Original Article

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:** In this study, we evaluated and compared the antibacterial activity of chlorine dioxide (ClO2) 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, ClO2 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 ClO2 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 ClO2, but not 100 ppm of NaClO, completely killed all tested strains. Taken together, these data suggest that ClO2 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 (ClO2) is a water-soluble and yellow gas with a strong oxidizing activity (5,6). Earlier studies have observed that ClO2 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 ClO2 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 ClO2 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 (ClO2; Cleverin L) was obtained from Taiko Pharmaceutical Co., Ltd. (Osaka, Japan), and sodium hypochlorite (NaClO) and sodium thiosulfate (Na2S2O3) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The concentrations of NaClO and ClO2 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...
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 OD625 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 (EN13772:2012) 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 ClO2 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 0.1 M Na2S2O3. 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 statistical analysis.

**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 (ClO2 and NaClO) for 60 s. ClO2 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 ClO2 was used, the initial count of approximately 107 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 ClO2 completely killed (below the detection limit) all the tested strains (Fig. 1B). With regard to MDRA, 10 ppm of ClO2 drastically reduced the number of all the strains tested whereas 100 ppm of ClO2 completely killed (below the detection limit) all the tested strains (Fig. 1B). Therefore, ClO2 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 ClO2 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 ClO2 caused a 2-log reduction in the bacterial number, and 100 ppm of ClO2 completely killed (below detection limit) even after a 15-s incubation (Fig. 2A). Similarly, 10 ppm and 100 ppm of ClO2 killed all of the bacteria (approximately 107 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 ClO2 significantly reduced the number of representative MDRA strain (ATCC1605) after a 15-s incubation (Fig. 2C). 10 ppm of ClO2 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 ClO2 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 ClO2 and NaClO against S. aureus (A), P. aeruginosa (B), and A. baumannii (C). Three strains of each bacterium were treated with the disinfectants for 60 sec at room temperature. Distilled water ( ); 10 ppm ClO2 ( ); 100 ppm ClO2 ( ); 10 ppm NaClO ( ); 100 ppm NaClO ( ). Values are given in mean log10 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 (*, P < 0.05 and **, P < 0.01).

DISCUSSION

In the present study, it was clearly demonstrated that ClO2 was more effective than NaClO in significantly reducing the number of colonies of MRSA, MDRP, and MDRA. Accordingly, 100 ppm of ClO2, 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 ClO2 as a disinfectant compared with NaClO was also reflected when a 10-fold lower concentration (10 ppm) of ClO2 was used and drastically reduced the number of all MDRP and MDRA strains, and most of the MRSA strains tested. Furthermore, 10 ppm of ClO2 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 ClO2 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 \textit{P. aeruginosa}, \textit{S. aureus}, and \textit{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{2} as a disinfectant on MDR strains including \textit{P. aeruginosa}, \textit{S. aureus}, and \textit{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\textsubscript{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\textsubscript{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\textsubscript{2} has a more potent antimicrobial activity than NaClO against MDR strains. Because ClO\textsubscript{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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\textbf{REFERENCES}