Decreased susceptibility to chlorhexidine and prevalence of disinfectant resistance genes among clinical isolates of

**Staphylococcus epidermidis**

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*Staphylococcus epidermidis* is a versatile agent, being both a commensal and a nosocomial pathogen usually with an opportunistic role in association with implanted foreign body materials. Pre-operative antiseptic preparation is an important strategy for reducing the risk of complications such as surgical site infection (SSI). Currently, the most widely used antiseptics are alcohols, quaternary ammonium compounds (QACs), and the bisbiguanide chlorhexidine. Occurrence of resistance to the latter agent has drawn increasing attention. The aim of this study was to investigate if decreased susceptibility to chlorhexidine among *S. epidermidis* was present in our setting, a Swedish university hospital.

*Staphylococcus epidermidis* (n = 143), retrospectively collected, were obtained from prosthetic joint infections (PJI) (n = 61), post-operative infections after cardiac surgery (n = 31), and the skin of the chest after routine disinfection prior to cardiac surgery (n = 27). In addition, 24 commensal isolates were included. Minimum inhibitory concentration (MIC) of chlorhexidine was determined on Mueller Hinton agar plates supplemented with serial dilutions of chlorhexidine. Five QAC resistance genes, *qacA/B*, *smr*, *qacH*, *qacJ*, and *qacG*, were detected using PCR. Decreased susceptibility to chlorhexidine was found in 54% of PJI isolates, 68% of cardiac isolates, 21% of commensal isolates, and 7% of skin isolates from cardiac patients, respectively. The *qacA/B* gene was present in 62/143 isolates (43%), *smr* in 8/143 (6%), and *qacH* in one isolate (0.7%). The *qacA/B* gene was found in 52% of PJI isolates, 61% of cardiac isolates, 25% of commensal isolates, and 19% of the skin isolates. In conclusion, decreased susceptibility to chlorhexidine, as well as QAC resistance genes, were prevalent among *S. epidermidis* isolates associated with deep SSIs.

Key words: *Staphylococcus epidermidis*; chlorhexidine; prosthetic joint infection; biocide resistance; nosocomial infection.

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The human skin and mucous membranes display a complex and heterogeneous flora of microorganisms. Coagulase-negative staphylococci (CoNS) constitute a major part of this commensal flora. During the past decades, CoNS were recognized as important nosocomial pathogens acting as opportunists in association with implanted foreign body materials (e.g., prosthetic joints and heart valves) or in highly immunocompromised patients (1).

Previously, CoNS have been regarded mainly as commensals, and the surveillance of the transmission of CoNS in hospital settings has been neglected. This may have contributed to the extensive transmission of specific methicillin as well as multidrug-resistant clones of CoNS in the hospital environment (1, 2). In addition, it has been reported that CoNS may act as a reservoir of resistance genes that can be transmitted to other bacterial species (1, 3).

Disinfectants are widely used in hospitals and other healthcare settings to prevent transmission of bacteria and thereby nosocomial infections. Pre-operative disinfection of the skin is an important strategy for reducing the risk of complications such as surgical site infections (SSIs). The currently most

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widely used antiseptic compounds are alcohols and chlorhexidine. Alcohols exhibit rapid broad-spectrum antimicrobial activity, but low levels of chlorhexidine are often included in the preparations to obtain a prolonged effect after evaporation of the alcohol (4). Chlorhexidine, a bisbiguanide, is bactericidal and the mechanism of action involves damages to both the outer cell wall layers and the cytoplasmic membrane (4). However, the efficacy of chlorhexidine may be diminished by the presence of biological material or biofilm, and the bactericidal efficacy is pH-dependent (4–6). Importantly, several gene-encoding resistance mechanisms common for quaternary ammonium compounds (QACs) and chlorhexidine have been identified and encode predominantly efflux pumps. The QAC resistance genes are harboured on plasmids, the qacA/B on large plasmids, and additional genes, such as smr (staphylococcal multidrug resistance, also known as qacC/D), qacG, qacH, and qacJ, are found on small plasmids (3, 4, 7). Presence of these QAC genes has been reported from both S. aureus, including methicillin-resistant Staphylococcus aureus (MRSA) (8–10), and various CoNS species (11–14).

In previous studies, we have investigated CoNS isolates causing infections following prostatic joint surgery as well as cardiac surgery, surgical procedures that are preceded by thorough strategies for disinfection (15–17). Despite these disinfection regimens, the prevalences of deep post-operative infections still remain at approximately 1% and 4%, respectively (15, 18). In a recent study (19), we were also able to isolate CoNS from the skin immediately before incision in cardiac patients, i.e., after disinfection with chlorhexidine in alcohol in the operating theatre. The aim of the present study was to investigate, by using both phenotypic and genotypic methods, if decreased susceptibility to chlorhexidine among S. epidermidis isolates was present in our setting.

**MATERIAL AND METHODS**

**Bacterial isolates**

The study included a total of 143 Staphylococcus epidermidis isolates, determined to species level by the ID32 Staph system (bioMérieux, Marcy l’Etoile, France), and in many cases, confirmed by sequencing of the rpoB gene (20). The origin of the isolates was as follows:

Sixty-one isolates were obtained from multiple tissue biopsies taken peri-operatively from 61 different patients during revision surgery for prosthetic joint infections (PJs) with extraction or exchange (hip (n = 46); knee (n = 13); elbow (n = 1); shoulder (n = 1)). The revisions were conducted from 1993 to 2008 (17).

From the LOGIP (15) and the LOGIX (16) trials, performed from 2000 to 2002 and from 2007 to 2009, respectively, 31 S. epidermidis isolates that caused deep surgical site infections (mediastinitis and/or sternitis) were examined. These trials investigated the effect of prophylactic use of locally administered gentamicin containing sponges (collatamp-G; Schering Plough, Stockholm, Sweden) with the end point post-operative infections after cardiac surgery.

From the ReCol study (19) performed between 2010 and 2011 and aimed to investigate the bacterial recolonization of the skin of patients undergoing cardiac surgery, S. epidermidis isolates (n = 27) cultured from the skin of the chest after routine pre-operative skin preparation with disinfection with 0.5% chlorhexidine solution in 70% ethanol (before incision) were included.

Commensal S. epidermidis isolates (n = 24) from healthy individuals without any recent contact with health care were also examined. These isolates were obtained from the nares (n = 13) collected in 2006 or the skin of the wrist (n = 11) collected in 2000.

**Determination of the MIC of chlorhexidine using agar dilution method**

Mueller Hinton (MH) agar plates [3.8% w/v BBL™ Mueller Hinton II Agar (BD Corporation, Franklin Lakes, New Jersey, USA)] were supplemented with 0.0025, 0.005, 0.01, 0.02, 0.04, and 0.08 mg/L chlorhexidine digluconate (Sigma-Aldrich, St Louis, MO, USA).

The isolates were subcultured in aerobic atmosphere at 37 °C overnight on MH agar plates without chlorhexidine digluconate. Approximately five to seven colonies were suspended in 2.5 mL of sterile saline solution (0.9% NaCl), and the density was adjusted to 0.5 McFarland using an Oxdox Turbidometer (Integrated Technologies Limited, Basingstoke, UK). An inoculum of 1 µL of the bacterial suspension was then inoculated on MH agar plates with serial dilutions of chlorhexidine digluconate by using a Multipoint Elite dispenser (Mast Group LTD, Merseyside, UK). As growth controls, MH agar plates without chlorhexidine digluconate were inoculated before and after the MH agar plates with serial dilutions of chlorhexidine digluconate. Bacterial growth on the agar plates was determined following 24-h and 48-h incubation in aerobic atmosphere at 37 °C.

**Isolation of DNA**

DNA was isolated from bacterial colonies by using the Bullet BUGS’n BEADS kit on the NorDiag Bullet extraction instrument, in accordance with the instructions from the manufacturer (NorDiag ASA, Oslo, Norway). All DNA preparations were stored at 4 °C prior to the PCR.

**Real-time PCR**

The amplification of the QAC resistance genes, qacA/B, smr, qacG, qacH, and qacJ were performed in a LightCycler PCR system 1.2 and 1.0 (Roche Molecular Biochemicals, Mannheim, Germany) using SYBR Green I fluorescence melting curve analysis to detect the specific amplicon. The PCR mixture contained 0.5 µM of respective primers (Scandinavian Gene Synthesis AB, Köping, Sweden), shown in Table 1, 3 mM MgCl₂, 1× LightCycler FastStart

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DNA Master SYBR Green I (Roche Molecular Biochemicals). The PCR programs started with a pre-incubation at 95 °C for 10 min, followed by 40 cycles of amplification (Table 1) and ended with melting curve analysis by a rapid heating to 95 °C, followed by 65 °C for 15 s, and then slowly raising the temperature to 95 °C with 0.1 °C/s. In each PCR run, one positive control (Table 1) and one negative control (water instead of DNA template) were included.

Initial confirmation of the PCR product was made by means of 2% agarose gel electrophoresis and DNA sequencing using the ABI PRISM BigDye Terminator v 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems).

**Antibiotic susceptibility testing**

Antibiotic susceptibility testing was performed on MH agar using the disc diffusion method (Oxoid, Cambridge, UK) and incubated in aerobic atmosphere at 35 °C for 16–20 hours. The breakpoints applied for antibiotic susceptibility were in accordance with the Nordic Committee on Antimicrobial Susceptibility Testing (www.nordicast.org). Norfloxacin 10 μg disc was used for screening for fluoroquinolone (FQ) resistance including ciprofloxacin.

**RESULTS**

**Chlorhexidine susceptibility among *S. epidermidis* isolates**

The distribution of MIC values for chlorhexidine after 24 and 48 h of incubation using agar dilution is shown in Table 2. *S. epidermidis* isolates obtained from post-operative infections (PJIs and cardiac surgery infections) displayed decreased susceptibility to chlorhexidine to a statistically significantly higher extent than commensals (Mann–Whitney; p = 0.0001). Decreased susceptibility to chlorhexidine was rare among isolates obtained in the ReCol study representing *S. epidermidis* still present on the skin following pre- and peri-operative disinfection. An increase in MIC values between 24-h and 48-h incubation was seen in 13 cases, in 8 from fully

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**Table 1.** Oligonucleotides sequences of primers used and PCR conditions for detection of *qac* genes in *Staphylococcus epidermidis*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward/reverse 5′–3′ primer</th>
<th>References</th>
<th>Product size</th>
<th>Amplification</th>
<th>Positive control (ref)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>qacA/B</em></td>
<td>GCTGCAATTATGACAATGTTTG AATCCCACCTACTAAAGCAG</td>
<td>3</td>
<td>630 bp</td>
<td>95 °C in 10 s 53 °C in 10 s 72 °C in 25 s</td>
<td><em>S. haemolyticus</em> NVH97A (14)</td>
</tr>
<tr>
<td><em>smr</em></td>
<td>ATAAAGTCTAGAAGTTATTGGAAGTT TTTCCAATAAGTTAACAAGTAACTA</td>
<td>24</td>
<td>286 bp</td>
<td>95 °C in 10 s 57 °C in 10 s 72 °C in 17 s</td>
<td><em>S. aureus</em> NVH99 (14)</td>
</tr>
<tr>
<td><em>qacG</em></td>
<td>CAAACAGAAATAATCGGAACTTACATTTAAGGCACTA</td>
<td>17</td>
<td>275 bp</td>
<td>95 °C in 10 s 45 °C in 10 s 72 °C in 12 s</td>
<td><em>S. warneri</em> ST94 (14)</td>
</tr>
<tr>
<td><em>qacH</em></td>
<td>ATATGTCAGTGAAGTTATTGGAAGTTAAGT</td>
<td>17</td>
<td>295 bp</td>
<td>95 °C in 10 s 45 °C in 10 s 72 °C in 12 s</td>
<td><em>S. saprophyticus</em> ST2H6 (14)</td>
</tr>
<tr>
<td><em>qacJ</em></td>
<td>CGTTATATTTAATAGGCAGATCCAAAAACGTTAAGA</td>
<td>17</td>
<td>306 bp</td>
<td>95 °C in 10 s 43 °C in 10 s 72 °C in 12 s</td>
<td><em>S. aureus</em> NVH01 (14)</td>
</tr>
</tbody>
</table>

**Table 2.** Cumulative distribution of growth *Staphylococcus epidermidis* isolates (n = 143) after 24-h and 48-h incubation, respectively, on agar plates with serial dilutions of chlorhexidine

<table>
<thead>
<tr>
<th>Chlorhexidine concentration</th>
<th>0 mg/L</th>
<th>0.0025 mg/L</th>
<th>0.005 mg/L</th>
<th>0.01 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>Commensals1 (n = 24)</td>
<td>24</td>
<td>24</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Wrist (n = 11)</td>
<td>11</td>
<td>11</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Nose (n = 13)</td>
<td>13</td>
<td>13</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Prosthetic joint infections (n = 61)</td>
<td>61</td>
<td>61</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td>Cardiac surgery infections (n = 31)</td>
<td>31</td>
<td>31</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>LOGIP (n = 20)</td>
<td>20</td>
<td>20</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>LOGIX (n = 11)</td>
<td>11</td>
<td>11</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>ReCol2 (n = 27)</td>
<td>27</td>
<td>27</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

1Isolates from persons with no recent health care contact.
2The ReCol study investigated bacterial recolonization of the skin of patients undergoing cardiac surgery following pre-operative skin disinfection.
susceptibility to decreased susceptibility. In five cases with decreased susceptibility, an additional increase in MIC value was noted following further incubation.

**Detection of QAC genes**

The distribution of QAC resistance genes, *qacA/B*, *smr*, *qacG*, *qacH*, and *qacJ*, appears in Table 3. The *qacA/B* gene was present in 62 of 143 isolates (43%), *smr* in 8/143 (6%), and *qacH* in one isolate (0.7%). None of the isolates was positive for *qacG* or *qacJ*. Two isolates were positive for both *qacA/B* and *smr*.

There was a statistically significant difference in the prevalence of *qacA/B* between *S. epidermidis* isolates from clinical infections compared with commensals (including ReCol) (Fisher exact test; *p* < 0.0001). This difference was also seen when comparing *S. epidermidis* isolates from deep cardiac post-operative infections with those obtained from the skin of patients who had undergone disinfection with the chlorhexidine-ethanol solution (Fisher; *p* = 0.001) as well as when comparing commensals with PJIs isolates (Fisher; *p* = 0.03).

**Correlation between presence of QAC genes and decreased susceptibility to chlorhexidine**

A decreased susceptibility to chlorhexidine was found more frequently among isolates carrying *qacA/B* genes compared with those not harbouring *qacA/B* genes, mean MIC value 0.0036 and 0.00056 mg/L, respectively (Fig. 1). This difference was statistically significant (Mann–Whitney; *p* = 0.001).

The presence of the *smr* gene did not correlate with a decreased susceptibility to chlorhexidine.

**Antibiotic susceptibility patterns and correlation with presence of QAC genes**

The results of the antimicrobial susceptibility testing of all *S. epidermidis* isolates (n = 143) are summarized in Fig. 2. Multi-drug resistance (MDR), i.e., resistance to ≥3 antimicrobial classes, was more prevalent among isolates obtained from PJIs (49/61) and SSI after cardiac surgery (18/31), respectively, compared with commensals (2/24) and isolates from the ReCol study (1/27). A strong statistical association between MDR and presence of *qacA/B* genes was also noted (p < 0.0001). In addition, 44 of 69 isolates resistant to FQ were positive for *qacA/B* and 44 of 62 *qacA/B* positive isolates were FQ-resistant.

**DISCUSSION**

In the present study, 143 isolates of *S. epidermidis* were investigated for the presence of gene-encoding resistance mechanisms for quaternary ammonium compounds as well as decreased susceptibility to chlorhexidine phenotypically.

The prevalence of gene-encoding resistance determinants was higher among the isolates obtained from surgical infections, i.e., PJIs and SSIs following prosthetic joint infections and cardiac surgical site infections as well as isolates sampled following routine pre-operative skin preparation with alcohol and chlorhexidine but before incision ("ReCol").

### Table 3. Prevalence of QAC resistance genes in *Staphylococcus epidermidis* isolates regarded as commensals, obtained from prosthetic joint infections and cardiac surgical site infections as well as isolates sampled following routine pre-operative skin preparation with alcohol and chlorhexidine but before incision ("ReCol").

<table>
<thead>
<tr>
<th></th>
<th><em>qacA/B</em></th>
<th><em>smr</em></th>
<th><em>qacH</em></th>
<th><em>qacJ</em></th>
<th><em>qacG</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Commensals (n = 24)</td>
<td>6 (25%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nose (n = 13)</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wrist (n = 11)</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Prosthetic joint infections (n = 61)</td>
<td>32 (52%)</td>
<td>3 (5%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cardiac surgical site infections (n = 31)</td>
<td>19 (61%)</td>
<td>3 (10%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>LOGIP (n = 20)</td>
<td>13 (65%)</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>LOGIX (n = 11)</td>
<td>6 (55%)</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ReCol (n = 27)</td>
<td>5 (19%)</td>
<td>2 (7%)</td>
<td>1 (4%)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

For explanations, see Table 2.

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cardiac surgery compared with commensals and S. epidermidis isolated from the skin following pre-operative disinfection (ReCol). Nevertheless, 25% of the commensals harboured qacA/B genes despite the fact that these isolates were obtained from 24 different, healthy individuals without any recent contact with a healthcare facility or hospital. A higher proportion of these isolates were obtained from wrists compared with nares. A plausible explanation for this may be that the flora on the wrists to a higher extent represents the transient flora, while CoNS colonizing the nose represent the persistent flora.

Quaternary ammonium compound resistance genes have previously been reported among S. aureus and predominantly among MRSA (8, 9, 11–13). However, reports regarding the prevalence of qac genes among CoNS and especially among S. epidermidis causing clinically significant infections are sparse. Yet, already, during the 1990s, qac genes were found among CoNS (11). Later, the distribution of these genes among various CoNS of bovine and caprine origin was reported (14). Recently, the prevalence of qacA/B and smr genes among hospital care workers and in a general population in Hong Kong has been reported (12) as well as among CoNS obtained from dialysis patients in Tunisia (13). In the latter study (13), presence of qacA/B, but not qacC, was uncommon among S. epidermidis isolates obtained from clinical samples. In our study, qacA/B was the most common disinfectant resistance determinant among S. epidermidis followed by a limited number of isolates comprising smr. Only one single isolate harboured qacH and no isolates carrying qacG or qacJ were found.

There is no accepted definition for resistance towards chlorhexidine (21). MIC determination by the broth microdilution method has been reported (22). We demonstrated, besides presence of genes encoding efflux pumps for quaternary ammonium compounds, such as qacA/B and smr, decreased susceptibility to chlorhexidine by using an agar dilution method. The results of this phenotypic method were in concordance with the genetic analysis.

The clinical relevance of decreased susceptibility to biocidal components has been questioned (23). When the bacteria are exposed to efficient concentrations of chlorhexidine, the bacteria may be killed by membrane damage. However, if the bacteria do have mechanism for counteracting chlorhexidine, e.g., efflux pumps, the concentration of chlorhexidine that the microbe is exposed to and the duration of exposure might be important.

Previous studies regarding MRSA have shown that efflux pump for biocidal components do exist (8–10, 12). It is possible that these nosocomial strains do have an advantage regarding survival in a hospital environment where they probably are exposed to residues of biocidal components. It is also possible that the accumulation of these genes encoding resistance against QAC covariate with the accumulation of genes encoding resistance against antimicrobial agents, such as an association between presence of qacA/B genes and FQ resistance observed.

In the present study, we found a strong correlation between presence of MDR and genes encoding qacA/B. These MDR strains were also associated with decreased susceptibility to chlorhexidine. MDR S. epidermidis was predominantly isolated...
from clinical infections, i.e., PJIs and SSIs following cardiac surgery, probably representing nosocomial strains that successively accumulate resistance genes including genes encoding resistance against QAC. Hospitalized patients may contract these strains during their hospital stay as otherwise healthy individuals or patients without any recent contact with health care do not carry these specific strains (24). Accordingly, the implementation of infection control measures and adherence to basic hygiene instructions is crucial to prevent transmission of MDR CoNS and thereby to circumvent colonization of the hospitalized patients.

Various concentrations of chlorhexidine, e.g., 0.5% and 2%, respectively, in 70% ethanol have been used and it has been claimed that the total amount of chlorhexidine that will be administered and retained in the keratinocytes is of importance (25). However, a recent Cochrane review (26) did not provide any clear evidence of benefit for pre-operative showering or bathing with chlorhexidine, over other wash products, to reduce surgical site infection.

In the present study, S. epidermidis isolated from the skin, following pre-operative preparation with showers three times with chlorhexidine soap and subsequent disinfection with chlorhexidine in alcohol immediately before incision, did not display a higher prevalence of genes encoding resistance against QAC than commensals. In addition, they did not display multi-drug resistance. Thus, pre-operative strategies to reduce post-operative infections by using chlorhexidine did not seem to select for isolates with decreased susceptibility against chlorhexidine, and the isolates present could be members of the commensal flora not completely eradicated by the disinfection procedure. However, this question has to be explored in further prospective studies.

A limitation of the present study is the fact that the isolates used were collected from various previous studies representing various time periods and that the number of isolates from the specific studies is limited.

In conclusion, in the present study, S. epidermidis isolated from clinical infections displayed higher prevalence of genes encoding resistance against QAC as well as decreased susceptibility against chlorhexidine compared with commensal strains.

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