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

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Summary (200/200)

In this study, we evaluated the antibacterial activity of chlorine dioxide (ClO_2) compared with sodium hypochlorite (NaClO) on various multidrug-resistant strains in the presence of bovine serum albumin and sheep erythrocytes to mimic the frequent blood contamination in clinical environment. The 3 most important species causing nosocomial infections, i.e., methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Pseudomonas aeruginosa* (MDRP) and multidrug-resistant *Acinetobacter baumannii* (MDRA) were evaluated, with 3 representative strains from each. At a 10 ppm-concentration, ClO_2 drastically reduced the number of all MDRP and MDRA, and 2 out of 3 MRSA strains, but NaClO was unable to cause any remarkable attenuation for any of the 9 strains tested in 60 seconds. Increased concentration of 100 ppm enabled ClO_2 to completely kill MRSA strains, whereas NaClO failed to significantly lower the number of 2 MRSA and 1 MDRA strains. A time-course experiment demonstrated that, within 15 seconds, 100 ppm of ClO_2 could kill completely all tested strains but NaClO at this concentration failed to do so. Together, these data suggest that ClO_2 is more effective than NaClO against MRSA, MDRP and MDRA, and 100 ppm could be a practical concentration of ClO_2 against these multidrug-resistant strains, which may cause fatal nosocomial infections.

Introduction

Multidrug-resistant (MDR) strains of bacteria 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. It is very difficult to treat the patients infected with these types of MDR strains as there are very limited options to select effective antimicrobial agents. MDR strains residing in the hospital environment can infect patients through health care apparatus or surgical instruments. Therefore, it is extremely important to eliminate MDR strains from health care apparatus and surgical instruments by using highly efficient disinfectant.

Sodium hypochlorite (NaClO) is one of the most widely used recommended disinfectants. However, NaClO has a strong irritating odor and has to be used in liquid form. Additionally, 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 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 bodies (12). However, there is a lack of extensive information whether ClO_2 has a strong antimicrobial activity against MDR strains such as MRSA, MDRP and MDRA.

In the context of above mentioned background, the present study has, therefore, evaluated and compared the antibacterial potential of ClO_2 and NaClO against the most important MDR strains causing clinical incidences, i.e., MRSA, MDRP and MDRA, in the presence of biological materials comparable to the contaminated blood and serum proteins, which may have some interferences, to mimic clinical settings.

Materials and methods

Reagent, strains and culture media

Chlorine dioxide (ClO_2; Cleverin L) obtained from Taiko pharmaceutical Co. Ltd (Osaka, Japan), and sodium hypochlorite (NaClO) and sodium thiosulfate ($Na_2S_2O_3$) purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) were used in this study. The concentration of NaClO (13) and ClO_2 (14) were estimated by iodometric method and spectrophotometric method, respectively. Defibrinated sheep blood was from Nippon Bio-Supp. Center (Tokyo, Japan). Tryptone was from Becton Dickinson (Franklin Lakes, NJ, USA). Sodium chloride was from Nacalai (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 bacterial cells grown on the plate after overnight incubation were suspended in sterile saline (0.85% NaCl, pH 7.4) and adjusted to 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 European standard (EN13727:2012) defined by Comité Européen de Normalisation

(CEN) using mixture of bovine serum albumin (BSA) solution-high concentration with sheep erythrocytes (SE) with some modifications. Briefly, bacterial suspension at OD_{625} of 0.35 was mixed with equal volume of mixture of 3% (w/v) BSA and 3% (v/v) SE in a diluent solution (0.1% [w/v] tryptone, 0.85% [w/v] NaCl /DW). One hundred micro liter of the bacterial suspension was treated with freshly prepared 400 µL of ClO_2 or NaClO at either 10 or 100 ppm at room temperature. One hundred micro liter aliquot of the treated samples were collected after 15, 30, 60 and 120-second incubation and neutralized by adding 900 µL of 50 mM $Na_2S_2O_3$. Then, 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 and Heart Infusion agar plates were used for *S. aureus*, and *P. aeruginosa* and *A. baumannii*, respectively. All of the experiments were done in triplicate for each of the individual strains.

Statistical analysis

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

Results

When MRSA strains were treated with 2 different concentrations (10 and 100 ppm) of each of the disinfectants (ClO_2 and NaClO) for 60 seconds, ClO_2 at a 100 ppm concentration was able to completely kill (below detection limit) all 3 strains tested but NaClO at this concentration was unable to significantly decrease their numbers except strain 0180900 (Fig. 1A). When 10 ppm of ClO_2 was used, about 10^7 cfu (initial count) of two MRSA strains (strains 3146529 and 0180900) were reduced ten times, whereas 10 ppm of NaClO did not reduce the number of any of the 3 MRSA strains tested. In the case of MDRP, even 10 ppm of ClO_2 completely killed (below detection limit) all the

tested strains (Fig. 1B). In the case of MDRA, 10 ppm of ClO_2 drastically reduced the number of all the tested strains whereas 100 ppm completely killed (below detection limit) as shown in Fig. 1C. However, in case of NaClO treatment using 10 ppm concentration, there was no considerable reduction in the number of any MDRP and MDPA strains tested, although 100 ppm of NaClO significantly reduced the number of all MDRP tested and 2 out of 3 MDRA strains (Fig. 1B and 1C). Therefore, according to these results, ClO_2 may be considered as a more potent disinfectant than NaClO for the selected important MDR strains.

Next we performed a time-course assay for the disinfectant activity of 2 different concentrations (10 and 100 ppm) of ClO_2 and NaClO against MRSA, MDRP, and MDRA. When the strain 3146529, as a representative MRSA strain, was examined, 10 and even 100 ppm of NaClO was unable to decrease its number after 120-second 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) approximately 10^7 CFU of this strain even after 15-second incubation (Fig. 2A). Similarly when the strain NGTPA4, as a representative MDRP strain, was examined, 100 and 10 ppm of ClO_2 was able to kill all of its cells (approximately 10^7 CFU) after 15- and 30-second incubation, respectively (Fig. 2A). However, when 10 ppm of NaClO was used, the number of MDRP strain NGTPA4 did not decrease significantly, although 100 ppm of NaClO was able to reduce the number of bacteria significantly (Fig. 2B). In the case of a representative MDRA strain (Strain ATCC1605), 100 ppm of ClO_2 caused a total reduction of its number (approximately 10^7 CFU) after 15-second incubation (Fig. 2C). On the other hand, 10 ppm of ClO_2 decreased the number of this bacterium in a time-dependent manner and could completely kill (below detection limit) all its treated cells

(approximately 10^7 CFU) after 120-second incubation. However, although 100 ppm of NaClO was able to reduce an equal number of this MDRA strain significantly after 120-second incubation, 10 ppm concentration of the disinfectant was incapable to cause a remarkable reduction in this bacterial number.

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

Discussion

In the present study it has been clearly demonstrated that ClO_2 is more effective than NaClO in killing all the cells or significantly reducing the number of MRSA, MDRP and MDRA strains. According to this study, a concentration of 100 ppm ClO_2, but not NaClO, is sufficient to kill all the 9 MDR strains tested, including 3 each of MRSA, MDRP and MDRA. The higher potential of ClO_2 than NaClO as a disinfectant is also reflected when a 10-fold lower concentration (10 ppm) of ClO_2 is used, i.e., a drastic reduction in the number of all MDRP and MDRA, and majority of the MRSA strains tested in case of ClO_2 but no remarkable reduction of any MDR strains by NaClO at this concentration. When 10 ppm of ClO_2 was used against MRSA, MDRP and MDRA strains in the absence of organic compounds such as blood, all MDR strains were completely killed (data not shown). Together, these data suggest that 100 ppm could be a practical concentration of ClO_2 to use as a disinfectant against these MDR strains in the presence of organic compounds. However, 10 ppm could be sufficient when ClO_2 was used as a disinfectant in the absence of organic compounds. Appropriate disinfection and sterilization procedures are required for control hospital-

acquired infections, which may often lead to fatal cases due to the opportunistic invasion of MDR strains, especially MRSA, MDRP and MDRA. The difficulty in effectively treating infections of highly resistant *P. aeruginosa*, *S. aureus* and *A. baumannii* is a serious medical problem (15). Infection routes of these pathogenic bacteria are usually via the health care staff or medical apparatus, including the life-supporting ventilators. Therefore, it is vital to maintain proper sanitary environment in hospitals, particularly in intensive care units. The present study supports that ClO_2 may be a superior disinfectant for the large scale usage in clinical facilities.

Among the several disinfectants used in hospitals, NaClO is often used and recommended for disinfection. However, there are several disadvantages of NaClO, e.g., it is irritant, toxic and effective in a limited pH range. In comparison, ClO_2 is also a better disinfectant but less toxic and irritant, effective in wide range of pH, can be used as both liquid and gas (16), and produce less trihalomethane (12). It has been demonstrated that the mode of action for ClO_2 is through denaturation of proteins involving covalent oxidative modification of their tryptophan and tyrosine residues (6). However, until now, there are no extensive efforts to evaluate the efficacy, as a disinfectant, of ClO_2 on MDR strains such as *P. aeruginosa*, *S. aureus*, and *A. baumannii.* In addition, clinical settings are often contaminated with blood and other biological substances and disinfectant is usually inactivated by biological substance such as protein and fatty acids. Therefore, in this study, 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 settings. Our pioneering study shows that ClO_2 is highly effective and better than NaClO in killing MRSA, MDRP and MDRA within 15

seconds, even in the presence of BSA and SE, if 100 ppm concentration of this chemical agent is used.

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

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Conflict of interest

HM, TK, TF, TM and TS are employed by Taiko Pharmaceutical Co. Ltd. This work was supported in-part by a consigned research fund from Taiko Pharmaceutical Co. Ltd. The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparations of the manuscript.

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Figure legends

Fig. 1. Disinfectant activity of ClO_2 and NaClO against *Staphylococcus aureus* (A), *Pseudomonas aeruginosa* (B) and *Acinetobacter baumannii* (C).

Three strains each of *S. aureus* (A), *P. aeruginosa* (B) and *A. baumannii* (C) 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 *Staphylococcus aureus* (A), *Pseudomonas aeruginosa* (B) and *Acinetobacter baumannii* (C).

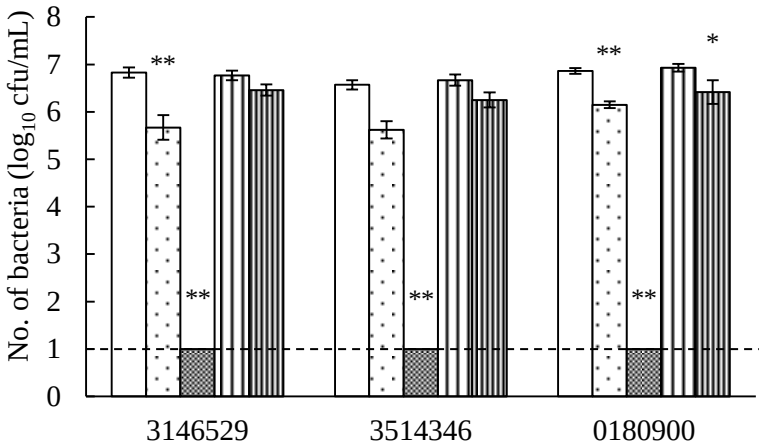
S. aureus strain 3146529 (A), *P. aeruginosa* strain NGTPA4 (B) and *A. baumannii* strain ATCC1605 (C) were treated with 10 (triangle symbols, dotted line) and 100 ppm (circle symbols, solid line) of ClO_2 (open symbols) and NaClO (closed symbols), respectively. Aliquots of samples were collected at 15, 30, 60, 120 sec at room temperature. Distilled water was used as negative control (open square). 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.

Table 1. Bacterial strains used in this study

Bacterial species	Strain	Origin	MDR patterns*
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, CFPN, 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

*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

(A)



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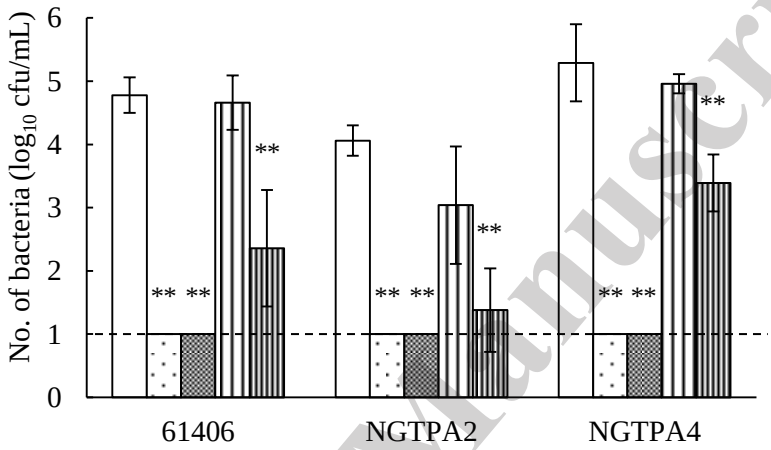

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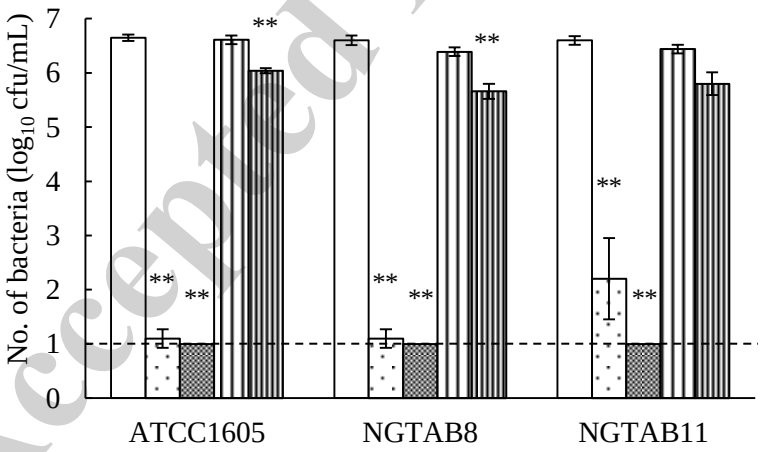
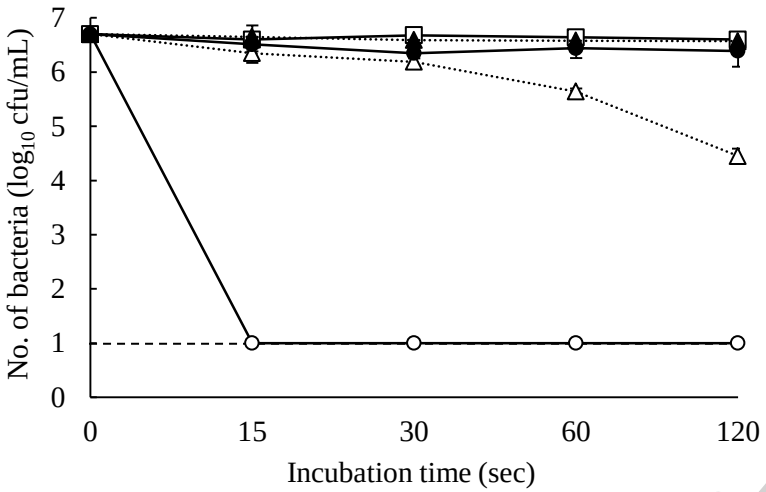


(A)



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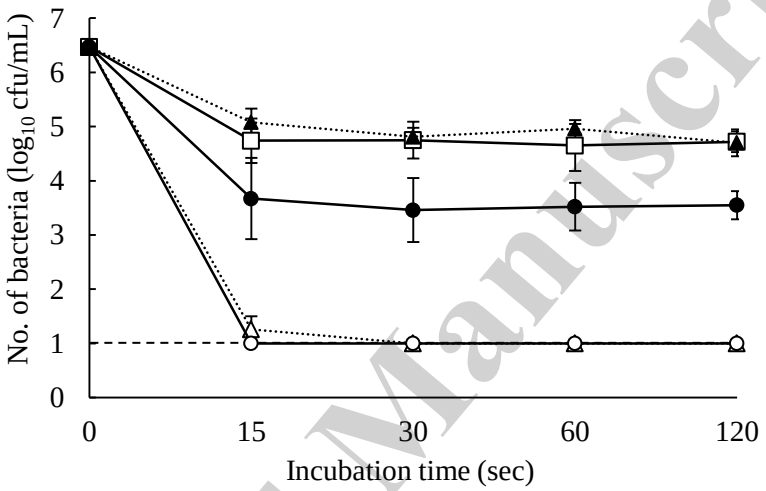

(C)