Susceptibility of livestock-associated methicillin-resistant Staphylococcus aureus (LA-MRSA) to chlorhexidine digluconate, octenidine dihydrochloride, polyhexanide, PVP-iodine and triclosan in comparison to hospital-acquired MRSA (HA-MRSA) and community-aquired MRSA (CA-MRSA): a standardized comparison

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Abstract

Background: Recent publications have raised concerns of reduced susceptibilities of clinical bacterial isolates towards biocides. This study presents a comparative investigation of the susceptibility of livestock-associated Methicillin-resistant Staphylococcus aureus (LA-MRSA), hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) to the commonly used antiseptics chlorhexidine (CHX), octenidine (OCT), polyhexanide (PHMB), PVP-iodine (PVP-I) and triclosan (TCX) based on internationally accepted standards.

Methods: In total, 28 (18 LA-, 5 HA- and 5 CA) genetically characterized MRSA strains representing a broad spectrum of hosts, clonal complexes and spa-types, as well as the reference methicillin-sensitive Staphylococcus aureus (MSSA) strain ATCC 6538, were selected. Minimal inhibitory concentration (MIC) and minimal microbicidal concentration (MBC) were determined in accordance with DIN 58940–7, 58940–8 and DIN EN ISO 20776-1. The microbicidal efficacy was determined in accordance with DIN EN 1040.

Results: Results from the MIC/MBC and quantitative suspension tests revealed differences between antiseptic substances but not between epidemiological groups of MRSA strains. OCT and PHMB were the most active substances with a minimal MIC of 1 mg/L, followed by CHX (2 mg/L), TCX (32 mg/L) and finally PVP-I (1024 mg/L). The MSSA reference strain showed a tendency to a higher susceptibility compared to the MRSA strains.

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Background
Antiseptic agents such as chlorhexidine digluconate (chlorhexidine, CHX), octenidine dihydrochloride (OCT), polyhexanide (polyhexamethylene biguanide, PHMB), PVP-iodine (Poly(vinylpyrrolidone)-iodine complex, PVP-I), and triclosan (5-chlorine-2-(2,4-dichlorphenoxy)-phenol, TCX) are widely used as topical antiseptics against colonization and infection of humans and animals with Methicillin-resistant Staphylococcus aureus (MRSA) [1–7]. The clinical benefits of decolonization of MRSA patients for prevention of nosocomial infections is well documented [8–10]. The antimicrobial properties of these agents against hospital acquired (HA) MRSA strains have been repeatedly shown [11–17]. However, to our best knowledge, there are no systematic investigations comparing the susceptibility of livestock-associated (LA) and community-associated (CA) strains versus HA-MRSA strains using standardized and harmonized test procedures. As CA- and LA-MRSA strains make up a growing proportion of MRSA strains in humans [18], such studies are quite pertinent. Our investigation was to test different antiseptics to selected MRSA strains reflecting stains that are prevalent in Germany with the main attention on LA-MRSA.

Methods
In order to provide reliable and reproducible information on the susceptibility of MRSA strains, the minimal inhibitory concentration (MIC), the minimal microbicidal concentration (MBC) (microdilution test; EN 58940) as well as the microbicidal efficacy (quantitative suspension test; EN 1040) of CHX, OCT, PHMB, PVP-I and TCX were determined in a comparative study under standardized conditions [19–22] using a spectrum of genetically characterized strains from different hosts.

Test strains
Strains were drawn from the national collection of the Robert Koch-Institute (RKI) to represent a broad spectrum of strains from different hosts that are prevalent in Germany, and from a collection of regional strains from northeastern Germany (HICARE Study) (Table 1) [23]. Methicillin-sensitive Staphylococcus aureus (MSSA) ATCC 6538 was used as the reference strain. The reference strain comes from the American Type Culture Collection (ATCC), a scientifically recognized source, and has defined resistance properties [24].

The S. aureus Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany) was used to test selected isolates for the presence of genes encoding quaternary ammonium compound efflux pumps (qac genes), as described elsewhere [25]. None of the tested strains in this comparison harbored qac genes.

Test preparations
Chlorhexidine digluconate (20% CHX solution, C 9394, Sigma-Aldrich Biochemie GmbH, Hamburg, Germany), octenidine dihydrochloride (Schülke & Mayr GmbH, Norderstedt, Germany), polyhexanide (20% PHMB solution, Fagron GmbH & Co. KG, Hamburg, Germany) and PVP-I (Betaisodona solution: 100 ml of the solution contains 10 g of poly(1-vinyl-2-pyrrolidone-)iodine-complex, with a content of 11% available iodine, Mundipharma GmbH, Limburg, Germany) were diluted in water of standardized hardness (WSH; according to DIN EN 1040 [19]) to the final test concentrations. As TCX (Irgasan, 72779, Fluka, Buchs, Switzerland) dissolves poorly in water, a stock solution of 50% TCX in 80% dimethylsulfoxide (DMSO) was diluted in several steps to obtain a final concentration of 1% TCX in 40% DMSO/WSH. A 40% DMSO/WSH solution was used in all dilution steps.

The suitability of 40% DMSO in WSH was demonstrated using the quantitative suspension test and the microdilution test as described previously [26].

The following solutions were used as neutralizing agents in accordance with DIN EN 1040 and 1275 [19, 20]:
- 3.0% (w/v) polysorbate 80 + 3.0% (w/v) saponin + 0.1% (w/v) L-histidine + 0.1% (w/v) cysteine for neutralizing CHX, OCT and PHMB
- 3.0% (w/v) polysorbate 80 + 0.3% (w/v) lecithin + 0.3% (w/v) L-histidine + 0.5% (w/v) sodium thiosulfate for neutralizing PVP-I
- 8.0% (w/v) polysorbate 80 + 2.0% (w/v) sodium dodecylsulfate (SDS) + 0.8% (w/v) lecithin + 1.0% (w/v) sodium thiosulfate + 6.0% (w/v) saponin for neutralizing TCX.

To determine the MICs and the MBCs, the substances were prepared in concentrations from 0.25 to 4096 mg/L (Table 2). Concentration ranges used in the quantitative suspension tests are summarized in Table 2.

Conclusions: This investigation of the susceptibility of a range of LA-, HA- and CA-MRSA strains using standardized conditions gave no indication that LA-MRSA strains are less susceptible to commonly used antiseptics compared to HA- and CA-MRSA strains.

Keywords: MRSA, Resistance, Decolonization, Antisepsis
Microdilution test

DIN EN 58940–7 [21] and 58940–8 [22] and the corresponding supplementary sheets were strictly followed to determine the MIC and MBC, as described previously [26]. Briefly, the test organisms were cultivated on CASO agar at 37 °C for 18 h; thereafter, four to five colonies were transferred into 1 ml of BBL Mueller Hinton Broth (BD, Becton Dickinson) and diluted to reach 5 × 10^5 cfu/ml. Tests were performed in 96-well microtiter plates. Each test was performed in duplicate. Each well was filled with 100 μl of defined antiseptic dilution and 100 μl of test organism suspension. The turbidity was visually evaluated as an indicator of bacterial growth and minimal inhibitory concentration after 24 h (MIC_{24}) and

Table 1 List of LA-MRSA, HA-MRSA and CA-MRSA strains with source, spa-type, SCCmec, resistance phenotype and provider

| LA-MRSA | Source | Spa-type | SCCmec; other | Resistance Phenotype | Provider |
|---------|--------|----------|---------------|----------------------|----------|
| CC398   | pig    | t034     | V             | PEN, OXA, ERY, CLI, TET, CIPi, SXT, OXA/Su | RKI      |
| CC398   | cow    | t011     | ND            | PEN, OXA, TET, OXA/Su | RKI      |
| CC398   | turkey | t034     | ND            | PEN, OXA, ERY, CLI, TET, SXTi, OXA/Su | RKI      |
| CC398   | poultry | t011    | ND            | PEN, OXA, ERY, CLI, TET, SXTi, OXA/Su | RKI      |
| CC398   | horse  | t011     | IV            | PEN, OXA, GEN, ERY, CLI, TET, CMP, SXT, OXA/Su | RKI      |
| CC398   | horse  | t6867    | IV            | PEN, OXA, GEN, TET, COX, OXA/Su | RKI      |
| CC398   | human  | t034     | V             | PEN, OXA, TET, SXTi, OXA/Su | RKI      |
| CC398   | human  | t899     | IV            | PEN, OXA, OXA/Su | RKI      |
| CC398   | human  | t2123    | ND            | PEN, OXA, GEN, TET, CIP, OXA/Su | RKI      |
| CC398   | human  | t2370    | ND            | ND                  | HICARE   |
| CC398   | human  | t456     | ND            | ND                  | HICARE   |
| CC398   | human  | t3275    | ND            | ND                  | HICARE   |
| CC398   | human  | t10721   | ND            | ND                  | HICARE   |
| CC130   | deer   | t843     | ND            | PEN, OXA, OXA/Su | RKI      |
| CC130   | horse  | t843     | ND            | PEN, OXA, OXA/Su | RKI      |
| CC130   | human  | t1773    | ND            | PEN, OXA, CIPi, OXA/Su | RKI      |
| CC9     | chicken | t1430   | ND            | PEN, OXA, CIP, MFL, OXA/Su | RKI      |
| CC9     | human  | t1430    | ND            | PEN, OXA, ERY, CLI, CIP, MFL, OXA/Su | RKI      |
| HA-MRSA | Source | Spa-type | SCCmec; other | Resistance Phenotype | Provider |
| CC22    | human  | t032     | IV            | PEN, OXA, ERY, CLI, CMP, CIP, MFL, OXA/Su | RKI      |
| CC22    | human  | t020     | ND            | PEN, OXA, ERY, CLI, CMP, MFL, OXA/Su | RKI      |
| CC22    | human  | t005     | ND            | PEN, OXA, OXA/Su | RKI      |
| C55 (ST225) | human | t003 | II | PEN, OXA, ERY, CLI, CMP, CIP, MFL, OXA/Su | RKI      |
| C55 (ST5) | human | t002 | ND | PEN, OXA, ERY, CLI, CMP, MFL, OXA/Su | RKI      |
| CA-MRSA | Source | Spa-type | SCCmec; other | Resistance Phenotype | Provider |
| CC1     | human  | t5100    | nd, lukPV, seh | PEN, OXA, GEN, TET, FUS, COX, OXA/Su | RKI      |
| CC8     | human  | t1476    | ND            | ND                  | HICARE   |
| CC8     | human  | t008     | IV, lukPV    | PEN, OXA, ERY, CIP, MFL, OXA/Su | RKI      |
| CC80    | human  | t044     | IV, lukPV    | PEN, OXA, TET, CIP, MUPi, FUS, OXA/Su | RKI      |
| CC59    | human  | t437     | nd, lukPV    | PEN, OXA, ERY, CLI, TET, CMP, OXA/Su | RKI      |

RKI Robert Koch-Institute, HICARE HICARE Study, ND not determined; Groups of strains were defined genetically by spa-typing, MLST, and SCCmec, as well as demonstration of luk-PV

Table 2 Concentration ranges used for determining MICs and MBCs in accordance with DIN EN 58940–7 and 58940–8 [18] and concentration ranges of the test preparations used in the quantitative suspension tests according to DIN EN 1040 [15]

| Antiseptic agent | Concentration range for MICs and MBCs determination [mg/L] | Concentration range for quantitative suspension tests [mg/L] |
|------------------|-----------------------------------------------------------|------------------------------------------------------------|
| CHX              | 0.25–4, 0.25–4                                           | 125–500, 20–40, 50–100, 5,000–10,000, 250–1,000           |
| OCT              | 0.25–4, 0.25–4                                           | 256–4096, 5,000–10,000, 250–1,000                          |
| CHX              | 16–256, 16–256                                           | 250–1,000                                                 |
| OCT              | 5–20, 5–20                                              | 5,000–10,000                                              |
| PHMB             | 50–100                                                   | 20,000–40,000                                             |
| PVP-I            | 50–100                                                   | 20,000–40,000                                             |
| TCX              | 250–1,000                                                | 5,000–10,000                                              |

Microdilution test

DIN EN 58940–7 [21] and 58940–8 [22] and the corresponding supplementary sheets were strictly followed to determine the MIC and MBC, as described previously [26]. Briefly, the test organisms were cultivated on CASO agar at 37 °C for 18 h; thereafter, four to five colonies were transferred into 1 ml of BBL Mueller Hinton Broth (BD, Becton Dickinson) and diluted to reach 5 × 10^5 cfu/ml. Tests were performed in 96-well microtiter plates. Each test was performed in duplicate. Each well was filled with 100 μl of defined antiseptic dilution and 100 μl of test organism suspension. The turbidity was visually evaluated as an indicator of bacterial growth and minimal inhibitory concentration after 24 h (MIC_{24}) and
after 48 h (MIC\textsubscript{48}). To determine the MBC, samples in the range of the threshold for turbidity after 24 h were transferred onto blood agar, as described in the standards, and evaluated for growth after 24 h incubation (MBK\textsubscript{24}).

**Quantitative suspension test**

DIN EN 1040 [19] was strictly applied to determine the bactericidal efficacy without organic load. Briefly, 0.1 ml of test organism suspension and 0.1 ml of WSH were mixed and left for 2 min. Afterwards, 0.8 ml of the respective antiseptic test substance were added. The resulting solutions were incubated for 5 and 30 min at 37°C. At the end of the contact time, 0.1 ml of the test solution was transferred to 0.8 ml of the respective neutralizing solution and 0.1 ml WSH and left for 5 min. Serial dilutions were prepared in neutralizer; 0.1 ml of each neutralized test dilution was spread onto nutrient agar plates in duplicates. After incubation for 24 h, the colonies were counted and the number of recoverable colonies (N\textsubscript{a}) in the test solution was calculated. The reduction factor (RF) was determined as the difference of the log number of cells in the test solution at the beginning of the contact time (N\textsubscript{0}) and log of N\textsubscript{a}.

In addition to the DIN EN, negative controls using 0.8 ml of WSH instead of test preparation were performed simultaneously in the first test run to exclude any bactericidal effects of WSH. In the water controls, no essential difference was observed compared to the N\textsubscript{0} values.

**Statistics**

Data were prepared using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) and analyzed using IBM SPSS Statistics 24 (IBM, Armonk, NY, USA). Strains were grouped as LA-, HA- and CA strains.

Robust nonparametric statistics were used to compare results from the microdilution tests and quantitative suspension tests [27]. A two-step procedure was chosen to avoid alpha-error inflation. Kruskal-Wallis tests were used as omnibus tests for multiple comparisons. If the omnibus tests indicated statistically significant differences between groups, Mann-Whitney tests were used for pairwise comparisons.

**Results**

**MIC and MBC**

Values of MICs and MBCs of tested substances showed marked differences between LA-, HA- and CA-MRSA (Table 3). OCT and PHMB were the most active substances with a minimum MIC of 1 mg/L followed by CHX, TCX and finally PVP-I. There was no significant difference between MIC\textsubscript{24} and MIC\textsubscript{48} of the same substances between LA-, HA- and CA-MRSA (Related-Samples-Wilcoxon-Signed-Rank Test, p = 1.00). There was a significant differences between MBC\textsubscript{24} and MIC\textsubscript{24} for all substances but TCX (Related-Samples-Wilcoxon-Signed-Rank-Test, p < 0.01). TCX showed the greatest range between minimum and maximum MIC and MBC values.

Values of MIC\textsubscript{24}, MIC\textsubscript{48} and MBC\textsubscript{24} differed significantly between groups of strains for PHBM (p = 0.003,
wise comparison of MIC 24, MIC 48 and MBC 24 of PHMB in the Independent-Samples Kruskal-Wallis test. Pairwise comparison of MIC_{24}, MIC_{48} and MBC_{24} of PHMB showed that this was caused by a higher susceptibility of the reference MSSA strain compared to the MRSA strains ($p < 0.02$). Pairwise comparisons for TCX showed that HA- and CA-MRSA strains were less susceptible to TCX than the reference strain ($p = 0.021$ and 0.01 respectively), and CA-MRSA was significantly less susceptible than LA-MRSA ($p = 0.035$).

**Quantitative suspension test**

The archived reduction factors show that all substances were used at or below the concentration needed to achieve the threshold set by DIN EN 1040 (at least a 5 log-step reduction) to be adequately bactericidal, as planned. As expected, reduction factors increased with contact time and concentration of the antiseptic (Fig. 1a-e). In contrast, the MSSA reference strain showed a higher susceptibility to CHX than did the MRSA strains, but the differences were not statistically significant in the omnibus test. All other tests for statistical significance were omitted due to the small absolute differences and the overlapping confidence intervals.

**Discussion**

The antibacterial activity of common antiseptics against a broad range of different pathogens has been well documented [26, 28]. Still, little is known about the differences in the susceptibility to antiseptics of LA-MRSA in comparison to HA-MRSA and CA-MRSA. While antiseptics show a broader antimicrobial spectrum compared to antibiotics and are less compromised by specific resistances, reduced susceptibility of various strains to antiseptics has been reported [29–33]. Besides antimicrobial agents other facts like metal-resistance genes might contribute to differences the susceptibility to antiseptics. For example Argudin et al. reported the occurrence of different metal-resistance genes among LA-MRSA [31]. Recent publications in particular have raised concerns of reduced susceptibilities of distinctive clinical isolates towards biocides and found associations with outbreaks [29, 33, 34].

Therefore, the susceptibility of LA-MRSA to antiseptics is an important issue, as LA-MRSA is an emerging problem and antiseptic agents are valuable drugs for prevention of MRSA infections [18, 35]. For example, antiseptic decolonization has been proven to control the spread of MRSA in intensive care healthcare settings [8] and to reduce surgical site infections [36]. Nevertheless, the effectiveness of these measures relies on the susceptibility of the targeted pathogens to the antiseptic products used.

Other mechanisms for reduced susceptibility to disinfectants in MRSA besides the qac gene coded efflux pumps have been described: reduced susceptibility to chlorhexidine can also result from mutations in the norA/norB genes which code for an efflux mechanism [29]. Reduced susceptibility to triclosan can be due to either enhanced expression of the target of this biocide, namely the enoyl-acyl carrier protein (ACP) reductase enzyme (FabI) [37], or acquisition of an additional sh-fabI allele derived from Staphylococcus haemolyticus by horizontal gene transfer [27]. We found no evidence of reduced susceptibility of LA-MRSA to CHX, OCT, PHMB, PVP-I and TCX in comparison to CA- and HA-MRSA. Differences in the susceptibility between the strains in MIC, MBC and microdilution assays were marginal. With a difference not greater than one dilution step, the range between the highest and lowest MIC and MBC between the groups of MRSA strains was at the same level or even smaller, as between the strains of the same group (one step for CHX, PHMB and OCT and up to two steps for PVP-I and TCX). The only exception was TCX in terms of LA-MRSA strains, which were significantly more susceptible than CA-MRSA.

Likewise, the results from the quantitative suspension assays were quite comparable between CA-, HA- and CA-MRSA strains. In contrast, the reference MSSA strain showed a tendency to higher susceptibility in the MIC, MBC and quantitative suspension assays. However, as only one reference strain was used, it is unclear whether this can be interpreted as higher susceptibility of MSSA in contrast to MRSA or as an attribute of the specific strain.

Our results are well comparable with those of other published studies. MICs reported by Koburger et al. for aureus ATCC 6538 almost matched our results, with the exception of PVP-I and TCX, which showed a markedly higher MIC{48} and MBC{24} in our tests [26]. The differences for PVP-I remain unexplained, while the reported higher MICs to TCX in comparison to Koburger et al. (0.125 versus 8 mg/L) can be explained by the fact that 8 mg/L was the lowest concentration used in our tests.

Furthermore, the tested MRSA-strain, a northern German epidemic strain, showed susceptibilities comparable to our results. Likewise, MICs to PHMB and TXC reported by Assadian et al. for MRSA, low level vancomycin-resistant (VISA) S. aureus strains and S. aureus ATCC 29213 correspond well to our results [11]. Interestingly, the MSSA reference strains showed a tendency to higher susceptibility to TCX in this two studies compared to MRSA.

It is important to bear in mind that the concentrations used in our study were well below the concentrations...
Fig. 1 (See legend on next page.)
recommended by the manufacturer. For example, PHMB is used at a concentration of 0.02% or 200 mg/L, which is 200 times greater than the MIC$_{24}$ for wound antisepsis.

The strength of the present study is the systematic approach based both on European standards for assessing the bactericidal effects in quantitative suspension assays and on industry standards to determine the MIC and MBC using the microdilution method [19, 21, 22, 26]. Our method can therefore easily be replicated by other researchers and for other strains. One point worthy of note is that parts of DIN 58940 have since been suspended and replaced by DIN EN ISO 20776-1:2007. However, this has no effects in terms of determining the MIC and MBC for antisepsics in this study.

Our study has limitations. For instance, we used only a limited number of strains and antisepsics for our analysis. It is well known that some strains express higher resistances to specific antisepsics. Resistance to antisepsics can arise through different mechanisms [38]. For example, efflux-mediated resistance to various biocides linked to qac-genes has been reported in different staphylococcal isolates in recent years [39–41]. However, this does not detract from our research question of whether LA-strains show a higher resistance to antisepsics compared to HA- and CA-strains per se, as qac-genes have been reported in HA-, CA- and LA-strains alike. Although we used a limited number of strains, all were genetically characterized and represented a broad spectrum of hosts, clonal complexes and spa-types. Most strains were drawn from the national collection of the Robert Koch Institute and were supplemented by regional strains from northeastern Germany as well as an ATCC reference strain. The aim of our study was to evaluate the susceptibility of LA-MRSA to different antisepsics in comparison to HA-MRSA and CA-MRSA. The MSSA reference strain serves as an intern control. The shown difference between the reference and the test strains should not be interpreted as evidence for a higher susceptibility of MSSA to MRSA strains in general.

Regarding the limited number of antisepsics used, we covered a broad spectrum of substances with different modes of action. Our selection included CHX, probably the most commonly used antiseptic agent worldwide, and OCT, PHMB, PVP-I and TCX. These substances are widely used in specific fields of application, such as antisepsis on skin and mucous membranes [1, 4], the eye [42, 43], acute and chronic wounds [2, 6] and sutures [44].

In summary, the present study gives no reason to doubt that the tested antisepsics can kill LA-MRSA at the concentrations recommended for use by the manufacturer. However, if the substances are diluted, which can happen deliberately as result of the usage (e.g., when irrigating wounds) or as part of the intended application (e.g., slow release of CHX from patches or TCX from sutures), the concentration may be reduced to levels that fall short of the MIC. As recent publications raise concerns about the increasing resistance of clinical isolates to antisepsics and disinfectants, this highlights the importance of safe and conscientious use of antisepsics.

**Conclusion**

This investigation of the susceptibility of a broad range of HA-, LA- and CA-MRSA strains using standardized and harmonized conditions provided no indication that LA-MRSA strains show reduced susceptibility to commonly used antisepsics compared to HA- and CA-MRSA strains.
Abbreviations
CA-MRSA: Community-acquired Methicillin-resistant Staphylococcus aureus; CHX: Chlorhexidine; HA-MRSA: Hospital-acquired Methicillin-resistant Staphylococcus aureus; LA-MRSA: Livestock-associated Methicillin-resistant Staphylococcus aureus; MBC: Minimal microbicidal concentration; MIC: Minimal inhibitory concentration; MRSA: Methicillin-resistant Staphylococcus aureus; MSSA: Methicillin-sensitive Staphylococcus aureus; OCT: Octenidine; PHMB: Polyhexanide; PVP-I: (Polyvinylpyrrolidone)-iodine complex; TCX: Triclosan

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Authors’ contributions
NOH and KD conceived the study and its original design. DT, TS and CC collected the data. TS, DT, GM and SH were responsible for the microbiological testing. KD, NH and GM drafted the manuscript supported by all authors. All authors read and approved the final manuscript.

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Availability of data and materials
The data and materials are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
For used human samples of the HICARE Study ethical approval from the ethics committee of the medical faculty of University Magdeburg (# 47/09) was sought. For the remaining human samples ethical approval was not necessary because these samples were transmitted of clinic-diagnostic laboratories.

Consent for publication
Not applicable.

Competing interests
Peter Pfaff is an employee of BBraun AG GmbH (Melsungen, Germany). The antiseptic compounds CHX and PHMB are part of some of the products of BBraun AG GmbH. The other authors declare that they have no competing interests. None of the authors holds stock or options in BBraun AG GmbH.

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