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Mutant selection window of disinfectants for *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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ABSTRACT

Objectives: The aim of this study was to determine the mutant selection window (MSW) of various disinfectants against *Staphylococcus aureus* and *Pseudomonas aeruginosa* clinical isolates to determine the tendency of these strains to acquire resistance to disinfectants.
Methods: A total of 60 *S. aureus* isolates [30 methicillin-resistant *S. aureus* (MRSA) and 30 methicillin-susceptible *S. aureus* (MSSA)] and 30 *P. aeruginosa*, including 2 multidrug-resistant *P. aeruginosa* (MDRP), were collected in Japan. The minimum inhibitory concentrations (MICs) and mutant prevention concentrations (MPCs) of disinfectants, including sodium hypochlorite (NaOCl), against these strains were established to determine the MSW.
Results: The MSW_{50}, MSW_{80} and MSW_{100} for sodium hypochlorite against *S. aureus* and *P. aeruginosa* were 4×, 8× and 16× MIC, respectively. Strains surviving in the sodium hypochlorite MSW remained at a concentration of ≤0.3% (≤3072 μg/mL).
Conclusions: This is the first evaluation of the bactericidal activity against *S. aureus* and *P. aeruginosa* strains surviving in the MSW of disinfectants. Environmental disinfection at low concentrations of sodium hypochlorite does not kill micro-organisms. Proper use of sodium hypochlorite shows a bactericidal effect against various pathogenic micro-organisms and is inexpensive, making it frequently used globally.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* can cause serious infections that are difficult to treat [1]. MRSA and *P. aeruginosa* are the cause of many nosocomial outbreaks [2–4], whereas methicillin-susceptible *S. aureus* (MSSA) often causes hospital-acquired infections in neonatal intensive care units [5,6]. Hand-washing and personal protective equipment can effectively prevent infections due to direct contact; however, it is also important to disinfect and sterilise surfaces in health facilities in order to eliminate potentially contagious bacteria from the environment. The most commonly used disinfectants include alcohol, chlorhexidine gluconate (CHG), benzalkonium chloride (BAC) and sodium hypochlorite (NaOCl). BAC-resistant *S. aureus* was first reported in Australia in 1984 [7], whilst other studies have reported CHG- and/or BAC-resistant Gram-negative bacteria [8–10]. Furthermore, a number of studies have detected CHG- and BAC-resistant MRSA in Australia, Japan and Europe [11,12]. The growing use of these disinfectants has reduced susceptibility in *S. aureus* and *P. aeruginosa*, which has become a problem in hospitals worldwide [13–15].

The antimicrobial effect of disinfectants is evaluated by the minimum inhibitory concentration (MIC) and minimal bactericidal concentration. Disinfectants and antiseptic agents are used at recommended concentrations, which are not determined using breakpoints as in the case of antimicrobial agents [16,17]. The effective concentration range of antibacterial agents for reducing

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pathogenic bacteria is based on pharmacokinetic/pharmacodynamic theory. The mutant prevention concentration (MPC) is defined as the concentration of an agent that prevents the selection of resistant mutants within a large susceptible bacterial population. Administration of an antimicrobial agent at concentrations greater than the MPC should not select for resistant bacteria. The mutant selection window (MSW) is the concentration range between the lower MPC and the upper MIC; strains that survive in the MSW are considered resistant.

Few studies have focused on the MSW of disinfectants. Therefore, the aim of this study was to determine the MSW of various disinfectants against *S. aureus* and *P. aeruginosa* clinical isolates. The tendency of these strains to acquire resistance to disinfectants was also analysed.

2. Materials and methods

2.1. Bacterial strains

A total of 60 *S. aureus* isolates (30 MRSA and 30 MSSA) and 30 *P. aeruginosa* isolates, including 2 multidrug-resistant *P. aeruginosa* (MDRP), were collected from clinical specimens from 13 hospitals in Japan in 2012. According to the Act on the Prevention of Infectious Diseases and Care for Patients with Infectious Diseases in Japan, the definition of MDRP includes strains that are resistant to three antibiotics, namely imipenem ($\geq$16 μg/mL), ciprofloxacin ($\geq$4 μg/mL) and amikacin ($\geq$32 μg/mL). Among the 60 *S. aureus* isolates, 29 (48%) were from sputum, 7 (12%) from blood, 3 (5%) from urine, 3 (5%) from central venous catheter samples and 18 (30%) were from other specimens (i.e. drains, vagina, etc.). The 30 *P. aeruginosa* isolates were also obtained from various samples, including 10 (33%) from urine, 9 (30%) from sputum, 2 (7%) from drains, 2 (7%) from nasal cavities and 7 (23%) from other specimens (i.e. blood, catheter, wound, etc.).

2.2. Minimum inhibitory concentration (MIC) determination for disinfectants

MIC determination for each disinfectant, including sodium hypochlorite (Milton, KYORIN Pharmaceutical Co., Ltd., Tokyo, Japan), CHG (HiBiTANE®; Sumitomo Dainippon Pharma Co., Ltd., Osaka, Japan) and BAC (Osvan® Disinfectant 10%; Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), was performed by the broth microdilution method.

2.3. Determination of the mutant prevention concentration (MPC) and mutant selection widow (MSW)

The MPC of various disinfectants was determined using a modification of a previously reported method [18–20]. Briefly, isolates were incubated on trypticase soy agar plates for 16 h at 37 °C. Bacterial cells were collected from the plates and were suspended in phosphate-buffered saline and then 100 μL aliquots containing $>10^{10}$ CFU were plated on Mueller–Hinton agar plates supplemented with disinfectant at concentrations of 1–4096 μg/mL and were incubated for 48–72 h at 37 °C. The MPC was recorded as the lowest disinfectant concentration that completely inhibited bacterial colony formation. The MSW was expressed as the concentration range between the lower MPC and the upper MIC.

2.4. Evaluation of the biocidal activity of disinfectants against mutant selection window (MSW)-surviving strains

The biocidal activity of each disinfectant against MSW-surviving strains was examined. Targeted strains were selected at the following MPCs: >2048 μg/mL sodium hypochlorite; >1024 μg/mL BAC; and >1024 μg/mL CHG. There were several MSW-surviving strains, including 5 MSSA, 8 MRSA, 7 *P. aeruginosa* and 1 MDRP in sodium hypochlorite; 5 *P. aeruginosa* and 1 MDRP in CHG; and 12 *P. aeruginosa* and 2 MDRP in BAC. The bactericidal effects of various disinfectants on these strains were measured by a modified quantitative suspension test using a protocol based on the American Society for Testing and Materials (ASTM) International [21] and European standard BS EN 1040 [22]. Briefly, a bacterial suspension was diluted in Mueller–Hinton broth and aliquots of the bacterial suspension (5.0×10^7 CFU/mL) were then treated with 1000 μg/mL CHG, 1000 μg/mL BAC or 200 μg/mL sodium hypochlorite. Colonies were counted following incubation at 37 °C for 0.1 h and 12 h. The limit of detection was 10^1 CFU/mL.

3. Results

3.1. Susceptibility of bacteria to disinfectants

The MIC distribution of sodium hypochlorite, CHG and BAC for the 60 *S. aureus* and 30 *P. aeruginosa* clinical isolates is shown in Table 1. The MICs of sodium hypochlorite, CHG and BAC against *S. aureus* (MSSA and MRSA) were 128–512, 0.5–2 and 1–4 μg/mL, respectively. The MICs of sodium hypochlorite, CHG and BAC against *P. aeruginosa* of 256–512, 4–64 and 32–128 μg/mL, respectively, were relatively higher than those against *S. aureus*. These results show that the antimicrobial activity of sodium hypochlorite did not differ against the tested bacterial species, and that CHG and BAC have excellent bactericidal activity against *S. aureus*. In addition, one MDRP strain showed a BAC MIC_{100} of 256 μg/mL.

3.2. Relationship between mutant selection window (MSW) and recommended concentration for each disinfectant

Fig. 1 shows the distribution of MICs and MPCs for various disinfectants against the tested strains compared with the recommended concentration range in Japan. The MSW_{50}, MSW_{80} and MSW_{100} for sodium hypochlorite against *S. aureus* and *P. aeruginosa* were 4×, 8× and 16× MIC, respectively. Sodium hypochlorite 0.05%, which is the recommended concentration in Japan, was inside the MSW for all strains (Fig. 1A). In addition, the MSW_{50}, MSW_{80} and MSW_{100} values for CHG against *P. aeruginosa*

Table 1
Susceptibility (in μg/mL) to disinfectants of clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Strain (*n*)	NaOCl			CHG			BAC		
	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
MSSA (30)	256–512	512	512	0.5–2	1	1	1–4	2	2
MRSA (30)	128–512	512	512	0.5–2	1	2	1–4	2	4
P. aeruginosa (28)	256–512	256	512	4–64	16	32	32–128	64	64
MDRP (2)	256–512	256	512	16–32	16	32	32–256	32	256

NaOCl, sodium hypochlorite; CHG, chlorhexidine gluconate; BAC, benzalkonium chloride; MIC, minimum inhibitory concentration; MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; MDRP, multidrug-resistant *P. aeruginosa*.

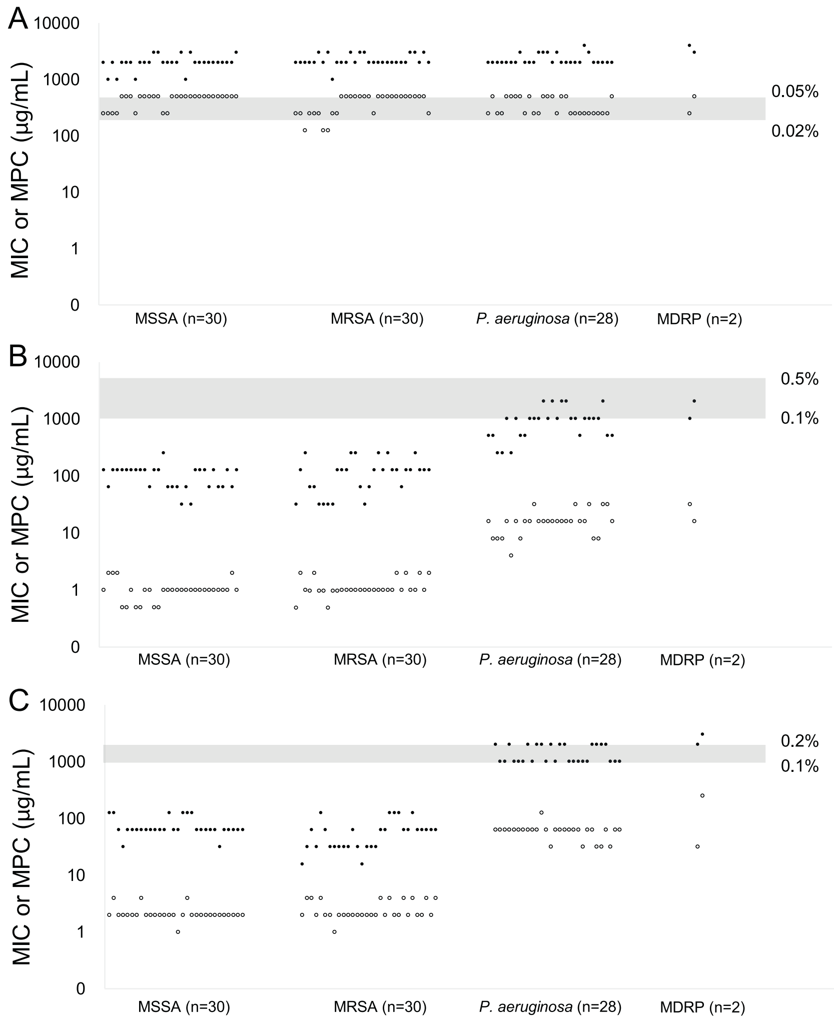

Fig. 1. Comparison of clinically recommended disinfectant concentrations in Japan with the minimum inhibitory concentration (MIC; ○) and mutant prevention concentration (MPC; ●) against *Staphylococcus aureus* and *Pseudomonas aeruginosa* for (A) sodium hypochlorite, (B) chlorhexidine gluconate (CHG) and (C) benzalkonium chloride. The grey zone shows, respectively, the recommended disinfectant concentrations in Japan. The mutant selection window (MSW) is the concentration range between the lower MPC and the upper MIC. MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; MDRP, multidrug-resistant *P. aeruginosa*.

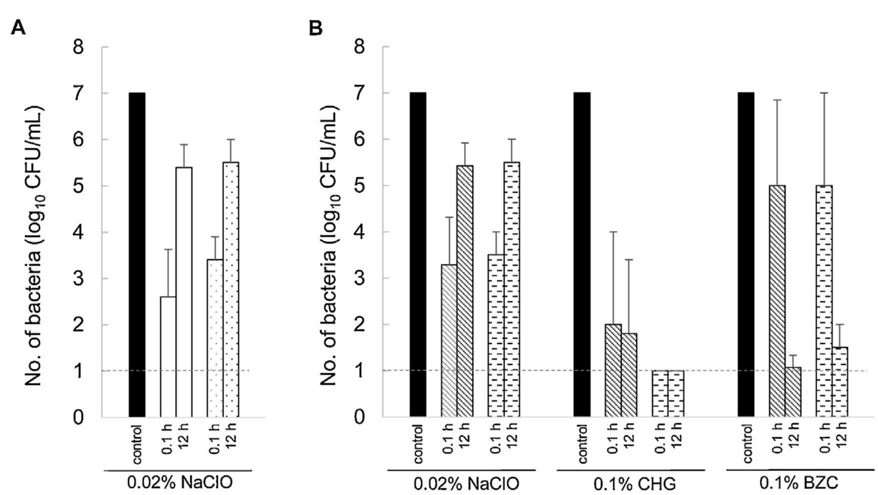

Fig. 2. Disinfectant activity against mutant selection window (MSW)-surviving strains. NaOCl, sodium hypochlorite; CHG, chlorhexidine gluconate; BAC, benzalkonium chloride. Bacteria were suspended in 0.02% NaOCl, 0.1% CHG or 0.1% BAC to achieve a final inoculum of 5.0×10^7 CFU/mL. Values are given as mean $\log_{10}$ CFU/mL. The dashed line indicates the limit of detection, and error bars indicate the standard deviation. Each bacterium was treated with the disinfectants for 0.1 h and 12 h. (A) *Staphylococcus aureus*, including methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA); and (B) *Pseudomonas aeruginosa*, including multidrug-resistant *P. aeruginosa* (MDRP). ■, control; □, MSSA; ⊡, MRSA; ⧅, *P. aeruginosa*; ⊟, MDRP.

were all 64× MIC, and the concentration of 0.5% exceeded all MSWs, but at 0.1% concentration 66.7% of *P. aeruginosa* were inside the MSW. In contrast, *S. aureus* exceeded all MSWs at any concentration of CHG (Fig. 1B). Finally, the MSW_{50}, MSW_{80} and MSW_{100} of BAC were 32× MIC against *P. aeruginosa*, and 53.3% of strains were inside the MSW at a concentration of 0.2% (Fig. 1C).

3.3. Bactericidal activity of disinfectants against mutant selection window (MSW)-surviving strains

Fig. 2 shows the bactericidal activity of each disinfectant against MSW-surviving strains. Since the MPC of CHG and BAC against *S. aureus* was <0.1% for each strain, the suspension was completely sterilised at this concentration. Whereas 0.02% sodium hypochlorite eliminated ca. 10^4 CFU/mL of *S. aureus* and *P. aeruginosa* at 0.1 h, which increased to 10^5–10^6 CFU/mL after 12 h for both strains. The bactericidal activity of CHG against *P. aeruginosa* was ca. 10^5 CFU/mL at 0.1 h, which remained constant even after 12 h, and it was below the detection limit for MDRP. In BAC solution, *P. aeruginosa* and MDRP decreased ca. by 10^2 CFU/mL at 0.1 h and by 10^4 CFU/mL at 12 h.

4. Discussion

Several studies have reported *S. aureus* and *P. aeruginosa* strains that are resistant to CHG and BAC [7–10]. These disinfectants are known to cause resistance at concentrations lower than the recommended values in clinical environments. In the present study, sodium hypochlorite-resistant *S. aureus* and *P. aeruginosa* survived sodium hypochlorite at levels above the recommended concentrations for environmental surface disinfection. In addition, resistant *P. aeruginosa* appeared by exposure to relatively high concentrations of CHG and BAC. Although clinical isolates with a high $MIC_{99.9}$ of each disinfectant have been reported [9], antimicrobial activities of disinfectants equivalent to those in this study have been reported globally [10,23–26].

Sodium hypochlorite is one of the most common disinfectants worldwide and is widely used for environmental disinfection in health facilities and nursing homes because of its efficient antimicrobial activity against norovirus [27]. Furthermore, there are reports stating that the detection rate of MRSA was reduced when sodium hypochlorite was used as an environmental disinfectant in hospitals in Scotland [28]. This evidence strengthens the fact that sodium hypochlorite is one of the most important disinfectants in infection control. Table 2 shows the recommended concentrations of sodium hypochlorite for environmental disinfection in Europe and the USA. In the USA, the recommended concentration of sodium hypochlorite is 0.525–0.615% (5250–6150 μg/mL) [32] and in Europe it is 0.5% (5000 μg/mL) [19], whereas in Japan it is 0.02–0.05% (200–500 μg/mL), which is much lower than the concentrations recommended in the USA and Europe. In this study, we first evaluated the bactericidal activity of sodium hypochlorite against MSW-surviving *S. aureus* and *P. aeruginosa*. Approximately 10^3 CFU/mL survived when these strains were inoculated into 0.02% sodium hypochlorite for 0.1 h. Therefore, when 0.02% sodium hypochlorite is used for environmental disinfection, *S. aureus* and *P. aeruginosa* may not be completely eliminated. In contrast, all strains were eliminated with 0.5% sodium hypochlorite (data not shown). The recommended concentration in Japan is thus considered inadequate for disinfection in health facilities and nursing homes, hence it is necessary to set the recommended concentration to the same level as in Europe and USA.

Table 2
Recommended concentrations for environmental disinfection in different countries.

Country	Concentration (%)[a]			Reference(s)
	NaOCl	CHG	BAC	
USA	0.525–0.615	N/A	0.0865–0.21	[29,32]
Europe	0.5	1–2	1–2	[19,30]
Japan	0.02–0.05	0.1–0.5	0.1–0.2	[31]

NaOCl, sodium hypochlorite; CHG, chlorhexidine gluconate; BAC, benzalkonium chloride; N/A, not applicable.
[a] 0.1% disinfectant concentration means 1000 μg/mL.

Moreover, sodium hypochlorite is used in bleach baths for antisepsis in atopy patients. The recommended concentration of sodium hypochlorite in these bleach baths is 0.005% [33,34]. In addition, 0.025% bleach bath therapy is administered for the treatment of MRSA infection in patients with burn injuries [35]. Since the concentration of sodium hypochlorite used in these bleach bath therapies is extremely low compared with those used for environmental disinfection, long-term exposure of sodium hypochlorite used at 0.005–0.025% may increase the appearance of sodium hypochlorite-resistant strains of *S. aureus* and *P. aeruginosa*. It is necessary to treat with care while paying attention to resistance because bleach baths are a treatment method effective for ameliorating the symptoms of patients with atopic dermatitis.

Sodium hypochlorite exerts a bactericidal effect by altering the permeability of the bacterial cell wall and cytoplasmic membrane, passing through these by passive diffusion, denaturing enzymes involved in glycolysis and the tricarboxylic acid (TCA) cycle, and destroying nucleic acids owing to its oxidising action [36]. Hypochlorous acid acts as a powerful oxidising agent and shows an instant sterilising effect. Bacteria do not produce substances that can inactivate hypochlorous acid, therefore in strains that survive in the MSW of sodium hypochlorite another mechanism might discharge or remove this substance. Furthermore, sodium hypochlorite MSW-surviving strains showed a slow growth rate, requiring ≥48 h for colonisation (data not shown), which might be related to the increase of H^+ concentration owing to the decomposition of hypochlorous acid, leading to a decrease in the pH level. We presume that ATP is used for the hydrolysis of H^+ to maintain the pH level in the bacterium [36], which slowed the growth rate by inhibiting growth and metabolism pathways.

The bactericidal capacity of CHG against MSW-surviving *P. aeruginosa* was observed as a decreased of 10^5 CFU/mL at the first 0.1 h, thus sterilisation was achieved in a relatively short time. In Japan and Europe, CHG is used for environmental disinfection; the recommended concentration in Japan is as low as 0.1–0.5% compared with 1–2% in Europe. As shown in Fig. 2B, MSW-surviving strains for CHG are sufficiently eliminated at 0.1%. In this study, the MPC was high but no resistant strains appeared. Therefore, the recommended concentration of 1–2% used in Europe is considered to be relatively high.

Pseudomonas aeruginosa strains that are resistant to BAC have been reported previously. The recommended concentration of BAC for environmental disinfection use in Europe is 1–2%, but in the USA and Japan it is ≤0.2%. In the current study, the bactericidal ability of 0.1% BAC against MSW-survived *P. aeruginosa* was reduced to the detection limit after 12 h, although it was not an immediate effect. Therefore, when using BAC as an environmental disinfectant it is important to consider not only increasing the concentration but also prolonging the sterilisation time to enhance the bactericidal effect.

5. Conclusions

This study evaluated for the first time the bactericidal activity against *S. aureus* and *P. aeruginosa* strains surviving in the MSW of disinfectants. Proper use of various disinfectants means using them at concentrations that both kill micro-organisms and inhibit resistance. In particular, sodium hypochlorite shows a bactericidal effect against various pathogenic micro-organisms and is inexpensive, making it frequently used globally. However, since it was found that resistant strains to sodium hypochlorite remained at a concentration of ≤0.3% (≤3072 μg/mL), environmental disinfection with a concentration of ≤0.3% should not be recommended. In contrast, CHG and BAC were found to be able to exert a sufficient bactericidal effect at a lower concentration than recommended levels used in Europe.

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Competing interests

None declared.

Ethical approval

Not required.

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