Reduction of disinfectant bactericidal activities in clinically isolated Acinetobacter species in the presence of organic material

Kumiko Kawamura-Sato1, Jun-ichi Wachino2, Takaaki Kondo1, Hideo Ito1 and Yoshichika Arakawa2*

1Department of Medical Technology, Nagoya University Graduate School of Health Science, 1-1-20 Daiko Minami, Higashi-ku, Nagoya 461-8673, Japan; 2Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan

Received 30 July 2007; returned 15 October 2007; revised 27 November 2007; accepted 28 November 2007

Objectives: In clinical Acinetobacter species, the reduction effects of organic material on bactericidal activities of four major disinfectants were investigated: chlorhexidine gluconate (CHX), benzethonium chloride (BZT), benzalkonium chloride (BZK) and alkyl diaminoethyl glycine hydrochloride (ADH).

Methods: The bactericidal activities of the four disinfectants against 283 strains of Acinetobacter species recovered from 97 Japanese hospitals in March 2002 were investigated by four different tests: MIC measurements, MBC measurements, time-killing assays and adaptation assays. Moreover, disinfectant efficacy was examined in the presence of BSA in two tests: MBC measurements and time-killing assays.

Results: No clinical isolates were able to withstand the in-use concentrations of the four disinfectants, although the MIC90 of ADH reached 100 mg/L. Strains for which MICs of at least two disinfectants were higher than MIC90 measured by the broth microdilution method were defined as isolates with ‘disinfectant reduced susceptibility (DRS)’. In the presence of 3.0% BSA, the MBCs of BZK, BZT and ADH for DRS isolates rose to 512 and 1024 mg/L, which were about half their in-use concentrations. Moreover, the times for bacterial complete killing were remarkably prolonged in DRS isolates even after a 10 min of exposure to 1000 mg/L of ADH, a half of its in-use concentration. The MICs of CHX for DRS isolates rose to 640 mg/L after repetitive passages in subinhibitory concentrations of CHX.

Conclusions: Given that the bactericidal effects of the four major disinfectants were considerably reduced in the presence of organic material (BSA) and DRS isolates tended to adapt to CHX, continuous surveys of the profiles of susceptibility to disinfectants among clinically isolated Acinetobacter species are very necessary from the standpoint of nosocomial infection control.

Keywords: susceptibility profiles, bovine serum albumin, adaptation

Introduction

Acinetobacter species have recently been recognized as one of the major hospital-acquired pathogens that cause opportunistic infections such as pneumonia, urinary tract infections, septicemia and surgical site infections, particularly in immunocompromised patients1-2 accommodated in intensive care units where they commonly undergo invasive medical procedures and tend to receive various broad-spectrum antimicrobial agents.3,4 Moreover, Acinetobacter species have rapidly developed multi-drug resistance capabilities over the past 10 years, and the increasing difficulties encountered in the treatment of infections caused by this opportunistic pathogen have become a serious clinical concern.5-7 The ability of this microbe to survive long-term in hospital environments even on dry surfaces has also been considered to play a crucial role in hospital-acquired infections.8,9 Biocides, including quaternary ammonium compounds (QACs) and bisbiguanides, have been assiduously used in hospitals and healthcare facilities, and have significantly contributed to maintaining sanitary conditions and preventing hospital-acquired infections.10 However, concerns have also been raised that widespread use of disinfectants could serve to select disinfectant-resistant
microbes among hospital-acquired pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*. Evidence for the reduced susceptibility due to exposure to disinfectants has been reported. For example, a steady increase in MICs of chlorhexidine for *P. aeruginosa* has resulted from the exposure to residual disinfectants at subinhibitory concentrations. It is possible that *Acinetobacter* species may also have developed insusceptibility to disinfectants because of their innate ability to survive long-term on body surfaces and in hospital environments in which various disinfectants have been consumed.

Another cause for concern is that organic materials are known to reduce the effectiveness of disinfectants and antiseptics. Because these agents have been extensively used in medical and healthcare facilities for the disinfection of mucous membranes and wounds, and for the sterilization of medical instruments and equipment surfaces, which often tend to be contaminated with organic materials, the influence of such materials should not be ignored from the viewpoint of practical use of disinfectants. Therefore, it is very important to understand the susceptibility status of *Acinetobacter* species against disinfectants, as well as the influences of organic materials upon reduction in their efficacies. Since little is known about the present susceptibility status of *Acinetobacter* species to disinfectants, the aim of this study was to assess the susceptibility profiles of clinically isolated *Acinetobacter* species, isolated in 2002. For the isolates with ‘disinfectant reduced susceptibility (DRS)’ selected from clinical isolates, the bactericidal activities of QACs, bisbiguanides and ampholytic detergents used widely in medical facilities were evaluated both with and without the organic materials. In addition, the adaptive resistance to four disinfectants was also investigated to predict the potential development of resistance to disinfectants in *Acinetobacter* species.

**Materials and methods**

**Bacterial strains and culture media**

In March 2002, 283 non-repetitive clinical isolates identified as *Acinetobacter* species were collected from 97 hospitals located in different geographical areas of Japan. Since these isolates were speculated to be major causative microbes of infection in each patient, they were subjected to identification and antibiotic-susceptibility tests. They were identified as 273 *Acinetobacter calcoaceticus*–*Acinetobacter baumannii* complex, 7 *Acinetobacter lwoffii* and 3 *Acinetobacter junii* by their biochemical identification using the API20NE system (bioMérieux Japan, Ltd, Tokyo, Japan) and a complementary test for the ability to grow at 37, 41 and 44°C. After re-identification, each isolate was grown on Luria–Bertani (LB) agar plates or in LB broth (Becton–Dickinson Diagnostic System, Sparks, MD, USA) for further studies.

**Disinfectants and susceptibility testing**

The disinfectants were obtained from the following sources: chlorhexidine gluconate (CHX), benzenthonium chloride (BZT), Wako Pure Chemical Industries, Ltd, Osaka, Japan; benzalkonium chloride (BZK), Kanto Chemical Co., Inc., Tokyo, Japan; alkyl diaminoethyl glycine hydrochloride [ADH; TEGO 51TM, 10% (w/v) solution]. The MICs of the disinfectants were determined by the broth microdilution method using Mueller–Hinton broth (Becton–Dickinson Diagnostic System) according to the protocol recommended by the CLSI (formerly NCCLS) in document M100-S14.

**Assay of bactericidal activity: quantitative suspension test**

The bactericidal effects of disinfectants on *Acinetobacter* species were measured using a slightly modified quantitative suspension test referring to the European Standard EN 1040. The neutralizer solution used in the suspension test contained the following: 10% Tween 80 (v/v), 3% lecithin (w/v), 0.1% histidine (w/v), 0.5% sodium thiosulphate (w/v) and phosphate-buffered saline (PBS, pH 7.4), all obtained from Wako Pure Chemical Industries.

Each isolate was cultivated in LB broth until its optical density (OD) of 0.90 at 660 nm was reached and washed once with PBS (pH 7.4). The bacterial test suspensions were then adjusted to an optical density of 0.08 at 660 nm (~10⁶ cfu/mL), and bacterial test suspensions were prepared for each strain. A 100 μL of test suspension was added to tubes containing 900 μL of disinfectant solutions at different concentrations and left for 3 min at 20 ± 2°C. Since the test results obtained at room temperature demonstrated poor reproducibility, all reactions were performed as much as possible at 20 ± 2°C in an incubator using bacterial suspensions, disinfectant solutions and neutralizer solutions preincubated at 20 ± 2°C. Aliquots of the reaction mixture (100 μL) of containing bacterial cells and disinfectant were then added to 900 μL of neutralizer solution at 20 ± 2°C for 3 min and serially diluted in PBS (pH 7.4). After dilution, 50 μL of the mixture was spread immediately onto LB agar plates and incubated for 18 h at 35°C. The numbers of colonies surviving on each plate were counted, and cell survival rates were calculated with those obtained by a test using a bacterial suspension treated with PBS (pH 7.4) instead of disinfectant as the control.

BSA (Sigma) was used to imitate organic soilings, thus ensuring that the ‘dirty’ test simulated practical ‘in-use’ conditions. The quantitative suspension tests with BSA were performed according to recommendation EN 1276. Each test was repeated to simulate ‘dirty’ conditions by mixing the test suspension with 0.3% or 3.0% (v/v) BSA solution before adding the 100 μL of test suspension to the 900 μL of disinfectant solution. The experiments were repeated three times on different days. In addition, the neutralizer was checked for its possible toxicity for the test organisms. Aliquots of a diluted bacterial suspension (100 μL) containing 1–3 × 10⁸ cfu/mL was added to 900 μL of neutralizer and left at 20 ± 2°C for 3 min. Two samples of 100 μL of mixture described above were then spread onto LB agar plates and incubated as above. The values obtained on each plate were 100–300 cfu/100 μL. Finally, the inactivation of the bactericidal activity of each disinfectant by neutralizer was also validated. Nine hundred microlitres of the solutions containing different concentrations of each disinfectant was added to 100 μL of the PBS (pH 7.4) and left at 20 ± 2°C for 3 min. Then, 100 μL of the mixture was transferred into 800 μL of neutralizer and left at 20 ± 2°C for 3 min. Aliquot of a diluted bacterial suspension (100 μL) containing 1–3 × 10⁸ cfu/mL was then added and left at 20 ± 2°C for 3 min. Two samples of 100 μL mixture, containing disinfectant, neutralizer and bacteria, were then spread onto LB agar plates and incubated as described above. The numbers of colonies grown on each plate ranged from 100 to 300 cfu/100 μL.

**MBC**

MBC was determined using the quantitative suspension test described above. The disinfectant solutions at different concentrations were made by serial 2-fold dilutions of each disinfectant.

**References**


2. CLSI (formerly NCCLS) in document M100-S14.


MBC was defined as the lowest concentration of disinfectants that completely suppressed bacterial growth in each disinfectant.

**Measurement of bactericidal activity: time-killing assay**

Time-killing assays were performed to evaluate the bactericidal effects of disinfectants using a modified quantitative suspension test referring to the European Standards EN1040 and EN1276. Bacterial cell suspension (100 μL) was added to 900 μL of disinfectant solution and 50 μL aliquots of the mixtures were sampled at 0.5, 1, 2.5, 5, 10, 20, 30, 60, 90 and 120 min, respectively. Each sample was immediately added to 450 μL of neutralizer solution at 20 ± 2°C and left for 3 min, then serially diluted in PBS (pH 7.4). Fifty microlitres of each diluent was spread onto LB agar plate and incubated for 18 h at 35°C. The numbers of colonies that grew on each plate were counted, and cell survival rates were calculated by similar methods using a bacterial suspension as the control treated with PBS (pH 7.4) instead of the mixture containing disinfectant and neutralizer solution. The test was repeated to simulate ‘dirty’ conditions using the test suspension containing a 0.3% or 3.0% (w/v) BSA by the same method employed in the MBC measurement. The experiments were repeated three times on different days.

**Adaptation to disinfectants**

Twelve strains, including five disinfectant-susceptible clinical isolates and seven DRS isolates, strains 1, 2, 4, 5, 6, 7 and 10 shown in Table 2, were subjected to the adaptation test. Five susceptible clinical isolates were randomly selected from the group of strains for which the MICs of all four disinfectants were less than MIC50, as shown in Table 1. Aliquots of the overnight culture (100 μL) were added to nutrient broth containing each agent at 1/2 of the MIC for each strain. The 1/2 MIC culture was incubated at 37°C with shaking for 72 h, and bacterial growth was assessed visually. When a culture density of higher than ~1 × 10^8 cfu/mL was observed, 100 μL of the 1/2 MIC culture was spread onto nutrient agar plates containing the same concentration of disinfectant, and the agar plates were incubated overnight at 37°C. The colonies grown on each agar plate were selected and cultured for further testing using nutrient broth containing 1/2 MIC disinfectant. An aliquot of cell culture was stored at −80°C as passage 1 (P1), and MICs and MBCs for P1 were re-determined. Any isolates that had shown an increase in MICs and MBCs were then inoculated into the broth media containing twice the original concentration of disinfectant, and the others not shown augmented MICs and MBCs were inoculated into media containing the disinfectant with the previous concentration. This procedure was repeated four times every 5 days (including 3 days for culture and 2 days for assay) after the selection of P1. If culture density was lower than 1 × 10^8 cfu/mL, the passage was not advanced to the next step, because no >5 log10 reduction could be detected in this condition. The stabilities of the MICs and MBCs for P5 isolates demonstrating highest disinfectant-reduced susceptible profile were checked four times every 5 days by repetitive culture with disinfectant-free broth. Moreover, the bactericidal activities of each disinfectant for these adapted isolates were evaluated by the quantitative suspension tests. The experiments were repeated three times on different days.

**Table 1.** Distributions of MICs of various disinfectants by the broth microdilution method

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>800</th>
<th>MIC50</th>
<th>MIC90</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHX</td>
<td>0</td>
<td>126^a</td>
<td>80</td>
<td>62</td>
<td>14^b</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>BZK</td>
<td>0</td>
<td>168^a</td>
<td>104</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>BZT</td>
<td>0</td>
<td>2</td>
<td>196^a</td>
<td>68</td>
<td>15</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>ADH</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>21</td>
<td>176^a</td>
<td>85</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

CHX, chlorhexidine gluconate; BZK, benzalkonium chloride; BZT, benzethonium chloride; ADH, alkyl diaminoethyl glycine hydrochloride.

^aNumbers indicate a group of clinical isolates demonstrating the phenotype susceptible to each disinfectant, and five strains used for adaptation tests were randomly selected in each group.

^bStandard MIC50 and MIC90 measurements (quantal measurement of 50% and 90% of the population) are given. Underlined numbers indicate number of isolates for which MICs of each disinfectant were higher than MIC50. MIC90 of ADH for all isolates was 100 mg/L. Fourteen isolates for which MICs of at least CHX, BZK and BZT were above MIC90 were defined as ‘isolates with reduced disinfectant susceptibility’ and selected as candidates for MBC and time-killing assays.

---

**Results**

**Susceptibility to disinfectants and selection of DRS isolates**

Distributions of MICs of CHX, BZK, BZT and ADH for 283 clinical isolates are shown in Table 1. *Acinetobacter* species tended to be susceptible to CHX, BZK and BZT, and MIC90s obtained by the broth microdilution method in the absence of BSA were ≤ 25 mg/L. However, MICs of ADH were relatively higher than those of the other three disinfectants, and the MIC90 of ADH obtained was 100 mg/L (Table 1).

The isolates with DRS possessing relatively high MICs of CHX, BZK, BZT and/or ADH were selected. In the present study, the DRS isolates were defined as those for which MICs of at least two among the four disinfectants were higher than MIC90 when measured by the broth microdilution method. As a result, 14 isolates (8 *A. baumannii*, 4 *A. calcoaceticus* and 2...
A. junii) out of 283 were provisionally defined as DRS isolates in the present study (Table 2).

**MBCs of four disinfectants**

To evaluate the phenotypes of DRS isolates selected, the MBC and bactericidal activity of the four disinfectants were measured under both the so-called ‘clean’ and ‘dirty’ conditions. Figure 1 shows cell survival rates of DRS isolates after exposure to CHX, BZK, BZT and ADH, with concentrations ranging from 1 to 1024 mg/L. The MBC values of the four disinfectants in the presence of BSA were higher than those in the absence of BSA, and MBC values of BZK, BZT and ADH obtained by the addition of 3.0% BSA showed high values (512 and 1024 mg/L), which were about half of the in-use concentration of these agents (Figure 1).

**Time-killing assay**

Time-killing assays were also performed to evaluate the bactericidal effects of the four disinfectants on DRS isolates from the viewpoint of exposure duration. As can be seen in Figure 2, at low concentrations (MIC50 of each disinfectant obtained by the broth microdilution method), the bacterial cell count in the absence of BSA reached a 5 log10 reduction after 10 min. On the other hand, the presence of 0.3% BSA simulating the ‘dirty’ condition elevated the cell survival rate, and, even after 120 min of exposure, reduction of bacterial cells was less than 1 log10 under the test conditions employed for BZK, BZT and ADH.

---

**Table 2. Susceptibility profiles of 14 isolates with ‘disinfectant-reduced susceptible’ properties**

<table>
<thead>
<tr>
<th>Strain number</th>
<th>MIC (mg/L) of disinfectanta</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHX</td>
<td>BZK</td>
</tr>
<tr>
<td>1b</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>2b</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>4b</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>5b</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>6b</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>7b</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>10b</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>13c</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>14c</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>

aMICs of disinfectants were determined using the broth microdilution method.  
bStrains 1, 2, 4, 5, 6, 7 and 10 were selected for adaptation tests.  
cStrains 13 and 14 were isolated from different patients in the same hospital.
Indeed, higher concentrations of disinfectants were reasonably more effective, especially under 'clean' conditions (Table 3). However, the bacterial cell numbers failed to show a 5 log₁₀ reduction after a 1 min of exposure to all four disinfectants at 1/5 of the in-use concentrations in the presence of 3.0% BSA. As for ADH, bacterial cells survived even after a 10 min of exposure to 1000 mg/L, a half of the in-use concentration, with 3.0% BSA (Table 3).

**Adaptation to disinfectants**

Five susceptible isolates and 7 DRS isolates selected from 283 isolates were subjected to an adaptation test. The adaptation profiles of these isolates are shown in Figure 3. For five susceptible strains, the MICs of BZK, BZT and ADH were not significantly elevated after repetitive passages through the broth media containing each disinfectant. These five susceptible isolates were most susceptible to CHX among the four disinfectants, but these isolates could adapt to only 2-fold higher concentrations of CHX after five passages. The MICs of BZK, BZT and ADH for DRS isolates were only slightly elevated (~2-fold) during five passages. However, MICs of CHX were significantly elevated (~10-fold, up to 640 mg/L) after the repeated passages. Similar findings were observed in the elevation of MBC values throughout the experimental process of passage as were seen in MIC (data not shown). The stability of disinfectant-reduced susceptible profile among strains demonstrating the highest adaptation ability was checked by culturing for 20 days in disinfectant-free broth. In some cases, the MIC or MBC of CHX for the adapted strains decreased approximately to 50%; however, no case declined to the level of parent strains (data not shown). Time-killing assays by suspension test were also performed to evaluate the bactericidal effect of CHX on both DRS and adapted isolates obtained through each passage process (Table 4). No considerable changes in the bactericidal activity of BZK, BZT and ADH for adapted isolates obtained after passage in CHX were observed (data not shown). On the other hand, an apparent elevation of the resistance level to CHX in the adapted isolates was observed by suspension test. As can be seen in Table 4, the bacterial cell counts of both DRS and adapted isolates showed a 5 log₁₀ reduction after a 1 min of exposure to 5000 mg/L CHX, that is, its in-use concentration. However, in P4 and P5 strains adapted for disinfectants, a 5 log₁₀ reduction of bacterial cells failed after a 1 min of exposure to 1000 mg/L CHX, 1/5 of its in-use concentration (Table 4).

**Discussion**

In healthcare settings, it has become more difficult to treat infections caused by Acinetobacter species because of their acquisition of consistent resistance to major groups of antimicrobial agents.5–7 Difficulties in the infection control practices as well as in chemotherapy of infectious diseases are due to the intrinsic capacities of Acinetobacter species for long-term survival in...
Adaptation to four disinfectants after persistent passages. Adaptations to disinfectants were generated by repeated subculture in nutrient broths containing each disinfectant (a) chlorhexidine gluconate (CHX); (b) benzalkonium chloride (BZK); (c) benzethonium chloride (BZT); (d) alkyl diaminoethyl glycine hydrochloride (ADH). Five disinfectant-susceptible clinical isolates selected randomly and seven DRS isolates (strains 1, 2, 4, 5, 6, 7, and 10 shown in Table 2) were subjected to this test. The 1/2 MIC cultures were incubated at 37°C with shaking for 72 h, and growth was assessed visually for cultures grown in media containing disinfectants. MICs were checked, and any isolates that showed an increase in MICs were then inoculated into media containing twice the original concentration. This procedure was repeated a total of five times every 5 days (including 3 days for culture and 2 days for assay). Stabilities of adaptive resistances of most resistant bacterial cells (P5) were determined by further passages through disinfectant-free broths and checked four times every 5 days. The experiments were performed three times on different days. White bars, five disinfectant-susceptible isolates selected; black bars, seven DRS isolates. Error bars represent standard deviations, and a significant difference is indicated by P < 0.05 (as determined by Dunnett’s and Bonferroni’s multiple-comparison tests).

Table 3. Bactericidal effects of four disinfectants on ‘disinfectant reduced-susceptible’ isolates

<table>
<thead>
<tr>
<th>Condition and exposure time</th>
<th>CHX (mg/L)</th>
<th>BZK (mg/L)</th>
<th>BZT (mg/L)</th>
<th>ADH (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5000b</td>
<td>2500</td>
<td>1000</td>
<td>500</td>
</tr>
<tr>
<td>0% BSA, 1 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
</tr>
<tr>
<td>0.3% BSA, 1 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>4.86 ± 0.08</td>
</tr>
<tr>
<td>3.0% BSA, 1 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>4.86 ± 0.09</td>
<td>3.89 ± 0.10</td>
</tr>
<tr>
<td>3.0% BSA, 10 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
</tr>
<tr>
<td>BZT (mg/L)</td>
<td></td>
<td>2000b</td>
<td>1000</td>
<td>400</td>
</tr>
<tr>
<td>0% BSA, 1 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
</tr>
<tr>
<td>0.3% BSA, 1 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>4.35 ± 0.09</td>
</tr>
<tr>
<td>3.0% BSA, 1 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>2.77 ± 0.16c</td>
<td>2.53 ± 0.12</td>
</tr>
<tr>
<td>3.0% BSA, 10 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>3.85 ± 0.12</td>
<td>2.72 ± 0.10</td>
</tr>
<tr>
<td>ADH (mg/L)</td>
<td></td>
<td>2000b</td>
<td>1000</td>
<td>400</td>
</tr>
<tr>
<td>0% BSA, 1 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
</tr>
<tr>
<td>0.3% BSA, 1 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>3.82 ± 0.10</td>
</tr>
<tr>
<td>3.0% BSA, 1 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>2.77 ± 0.16c</td>
<td>2.53 ± 0.12</td>
</tr>
<tr>
<td>3.0% BSA, 10 min</td>
<td>&gt;5.23</td>
<td>&gt;5.23</td>
<td>3.85 ± 0.12</td>
<td>2.72 ± 0.10</td>
</tr>
</tbody>
</table>

CHX, chlorhexidine gluconate; BZK, benzalkonium chloride; BZT, benzethonium chloride; ADH, alkyl diaminoethyl glycine hydrochloride. The bacterial cell concentration exposed to each disinfectant was 3.40 ± 0.81 × 10⁷ cfu/mL. aShown as log₁₀ reduction in bacterial cell number in both ‘clean’ (0% BSA) and ‘dirty’ (0.3% and 3% BSA) conditions; mean ± SD. bNumbers indicate ‘in-use’ concentration (mg/L) of each disinfectant. cDisinfectants failed to provide a 5 log₁₀ reduction in live bacterial cell counts in the presence of BSA.

Figure 3. Adaptation to four disinfectants after persistent passages. Adaptations to disinfectants were generated by repeated subculture in nutrient broths containing each disinfectant (a) chlorhexidine gluconate (CHX); (b) benzalkonium chloride (BZK); (c) benzethonium chloride (BZT); (d) alkyl diaminoethyl glycine hydrochloride (ADH). Five disinfectant-susceptible clinical isolates selected randomly and seven DRS isolates (strains 1, 2, 4, 5, 6, 7, and 10 shown in Table 2) were subjected to this test. The 1/2 MIC cultures were incubated at 37°C with shaking for 72 h, and growth was assessed visually for cultures grown in media containing disinfectants. MICs were checked, and any isolates that showed an increase in MICs were then inoculated into media containing twice the original concentration. This procedure was repeated a total of five times every 5 days (including 3 days for culture and 2 days for assay). Stabilities of adaptive resistances of most resistant bacterial cells (P5) were determined by further passages through disinfectant-free broths and checked four times every 5 days. The experiments were performed three times on different days. White bars, five disinfectant-susceptible isolates selected; black bars, seven DRS isolates. Error bars represent standard deviations, and a significant difference is indicated by P < 0.05 (as determined by Dunnett’s and Bonferroni’s multiple-comparison tests).
<table>
<thead>
<tr>
<th>Passage</th>
<th>Bacterial cells exposed to disinfectant</th>
<th>CHX (5000 mg/L)</th>
<th>CHX (1000 mg/L)</th>
<th>CHX (500 mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>exposure time (30 s)</td>
<td>exposure time (60 s)</td>
<td>exposure time (180 s)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>viable cell concentration</td>
<td>log_{10} reduction</td>
<td>viable cell concentration</td>
</tr>
<tr>
<td>Parent</td>
<td>5.36 ± 1.20 × 10^7</td>
<td>NC</td>
<td>&gt;5.43</td>
<td>NC</td>
</tr>
<tr>
<td>P1</td>
<td>4.43 ± 1.73 × 10^7</td>
<td>2.54 ± 1.05 × 10^3</td>
<td>5.24</td>
<td>9.80 ± 0.78 × 10^3</td>
</tr>
<tr>
<td>P2</td>
<td>8.00 ± 1.00 × 10^7</td>
<td>5.54 ± 1.25 × 10^3</td>
<td>5.16</td>
<td>3.60 ± 0.68 × 10^7</td>
</tr>
<tr>
<td>P3</td>
<td>8.85 ± 2.06 × 10^7</td>
<td>3.10 ± 1.43 × 10^3</td>
<td>4.46</td>
<td>NC</td>
</tr>
<tr>
<td>P4</td>
<td>6.34 ± 1.73 × 10^7</td>
<td>1.88 ± 0.88 × 10^3</td>
<td>4.53</td>
<td>3.80 ± 0.92 × 10^2</td>
</tr>
<tr>
<td>P5</td>
<td>3.37 ± 0.70 × 10^7</td>
<td>3.64 ± 0.89 × 10^3</td>
<td>3.97</td>
<td>4.37 ± 1.31 × 10^2</td>
</tr>
</tbody>
</table>

CHX, chlorhexidine gluconate.

*Strains were obtained throughout adaptation process passaged in increasing concentrations of CHX as shown in Figure 3.

CHX, chlorhexidine gluconate.

For seven DRS isolates, the experiments were repeated three times on different days. First, the viable cell counts of each isolate were calculated by the average of three test results performed on different days, and then average and distribution of the viable cell counts of the seven DRS isolates were again calculated.

NC indicates negative culture. Viable cell counts were estimated to be <2 × 10^7 cfu/mL in all three tests for seven DRS isolates selected.

Concentration of CHX to which bacterial cells were exposed before inoculation onto agar plate for counting surviving bacterial colonies.

Average viable cell concentration (cfu/mL ± SE) after the exposure to disinfectant.

Since no colonies grew on several plates, viable cell count became below the detection limit after the statistical analysis.
hospital environments and transmission among patients. Therefore, performance of contact-precautions including hand-hygiene and disinfection is crucial to interrupt patient-to-patient transmission of this microbe. Indeed numerous studies have focused on the issues of Acinetobacter species resistant to antimicrobial agents,17,18 but only a few have so far explored the susceptibility profiles to antiseptics and disinfectants in this microbe. Thus, investigations on the susceptibility status of clinically isolated Acinetobacter species’ susceptibility to antiseptics and disinfectants assessed by four different test methods, MIC measurements, MBC measurements by quantitative suspension tests, time-killing assays and adaptation tests, would provide instructive new insights into coping with Acinetobacter species in various healthcare settings.

In the present study, the MIC90 of the four disinfectants tested for 283 Acinetobacter species isolates were ≤100 mg/L, which is lower than the actual in-use concentration of each disinfectant. Moreover, the 14 DRS isolates selected were also confirmed to be susceptible to disinfectants by multiple tests, including MBC measurement, and time-killing assay, in the absence of organic materials. The results are consistent with those of Martró et al.19 and Wisplinghoff et al.20 who found no apparent development of resistance to disinfectants among clinically isolated Acinetobacter species. They assessed the susceptibility of A. baumannii to respective disinfectants and antiseptics by suspension test without adding organic materials. However, since Acinetobacter species inhabit hospital environments often contaminated with a variety of organic materials and colonize various body sites of patients, one must never fail to take into account the reduction effects of organic materials on antiseptics and disinfectants in practical use. Therefore, we further extended their studies to evaluate the properties of DRS isolates against antiseptics and disinfectants by MBC measurements and time-killing assays in the presence of BSA. The 10 min of exposures of DRS isolates to CHX at much lower than its in-use concentration in the presence of 3.0% BSA provided a 5 log10 reduction in bacterial cell numbers, whereas 10 min of exposure of these isolates to 200 mg/L BZK or 400 mg/L BZT failed to produce a 5 log10 reduction of bacterial cells in the presence of 3.0% of BSA. In addition, the 10 min of exposure to 1000 mg/L ADH, half of its in-use concentration, failed to eliminate the live bacterial cells in the presence of 3.0% BSA. This finding is crucial, because ADH is one of the most frequently used disinfectants for medical instruments and hospital environments.

Acinetobacter species usually cause hospital-acquired infections, including urinary- and respiratory-tract infections, and particularly ventilator-associated pneumonia, especially in debilitated individuals.1,2,21 Indeed, no apparent resistance properties of these DRS isolates against disinfectants were observed from the viewpoints of MIC and MBC measurements in the absence of organic materials, but the results obtained by the suspension test in the presence of BSA suggested that these DRS isolates may well survive in conditions of contamination by organic materials such as blood and exudation. Thus, care should be taken in monitoring the susceptibility profile of Acinetobacter species against disinfectants, especially when this microbe is frequently or continuously isolated from clinical samples.

To our knowledge, no adaptive resistance to disinfectants in strains belonging to Acinetobacter species has been reported to date. Our results demonstrate that repeated exposure to subinhibitory concentrations of CHX gradually elevated its MIC (at most 10-fold, up to 640 mg/L) for the DRS isolates. Furthermore, P4 and P5 strains obtained after several passages in 1/2 MICs of CHX survived after a 1 min of exposure to 1000 mg/L CHX, 20% of its in-use concentration (0.5% = 5000 mg/L), in suspension test. CHX has been demonstrated to have a persistent or residual effect after applications to skin22 and mucous membranes.23

Irizarry et al.24 suggested that environmental residues of CHX and cetylpyridinium chloride might confer some selective advantage on MRSA, an organism that has also been known as relatively resistant to CHX on dry surfaces. A recent study by Thomas et al.18 gives some experimental support to the idea that repetitive exposure to subinhibitory concentrations of CHX results in a stable increase in MICs of CHX for P. aeruginosa. Acinetobacter species have also become very common in hospital environments as well as MRSA and P. aeruginosa known as the major hospital-acquired pathogens. Our results suggest that the DRS isolates, which have been exposed to some extent to subinhibitory amounts of disinfectants remaining on environmental surfaces or even on the skin, would also develop the properties of adaptive resistance to CHX. It would therefore be necessary to carefully screen and select appropriate disinfectants based on a sufficient understanding of each for use in medical facilities.

In conclusion, no resistance to CHX, BZX, BZT and ADH was detected among clinically isolated Acinetobacter species by MIC measurements. However, the bactericidal effects of BZK, BZT and ADH, especially on the DRS isolates, were remarkably reduced in the presence of an organic material (3% BSA). Furthermore, the DRS isolates tended to adapt a higher concentration of CHX after repetitive passages in 1/2 MIC concentrations of CHX. To prevent hospital-acquired infections caused by this kind of microbe, the profile of susceptibility to disinfectants, as well as to antimicrobial agents, must be carefully monitored and checked among Acinetobacter species isolated from both clinical specimens and environments. Disinfectants are indispensable to perform appropriate infection control. Hence, this study highlights the need to ensure that these agents are being used appropriately in practice at the correct concentrations and for adequate contact times.

Acknowledgements

We are grateful to all the medical institutions that submitted bacterial isolates to the National Reference Laboratory.

Funding

This work was supported by an H18-Shinkou-11 grant from the Ministry of Health, Labour and Welfare, Japan and in part an H17-Gakushin grant from Nagoya University Graduate School.

Transparency declarations

None to declare.
References