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Original Article

Chlorine Dioxide is a Better Disinfectant than Sodium Hypochlorite against Multi-Drug Resistant *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Acinetobacter baumannii*

Atsushi Hinenoya[1], Sharda Prasad Awasthi[1], Noritomo Yasuda[1], Ayaka Shima[1], Hirofumi Morino[2], Tomoko Koizumi[2], Toshiaki Fukuda[2], Takanori Miura[2], Takashi Shibata[2], and Shinji Yamasaki[1]*

[1]*Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 598-8531; and* [2]*Taiko Pharmaceutical Co., Ltd., Osaka, Japan*

SUMMARY: In this study, we evaluated and compared the antibacterial activity of chlorine dioxide (ClO_2) and sodium hypochlorite (NaClO) on various multidrug-resistant strains in the presence of bovine serum albumin and sheep erythrocytes to mimic the blood contamination that frequently occurs in the clinical setting. The 3 most important species that cause nosocomial infections, i.e., methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Pseudomonas aeruginosa* (MDRP), and multidrug-resistant *Acinetobacter baumannii* (MDRA), were evaluated, with three representative strains of each. At a 10-ppm concentration, ClO_2 drastically reduced the number of bacteria of all MDRP and MDRA strains, and 2 out of 3 MRSA strains. However, 10 ppm of NaClO did not significantly kill any of the 9 strains tested in 60 seconds (s). In addition, 100 ppm of ClO_2 completely killed all MRSA strains, whereas 100 ppm of NaClO failed to significantly lower the number of 2 MRSA strains and 1 MDRA strain. A time-course experiment demonstrated that, within 15 s, 100 ppm of ClO_2, but not 100 ppm of NaClO, completely killed all tested strains. Taken together, these data suggest that ClO_2 is more effective than NaClO against MRSA, MDRP, and MDRA, and 100 ppm is an effective concentration against these multidrug-resistant strains, which cause fatal nosocomial infections.

INTRODUCTION

Multidrug-resistant (MDR) bacterial strains have been increasingly recognized as a serious problem in clinical settings (1–4). Among the resistant strains, methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Pseudomonas aeruginosa* (MDRP), and multidrug-resistant *Acinetobacter baumannii* (MDRA) are the leading causes of hospital-borne infections, which are often fatal to immunocompromised patients. The treatment of patients infected with these MDR strains is inadequate because of the limited options of antimicrobial agents. In addition, the MDR strains found in the hospital environment can infect patients through medical and surgical instruments. Therefore, it is extremely important to eliminate MDR strains from these instruments by using highly efficient disinfectants.

Sodium hypochlorite (NaClO) is one of the most widely used disinfectants. However, it has a strong irritating odor and has to be used in liquid form. In addition, NaClO is easily inactivated in the presence of biological materials such as blood cells and plasma proteins. In comparison, chlorine dioxide (ClO_2) is a water-soluble and yellow gas with a strong oxidizing activity (5,6). Earlier studies have observed that ClO_2 has a potent antimicrobial activity against bacteria, fungi, protozoa, and viruses (7–11). This chemical agent has been also utilized for the disinfection of supplied water in European countries (maximum 0.5 ppm) and the United States (maximum 0.8 ppm) because of its low production of trihalomethane compounds (12). However, there is limited data on whether ClO_2 has a strong antimicrobial activity against MDR strains, including MRSA, MDRP, and MDRA.

Therefore, the present study aimed to evaluate and compare the antibacterial activity of ClO_2 and NaClO against the most clinically important MDR strains i.e., MRSA, MDRP, and MDRA, in the presence of biological materials comparable to contaminated blood and serum proteins, which interfere with antimicrobial activity in the clinical setting.

MATERIALS AND METHODS

Reagents, strains, and culture media: Chlorine dioxide (ClO_2; Cleverin L) was obtained from Taiko Pharmaceutical Co., Ltd. (Osaka, Japan), and sodium hypochlorite (NaClO) and sodium thiosulfate ($Na_2S_2O_3$) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The concentrations of NaClO and ClO_2 were estimated using an iodometric method (13) and spectrophotometric method (14), respectively. Defibrinated sheep blood was obtained from Nippon Bio-Supp. Center (Tokyo, Japan). Tryptone was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Sodium chloride was purchased from Nacalai

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*Corresponding author: Mailing address: Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58, Rinku ourai-kita, Izumisano, Osaka 598-8531, Japan. Tel/Fax: +81-72-463-5653, E-mail: shinji@vet.osakafu-u.ac.jp

Table 1. Bacterial strains used in this study

Bacterial species	Strain	Origin	MDR pattern[1)]
Staphylococcus aureus	3146529	Clinical	MPIPC, CEZ, CMZ, IPM, GM, EM, CLDM, MINO, LVFX
	3514346	Clinical	MPIPC, CEZ, CMZ, IPM, GM, EM, CLDM, MINO
	0180900	Clinical	MPIPC, CEZ, CMZ, EM, LVFX
Pseudomonas aeruginosa	61406	Clinical	CEZ, CTM, CFDN, CTRX, CFPM, MEM, AMK, DOXY, ST
	NGTPA2	Clinical	ABPC, CAZ, IPM, SM, KM, NFLX, CM
	NGTPA4	Clinical	ABPC, CAZ, IPM, SM, KM, NFLX, CM
Acinetobacter baumannii	ATCC1605	Clinical	TIPC, PIPC, AZT, CAZ, CFPM, IPM, MEM, GM, CPFX
	NGTAB8	Clinical	ABPC, SM, NFLX, CM
	NGTAB11	Clinical	ABPC, SM, NFLX, CM

[1)]: ABPC, ampicillin; MPIPC, oxacillin; TIPC, ticarcillin; PIPC, peperacillin; AZT, aztreonam; CEZ, cefazolin; CTM, cefotiam; CAZ, ceftazidime; CMZ, cefmetazole; CFDN, cefdinir; CTRX, ceftriaxone; CFPM, cefepime; IPM, imipenem; MEM, meropenem; AMK, amikacin; SM, streptomycin; KM, kanamycin; GM, gentamicin; EM, erythromycin; DOXY, doxycycline; CLDM, clindamycin; MINO, minocycline; LVFX, levofloxacin; NFLX, norfloxacin; CPFX, ciprofloxacin; CM, chloramphenicol; ST, sulfamethoxazole-Trimethoprim.

(Kyoto, Japan). Mannitol salt agar with egg yolk (MSEY) and heart infusion agar plates were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). The bacterial strains used in this study are listed in Table 1. The strains were cultured on heart infusion agar plates at 37°C overnight. The grown bacterial cells were suspended in sterile saline (0.85% NaCl, pH 7.4) and adjusted to an OD_{625} of 0.35 for use in the disinfection assay.

In vitro disinfection assay: The disinfection assay was performed using an established protocol based on the European standard (EN13727:2012) defined by the Comité Européen de Normalisation using a mixture containing a high concentration of bovine serum albumin (BSA) and sheep erythrocytes (SE), with some modifications. Briefly, the bacterial suspension described above was added to an equal volume of a mixture containing 3% (w/v) BSA and 3% (v/v) SE in a diluent solution (0.1% [w/v] tryptone, 0.85% [w/v] NaCl/DW). A 100 μL aliquot of the bacterial suspension was treated with 400 μL of a freshly prepared solution of ClO_2 or NaClO at either 10 ppm or 100 ppm at room temperature. A 100 μL aliquot of each treated sample was collected after a 15, 30, 60, and 120-second (s) incubation and neutralized by adding 900 μL of 50 mM $Na_2S_2O_3$. Subsequently, the mixture was serially diluted (10-fold) and spread on agar plates. After incubation at 37°C for 24 to 48 h, the number of colonies was counted. MSEY was used for *S. aureus* and heart infusion agar plates were used for *P. aeruginosa* and *A. baumannii*. All of the experiments were done in triplicate for each bacterial strains.

Statistical analysis: Scheffe's F test was used for the statistical analysis.

RESULTS

Each MRSA strain was treated with 2 different concentrations (10 ppm and 100 ppm) of each of the disinfectants (ClO_2 and NaClO) for 60 s. ClO_2 at 100 ppm completely killed (below the detection limit) all 3 strains tested. However, NaClO at this concentration did not significantly decrease the number of bacteria except for strain 0180900 (Fig. 1A). When 10 ppm of ClO_2 was used, the initial count of approximately 10^7 cfu of two MRSA strains (strains 3146529 and 0180900) decreased 10 times, whereas 10 ppm of NaClO did not significantly kill any of the MRSA strains tested. With regard to MDRP, even 10 ppm of ClO_2 completely killed (below the detection limit) all the tested strains (Fig. 1B). With regard to MDRA, 10 ppm of ClO_2 drastically reduced the number of all the strains tested whereas 100 ppm of ClO_2 completely killed (below the detection limit) these strains as shown in Fig. 1C. By contrast, 10 ppm of NaClO did not significantly reduce the number of any MDRP and MDPA strains tested, although 100 ppm of NaClO significantly reduced the number of all MDRP strains tested and 2 out of 3 MDRA strains (Fig. 1B and 1C). Therefore, ClO_2 may be considered as a more potent disinfectant than NaClO for the MDR strains evaluated.

Subsequently, we performed a time-course assay to evaluate the antimicrobial activity of 2 different concentrations (10 ppm and 100 ppm) of ClO_2 and NaClO against MRSA, MDRP, and MDRA. When a representative MRSA strain (strain 3146529) was evaluated, 10 ppm or even 100 ppm of NaClO did not decrease its number after a 120-s incubation whereas 10 ppm of ClO_2 caused a 2-log reduction in the bacterial number, and 100 ppm of ClO_2 completely killed (below detection limit) even after a 15-s incubation (Fig. 2A). Similarly, 10 ppm and 100 ppm of ClO_2 killed all of the bacteria (approximately 10^7 cfu) of a representative MDRP strain (NGTPA4) after a 30- and 15-s incubation, respectively (Fig. 2B). By contrast, 10 ppm of NaClO did not significantly decrease the number of MDRP strain NGTPA4, although 100 ppm of NaClO reduced the number of bacteria significantly (Fig. 2B). Furthermore, 100 ppm of ClO_2 significantly reduced the number of representative MDRA strain (ATCC1605) after a 15-s incubation (Fig. 2C). 10 ppm of ClO_2 decreased the number of bacteria in a time-dependent manner and killed all the treated cells (below the detection limit) after a 120-s incubation. However, although 100 ppm of NaClO reduced a number (1 log) of this MDRA strain after a 120-s incubation, a 10-ppm concentration of this disinfectant was incapable to cause a remarkable reduction in this number.

Taken together, these data suggest that ClO_2 is a more effective bactericidal agent compared with NaClO, particularly against MRSA, MDRP, and MDRA, which are the most important bacterial pathogens associated with

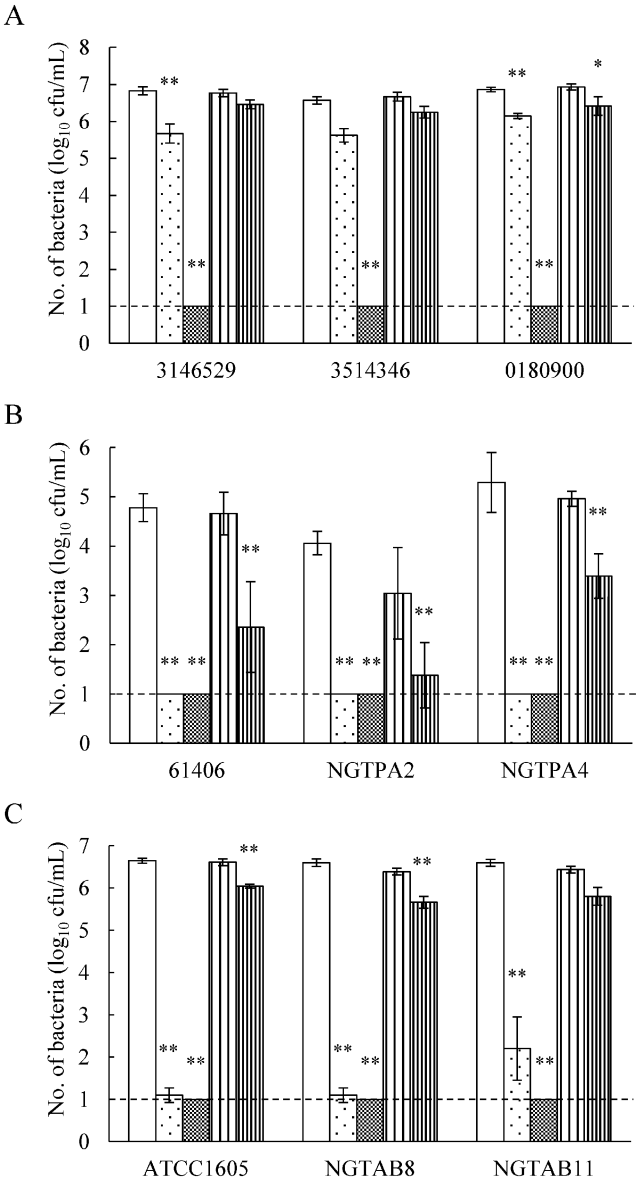

Fig. 1. Disinfectant activity of ClO_2 and NaClO against *S. aureus* (A), *P. aeruginosa* (B), and *A. baumannii* (C). Three strains each of bacteria were treated with the disinfectants for 60 sec at room temperature. Distilled water (□); 10 ppm ClO_2 (⬚); 100 ppm ClO_2 (▨); 10 ppm NaClO (▥); 100 ppm NaClO (▥). Values are given in mean $\log_{10}$ cfu/mL (n = 3). In all cases, dashed lines indicate the limit of detection, and error bars indicate standard deviations. The bars denoted with asterisks represent significant differences from negative controls treated with distilled water (*, $P < 0.05$ and **, $P < 0.01$).

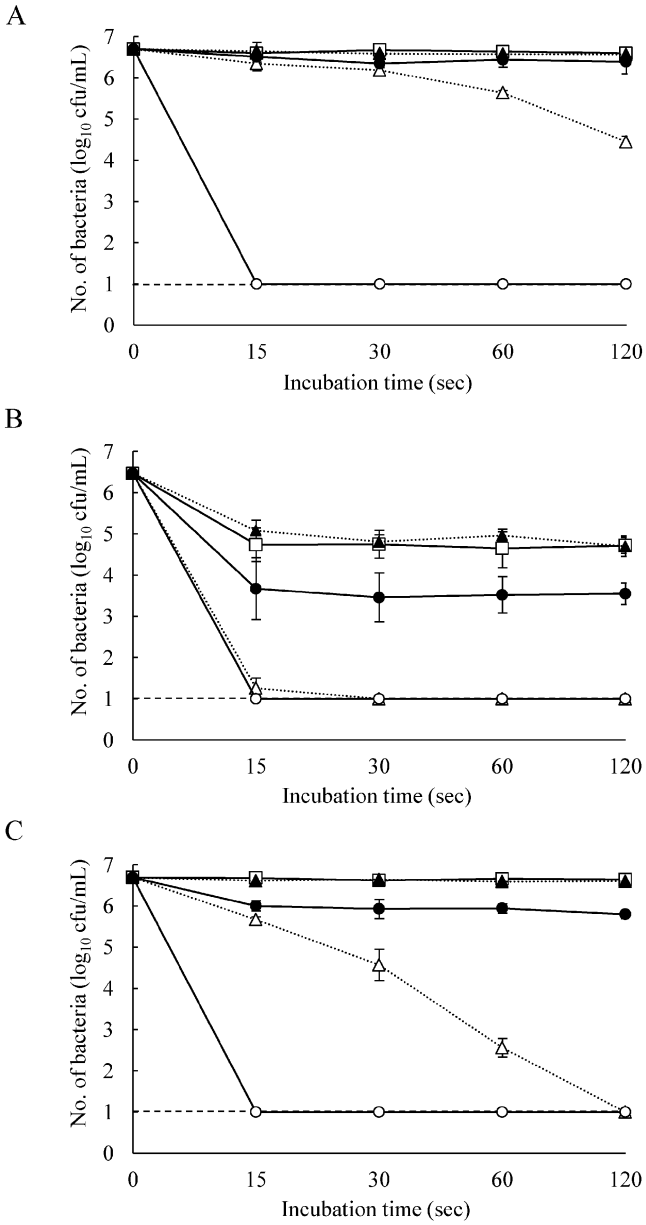

Fig. 2. Time course study for the disinfectant activity of various concentrations of ClO_2 and NaClO against *S. aureus* strain 3146529 (A), *P. aeruginosa* strain NGTPA4 (B) and *A. baumannii* strain ATCC1605 (C). Cells were treated with 10 ppm (triangle symbols, dotted line) and 100 ppm (circle symbols, solid line) of ClO_2 (open symbols) and NaClO (closed symbols), respectively. Aliquots of samples were collected at 15, 30, 60, 120 s at room temperature. Distilled water was used as negative control (open squares). Values are given in mean $\log_{10}$ cfu/mL (n = 3). In all cases, dashed lines indicate the limit of detection, and error bars indicate standard deviations.

nosocomial infections.

DISCUSSION

In the present study, it was clearly demonstrated that ClO_2 was more effective than NaClO in significantly reducing the number of colonies of MRSA, MDRP, and MDRA. Accordingly, 100 ppm of ClO_2, but not 100 ppm of NaClO, was sufficient to kill all the 9 MDR strains tested, including 3 each of MRSA, MDRP, and MDRA. The higher potential of ClO_2 as a disinfectant compared with NaClO was also reflected when a 10-fold lower concentration (10 ppm) of ClO_2 was used and drastically reduced the number of all MDRP and MDRA strains, and most of the MRSA strains tested. Furthermore, 10 ppm of ClO_2 killed all the MDR strains tested in the absence of organic compounds such as blood (data not shown). However, 10 ppm of NaClO did not significantly reduce the number of any MDR strain tested in this manner. Together, these data suggest that 100 ppm of ClO_2 can be used as a disinfectant against these MDR strains in the presence of organic compounds, and 10 ppm may be sufficient in the absence of organic compounds. Appropriate disinfection

and sterilization procedures are required for the control of hospital-acquired infections, which often lead to fatal cases due to opportunistic infections with MDR strains, particularly MRSA, MDRP, and MDRA. The difficulty in effectively treating infections due to highly resistant *P. aeruginosa*, *S. aureus*, and *A. baumannii* is a serious clinical problem (15). The infection routes of these pathogenic bacteria are usually through contact with infected humans and instruments, including life-supporting ventilators. Therefore, it is vital to maintain a proper sanitary environment in hospitals, particularly in intensive care units. The present study supports the hypothesis that ClO_2 may be a superior disinfectant for large-scale usage in clinical facilities.

Among the several disinfectants used in hospitals, NaClO is often used and recommended for disinfection. However, the use of NaClO brings several disadvantages including its irritating and toxic effects and efficacy in a limited pH range. In contrast, ClO_2 is an efficient disinfectant and is less toxic, less irritant, effective in a wide pH range, can be used as both liquid and gas (16), and produces fewer trihalomethane compounds (12). It has been demonstrated that the mode of action of ClO_2 is via protein denaturation and involves the covalent oxidative modification of tryptophan and tyrosine residues (6). However, until date, little effort has been devoted to evaluating the efficacy of ClO_2, as a disinfectant on MDR strains including *P. aeruginosa*, *S. aureus*, and *A. baumannii*. In addition, clinical settings are often contaminated with blood and other biological substances, and disinfectants are usually inactivated by biological substances such as proteins and fatty acids. Therefore, in this study, a comparative evaluation of the effects of ClO_2 and NaClO on MDR strains was conducted in the presence of BSA and SE to mimic the clinical setting. Our pioneering study showed that ClO_2 was highly effective and better than NaClO in killing MRSA, MDRP, and MDRA within 15 s, even in the presence of BSA and SE, when a concentration of 100 ppm was used.

In conclusion, ClO_2 has a more potent antimicrobial activity than NaClO against MDR strains. Because ClO_2 is less irritating and less toxic than NaClO, it can be a more suitable and effective disinfecting agent against MDR strains such as MRSA, MDRP, and MDRA, which cause fatal opportunistic infections in hundreds of thousands of hospitals throughout the world, including advanced medical centers in developed countries.

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