Effects of alcohols, povidone-iodine and hydrogen peroxide on biofilms of Staphylococcus epidermidis

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Objectives: To test the effects of several biocides [N-propanol, a commercially available propanol/ethanol/chlorhexidine mixture, polyvinylpyrolidone (povidone-iodine) and hydrogen peroxide] on established biofilms of Staphylococcus epidermidis isolated from patients with cardiac implant infections and catheter-related bacteraemia.

Methods: Biofilms were grown in microtitre plates for 24 h, dyed and stained with Crystal Violet. The mean optical density (OD) and the OD ratio (ODr = OD of the treated biofilm/OD of the untreated biofilm) were used for quantification. Biofilms were incubated with 60% (v/v) N-propanol, the mixture of propanol/ethanol/chlorhexidine, hydrogen peroxide at three concentrations (0.5%, 3% and 5%, v/v) and povidone-iodine for 1, 5, 15, 30 and 60 min. Unstained biofilms were sonicated and plated on Columbia agar for time–kill curves. S. epidermidis skin isolates from healthy volunteers were used as controls.

Results: Biofilm ODs of the clinical S. epidermidis isolates and the isolates from the healthy volunteers were significantly different (1.17 ± 0.512 versus 0.559 ± 0.095, respectively; mean ± SD) (P < 0.01). No viable S. epidermidis was detected in biofilms treated with the alcohols, N-propanol or the propanol/ethanol/chlorhexidine mixture. Incubation with povidone-iodine and hydrogen peroxide 3% and 5% led to a log reduction of the viable cells of >5 after incubation for 5 min, however, up to 103 viable cells were detected in four isolates after 30 min of incubation with povidone-iodine.

Conclusions: S. epidermidis obtained from infected implants forms thicker biofilms than that of healthy volunteers. Hydrogen peroxide, at a concentration of 3% and 5%, and alcohols rapidly eradicate S. epidermidis biofilms, whereas povidone-iodine is less effective.

Keywords: S. epidermidis, biocides, staphylococci

Introduction

Infections of implants, i.e. cardiac pacemakers or prosthetic heart valves, are difficult-to-treat infections.1 Although usually less serious in outcome, bacteraemia originating from intravascular catheters is common, leading to prolonged hospitalization and to increased costs.2 Staphylococcus epidermidis, a normal inhabitant of the human skin and mucosa, is the most frequent cause of catheter-associated sepsis and implant infections.3 Biofilm, a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface, is a natural form of existence of S. epidermidis. Biofilm formation is a renowned virulence factor of S. epidermidis.4 Treating biofilm-associated infections with antibiotics is often inefficient and leads to the explantation of the implant.

To assess whether S. epidermidis isolates from implant infections and patients with catheter-associated bacteraemia (CAB) were susceptible to the biocides used in the clinical practice for
pre-operative skin and hand disinfection, we tested the effects of hydrogen peroxide, povidone-iodine solution, 60% (v/v) N-propanol and a commercially available mixture of propanol/ethanol/chlorhexidine on established bacterial biofilms.

Materials and methods

Bacterial isolates investigated

Thirty isolates of S. epidermidis, including 10 isolates from patients with implanted cardiac devices (pacemakers, implanted defibrillators and prosthetic valves), 10 isolates from patients with intravascular CAB and 10 isolates from the skin of healthy volunteers, were investigated. Selection criteria of the healthy volunteers included having had no hospital contact and not receiving any antibiotics for 3 months. They were used as controls with regard to biofilm formation or effects of the biocide treatment of the biofilms. As a control strain, S. epidermidis DSM 3269 (Deutsche Sammlung von Mikroorganismen und Zellkulturen/German Collection of Microorganisms and Cell Cultures; http://www.dsmz.de/) was tested. The isolates were identified as strains using the pulsed field electrophoresis genotyping method as described previously.6 The isolates were identified using routine laboratory methods and stored at −70°C.5 Susceptibility testing was performed using the routine laboratory methods according to the recommendation of the CLSI (formerly NCCLS) (M02-A6). Antibiotic susceptibility was determined by the disc diffusion method on cation-adjusted Mueller–Hinton agar (bioMerieux, l’Étoile, France). The isolates were identified as strains using the pulsed field electrophoresis genotyping method as described previously.6

Definitions of infections

Definitions of the infections were as follows: implant infection [foreign body infection (FBI)] was defined as the presence of an implanted foreign body, signs and symptoms of systemic infection and the presence of S. epidermidis in at least two blood cultures and—if explanted—on the foreign body after removal. CAB was tested according to the criteria of the International Sepsis Forum Consensus Conference.7

Disinfectants and antimicrobial solutions

Two biocides used for rinsing of wounds and other infected sites accessible from the body surface were investigated: a commercially available povidone-iodine solution (100 mL contains 10 g of povidone-iodine, glycerol, citric acid, sodium hydroxide, potassium iodate, aqua destillata; Betaisodona®, Braun, Austria) and hydrogen peroxide (Merck, Germany) at concentrations of 0.5%, 3% and 5% prepared in sterile water. Two skin disinfectants were evaluated: 60% (v/v) N-propanol and a commercially available mixture of 2-propanol, 1-propanol, ethanol and chlorhexidine gluconate (Biotensid®, Schülke & Mayr, Austria).

Biofilm model

Biofilms were studied using the static microtitre plate model established by Christensen et al.5 Briefly, the S. epidermidis isolates were prepared in brain heart infusion broth (BHI; Oxoid, Germany) at a density equivalent to that of a 0.5 McFarland standard and diluted 1:100 with BHI. Biofilms were formed on a 96-well polystyrene flat-bottomed microtitre plate (Nunc, Germany) incubated for 24 h in ambient air at 35°C. Media and planktonic cells were removed. Subsequently, the biofilms in the wells were fixed and stained with Crystal Violet. After washing, the biofilms were quantified using the mean optical density (OD) at 550 nm wavelength in a microtitre plate-reader. All biofilm experiments were performed five times. Biofilm formation was verified in the Low Vacuum mode of an environmental scanning electron microscope (FEI Quanta 600 FEI).

To test the anti-biofilm effects of the biocides, the biofilms were incubated with 100 µL of the solutions for 1, 5, 15, 30 and 60 min at 35°C ambient air. Five wells per isolate were tested for each concentration and each substance. For calculation of the decrease of the biofilm OD, a ratio of the biofilm OD of the isolate incubated with antibiotic to the biofilm OD of the same isolate without antibiotic (control) was calculated. This OD ratio (ODr = OD of the treated biofilm/OD of the untreated biofilm) was used to measure changes in the thickness of the biofilms over time. For time–kill curves, the biofilms were not stained, but scraped off and re-suspended in MHB, seeded to Columbia agar and examined for growth. The viable count of S. epidermidis in suspension was assessed by serial dilutions, and 10 µL of each dilution was plated onto blood agar plates. The plates were then incubated at 35°C in ambient air and read after 48 h. The efficacy was expressed by the bacterial log reduction, which is the ratio of pre-values (number of cfu sampled before treatment) and post-values (number of cfu sampled after treatment) expressed by the decimal logarithm. Significance of differences (P < 0.05) was assessed using the Mann–Whitney U-test.

Results

Overall, biofilm ODs of the clinical S. epidermidis isolates and the isolates from the healthy volunteers were significantly different (1.17 ± 0.512 versus 0.559 ± 0.095, respectively; mean ± SD) (P < 0.01). Biofilm ODs of the isolates from patients with implant infections were not significantly different to those of isolates from patients with CAB, 1.36 ± 0.593 versus 0.973 ± 0.431, respectively.

Hydrogen peroxide significantly reduced the biofilm OD after 1 min of incubation; no further reduction was seen at the later time points (Figure 1). Hydrogen peroxide 3% reduced the biofilm (ODs, baseline: 1) significantly compared with hydrogen peroxide 0.5% (ODs, 1 min: 0.366 ± 0.221 versus ODs, 1 min: 0.783 ± 0.261) (P < 0.001). Increasing the concentration of hydrogen peroxide to 5% (ODs at 1 min: 0.312 ± 0.188) did not result in a further reduction of the biofilm OD. The performance of hydrogen peroxide was not different on the biofilms in all three groups; FBI, CAB and controls. Treating the biofilms with the 60% N-propanol, the propanol/ethanol/chlorhexidine mixture, or with povidone-iodine showed no reduction of the OD (Figure 1). The ODs values of the biofilms of the control strain S. epidermidis DSM 3269 are given in Table 1.

In the time–kill cultures, no viable S. epidermidis was detected in biofilms treated with the alcohols, N-propanol or the propanol/ethanol/chlorhexidine mixture. Incubation with povidone-iodine and hydrogen peroxide 3% and 5% led to a log reduction of the viable cells of >5 after incubation for 5 min. After incubation of the biofilms for 1 min, hydrogen peroxide at concentrations of 3% and 5% was bactericidal in 90% and 76%, respectively. Povidone-iodine reduced the mean log count of viable cells by 5 but up to 103 viable cells were detected even after an incubation for 30 min in four isolates. With regard to the control strain S. epidermidis DSM 3269; 60% N-propanol,
the propanol/ethanol/chlorhexidine mixture, povidone-iodine and hydrogen peroxide 5% were bactericidal after incubation for 1 min. Hydrogen peroxide 0.5% was not bactericidal at all.

Discussion

In clinical practice, the management of a biofilm-associated implant infection combines both medical and surgical treatments, preferentially with removal of the implant. However, if removal of the infected implant is not feasible, the therapy has to rely on treatment with antibiotics alone. The present study shows that *S. epidermidis* obtained from patients with implant or vascular catheter infections are presumably thicker than the isolates of healthy volunteers as expressed by the measured OD. Within these biofilms formed probably as a reaction to an aggressive environment set up by the immune response and/or low levels of antibiotics, the *S. epidermidis* cells undergo changes in phenotype and metabolism resulting in increased resistance to standard antibiotic treatment.

Alcoholic disinfectants such as *N*-propanol and ethanol, but also mixtures of alcohols with the biguanide chlorhexidine, are the backbone for disinfection of skin and hands. Alcohols are most commonly used for disinfection and pre-operative preparation of the skin. However, resistance to commonly used biocides has been described. In the present study, the effectiveness of 60% *N*-propanol and the propanol/ethanol/chlorhexidine mixture on *S. epidermidis* strains isolated from patients with FBI and CAB was tested. The effectiveness was confirmed by an excellent bactericidal activity in time–kill curves. Hydrogen peroxide at concentrations of 3% and 5% was the most effective to reduce the biofilm density in the elimination of biofilms and killing of the bacteria. Both elimination of the biofilms and killing of the bacteria were achieved. Thus, hydrogen peroxide at a concentration of 3% (v/v) may be used on biofilms on implants, on the implant-surrounding tissue, the skin surface or on infected wounds without device (Figure 1). However, a drawback of hydrogen peroxide is toxicity and irritation of the skin or tissue when used for longer contact times. Therefore, the use of hydrogen peroxide as a rinsing solution for wounds or fistulas has to be carefully considered before application. Povidone-iodine has been widely used as a rinsing solution in surgery. In the present study, on incubation with povidone-iodine, a low number of viable bacteria persisted in spite of a 5 log bacterial reduction within the biofilms. Thus, the

Table 1. OD₄ of the biofilms of the control strain *S. epidermidis* DSM 3269 (mean ± SD)

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>1</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% H₂O₂</td>
<td>0.530±0.204</td>
<td>0.503±0.111</td>
<td>0.536±0.115</td>
<td>0.506±0.147</td>
<td>0.627±0.065</td>
</tr>
<tr>
<td>3% H₂O₂</td>
<td>0.306±0.059</td>
<td>0.391±0.118</td>
<td>0.412±0.044</td>
<td>0.339±0.139</td>
<td>0.513±0.087</td>
</tr>
<tr>
<td>5% H₂O₂</td>
<td>0.249±0.059</td>
<td>0.316±0.083</td>
<td>0.331±0.065</td>
<td>0.216±0.167</td>
<td>0.508±0.055</td>
</tr>
<tr>
<td>60% N-propanol</td>
<td>0.757±0.189</td>
<td>1.037±0.558</td>
<td>0.957±0.347</td>
<td>0.597±0.520</td>
<td>1.323±0.231</td>
</tr>
<tr>
<td>Alcohol mixture</td>
<td>1.064±0.258</td>
<td>1.064±0.237</td>
<td>1.058±0.301</td>
<td>1.173±0.288</td>
<td>0.854±0.318</td>
</tr>
<tr>
<td>Povidone-iodine</td>
<td>0.620±0.222</td>
<td>0.659±0.161</td>
<td>0.974±0.142</td>
<td>0.781±0.276</td>
<td>1.221±0.200</td>
</tr>
</tbody>
</table>

OD₄ untreated biofilm=1.
use of povidone-iodine solution for local treatment of biofilm may result in a certain number of persisting bacteria.

In conclusion, alcohols and hydrogen peroxide at concentrations of 3% and 5% rapidly eradicate \textit{S. epidermidis} biofilms, whereas povidone-iodine is less effective. Thus, hydrogen peroxide and alcohols can be used to fight \textit{S. epidermidis} biofilms on surfaces. However, in deep infections of cardiac implants or vascular catheters the use of the tested substances is not feasible, and efforts have to be focused on the development of new parenteral antibiotics with antibiofilm properties.

**Transparency declarations**

None to declare.

**References**


