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Inactivation of Airborne Bacteria and Viruses Using Extremely Low Concentrations of Chlorine Dioxide Gas

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Key Words
Chlorine dioxide · Gas · Bacteria · Virus · Airborne · Bactericidal · Nursing home · Operating room · Disinfection · Hospital

Abstract
Infectious airborne microbes, including many pathological microbes that cause respiratory infections, are commonly found in medical facilities and constitute a serious threat to human health. Thus, an effective method for reducing the number of microbes floating in the air will aid in the minimization of the incidence of respiratory infectious diseases. Here, we demonstrate that chlorine dioxide (ClO_2) gas at extremely low concentrations, which has no detrimental effects on human health, elicits a strong effect to inactivate bacteria and viruses and significantly reduces the number of viable airborne microbes in a hospital operating room. In one set of experiments, a suspension of *Staphylococcus aureus*, bacteriophage MS2, and bacteriophage ΦX174 were released into an exposure chamber. When ClO_2 gas at 0.01 or 0.02 parts per million (ppm, volume/volume) was present in the chamber, the numbers of surviving microbes in the air were markedly reduced after 120 min. The reductions were markedly greater than the natural reductions of the microbes in the chamber. In another experiment, the numbers of viable airborne bacteria in the operating room of a hospital collected over a 24-hour period in the presence or absence of 0.03 ppm ClO_2 gas were found to be 10.9 ± 6.7 and 66.8 ± 31.2 colony-forming units/m^3 ($n = 9$, $p < 0.001$), respectively. Taken together, we conclude that ClO_2 gas at extremely low concentrations (≤0.03 ppm) can reduce the number of viable microbes floating in the air in a room. These results strongly support the potential use of ClO_2 gas at a non-toxic level to reduce infections caused by the inhalation of pathogenic microbes in nursing homes and medical facilities.

Introduction

The occurrence of airborne nosocomial infection has long been a threat to patients and staff at nursing homes and hospitals. Hospital-acquired infections are an especially important public health problem associated with significant rates of morbidity and mortality [1]. The common causative agents of airborne infections include bacteria, viruses and fungi. Among the bacteria that cause respiratory infections, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus*

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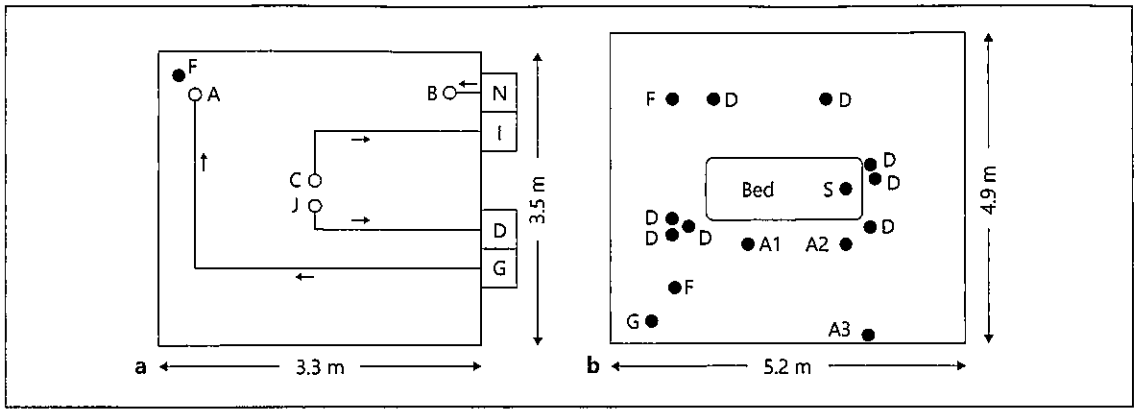

Fig. 1. Layouts of the exposure chamber and hospital operating room of a hospital that were used in our experiments. **a** Layout of the exposure chamber. The locations of the ClO_2 generator (G), air impinger (I), ClO_2 gas analyzer (D), and bacterial nebulizer (N) are shown. The orifices for ClO_2 gas release (A), the gas analyzer (J), the air impinger (C) and the bacterial nebulizer (B) are also indicated by open circles. (F) indicates the location of the fan that was placed on the floor. The arrows indicate the direction of airflow. **b** The layout of the hospital operation room used in our experiments. The locations of the fan (F), ClO_2 gas analyzer (D), ClO_2 gas generator (G), thermohygrometer (S) and air-samplers (A1, A2, and A3) are shown. Medical instruments were present in the room but are not shown except the bed in this scheme.

pneumoniae, *Haemophilus influenzae* and *Moraxella catarrhalis* are frequently found in upper and lower respiratory tract infections [2, 3]. Most airborne infections are transmitted by microbe-containing droplets that are expelled from infected persons [4]. However, some infections result from the inhalation of pathogen-contaminated particulate matter floating in the air [5]. Thus, the rigorous control of these airborne pathogens is important in nursing homes and medical facilities to reduce the occurrence of respiratory tract infections.

Chlorine dioxide (ClO_2) is a yellow gas at room temperature and has a characteristic odor [6]. ClO_2 has one unpaired electron in its molecular orbital and is a relatively stable free radical [7]. ClO_2 is a powerful oxidizing agent and denatures proteins by oxidizing their constituent tryptophan and tyrosine residues [8]. Due to this strong oxidizing ability, ClO_2 is used to disinfect tap water in some countries [2, 9, 10]. ClO_2 has also been used in the form of a gas at very high concentrations (i.e. 7–30 mg/l) as a disinfectant to kill microbes in enclosed spaces [11]. However, because such high concentrations of ClO_2 gas are toxic, the room being disinfected must be evacuated before disinfection [12].

In 2012, Akamatsu et al. [12] demonstrated that whole-body exposure to ClO_2 gas at 0.1 parts per million (ppm, volume/volume) has no toxic effect on rats during or after an exposure period of 6 months. Ogata et al. [13] also confirmed that whole-body exposure to ClO_2 gas at 1.0 ppm has no toxic effect on rats during or after an exposure period of 5 h per day for 5 days per week over 10 weeks. The American Occupational Safety and Health Administration states that human exposure to 0.1 ppm ClO_2 gas as an 8-hour time-weighted average is permissible [14]. Based on these findings, we assumed that exposure to extremely low concentrations of ClO_2 gas (i.e. below 0.1 ppm) would not elicit any harmful effects in humans, but could be potentially useful for eradicating infectious airborne microbes. A specific advantage of disinfecting rooms with such low concentrations of ClO_2 gas is that the area need not be evacuated prior to treatment. However, such applications require the development of a device that enables the release of very low concentrations of ClO_2 gas in a precisely controlled manner. The recent fabrication of sophisticated ClO_2 gas-generating instruments that can be operated in such a manner motivated us to initiate this study. Here, we demonstrate that ClO_2 gas at or below 0.03 ppm effectively reduces the number of viable airborne microbes.

Materials and Methods

Effect of ClO_2 Gas in an Exposure Chamber

An exposure chamber of 25-m^3 (3.3 × 3.5 × 2.2 m) with walls covered with stainless steel plates (Amenity Technology, Kawasaki, Japan) was used to test the efficacy of the inactivation of microbes with ClO_2 gas (fig. 1a). During this experiment, the door of the chamber was tightly closed, and the air inside the chamber was constantly circulated to maintain a homogeneous concentration of ClO_2 gas using a fan (BS-B-25: Yamazen, Osaka, Japan) placed on the floor. After the completion of each experiment, the residual

microorganisms in the chamber were disinfected via exposure to ultraviolet irradiation.

After incubation for 24 h at 35°C in tryptic soy agar (TSA: Difco, Becton, Dickinson and Company, Md., USA), *S. aureus* NBRC 1273 was collected and suspended in sterile ion-exchanged water at 1.2×10^{10} colony-forming units (CFUs)/ml. ClO_2 gas was introduced into the chamber through a Teflon tube (6-mm outer diameter and 4-mm inner diameter) at site A near fan F (fig. 1a) from a ClO_2 generator (Lispass S™; Taiko Pharmaceutical, Osaka, Japan) placed outside of the exposure chamber (G; fig. 1a). The ClO_2 gas concentration in the chamber was measured continuously by drawing air from site J located 1.5 m above the chamber floor via a Teflon tube into a precisely calibrated ClO_2 analyzer (D; fig. 1a; MIDAS-E-BR2: Honeywell, N.J., USA; FC303: Riken Keiki, Tokyo, Japan). The ClO_2 gas concentration was maintained at 0.01, 0.02 or 0.1 ppm (280 μg/m^3) during the course of the experiment. To confirm the homogeneity of the gas distribution in the chamber, we also sampled air with ClO_2 analyzers from 4 other locations within the chamber (i.e. the usual test site J plus the 4 corners of the chamber 1.0 m above the floor; fig. 1a).

After stabilizing the ClO_2 gas concentration, the bacterial suspension was released into the chamber using a nebulizer for 1 min at a flow rate of 0.2 ml/min and pressure of 230 kPa as aerosol particles of 0.3–3.0 μm in diameter. After 1 min, the released bacteria were intermittently collected for up to 120 min by aspirating the air from the chamber for 2 min (5 ml/min) with a glass impinger containing 20 ml of collection fluid (physiological saline containing 0.015% sodium thiosulfate). After the airborne bacteria in the chamber had been collected, a viable cell count was determined by incubation on a nutrient agar plate (SCD medium plate) for 48 h at 35°C. The results are expressed as CFU/m^3 of air collected. Control experiments were performed by repeating the experiment in the absence of ClO_2 gas (0 ppm). The temperature and relative humidity in the chamber were measured with a thermohygrometer (Ondotori TR-72U: T&D, Nagano, Japan) between 0 and 120 min after initiating the ClO_2 gas release. The temperature and relative humidity during this period were 22.6 ± 0.3°C and 62.9 ± 0.9%, respectively.

To test the effects of ClO_2 gas against viruses, *Escherichia coli* bacteriophage MS2 (NBRC 102619; a non-enveloped single-stranded RNA bacteriophage) and ΦX174 (ATCC 13706-B1; a non-enveloped single-stranded circular DNA bacteriophage) were used. MS2 was grown using *E. coli* strain NBRC106373 and ΦX174 was grown using *E. coli* strain ACTT13706. Suspensions of the bacteriophages (2.7×10^{10} plaque-forming units (PFUs)/ml for MS2 and 1×10^{10} PFUs/ml for ΦX174), were made, and released into the exposure chamber as an aerosol as described earlier in the presence or absence of 0.01 or 0.02 ppm ClO_2 gas. The bacteriophages were collected with an impinger as described earlier, and the viable bacteriophages were recovered by infection to *E. coli* using semisolid agar medium overlaid on a hard agar plate. The numbers of viable bacteriophages are expressed as PFU/m^3 of aspirated air. The temperature and relative humidity during the period of the experiment were 22.0 ± 0.4°C and 63.4 ± 1.5%, respectively.

Effect of ClO_2 Gas on the Viability of Airborne Bacteria in an Operating Room

An operating room in a hospital located in the Osaka district was used during a spring long-term holiday season to test the effects of low concentrations of ClO_2 gas on airborne microbes. The room was out of service during the experiment, and no one was present in the room during the gas-exposure sessions of the experiment. All medical instruments in the operating room were covered with plastic sheets and left as they were normally placed in the operating room. All doors were kept closed throughout the experiment. The room was 64 m^3 (2.5 × 4.9 × 5.2 m; fig. 1b). A ClO_2 generator, ClO_2 analyzers and thermohygrometers were placed in the operating room (fig. 1b). ClO_2 gas was then released into the operating room for 24 h at a time-weighted average concentration of 0.03 ppm. The bacteria naturally floating in the room were collected with air samplers (MAS-100Eco, Merck Millipore, Darmstadt, Germany) that were placed in the room during the 24-hour period. The bacteria collected by the air samplers, which were operating at a flow rate of 100 l/min, over 10 min were then cultured using nutrient agar plates, and the numbers of collected viable bacteria were counted and are expressed as CFU/m^3 air aspirated. As a control, bacteria were collected over a 24-hour period as described earlier in the absence of ClO_2 gas. The control experiment was performed before the ClO_2-release session.

Statistical Analysis

The average values of the 2 groups were statistically compared with 2-tailed Student's t tests. The differences in the averages were considered significant when $p < 0.05$.

Results

Bactericidal Effect of ClO_2 Gas in an Exposure Chamber

The bactericidal effect of exposure to ClO_2 gas at a concentration of 0.01 ppm was examined. The numbers of viable bacteria decreased from 2.0×10^6 to 1.3×10^4 CFU/m^3 after 60 min at this concentration (fig. 2, open triangles), and this decrease was more pronounced at the ClO_2 gas concentrations of 0.02 and 0.1 ppm (fig. 2, filled circles and triangles, respectively). The observed decrease in the number of viable bacteria was much lower in the absence of the ClO_2 gas (i.e. 'natural' reduction; fig. 2 open circles). These results clearly demonstrated that the ClO_2 gas exhibited a bactericidal effect against the airborne bacteria.

Effect of ClO_2 Gas against Viruses in an Exposure Chamber

To test the effect of the ClO_2 gas against viruses, we used the *E. coli* bacteriophages MS2 and ΦX174 as model viruses. As illustrated in figure 3, both of the bacteriophages were inactivated at ClO_2 gas concentrations of 0.01 and 0.02 ppm. The reductions in the numbers of viable bacteriophages were greater than 2 $\log_{10}$ after 180 min in both of the experiments (fig. 3).

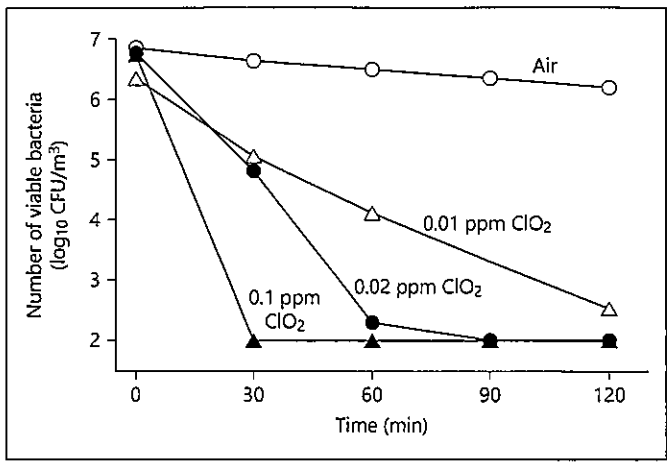

Fig. 2. Numbers of viable bacteria collected from the exposure chamber. A *S. aureus* suspension was released into the chamber as an aerosol in the presence of 0.01 ppm (open triangles), 0.02 ppm (filled circles) or 0.1 ppm (filled triangles) ClO_2 gas. The bacteria floating in the chamber air were collected over 2-min periods using an air impinger after various time intervals. As a negative control, the experiment was repeated with only air present in the chamber (open circles). The numbers of viable bacteria were counted after culturing and are presented as CFUs/m^3 of aspirated air.

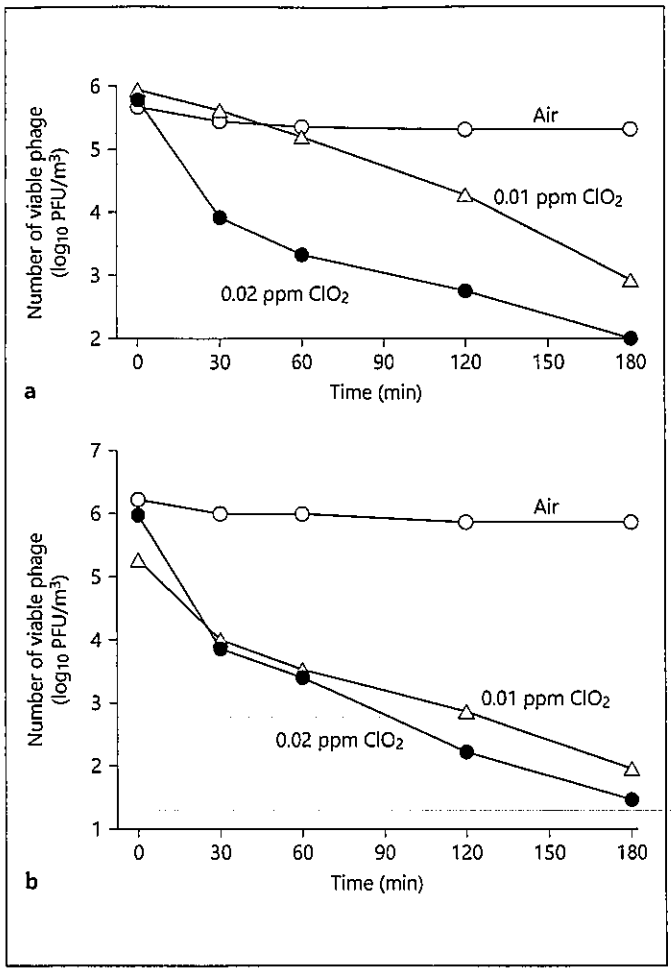

Fig. 3. Numbers of viable bacteriophages collected from the exposure chamber. Bacteriophage MS2 (**a**) and ΦX174 (**b**) were exposed to 0.01 (open triangles) or 0.02 ppm (filled circles) ClO_2 gas in the exposure chamber. The air in the chamber was aspirated for 2-min periods after various time intervals. As a negative control, the experiment was repeated with only air present in the chamber (open circles). The numbers of viable bacteriophages were counted as PFUs/m^3 of aspirated air after infection to *E. coli*.

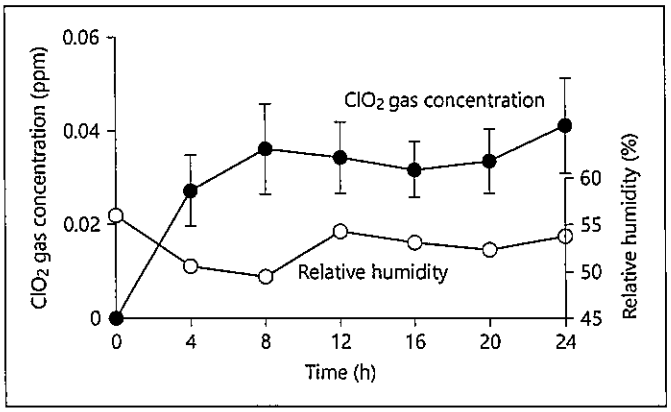

Fig. 4. ClO_2 gas concentration and relative humidity in the hospital operating room used in our experiments. The ClO_2 gas concentrations (filled circles) and relative humidities (open circles) are presented. Each ClO_2 gas concentration data point is represented by the mean ± SD of the data obtained from 7 ClO_2 analyzers located in the operating room (fig. 1b). Each relative humidity data point represents the mean of 2 measurements.

Control of the ClO_2 Gas Concentration in an Operating Room

The ClO_2 gas was released from the ClO_2 gas generator into the operating room (fig. 1b), and its concentration was kept nearly constant in the room with the exception of the initial 4-hour period (fig. 4, filled circles). The time-weighted average concentration over 24 h was 0.029 ± 0.013 ppm (n = 7). The relative humidity in the room during this period was 52.6 ± 2.0% (n = 144). The temperature of the room during this period was 26.5 ± 0.1°C (n = 144).

Table 1. Numbers of bacterial colonies recovered from the operating room

Air-sampling point[a]	ClO_2 gas concentration		p value (0 vs. 0.03 ppm)
	0 ppm	0.03 ppm	
A1	98.3±37.3	15.0±8.7	0.020
A2	56.3±4.6	9.7±4.0	0.00019
A3	45.7±12.7	8.0±7.0	0.011
Average[b]	66.8±31.2	10.9±6.7	0.00058

Airborne bacteria were collected in an operating room over a 24-hour period at points A1, A2 and A3 (each n = 3). The captured bacteria were then cultured, and the numbers of viable bacteria were counted and are expressed as CFUs/m^3 of air collected. The data are presented as the means ± SD.

[a] The air-sampling points are illustrated in figure 1b.

[b] Average of all of the individual data from A1, A2 and A3 (n = 9).

Bactericidal Effect of ClO_2 Gas in an Operating Room

Next, the bactericidal effect of the ClO_2 gas was examined in an actual operating room (fig. 1b). The numbers of viable airborne bacteria (i.e. naturally floating in air) collected from the room air over a 24-hour period were always lower when the ClO_2 gas was present at a concentration of 0.03 ppm ($n = 9$, $p < 0.001$) at all 3 of the air-sampling locations (fig. 1b; table 1). Based on these results, we conclude that ClO_2 gas at an extremely low concentration, which is non-toxic to humans, exhibits a significant bactericidal effect. Therefore, ClO_2 gas could be a convenient and efficient means of disinfecting rooms within hospital environments.

Discussion

It is technically difficult to release ClO_2 gas at extremely low concentrations and precisely maintain those concentrations in a controlled manner. However, the advent of the new ClO_2 generating device (Lispass S™) that was used in our experiment facilitated the desired release of low concentrations of ClO_2 gas (fig. 4). ClO_2 gas at concentrations below 0.1 ppm has been demonstrated to exert no toxic effects in humans [14] or animals [12, 13], although higher concentrations of the gas have been reported to cause damage to the respiratory system [15]. Therefore, it is essential to maintain the gas concentration below 0.1 ppm if it is to be used to control microbial infections in rooms occupied by people. Notably, Morino et al. [16] found that ClO_2 gas at 0.05 ppm can inactivate *S. aureus* and *E. coli* attached to glass surfaces. This result indicates that ClO_2 can inactivate both gram-positive *(S. aureus)* and gram-negative *(E. coli)* bacteria. Ogata and Shibata [17] also demonstrated that low-concentration ClO_2 gas (0.03 ppm) can protect mice against influenza virus infection, which suggests that this gas could also be used to control viral infections. This finding also suggests that ClO_2 can inactivate viruses with envelopes, such as influenza virus. Indeed, the presence of low concentrations of ClO_2 gas has been found to decrease the occurrence of respiratory diseases in an army base in Japan [18], and decrease absenteeism among children in an elementary school [19].

Ogata demonstrated that the mechanism of the antimicrobial effect of ClO_2 is due to the oxidation of a critical tryptophan residue of the viral protein hemagglutinin [20]. Indeed, these findings are consistent with the observation that ClO_2 can oxidize and denature proteins by modifying their tryptophan and tyrosine residues [8]. Therefore, we postulate that the antimicrobial action of ClO_2 arises because the constituent proteins of the microbes are oxidatively denatured by the gas. It should also be noted that Benarde et al. [21] demonstrated that ClO_2 kills bacteria by blocking the biosynthesis of proteins. However, Cho et al. [22] revealed that ClO_2 oxidizes lipids and consequently increases the permeability of the bacterial membrane, which leads to the release of proteins from the bacteria. In contrast, Berg et al. [23] reported that ClO_2 causes a loss of control of the permeability of K^+ and nonspecific oxidative damage to the bacterial outer membrane in *E. coli*, which subsequently results in the destruction of the trans-membrane ionic gradient and impairment of respiration. However, no significant leakage of intracellular macromolecules from the bacteria was observed in these experiments [23]. In conclusion, all of or at least some combination of the above-mentioned effects may explain the observed antimicrobial action of low concentrations of ClO_2 gas in our experiments.

Ultraviolet irradiation is occasionally used to disinfect operating rooms in some hospitals [24, 25]. The disadvantage of this system is that people have to be evacuated from the room to avoid damage to their eyes and skin [25]. Human exposure to ultraviolet rays may cause keratoconjunctivitis in the eyes and erythema in the skin, and thus, ultraviolet irradiation requires protection of skin and eyes [25]. Moreover, some areas of the operating room that are unlikely to be directly exposed to the ultraviolet rays may not be sufficiently disinfected based on the mechanisms of such systems. In contrast, our present findings indicate that the use of the low-concentration ClO_2 gas can disinfect almost all of the areas of operating rooms that are occupied by people.

Disclosure Statement

This work was supported by Taiko Pharmaceutical Co., Ltd. All authors except H. Oka are employees of this company.

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