Susceptibilities of Methicillin-Resistant Staphylococcus aureus Isolates to Seven Biocides

Koji Narui, Mitsuo Takano, Norihisa Noguchi, and Masanori Sasatsu

Department of Microbiology, School of Pharmacy, Tokyo University of Pharmacy and Life Science; 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan; and Department of Pharmacy, Hokushin General Hospital; 1-5-63 Nishi, Nakano, Nagano 383-8505, Japan.

Received September 27, 2006; accepted December 18, 2006; published online December 19, 2006

Minimum bactericidal concentrations (MBCs) of seven biocides for 42 methicillin-resistant Staphylococcus aureus (MRSA) isolates at 5, 30, or 180 min, for hand scrubs or soaks, isolated in 2003 in Japan were determined. The MBC values of glutaraldehyde, povidone iodine, and ethanol were lower than the user concentrations in all exposure times. However, at 5 min exposure of sodium hypochlorite, benznazolium chloride, alkyl-diaminoethylglycine hydrochloride, and chlorhexidine digluconate some strains showed higher MBC values than the user concentrations. These results indicated the possibility that MRSA survived under proper user concentration conditions and exposure time.

Key words methicillin-resistant Staphylococcus aureus; minimum bactericidal concentration; biocide; user concentration; exposure time

Meticillin-resistant Staphylococcus aureus (MRSA) is a major nosocomial pathogen, and biocides including antiseptics and disinfectants have been used in order to prevent its infections and spreading. Biocides have a wide variety of uses, and their concentrations and exposure times vary according to usage. Recently, MRSA isolates with decreased biocide susceptibilities have been isolated from clinical samples, and MRSA isolates carrying antiseptic-resistance gene(s) have been prevalent worldwide. It is important for infection control to know the susceptibilities of MRSA to various biocides. Consequently, we determined the minimum bactericidal concentrations (MBCs) of biocides for the clinical isolates of MRSA at different exposure times.

MATERIALS AND METHODS

Strains, Biocides and Media A total of 42 MRSA isolates were used in this study. They were isolated from sputum, urine, or wounds of 24 male and 18 female patients aged 76.8 ± 10.0 years in a hospital. PFGE of SmaI-digested chromosomal DNA was performed as described previously. All isolates were not genetically identical (Fig. 1). S. aureus ATCC29213 was used as a susceptibility test standard. The biocides were selected the chemical disinfectants recommended for patient-care items and instruments. Sodium hypochlorite solution (5%), benznazolium chloride, alkyl-diaminoethylglycine hydrochloride solution (40%), chlorhexidine gluconate solution (20%), and ethanol (99.5%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glutaraldehyde solution (50%) and povidone iodine solution (10%) were purchased from Sigma-Aldrich (Tokyo, Japan) and Meiji Seika Kaisha, Ltd. (Tokyo, Japan), respectively. Neutralization medium [3% polysorbate (Kanto Chemical Co., Inc., Tokyo, Japan), 0.3% lecithin (Wako), 0.5% sodium thiosulfate (Wako), 0.1% L-histidine (Wako), 3% saponin (Merck Ltd., Tokyo, Japan), 0.1% tryptone (Difco laboratory, Detroit, MI, U.S.A.), 0.9% sodium chloride (Wako)] as described by EN1276 was used. MRSA isolates were grown on Trypto-soy agar (TS agar; Eiken Chemical Co., Ltd., Tokyo, Japan). Mueller-Hinton medium (MH, Difco) was used for susceptibility testing.

Antiseptic and Disinfectant Susceptibilities Tests All isolates were grown on TS agar for 24 h at 37 °C, and suspended in MH medium to McFarland Standard 4 [1.2—1.0 × 10^9 colony-forming units (cfu/ml)]. One microliter of the cell suspension (approximately 10^7 cfu) was inoculated into 0.1 ml of water containing the biocides and was exposed for 5, 30, and 180 min, respectively [the exposure times were for hand scrubs (5 min) and soaks (60 and 180 min)]. To inactivate the remaining biocides, 1 μl of the bacteria–biocide mixture was transferred into 0.1 ml of the neutralization medium. Then, 1 μl of the mixture (approximately 10^3 cfu)
was inoculated into an MH broth without biocide. Bacteria growth was observed after incubation at 35 °C for 24 h. Inoculations on each stage of work were carried out at 20 °C and with an auto-inoculater MIC-2000 System (Dynatech laboratories, Inc, Alexandria, VA, U.S.A.). MBCs were determined by three times test. The common user concentrations and exposure times of the biocides are listed in Table 1.

RESULTS

The MBCs of the seven biocides for the 42 MRSA isolates at 5, 30, or 180 min were determined (Fig. 2).

The MBCs of sodium hypochlorite at 5 min for a few isolates were 64 µg/ml, although sodium hypochlorite for mucosa and wounds was 50 µg/ml (Table 1). The MBCs of sodium hypochlorite at 30 and 180 min, however, were distributed less than the common user concentration of 125 µg/ml. The MBCs of glutaraldehyde at 30 and 180 min were distributed less than the minimum user concentration of 5000 µg/ml, and there was a correlation between exposure time and disinfection efficacy. Benzalkonium chloride required antiseptic efficacy at short times and low concentrations because of its use as a hand scrub or application to mucosa, wounds, or medical instruments (Table 1). However, MBCs of benzalkonium chloride for a few isolates at 5 min were higher than 100 µg/ml. MBCs of alkyldiaminoethylglycine hydrochloride at all exposure times were distributed higher than 100 µg/ml. At 5 min, 90% of the isolates were higher. The minimum user concentration of chlorhexidine digluconate was higher than those of benzalkonium chloride and chlorhexidine digluconate, however MBCs of chlorhexidine digluconate, as well as benzalkonium chloride and alkyldiaminoethylglycine hydrochloride, for a few strains were higher than the common user concentration. MBCs of povidone iodine and ethanol at all exposure times were lower than the common user concentration, and correlations between exposure time and disinfection efficacy in ethanol, as well as glutaraldehyde, were observed. Meanwhile, MBCs for methicillin-susceptible Staphylococcus aureus (MSSA) ATCC29213 are shown in Table 2. MBCs for MSSA were distributed in par or low concentration range.

DISCUSSION

It was difficult to evaluate disinfection efficacy because the biocides had a wide variety of uses. The disinfection efficacy of biocides change by contact with organic material, and biocides differ in disinfection time dependent upon use. The agar doubling dilution, broth microdilution, and disk diffusion methods as susceptibility tests are well-known, but these methods are not appropriate susceptibility tests for biocides because biocides come in contact with medium, and disinfection time is incubation time in these methods. Therefore, in this study, we developed a simple and rapid method that solved these problems for biocide susceptibility testing and evaluated the susceptibilities of the 42 MRSA isolates with this method. The MBCs at 5, 30, and 180 min represented the

---

### Table 1. User Concentration and Soak Time of Biocide

<table>
<thead>
<tr>
<th>Agent</th>
<th>User concentration (soak time)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hand scrub</td>
</tr>
<tr>
<td>NaClO</td>
<td>100—500</td>
</tr>
<tr>
<td>GLA</td>
<td>N.A.</td>
</tr>
<tr>
<td>BKC</td>
<td>500—1000</td>
</tr>
<tr>
<td>AEG</td>
<td>500—2000</td>
</tr>
<tr>
<td>CHG</td>
<td>1000—5000</td>
</tr>
<tr>
<td>PVI</td>
<td>75000</td>
</tr>
<tr>
<td>EtOH</td>
<td>76.9—81.4</td>
</tr>
</tbody>
</table>

NaClO, Sodium hypochlorite; GLA, glutaraldehyde; BKC, benzalkonium chloride; AEG, alkyldiaminoethylglycine hydrochloride; CHG, chlorhexidine digluconate; PVI, povidone iodine; EtOH, ethanol; N.A., not applicable. The numbers in parenthesis are soak times (min). The units of EtOH or the others are shown as % or µg/ml, respectively.

---

### Fig. 2. MBCs of Biocides for 42 MRSA Isolates in Each Exposure Time

NaClO, Sodium hypochlorite; GLA, glutaraldehyde; BKC, benzalkonium chloride; AEG, alkyldiaminoethylglycine hydrochloride; CHG, chlorhexidine digluconate; PVI, povidone iodine; EtOH, ethanol. The solid, broken, and dotted lines indicate MBCs for 42 MRSA isolates at 5, 30, and 180 min, respectively. The double lines indicate minimum user concentration.
isolates of biocides. The results suggest that biocide resistance is not caused by a specific resistant mechanism, and the biocide concentration. It is necessary that these correlations in other strains and agents be researched as the number of compromised hosts increase.

Acknowledgements The authors wish to thank H. Nakaminami, E. Obata, and Y. Takikawa for their expert technical assistance. This work was supported by grants for private universities provided by the Ministry of Education, Culture, Sports, Science and Technology and by the Promotion and Mutual Aid Corporation for Private Schools of Japan.

Table 2. MBCs of Biocides for MSSA ATCC29213 Isolates in Each Exposure Time

<table>
<thead>
<tr>
<th>Agent</th>
<th>MBC (µg/ml) at following soak time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>NaClO</td>
<td>64</td>
</tr>
<tr>
<td>GLA</td>
<td>512</td>
</tr>
<tr>
<td>BKC</td>
<td>64</td>
</tr>
<tr>
<td>AEG</td>
<td>512</td>
</tr>
<tr>
<td>CHG</td>
<td>64</td>
</tr>
<tr>
<td>PVI</td>
<td>1024</td>
</tr>
<tr>
<td>EtOH</td>
<td>40</td>
</tr>
</tbody>
</table>

NaClO, sodium hypochlorite; GLA, glutaraldehyde; BKC, benzalkonium chloride; AEG, alkyldiaminoethylglycine hydrochloride; CHG, chlorhexidine digluconate; PVI, povidone iodine; EtOH, ethanol.

hand scrub or soak times were determined by our method. Unlike with the European suspension test, EN1276, the MBC was the lowest concentration of biocide that completely inhibited growth of the MRSA in microdilution wells as detected by the unaided eye.

In all agents, the MBC values at the same time interval for the 42 MRSA isolates varied. Although biocides include various compounds with different chemical structures, the isolates that exhibited low-level susceptibility to an agent tended to have low-level susceptibilities to other agents. All isolates were disinfected by glutaraldehyde, povidone iodine, and ethanol at a common exposure time and user concentration. However, the MBCs of benzalkonium chloride at 5 min for a few isolates were higher than the user concentration. These results indicate the possibility that MRSA survived in the proper user concentration and exposure time. MBC of alkyldiaminoethylglycine hydrochloride at 5 min for MSSA ATCC29213 was higher than the user concentration (Table 2). The results suggest that biocide resistance is not caused by a specific resistant mechanism, and the biocide concentrations that are necessary for disinfection of S. aureus were higher than our recognition. Antiseptic resistance genes, qacA, qacB, smr, norA, sepA, and sdm are found mainly in clinical isolates of S. aureus and are associated with resistance to biocides. The qacA, qacB, and smr are mainly found on plasmids, and norA, sepA, and sdm are located on the S. aureus chromosome. Antiseptic resistance in S. aureus is caused by proton motive force-dependent multidrug efflux. Some isolates that survived in user concentration had neither qacA, qacB, nor smr (data not shown). Further studies of the resistance mechanism(s) including chromosome resistance genes are necessary. Since this experiment was carried out under laboratory conditions, the actual disinfection efficacy of biocides may vary under clinical conditions. Our results suggest that MRSA survived under lower concentrations than under MBCs after taking into consideration the influences of body fluid and blood. Therefore, current user concentrations must be amended to avoid further infection and bacteria circulation.

In this study, we investigated the correlations between biocide susceptibility to MRSA, exposure time, and user concentration. It is necessary that these correlations in other strains and agents be researched as the number of compromised hosts increase.

REFERENCES

10) European Committee for Standardization, “European Standard EN 1276: chemical disinfectants and antiseptics—Quantitative suspension test for the evaluation of bactericial activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Test method and requirements (Phase 2, Step1),” British Standards Institution, London, 1997.