueller-Hinton broth. Overnight

303 culture at 37°C was diluted to deliver the final inoculum of 1.5 x 10^6 CFU/ml into each

304 tube containing different polyhexanide concentrations (MHB containing 0, 0.25, 0.5, 1,

305 2 and 4 µg/ml of polyhexanide). After incubation for 24-48h at 37°C, MIC was defined

306 as the lowest concentration allowing bacterial growth. Three independent MIC

307 determinations were performed for each isolate. Modal MICs for each isolate are

308 represented. Chlorhexidine MIC was determined as described above but using

309 chlorhexidine diluted to a final concentration of 0.12-16 µg/ml.

310 ***In vitro* selection of polyhexanide and chlorhexidine mutants with reduced**

311 **susceptibility.** The selected MRSA strains COL, MRSA134947 and MRSA128822

312 showing an initial polyhexanide MIC = 0.5, 1 and 1 µg/ml, respectively, were serially

313 passaged onto increasing concentrations of polyhexanide. Briefly, 100 ml of Muller

314 Hinton Broth (CAMHB) containing MIC concentration (0.5 or 1 µg/ml) of

315 polyhexanide was inoculated with overnight bacteria at a concentration of 1 x 10^9

316 bacteria/ml. After incubation for 2 days at 37°C, bacteria growing at concentration of

317 polyhexanide 0.5 or 1 µg/ml, were used for a second step passage in increased

318 polyhexanide concentrations. Further stepwise passages were done when indicated.

319 After passages bacteria were collected and macrodilution MIC was determined. An

320 identical methodology was used for selection of chlorhexidine reduced-susceptible

13

321 mutants of strains COL, MRSA134947 and MRSA128822, showing an initial

322 chlorhexidine MIC of 2, 4 and 8 µg/ml, respectively.

323 **Antimicrobial susceptibility testing.** The bacterial inoculum suspension was

324 prepared by selecting several colonies from overnight growth (16–24 h of incubation)

325 on Columbia Agar plates with a cotton swab and suspending the colonies in sterile

326 saline (0.85% NaCl w/v in water) to the density of a 0.5 McFarland standard,

327 corresponding to 3-4 x 10^8 CFU/ml. The inoculum was spread over the entire surface of

328 the Mueller–Hinton agar plate by swabbing in three directions, and the plates were

329 incubated in a humid atmosphere at $35 \pm 1°C$ for 18 ± 2 h. Antibiotic resistance profiles

330 were tested using disc diffusion assays according to EUCAST methods.

331 **Genome sequencing.** High-throughput sequencing was used to sequence the

332 genomes of all isolates. Genomic DNA from each isolate was purified by using DNeasy

333 columns (Qiagen), and then sequenced on the Illumina HiSeq 2500 (Illumina, San

334 Diego, CA, USA) using 100 bases paired-ends and barcodes according to the Nextera

335 XT kit (Illumina). Read sequence quality was assessed with the Fastqc program

336 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and filtered using the

337 fastq-mcf program (Ea-utils: http://code.google.com/p/ea-utils). Genome assembly was

338 performed using the Edena v3 assembler (32). Assembled genomes were annotated

339 using the Prokka v1.10 program (33). The phylogenetic relationships of isolates were

340 investigated by genomic single-nucleotide polymorphism (SNP)-based analysis using

341 the Parsnp v1.0 program (34). The proteome comparison of all isolates was performed

342 using the "CGView Comparison Tool" program (35). The BlastP analysis was used to

343 detect non-synonymous mutations.

344

14

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POTENTIAL CONFLICT OF INTEREST.

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478

479 **Table 1 : Polyhexanide and chlorhexidine resistant profiles of in vitro selected antiseptic mutants**

Strain	Antiseptic selection	Number of passages	Polyhexanide[a] MIC (µg/ml)	Chlorhexidine[a] MIC (µg/ml)
COL	-		0.5	2
COL P3	Polyhexanide	3	2	2
COL P5	Polyhexanide	5	4	2
134947	-		1	4
134947 P6	Polyhexanide	6	8	4
128822	-		1	8
128822 P6 [b]	Polyhexanide	6	1	8
COL	-		0.5	2
COL P10	Chlorhexidine	10	0.5	8
COL P7	Chlorhexidine	7	0.5	8
134947	-		1	4
134947 P10	Chlorhexidine	10	1	8
128822	-		1	8
128822 P10	Chlorhexidine	10	1	8

[a] Polyhexanide and chlorhexidine MIC are measured by macrodilution method

[b] Selection of Polyhexanide step-wise mutants was impossible with this strain

480

481 **Table 2 :** Antibiotic resistant profiles and single nucleotide polymorphisms present in MRSA antiseptic selected mutants compared to their corresponding wild-type strains

Strain	Antiseptic selection	Polyhexanide MIC (ug/ml)	Chlorhexidine MIC (ug/ml)	Antibiotic susceptibility pattern (MIC)[a]	ST type	Gene name	SNPs	AA changes	Gene Functions
COL		0.5	2	PenR, OxaR, ACR, CefR, VanS(1.5), Tei(1.5), Dap(0.5)	250				
COL P3	Polyhexanide	2	2	PenR, OxaR, ACR, CefR, VanR(3), TeiR(3), DapR(3)	250	mprF	C1010T	L337S	Phosphatidylglycerol lysyltransferase
COL P5	Polyhexanide	4	2	PenR, OxaR, ACR, CefR, TeiR(4), DapR(4)	250	mprF	C1010T	L337S	Phosphatidylglycerol lysyltransferase
134947		1	4	PenR, OxaR, ACR, CefR, CipR, CliR, EryR	228				
134947 P6	Polyhexanide	8	4	PenR, OxaR, ACR, CefR, CipR, CliR, EryR, DapR(1.5-2)	228	purR	T686G	V229G	Pur operon repressor
						mprF	C884T	S295L	Phosphatidylglycerol lysyltransferase
128822		1	8	PenR, OxaR, ACR, CefR, CipR, CliR, EryR, GenR	228				
128822 P6	Polyhexanide	1	8	PenR, OxaR, ACR, CefR, CipR, CliR, EryR, GenR	228	ND			

Strain	Antiseptic selection	Polyhexanide MIC (ug/ml)	Chlorhexidine MIC (ug/ml)	Antibiotic susceptibility pattern (MIC)[a]	ST type	Gene name	SNPs	AA changes	Gene Functions
COL		0.5	2	PenR, OxaR, ACR, CefR	250				
COL P10	Chlorhexidine	0.5	8	PenR, OxaR, ACR, CefR, CipR(D17)	250		T to A		Intergenic region (position 775770) between SACOLSACOL_RS03870 and NorA
							G to A		Intergenic region (position 814686) between SACOL_RS04065 and NrdL
							T to A		Intergenic region (positions 1227842) upstream SACOL_RS06240
							G to A		Intergenic region (position 1227910) upstream SACOL_RS06245SACOL_RS06245
COL P7	Chlorexidine	0.5	8	PenR, OxaR, ACR, CefR	250	mepA	C127T	T376I	Multidrug export protein MepA
						purR	G403T	V135F	Pur operon repressor
134947		1	4	PenR, OxaR, ACR, CefR, CipR, CliR, EryR	228				
134947 P10	Chlorhexidine	1	8	PenR, OxaR, ACR, CefR, CipR, CliR, EryR	228	ND			
128822		1	8	PenR, OxaR, ACR, CefR, CipR, CliR, EryR, GenR	228				
128822 P10	Chlorhexidine	1	8	PenR, OxaR, ACR, CefR, CipR, CliR, EryR, GenR, DapR(2)	228	pldB	G89T	G30V	Lysophospholipase L2
						glpD	C293A	T98K	Aerobic glycerol-3-phosphate dehydrogenase
						mprF	C1001T	P334L	Phosphatidylglycerol lysyltransferase

482

483 [a] Antibiotic susceptibility was measured by disc diffusion assays for all antibiotics except vancomycin, teicoplanin and daptomycin (D corresponds to diameter size measurement;
484 EUCAST ciprofloxacin susceptible diameter = 19-20). For vancomycin, teicoplanin and daptomycin, MIC values were determined by Etest assays. EUCAST susceptibility
485 breakpoints for vancomycin ≤2; teicoplanin ≤2; daptomycin ≤1. Changes in antibiotic resistance pattern compared to wild-type strains are shown in bold. Pen=penicillin;
486 Oxa=oxacillin; AC=amoxicillin-clavulanate; Cef=cefoxitine; Cip=ciprofloxacin; Cli=clindamycin; Ery=erythromycin; Gen= gentamicin; Van=vancomycin; Tei=teicoplanin;
487 Dap=daptomycin.

488　**Table 3** : Chlorhexidine and Polyhexanide MIC values of 54 MRSA clinical strains isolated before and

489　after Polyhexanide patient decolonization.

Strain number (Day 0)[*]	MIC (µg/ml)		Strain number (Day 28)[*]	MIC (µg/ml)	
	Chlorhexidine	Polyhexanide		Chlorhexidine	Polyhexanide
1	4	0.5	1a	4	0.5
2	< 0.5	0.5	2a	1 / 0.5	1
3	4	0.5	3a	4	1
4	< 0.5	0.5	4a	< 0.5	0.5
5	4	0.25	5a	2 / 4	0.5
6	4	0.5	6a	2	0.5
7	4	0.5	7a	4	0.5
8	1	0.5	8a	1	0.5
9	< 0.5	0.5	9a	< 0.5	0.5
10	4	0.5	10a	4	0.5
11	**4**	**0.5**	**11a**	**1**	**0.5**
12	4	0.25	12a	4	0.5
13	**4**	**1**	**13a**	**< 0.5**	**0.5**
14	4	0.5	14a	2	1
15	**1**	**1**	**15a**	**4**	**0.5**
16	2	0.5-1	16a	4	0.5
17	< 0.5	1	17a	< 0.5	0.5
18	4	0.5	18a	4	0.25
19	4	0.5	19a	4	0.5
20	2	0.5	20a	2	0.5
21	2	0.25	21a	2	0.25
22	< 0.5	0.5	22a	1	1
23	2	1	23a	1	1
24	4	0.5	24a	4	0.5
25	**4**	**0.5**	**25a**	**1**	**0.5**
26	4	0.5	26a	4	0.5
27	**2**	**1**	**27a**	**4**	**0.25**

* MRSA clinical strains isolated prior to Polyhexanide decolonization (Day 0) or after 28 days of polyhexanide treatment (D 28)

(a) denoted MRSA Day 28 bacteria clonally-related to Day 0 bacteria, isolated from the same patient

490　Bold font is used for pairs of strains showing the most important changes