Inactivation of Feline Calicivirus, a Norovirus Surrogate, by Chlorine Dioxide Gas

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The efficacy of gaseous chlorine dioxide (ClO₂) against feline calicivirus (FCV), a norovirus surrogate, in the dry and the wet states on a hard surface was evaluated. We demonstrated that low-concentration ClO₂ gas (mean 0.08 ppm, 0.22 µg/l) could inactivate FCV in the wet state with 0.5% fetal bovine serum (FBS) within 6 h in 45 to 55% relative humidity (RH) (>3 log₁₀ reductions) and FCV in the dry state with 2% FBS (percentage of FBS in the viral suspension) within 10 h in 75 to 85% RH (>3 log₁₀ reductions) at 20°C, respectively. Furthermore, a <0.3 ppm concentration of ClO₂ gas (mean 0.26 ppm, 0.73 µg/l) could inactivate (below the detection limit) FCV in the dry state with 5% FBS within 24 h in 75 to 85% RH at 20°C. In contrast, in 45 to 55% RH at 20°C, ClO₂ gas had little effect even when the FCV in the dry state was exposed to high-concentration ClO₂ (mean 8 ppm, 22.4 µg/l) for 24 h. These results suggest that humidity plays an important role in the inactivation by ClO₂ gas of FCV in the dry state. According to the International Chemical Safety Card, threshold limit values for ClO₂ gas are 0.1 ppm as an 8-h time-weighted average and 0.3 ppm as a 15 min short-term exposure limit. From these data, we propose that the treatment of wet areas of human activity such as kitchens, toilets, etc., with low-concentration ClO₂ gas would be useful for reducing the risk of infection by noroviruses (NV) without adverse effects. In addition, we believe that the application of a combination of a <0.3 ppm concentration of ClO₂ gas and a humidifier in places without human activity may make it possible to inactivate NV in the dry state on any surface within a contaminated room without serious adverse effects.

Key words: Chlorine dioxide/Gas/Feline Calicivirus/Norovirus/Disinfectant.

INTRODUCTION

Noroviruses (NV) belong to the family Caliciviridae and have a single-stranded RNA genome. NV are one of the most important cause of acute gastroenteritis throughout the world and have caused outbreaks in hospitals, nursing homes, residential facilities, schools, cruise ships, and hotels so on (Fankhauser et al., 1998; Lopman et al., 2003; Koopmans et al., 2003). NV infection can be spread by person-to-person contact, contaminated food or water, aerosols and environmental surfaces (Glass et al., 2000; Marks et al., 2000). A current study indicates that the sources of NV infection vary widely including environmental surfaces such as carpets, toilets, light fittings, curtains and lockers (Lopman et al., 2002). Therefore, conventional liquid disinfectants cannot adequately prevent NV infection and it is hoped that a new method for reducing the risk of infection by NV will be developed. Although an in vitro cell culture infectivity assay for human NV using an organoid model of human small intestinal epithelium has recently been reported (Straub et al., 2007), NV are
very difficult to grow in monolayer cell cultures. Feline calicivirus (FCV) has been used as a surrogate for NV in several disinfection studies (Doultree et al., 1999; Nuanualsuwan et al., 2003; Duizer et al., 2004; Urakami et al., 2007). FCV has a genome organization and capsid architecture similar to those of NV, and can easily be grown in cell culture (Jiang et al., 1993). Therefore, we used FCV to evaluate the anti-NV activity of the disinfectant described here.

Chlorine dioxide (ClO₂, CAS no. 10049-04-4), which is a strong oxidant gas at room temperature (Budavari et al., 2001), has been used as a water disinfectant in North America (Gates, 1998). Gaseous ClO₂ dissolves readily in water, and there are many reports of the antibacterial and antifungal activity of the solution (Benarde et al., 1965; Takayama et al., 1995). Recently, antibacterial and antifungal activity has been reported for gaseous ClO₂ (Wilson et al., 2005; Sy et al., 2005; Lee et al., 2004). However, little is known about whether gaseous ClO₂ has antiviral activity. The gaseous agents show features of excellent diffusibility and penetrability, making it possible to access sites that are difficult to disinfect with conventional liquid agents. In other words, these features make it possible for the gas to disinfect widely dispersed sources of NV infection. Therefore, we evaluated the possibility of using gaseous ClO₂ as a virucidal agent against FCV, surrogate of NV.

The purpose of this study was to investigate the possibility of using gaseous ClO₂ as a new method for reducing the risk of infection by NV.

**MATERIALS AND METHODS**

**Cells and virus**

Crandell Reese feline kidney (CRFK) cells were obtained from the American Type Culture Collection (ATCC; CCL-94). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, Mo.), 100 U/ml penicillin and 100 µg/ml streptomycin in 175 cm² disposable tissue culture flasks. The maintenance medium contained DMEM supplemented with 2% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. FCV strain F9 was obtained from ATCC (VR-782; Manassas, Va). CRFK cells were washed three times with Dulbecco’s phosphate buffer saline (D-PBS) and FCV was inoculated into a monolayer of cells in the flasks, allowed to adsorb for 90 min, then incubated in the flasks at 37°C for 17 h in a humidified atmosphere containing 5% CO₂ with the maintenance medium. After 17 h incubation, the viruses were released from the cells by freezing and thawing, and then clarified at 12,000 × g for 45 min to remove the cell debris. The supernatant was filtered through a 0.45 µm membrane filter (SCHVU11RE, Millipore Co., Billerica, MA) and ultra centrifuged at 70,000 × g for 3 h at 5°C. The precipitate was suspended in D-PBS and the suspension was centrifuged at 13,800 × g for 10 min, and the supernatant was stored at -80°C as virus stock (10⁷ TCID₅₀/50 µl, 370 µg protein/ml). The protein concentrations of the viral suspensions were determined by using the Bio-Rad Protein Assay (Bio-Rad, CA).

**Virus assay**

The infectivity titers of FCV were determined as the 50% tissue culture infectious dose (TCID₅₀)/50 µl according to the Spearman Kärber method (Hierholzer et al., 1996). CRFK cells were seeded into 96-well flat-bottomed micro titer plates (FALCON, Franklin Lakes, NJ, USA) and allowed to grow to confluence. CRFK cells in 50 µl of DMEM with 10% FBS per well were infected by the addition of 50 µl of serial 10-fold dilutions of virus (in the maintenance medium) per well, with four replicates per dilution.

**The preparation of FCV in the dry and the wet states**

FCV (100 µl, 10⁻³ TCID₅₀/50 µl) in D-PBS with 0 to 5% FBS were placed on glass dishes, respectively. For FCV in the dry state, the dishes were dried in a desiccator with silica gel for 2 h at 20°C after being air-dried for 1 h in a biological safety cabinet (FCV in the dry state with 0 to 5% FBS), but this drying was not performed for FCV in the wet state. These preparations were placed in the model space described below and were exposed to ClO₂ gas for 0, 2, 4, 6, 10, 16, 24 and 48 h. 100 µl of the maintenance medium was added to the dishes after exposure to ClO₂ gas or air, and then a sample was collected with a cell scraper (179693, Nunc). In the case of the wet state, the final volume was adjusted to 100 µl.

**Experimental setup**

To evaluate the efficacy of ClO₂ gas against FCV in the dry and the wet states, a model space was made by using a Tedlar bag (150 l, polyvinyl fluoride film, DuPont) with a spin bar (Fig. 1). The ClO₂ gas was produced by injection of a ClO₂ solution or a high-concentration of ClO₂ gas into the bag. The experiments were performed in the dark to prevent photo inactivation of ClO₂ gas. The ClO₂ gas in the model space was stirred by a spin bar during the evaluation. The concentration of ClO₂ gas in the model space
was measured by a ClO₂ measuring device (0-1000 ppb, Model 4330-SP, Interscan corporation). Similarly, a model space without ClO₂ gas was set up as a control. In the case of high concentration ClO₂ gas (more than 1 ppm), instead of the ClO₂ measuring device, the concentration of ClO₂ gas in the model space was measured with a gas detector tube (23M, GASTEC).

Control of Relative Humidity (RH)

The humidity in the model space was controlled using a saturated salt solution (Greenspan, 1977). In addition, the humidity in the model space was adjusted by using dry or wet air if the RH was not in the expected range. To avoid consumption of ClO₂ gas, the saturated salt solution (K₂CO₃: 43.2%, KCl: 85.1% at 20°C) was placed outside of the Tedlar bag (Fig. 1). The saturated salt solution and the Tedlar bag were placed in a polyethylene bag (150 × 100 cm) with low moisture permeability. The Tedlar film can slowly pass moisture at normal temperatures and pressures, but not ClO₂ gas. This enabled the humidity to be controlled in the range of 45 to 55% for "moderate" RH (absolute humidity 6.5 to 8.0 g/kg dry air) and 75 to 85% for "high" RH (absolute humidity 11.0 to 12.4 g/kg dry air) at 20°C. The RH and temperature in the model space were recorded by a portable hygrometer (DL-8829, CUSTOM, accuracy ±3 %RH) under air and ClO₂ gas.

Materials

A ClO₂ solution was prepared according to the method described earlier (Ogata, 2007). All other reagents were of the highest grade commercially available.

RESULTS

The concentration of ClO₂ gas in the model space (low-concentration ClO₂ gas)

The time course of changes in the concentration of ClO₂ gas in the model space is shown in Fig. 2. The average value of ClO₂ gas concentration in the model space was 0.08 ppm and the minimum and maximum values were 0.08 ppm and 0.09 ppm, respectively, except for the value at onset. Similar results were obtained in the case of high-concentration ClO₂ gas.

Inactivation of FCV in the dry and the wet states

We evaluated the efficacy of low-concentration ClO₂ gas against FCV in the dry and the wet (without any drying process) states on a hard surface. When the preparations of FCV in the dry state without organic matter were placed in the model space with low-concentration ClO₂ gas (mean 0.08 ppm) for 0, 6, 24 and 48 h in moderate RH at 20°C, the effect of low-concentration ClO₂ gas was little observed even after 48 h (Fig. 3A). In contrast, when preparations of FCV in the wet state without organic matter were placed in the model space with ClO₂ gas (mean 0.08 ppm) for 0, 2, 4, 6, 24 and 48 h in moderate RH at 20°C, virus infectivity was reduced by 2.5 and 4.0 log10 at 2 and 4 h and was below the detection limit after 6 h (Fig. 3B). The reduction in the infectivity of the virus was little observed in ordinary air even after 48 h (Fig. 3AB).

Effect of organic load on the inactivation of FCV in the wet state by low-concentration ClO₂ gas with moderate RH
Effect of humidity on the inactivation of FCV in the dry state by ClO₂ gas

The inactivation of FCV in the wet state by low-concentration ClO₂ gas under moderate RH was observed, but not in the dry state as described above. Therefore, we investigated the effect of humidity on the inactivation of FCV in the dry state by ClO₂ gas. Preparations of FCV in the dry state with and without 5% FBS were placed in the model space with various ClO₂ gas concentrations under moderate and high RH at 20°C for 24 h. Inactivation of FCV in the dry state with and without 5% FBS by ClO₂ gas (with 5% FBS: >0.26 ppm, without 5% FBS: >0.08 ppm) was observed in high RH (>4 log₁₀ reductions), but only slight inactivation was noted in moderate RH (Table 2).

The time course of the inactivation of FCV in the dry state with organic matter by ClO₂ gas of various concentrations in high RH

When preparations of FCV in the dry state with 0, 1, 2 and 5% FBS were placed in the model space with low-concentration ClO₂ gas (mean 0.08 ppm) for 0 to 24 h in high RH at 20°C, virus infectivity fell below the detection limit after 10 h (0% FBS) and after 24 h (1% FBS), and was reduced by 3.2 log₁₀ at 10 h (2% FBS) and 2 log₁₀ at 24 h (5% FBS) (Fig. 4A). When preparations of FCV in the dry state with 5% FBS were placed in the model space with ClO₂ gas of various concentrations (>0.26 ppm) for 0 to 24 h in high RH at 20°C, virus infectivity was below the detection limit after 6 h (mean 0.8 and 8 ppm) and after 24 h at a mean concentration 0.26 ppm (Fig. 4B). In the case of <20°C, low-concentration ClO₂ gas (mean 0.08 ppm, 0.22 μg/l) could inactivate FCV in the dry state with 2% FBS within 24 h in 75 to 85% RH (>5 log₁₀ reductions) at 15°C (data not shown).

TABLE 1. Effect of organic load on the inactivation of FCV in the wet state by low-concentration ClO₂ gas under moderate RH.

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>FBS concentration in virus suspension (%)</th>
<th>Infectivity (log₁₀[TCID₅₀/50 μl])</th>
<th>Log₁₀ reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Air (Control)</td>
<td>ClO₂ gas (Mean 0.08 ppm)</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>6.2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>6.3</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>6.3</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>6.4</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>6.4</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.4</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Values are the mean of four experiments.

⁺⁺Values indicate titer reductions of >3 log₁₀ as compared with control values (in ordinary air).
TABLE 2. Effect of humidity on the inactivation of FCV in the dry state by ClO₂ gas.

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>ClO₂ gas concentration (ppm)</th>
<th>45 to 55 RH (%)</th>
<th>5% FBS (−)</th>
<th>5% FBS (+)</th>
<th>75 to 85 RH (%)</th>
<th>5% FBS (−)</th>
<th>5% FBS (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.08</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>6.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.26</td>
<td>1.0</td>
<td>0.6</td>
<td>6.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>4.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.3</td>
<td>2.1</td>
<td>4.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean of four experiments. Reduction in viral infectivity was little observed in air under moderate or high RH after 24 h. Values indicate titer reductions of >4 log<sub>10</sub> as compared with control values (in ordinary air).

FIG. 4. The time course of the inactivation of FCV in the dry state with organic matter by ClO₂ gas of various concentrations in high RH. (A) Glass dishes with FCV (○, 0% FBS; □, 1% FBS; △, 2% FBS; ◊, 5% FBS) were placed in the model space with low-concentration ClO₂ gas (mean 0.08 ppm) for 0, 6, 10 and 24 h in 75 to 85% RH at 20°C. (B) Glass dishes with FCV (5% FBS) were placed in the model space with various concentrations (○, 0.26 ppm; △, 0.8 ppm; □, 8 ppm) of ClO₂ gas for 0 to 24 h in 75 to 85% RH at 20°C. There were no differences in infectivity among preparations of FCV in the dry state with FBS of various concentrations at each time point in air conditions as control. *Infectivity is below the detection limit (<10⁻⁶ TCID₅₀/50 μl). ○, air (control).

DISCUSSION

In the present study, we demonstrated that low-concentration ClO₂ gas (mean 0.08 ppm) could inactivate FCV in the wet state with 0.5% FBS within 6 h in moderate RH (>3 log<sub>10</sub> reductions) and FCV in the dry state with 2% FBS within 10 h in high RH (>3 log<sub>10</sub> reductions) at 20°C. According to the International Chemical Safety Card, the threshold limit values for ClO₂ gas are 0.1 ppm as an 8-h time-weighted average and 0.3 ppm as a 15 min short-term exposure limit (Dobson et al., 2002). These data prompt us to consider that treatment of wet areas of human activity such as kitchens, toilets, etc., with low-concentration ClO₂ gas would be a useful for reducing the risk of infection by NV without adverse effects.

ClO₂ gas showed little effect against FCV in the dry state with 5% FBS in moderate RH at 20°C, even at a high concentration (mean 8 ppm) for 24 h. In contrast, in high RH at 20°C for 24 h, a concentration of 0.26 ppm ClO₂ gas inactivated (below the detection limit) FCV in the dry state with 5% FBS. These results suggest that humidity plays an important role in the inactivation of FCV in the dry state by ClO₂ gas. A recent study showed that ozone gas, which is as strong an oxidant as ClO₂, could inactivate FCV and NV in the dry state in high RH (>70%) (Hudson et al., 2007). We think that humidity is indispensable for an oxidant gas to inactivate FCV in the dry state.

For the inhalation toxicity of ClO₂ gas, there are no quantitative human data up to this moment. However, a study of inhalation exposure in rats has been reported for a single exposure, the calculated mean 50% lethal concentration (LC₅₀) was 32 ppm (90 μg/l) and for short-term exposure, there were no signs of toxicity related to exposure at 5 ppm (Dobson et al., 2002). From these data, the major disadvantage of gaseous ClO₂ is its potential toxicity at high concentrations, which precludes its use in places where people are active. In practice, this means that it can only be used in rooms that can be sealed off or quarantined for the duration of the treatment. Sources of NV infection are widely and generally distributed on environmental surfaces such as carpets, light fittings,
curtains, and lockers (Lopman et al., 2002), indicating the possibility that NV persists in the dry state and in low RH. We believe that the use of both a ClO₂ gas generator and a humidifier in places without human activity could inactivate NV in the dry state on any surface within a contaminated room. After treatment with the ClO₂ gas, the residual ClO₂ gas in the room can be removed by circulating the room air through a scrubbing solution or activated charcoal. In addition, the ClO₂ gas is readily detected by its chlorine-like odor at 0.1 ppm concentration, which is equal to the permissible exposure limit and hence can be avoided. In previous reports, the concentration of ClO₂ gas used to inactivate bacteria and fungi was extremely high (250 to 3500 ppm) (Wilson et al., 2005; Sy et al., 2005; Lee et al., 2004) which was much higher than the LC₅₀ value in rats. In the present study, the concentration of ClO₂ gas needed to inactivate FCV in the dry state with 5% FBS was much lower than these values. Here, we propose that a <0.3 ppm concentration of ClO₂ gas would also be a useful for reducing the risk of infection by NV without serious adverse effects.

FCVs is a highly infectious pathogen affecting cats. The viruses typically cause a moderate self-limiting acute oral and upper respiratory tract disease. Although morbidity is high, mortality is usually low except in kittens (Radford et al., 2007). In addition, vaccination against FCV has effectively reduced the incidence of clinical disease for many years. However, vaccination does not completely protect cats against infection because of FCV’s high-variability (Radford et al., 2007). Furthermore, a highly virulent strain of FCV (the so-called virulent systemic (VS) FCV) has emerged that is associated with outbreaks of disease with high mortality (>40%) even among vaccinated cats, and a new range of clinical features (Pesavento et al., 2004; Ossiboff et al., 2007; Hurley et al., 2004). All things considered, we think that the low and <0.3 ppm of concentrations of ClO₂ gas would be effective in protecting pet cats against infection with FCV and VS-FCV in homes, animal hospitals, pet shops, catteries, etc.

The mechanism of inactivation of FCV by ClO₂ has not been reported to date. However, the mechanism of inactivation of other viruses such as bacterial virus f2, poliovirus 1, and hepatitis A virus by ClO₂ has frequently been reported (Noss et al., 1985; Alvarez et al., 1982; Li et al., 2004). The reactivity of ClO₂ towards proteins, and the role of these reactions in the inactivation of the bacterial virus f2 have been evaluated (Noss et al., 1985). The report discussed the fact that f2 virus was inactivated by ClO₂ reacting with tyrosine residues on the capsid protein so that viral adsorption to the host bacteria became impossible. With respect to the denaturation of protein by ClO₂, a biochemical study using model proteins has clearly demonstrated that their denaturation is caused by the oxidative modification of their tryptophan and tyrosine residues (Ogata, 2007). That report concluded that microbes were inactivated by ClO₂ owing to the denaturation of constituent proteins critical to their integrity and/or function. From these reports, we speculate that FCV is inactivated by ClO₂ gas reacting with the tryptophan and tyrosine residues on key proteins that are indispensable to the infectivity of FCV. For example, the P2 domain, which is located at the outer surface of the FCV capsid protein VP1, could be a target of ClO₂. The P2 domain is essential for virus-host interactions for FCV infection (Bhella et al., 2008). Feline junctional adhesion molecule 1 (fJAM-1) was recently identified as a functional receptor for FCV (Makino et al., 2006). Bhella et al. have shown that a serine residue (S91) of the fJAM-1, which was identified as possibly important for FCV binding (Makino et al., 2006), comes into contact with a conserved tyrosine residue (Y435) of the P2 domain, using cryo-electron microscopy and three-dimensional image reconstruction. It may be involved, at least in part, in the mechanism of inactivation of FCV by ClO₂ that the ability of FCV to bind the receptor, fJAM-1, is abolished by ClO₂ reacting with the tyrosine residue (Y435) of the P2 domain.

In summary, we have demonstrated that low-concentration ClO₂ gas (mean 0.08 ppm) can inactivate FCV in the wet state with 0.5% FBS within 6 h in moderate RH (>3 log₁₀ reductions) and FCV in the dry state with 2% FBS within 10 h in high RH (>3 log₁₀ reductions) at 20°C, and a <0.3 ppm concentration of ClO₂ gas (mean 0.26 ppm) inactivates (below the detection limit) FCV in the dry state with 5% FBS within 24 h in 75 to 85 RH at 20°C. To our knowledge, this is the first study reporting FCV inactivation by ClO₂ in the gas form. We propose that the low and <0.3 ppm concentrations of ClO₂ gas may be suitable as a disinfectant to prevent NV infection.

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References


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