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Inactivation Kinetics and Mechanism of a Human Norovirus Surrogate on Stainless Steel Coupons via Chlorine Dioxide Gas

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Acute gastroenteritis caused by human norovirus is a significant public health issue. Fresh produce and seafood are examples of high-risk foods associated with norovirus outbreaks. Food contact surfaces also have the potential to harbor noroviruses if exposed to fecal contamination, aerosolized vomitus, or infected food handlers. Currently, there is no effective measure to decontaminate norovirus on food contact surfaces. Chlorine dioxide (ClO_2) gas is a strong oxidizer and is used as a decontaminating agent in food processing plants. The objective of this study was to determine the kinetics and mechanism of ClO_2 gas inactivation of a norovirus surrogate, murine norovirus 1 (MNV-1), on stainless steel (SS) coupons. MNV-1 was inoculated on SS coupons at the concentration of 10^7 PFU/coupon. The samples were treated with ClO_2 gas at 1, 1.5, 2, 2.5, and 4 mg/liter for up to 5 min at 25°C and a relative humidity of 85%, and virus survival was determined by plaque assay. Treatment of the SS coupons with ClO_2 gas at 2 mg/liter for 5 min and 2.5 mg/liter for 2 min resulted in at least a 3-log reduction in MNV-1, while no infectious virus was recovered at a concentration of 4 mg/liter even within 1 min of treatment. Furthermore, it was found that the mechanism of ClO_2 gas inactivation included degradation of viral protein, disruption of viral structure, and degradation of viral genomic RNA. In conclusion, treatment with ClO_2 gas can serve as an effective method to inactivate a human norovirus surrogate on SS contact surfaces.

Human norovirus (NoV) is the most prevalent cause of foodborne illnesses worldwide (1–3). This etiological agent accounts for more than 58% of all foodborne illnesses, causing 5.5 million cases of acute gastroenteritis in the United States annually (1). It is estimated that human NoV is responsible for more than 95% of nonbacterial acute gastroenteritis. Human NoV transmission occurs primarily through the fecal-oral route, either via person-to-person contact or contaminated food, water, fomites, and environmental surfaces (4, 5), and airborne transmission of viral particles may also be possible due to aerosolized vomitus or fecal material (6). Human NoV is highly contagious, with an infectious dose as low as 10 particles, and outbreaks often occur in confined settings, including restaurants, coach buses, hotels, nursing homes, hospitals, and cruise ships (7–11). Although human NoV causes significant health and emotional burdens, research on human NoV has been hampered due to the lack of an *in vitro* cell culture method and a small animal model (12). Therefore, we must rely on proper surrogates to study the survival of human NoV. Currently, cultivable animal caliciviruses, such as murine norovirus (MNV), feline calicivirus (FCV), and Tulane virus (TV), are used as surrogates for the study of human NoV (13, 14). Studies have shown that MNV is more resistant to acid, heat, and environmental stresses than FCV (15). While MNV and TV have similar long-term storage stability and resistance to heat treatment, MNV was found to be more stable than TV when treated with low concentrations ($<$2 ppm) of chlorine (16). Therefore, MNV is a better surrogate to study human NoV inactivation by gaseous chlorine dioxide (ClO_2) than either FCV or TV.

Environmental contact surfaces are often contaminated with human NoV (17, 18). Surface contamination by human NoV may occur in restrooms, when vomitus or fecal excrements get aerosolized (6), or outside restrooms where poor hand hygiene and ineffective sanitation further spread the virus to surfaces (18). Individuals infected with human NoV can shed up to 9.5×10^{10} viral genomic RNA copies/g of feces, and viral shedding can last up to 28 days (19). Viral shedding lasts long after symptoms have subsided; moreover, asymptomatic individuals have also been shown to shed high levels of human NoV RNA (19, 20). Individuals lacking symptoms of an active human NoV infection, therefore, often unknowingly spread the virus to foods and contact surfaces (21). With regard to the food industry, areas susceptible to contamination include the processing plant, equipment, common surfaces, and food service utensils (such as plates, forks, and glasses), and this may occur through a number of routes, including exposure to infected food handlers and high-risk food items, such as oysters and fresh produce.

Sanitization of contact surfaces, regardless of whether directly or indirectly in contact with food, is a common practice in the food industry. Most routine sanitation procedures involve the use of liquid sanitizers, such as soap and water, chlorine, sodium hypochlorite, peracetic acid, hydrogen peroxide, ozone, and quater-

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nary ammonium compounds (22, 23). However, these sanitizers are not effective in inactivating and removing foodborne viruses from contact surfaces at Environmental Protection Agency (EPA)- and Food and Drug Administration (FDA)-permitted concentrations. Sodium hypochlorite and quaternary ammonium sanitizers achieved only a 0.5-log reduction in MNV-1 titer when tableware and food preparation utensils (plates, drinking glasses, and stainless steel forks) were contaminated with MNV (24). Development of an effective sanitizer for the inactivation of human NoV, therefore, is urgently needed.

ClO_2 gas is a strong oxidizer and is one of the emerging technologies for decontamination of contact surfaces and fresh produce. Besides being an effective biocidal agent (25, 26), ClO_2 is dispersed through the air and can be distributed to areas beyond the reach of liquid sanitizers. It has been shown that gaseous ClO_2 is a rapid and effective sterilizing agent active against bacteria, yeasts, and molds; however, its ability to inactivate foodborne viruses is less established. The objectives of this study, therefore, were to determine the kinetics of MNV-1 inactivation on stainless steel (SS) coupons when exposed to different concentrations (1, 1.5, 2, 2.5, and 4 mg/liter) of ClO_2 gas and to determine the mechanism of viral inactivation by ClO_2 gas.

MATERIALS AND METHODS

Cell culture and virus propagation. Murine norovirus strain 1 (MNV-1) was kindly provided by Herbert W. Virgin IV of the Washington University School of Medicine. MNV-1 was propagated in confluent monolayers of the murine macrophage cell line RAW 264.7 (ATCC, Manassas, VA) and maintained in Gibco Dulbecco's modified Eagle medium with GlutaMax (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Scientific, Lenexa, KS). For viral stock preparation, MNV-1 was inoculated into confluent RAW 264.7 cells at a multiplicity of infection of 0.01 and incubated at 37°C in 5% CO_2 for 48 h. When extensive cytopathic effects were observed, MNV-1 was harvested by freeze-thawing three times. After centrifugation at 3,000 rpm for 15 min, the supernatant was collected and stored at −80°C for later use.

Virus purification. Purified MNV-1 stock was prepared using the method previously described by Lou et al. (63). Large stocks of MNV-1 were generated by inoculation of 8 to 10 confluent T150 flasks of RAW 264.7 cells, as described above. Following harvest, the viral suspension was centrifuged at 8,000 × *g* for 15 min to pellet cellular debris in a Sorvall SS-34 rotor (Kendro Laboratory Products, Germany). The supernatant was collected and incubated with DNase I (10 μg/ml) and $MgCl_2$ (5 mM) at room temperature. After 1 h of incubation, 10 mM EDTA and 1% lauryl sarcosine were added to stop DNase activity. Subsequently, MNV-1 was concentrated by centrifugation at 82,000 × *g* for 6 h at 4°C in a Ty 50.2 rotor (Beckman Coulter, Fullerton, CA). Pelleted virus was resuspended in phosphate-buffered saline (PBS) and further purified by centrifugation at 175,000 × *g* for 6 h at 4°C with a sucrose gradient (7.5% to 45%) in an SW55 Ty rotor (Beckman Coulter). Finally, 100 μl of PBS was used to resuspend the final virus-containing pellets. The viral titer was determined by plaque assay on RAW 264.7 cells.

Viral quantification by plaque assay. RAW 264.7 cells were seeded in multiwell 6-well tissue culture plates (BD Falcon, Franklin Lakes, NJ) at a density of 2×10^6 cells per well. After 24 h of incubation, cell monolayers were infected with 400 μl of a 10-fold dilution series of the virus. The plates were then incubated for 1 h at 37°C with gentle shaking every 10 min during the 1-h incubation period. After incubation, cells were overlaid with 2.5 ml of Gibco minimum essential medium (Invitrogen) containing 1% UltraPure low-melting-point (LMP) agarose (Invitrogen), 2% FBS, 1% sodium bicarbonate, 0.1 mg of kanamycin/ml, 0.05 mg of gentamicin/ml, 15 mM HEPES (pH, 7.7; Sigma-Aldrich, St. Louis, MO), and 2 mM L-glutamine (Invitrogen). After incubation at 37°C and 5% CO_2 for 48 h, the plates were fixed with 2 ml of 10% formaldehyde for a minimum of 2 h, followed by 30 min of staining with crystal violet.

Preparation of SS coupons. SS coupons (dimensions, 1.3 cm by 5 cm; area, 6.5 cm^2) were completely submerged in 5.25% sodium hypochlorite for 10 min, followed by rinsing with deionized water. Rinsed coupons were wiped dry with paper towels, wrapped in aluminum foil, and then autoclaved.

Inoculation of MNV-1 on SS coupons. In a class II biosafety cabinet, sterile coupons were transferred into petri dishes. One hundred microliters of MNV-1 (approximately 10^8 PFU/ml) was inoculated onto the surface of each stainless steel coupon to achieve a final concentration of 10^7 PFU/coupon. The inoculum was spread out evenly across the coupon surface with the tip of a pipette. Each coupon was air dried for 15 min in the biosafety cabinet. After air drying, coupons were transferred to individual centrifuge tubes using sterile forceps. Coupon-containing centrifuge tubes were stored in a polystyrene cooler along with prefrozen refrigerant gel packs for temperature maintenance before transport to Purdue University for ClO_2 gas treatment. All samples (including control) were transported and stored under the same conditions before, during, and after treatments.

Treatment of MNV-1 on stainless steel coupons by ClO_2 gas. The ClO_2 gas was generated based on a method described by Trinetta et al. (27). ClO_2 gas treatments at concentrations of 1, 1.5, 2, 2.5, and 4 mg/liter were used to treat SS coupons inoculated with MNV-1 for 1 to 5 min inside a closed gas chamber. SS coupons were preconditioned for 10 min at 25°C and 85% relative humidity prior to gas treatment. SS coupons were taken out of the gas chamber after exposure to the desired ClO_2 concentration for the specified time and were immediately transferred into another 50-ml centrifuge tube containing 10 ml of PBS supplemented with 1% sodium pyruvate for elution. After vortexing, SS coupons were removed from the elution liquid. Surviving viral particles in the elution liquid were quantified using the plaque assay method described above. The 0-min time point serves as the recovery control and was used as the starting MNV-1 titer to determine the *D* value. There was an approximate 0.7-$\log_{10}$ PFU/coupon loss of MNV-1 in the recovery control compared to the initial inoculum level of 7.0 $\log_{10}$ PFU/coupon.

Treatment of purified MNV-1 in liquid medium by ClO_2 gas. Aliquots (100 μl) of highly purified MNV-1 suspension (approximately 10^{10} PFU/ml) were prepared in 1.5-ml Eppendorf tubes. The tubes were prepared and transported the same way as previously described in the inactivation study section. Purified MNV-1 was treated by ClO_2 gas at concentrations of 0.1 mg/liter for 30 s or 2 mg/liter for 5 min. Treated samples were subjected to transmission electron microscopy (TEM), SDS-PAGE, Western blot, and reverse transcription (RT)-PCR analysis.

Transmission electron microscopy. The highly purified MNV-1 particles were visualized using negative-staining electron microscopy. A total of 20 μl each of the purified, untreated, and treated MNV-1 samples was fixed to copper grids with Formvar film (FF300-Cu; Electron Microscopy Sciences, Hatfield, PA) and negatively stained with 1% ammonium molybdate. Viral particles were then visualized under the Tecnai G2 spirit transmission electron microscope (FEI Electron Microscopes, Hillsboro, OR) at 80 kV at the Microscopy and Imaging Facility at The Ohio State University, and images were captured on a MegaView III side-mounted charge-coupled device (CCD) camera (Soft Imaging System, Lakewood, CO).

RT-PCR. Viral RNA was extracted from the purified MNV-1 suspension using the RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A OneStep RT-PCR kit (Qiagen) was used to perform RT-PCR. Two primers (forward, 5′-ATGAGGATGAGTGATG GCGC-3′; reverse, 5′-TTATTGTTTGAGCATTCGGCC-3′) were designed to amplify the MNV-1 major capsid protein (VP1) gene. The amplified products were analyzed on 1% agarose gel electrophoresis.

Analysis of viral protein by SDS-PAGE. Purified MNV-1 suspensions (untreated and treated) were mixed with loading buffer containing 1%

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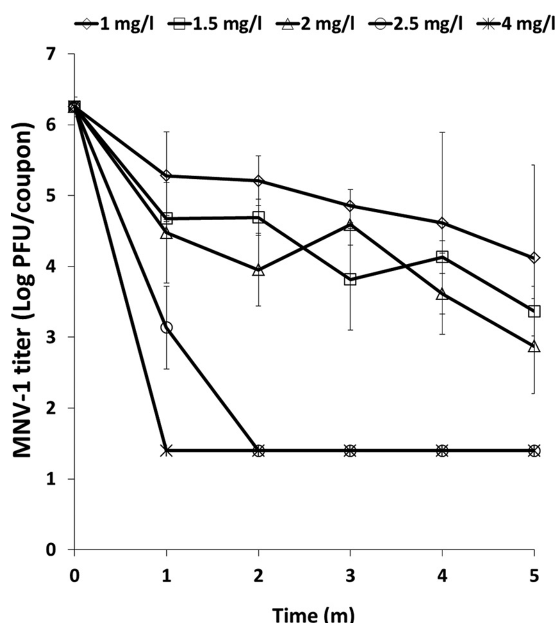

FIG 1 Effect of ClO_2 gas concentrations on inactivation of MNV-1 on stainless steel coupons. Stainless steel coupons inoculated with10^7 PFU of MNV-1 were exposed to ClO_2 gas with concentrations ranging from 1 mg/liter to 4 mg/liter for 5 min at 25°C and a relative humidity of 85%. The survival of MNV-1 was determined by plaque assay. Data are the means of three replicates. Error bars represent the means ± standard deviations.

SDS, 2.5% β-mercaptoethanol, 6.25 mM Tris-HCl (pH, 6.8), and 5% glycerol and boiled for 5 min. Viral proteins were separated on a 12% polyacrylamide gel and were visualized by Coomassie blue staining. For densitometric quantitation, all protein gels were scanned using a Typhoon 9210 scanner (GE Healthcare, Piscataway, NJ), and the intensities of protein bands were determined using ImageQuant TL software (GE Healthcare). For each protein band, background was subtracted, and the intensities of ClO_2 gas-treated protein bands were normalized to the value for untreated controls. The percentage of protein remaining was calculated for each treatment time.

Western blotting. Purified MNV-1 suspensions (untreated and treated) were separated by 12% polyacrylamide gel and transferred onto a Amersham Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (GE Healthcare, Pittsburgh, PA) in a mini trans-blot electrophoretic transfer cell (Bio-Rad, Hercules, CA). The blot was probed with rabbit anti-MNV VP1 polyclonal antibody (a gift from Herbert W. Virgin IV) at a dilution of 1:10,000 in blocking buffer (5% skim milk), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:20,000. The blot was developed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and exposed with GeneMate blue basic autorad film (Bioexpress, Kaysville, UT). The intensities of protein bands were determined using ImageQuant TL software (GE Healthcare), as described above.

Statistical analysis. All experiments were carried out in triplicate. Virus survival was expressed as the mean $\log_{10}$ PFU ± 1 standard deviation. For samples in which no MNV-1 was detected, the detection limit (2.5×10^1 PFU/coupon) was used for data analysis. The best-fitted linear regression was determined for each treatment curve, and the slope representing the rate of MNV-1 inactivation was compared using Prism 6 (GraphPad Software, Inc.). A P value of <0.05 was considered statistically significant.

RESULTS

Kinetics of MNV-1 inactivation on SS coupons using ClO_2 gas. SS coupons were chosen as a model for MNV-1 inactivation on food contact surfaces. Figure 1 shows the kinetics of MNV-1 inactivation using ClO_2 gas concentrations of 1, 1.5, 2, 2.5, and 4 mg/liter at 25°C and a relative humidity of 85%. The D values for MNV-1 inactivation by ClO_2 gas treatments at 1, 1.5, and 2 mg/liter were 2.788 min, 2.068 min, and 1.874 min, respectively (Table 1), and the values were not significantly different. At higher treatment concentrations (2.5 and 4 mg/liter), the effectiveness of MNV-1 inactivation was significantly enhanced. ClO_2 gas at 2.5 mg/liter was found to reduce 3.02 log of MNV-1 after 1 min, and inactivation below the detection limit (≤1.4 log) was achieved after 2 min of treatment (Fig. 1). The concentration of 4-mg/liter ClO_2 gas, on the other hand, was able to reduce the MNV-1 titer below the detection limit after 1 min of treatment. ClO_2 gas treatments at concentrations of 2, 2.5, and 4 mg/liter were able to achieve 3-log reductions in MNV-1 titers on SS coupons following 5, 2, and 1 min of exposures, respectively. The time required to reach a 3-log reduction in MNV-1 titer, therefore, was reduced as the concentration of ClO_2 gas used for treatment increased.

ClO_2 disrupts viral particles. To understand the mechanism of MNV-1 inactivation by ClO_2 gas, highly purified MNV-1 suspensions were subjected to ClO_2 treatment under two different conditions: 0.1 mg/liter for 30 s and 2 mg/liter for 5 min. The first treatment condition was selected because it achieved an approximate 0.5-log reduction and thus permitted the detection of a mixture of inactivated and infectious viruses. In contrast, MNV-1 was completely inactivated at 2 mg/liter for 5 min, and this allowed the determination of the mechanism of virus inactivation under lethal doses of ClO_2. MNV-1 particles in the untreated control were small and round structured virions, approximately 30 to 38 nm in diameter (Fig. 2). When exposed to gaseous ClO_2 at 0.1 mg/liter for 30 s, the number of intact virus particles was significantly reduced, and some irregularly shaped particles were observed. In some cases, broken particles with an empty viral capsid, resembling virus-like particles, were observed. On the other hand, at a ClO_2 gas concentration of 2 mg/liter for 5 min, no intact virus particles were found. Rather, a large amount of aggregated protein debris and irregularly shaped particles (18 to 27 nm in diameter) were observed. These results demonstrated that ClO_2 gas damaged the viral capsid and disrupted virion structure.

ClO_2 degrades viral capsid protein. Although it is known that ClO_2 is an oxidizing agent, the impact of ClO_2 on the viral capsid protein is poorly understood. To gain mechanistic insight into viral inactivation by ClO_2, we determined whether viral proteins were degraded. As shown in Fig. 3, a 58-kDa protein band was observed in the untreated sample, which is consistent with the size

TABLE 1 Summary of parameters for best-fitted linear line under various ClO_2 gas treatment concentrations at 25°C and 85% relative humidity

Concn (mg/liter)	Best-fit line equation	D value, − 1/slope (min)	R^{2a}	P
1	$y = -0.359x + 5.923$	2.788	0.4444	0.0025
1.5	$y = -0.484x + 5.698$	2.068	0.7391	<0.0001
2	$y = -0.534x + 5.632$	1.874	0.6425	0.0001
2.5	$y = -2.425x + 6.021$	0.412	0.9525	<0.0001
4	$y = -0.359x + 5.923$	0.206	0.9989	<0.0001

[a] Coefficient of determination (R^2) for the best-fit line equation. An R^2 of 1 indicates that viral inactivation is linear with increasing treatment time, while an R^2 of 0 indicates that viral inactivation is not linear with increasing treatment time.

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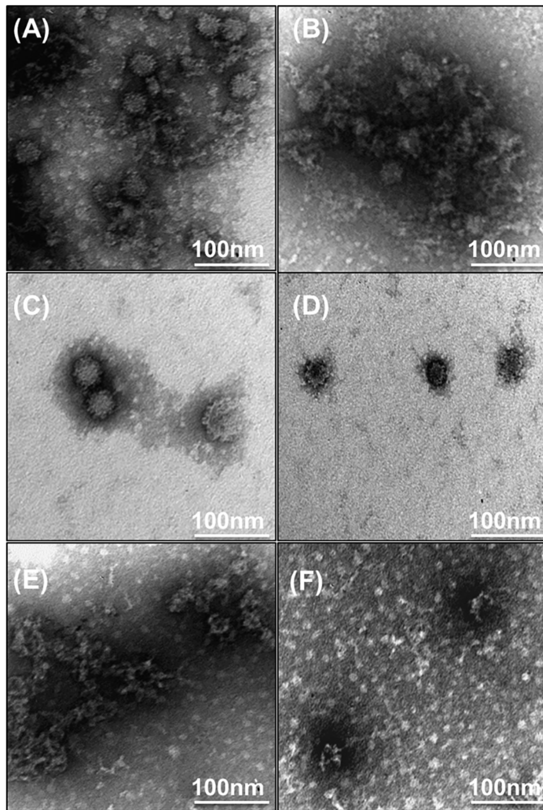

FIG 2 The effect of ClO_2 gas on the structure of MNV-1 particles. Purified MNV-1 was treated with ClO_2 gas at 0.1 mg/liter for 30 s and at 2 mg/liter for 5 min, and samples were observed by TEM. (A, B) Untreated MNV-1 virion. (C, D) ClO_2 gas-treated MNV-1 at 0.1 mg/liter for 30 s. (E, F) ClO_2 gas-treated MNV-1 at 2 mg/liter for 5 min.

of viral major capsid protein (i.e., VP1). After treatment with ClO_2 gas at 0.1 mg/liter for 30 s, the amount of the VP1 protein was reduced to approximately 30% compared to the amount in untreated control. (Fig. 3, lanes 2 and 3). Interestingly, the VP1 protein was undetectable when MNV-1 was treated at 2 mg/liter for 5 min (Fig. 3, lane 4), suggesting that the VP1 protein was completely degraded under this condition. To determine whether the treated viral protein was still able to react with antibodies, Western blot analysis was performed using a polyclonal antibody against the VP1 protein. As shown in Fig. 4, the amount of VP1 protein detected by Western blotting was significantly diminished (approximately 30%) after treatment of ClO_2 at 0.1 mg/liter for 30 s. Consistent with SDS-PAGE analysis, VP1 protein was undetectable under the condition of 2 mg/liter for 5 min. Taken together, these results demonstrated that ClO_2 treatment degraded viral capsid protein as the ClO_2 concentration and treatment time increased. In addition, the remaining VP1 protein after treatment of 0.1 mg/liter for 30 s was still able to react with norovirus-specific antibody.

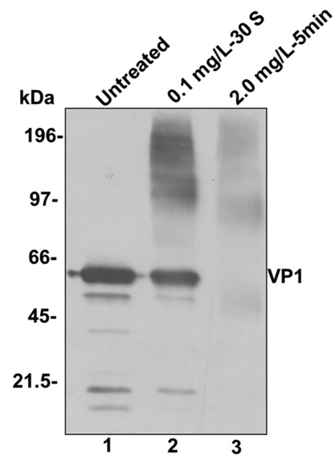

FIG 4 Western immunoblotting analysis of MNV-1 major capsid protein. Purified MNV-1 was treated with ClO_2 gas at 0.1 mg/liter for 30 s and at 2 mg/liter for 5 min. Identical amounts of untreated and treated samples were separated by SDS-PAGE and subjected to Western blot analysis using rabbit anti-MNV VP1 polyclonal antibody.

ClO_2 degrades viral genomic RNA. We next determined whether ClO_2 damages viral genomic RNA. As shown in Fig. 5, a

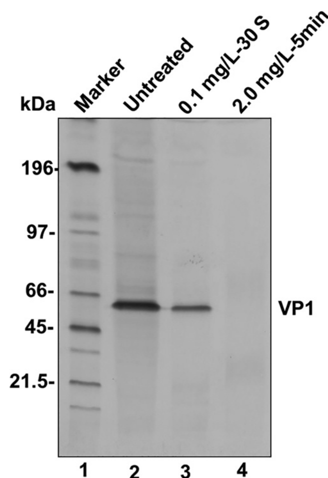

FIG 3 Detection of MNV-1 major capsid protein using SDS-PAGE. Purified MNV-1 was treated with ClO_2 gas at 0.1 mg/liter for 30 s and at 2 mg/liter for 5 min. Major capsid protein (VP1) of untreated and treated viruses was analyzed by 12% SDS-PAGE, followed by Coomassie staining.

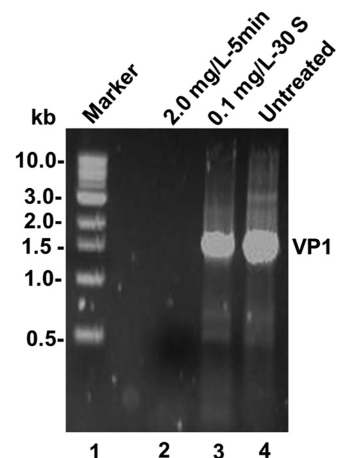

FIG 5 Effect of ClO_2 gas on MNV-1 genomic RNA. MNV-1 genomic RNA was extracted from either untreated or ClO_2 gas-treated MNV-1. The VP1 gene of MNV-1 was amplified by one-step RT-PCR. The PCR product was then visualized on a 1% agarose gel with ethidium bromide staining.

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1.5-kb band was observed in the untreated control, which is consistent with the size of the MNV-1 VP1 gene. The VP1 gene was also amplified in RNA samples extracted from MNV-1 treated by ClO_2 at 0.1 mg/liter for 30 s; however, the abundance of the VP1 gene decreased compared with the untreated control. The VP1 gene was not detectable in the RNA extracted from MNV-1 stock treated by ClO_2 at 2 mg/liter for 5 min in which MNV-1 was completely inactivated, suggesting that MNV-1 RNA was completely degraded under this condition.

DISCUSSION

Environmental and food contact surfaces are one of the most common routes for human NoV transmission (4, 7, 28–32). FDA- and EPA-approved sanitizers, however, are not effective for the inactivation and removal of human NoV from contact surfaces. Research efforts investigating the virucidal effects of ClO_2 gas on human NoV and its surrogates have been very limited. In this study, we used MNV-1 as a human NoV surrogate to determine the stability of the virus on the surface of stainless steel coupons when exposed to ClO_2. We found that MNV-1 can be effectively inactivated by ClO_2 gas at a relatively low concentration (less than 4 mg/liter) for a short treatment time (less than 5 min). Therefore, ClO_2 gas can be used to inactivate MNV-1 on stainless steel surfaces.

Kinetics of MNV-1 inactivation by ClO_2 gas. The efficacy of viral inactivation using the ClO_2 gas system depends on many factors, including gas concentration, exposure time, temperature, relative humidity, and the type of surface and food matrix. In general, ClO_2 inactivated MNV-1 in a dose- and time-dependent manner. There was no significant difference in virus inactivation induced by low ClO_2 concentrations (1, 1.5, and 2 mg/liter). However, virus inactivation was significantly enhanced when higher ClO_2 concentrations (2.5 and 4 mg/liter) were used ($P < 0.05$). To achieve a minimum 3-log reduction, only a 5-min treatment time was needed using a gas concentration of 2 mg/liter at a relative humidity of 85% and a temperature of 25°C. At a concentration of 4 mg/liter, 1 min was sufficient to achieve complete virus inactivation (>5-log reduction).

Previously, it was reported that different viruses have different susceptibilities to ClO_2 gas (33–36). A systematic study compared the efficacy of ClO_2 and sodium hypochlorite solutions against several viruses, including FCV, human influenza virus, measles virus, canine distemper virus, human herpesvirus, human adenovirus, canine adenovirus, and canine parvovirus (33). Overall, the study found that the efficacy of ClO_2 against the viruses was approximately 10 times higher than that of sodium hypochlorite (33). In general, the enveloped viruses (influenza virus, measles virus, and herpesvirus) were more sensitive to ClO_2 than nonenveloped viruses (FCV, adenoviruses, and parvoviruses) were (33). In another study, a low concentration of ClO_2 gas (0.05 ppm) was able to cause a 6.5-log reduction of influenza A virus after 3 h of treatment. In contrast, only a 2-log reduction of FCV was observed even after 4 h of exposure time (35). This is possibly due to the viral structure, with an enveloped virus (such as influenza virus) being more sensitive to ClO_2 than a nonenveloped virus (such as FCV). In addition, different virus strains within the same genus have also been shown to have different sensitivities to ClO_2. A 3-log reduction in simian rotavirus SA-11 was achieved following treatment with the 0.2-mg/liter ClO_2 solution after 60 s. However, in order to achieve the same log reduction in human rotavirus strain Wa, the treatment time needed to be increased to 120 s (36). There also remains the possibility that viral quasispecies from the same viral population may have more resistance to ClO_2 treatment than the rest of the population or that aggregation of viral particles may protect a subset of the viral population from ClO_2 inactivation. Though not directly investigated in this study, this may explain the variability in MNV-1 inactivation observed at low ClO_2 concentrations.

Vegetative bacterial cells commonly associated with foodborne disease and food spoilage can be effectively inactivated by ClO_2 gas either at a high concentration for a short treatment time or at a low concentration for a longer treatment time (25, 35). However, in order to eliminate bacterial spores, elevated gas concentrations and longer treatment times are necessary (37–39). Overall, fungi have been shown to be more resistant than vegetative bacteria to ClO_2 gas treatment. ClO_2 gas treatment at lower concentrations (1.29 mg/liter to 1.74 mg/liter) only reduced yeast populations by less than 1 log (40, 41). However, ClO_2 gas treatments at higher concentrations or longer exposure times were shown to be capable of reducing yeast and mold populations (42, 43). Based on our results, it appears that MNV-1 is more resistant than vegetative bacteria and fungus to ClO_2 gas treatment, although this was not directly compared in the study. It has been well established that, in general, nonenveloped viruses are more resistant than vegetative bacteria and fungi to disinfectants, which also appears to be the case for ClO_2 gas.

Overall, the virucidal effect of aqueous ClO_2 is well established compared to that of the gaseous form. At 20°C, a 0.255-mg/liter ClO_2 solution was able to produce a 3-log reduction of MNV-1 after a contact time of 4.64 min (44). Similarly, treatment with 0.255- and 0.8-mg/liter ClO_2 solutions with contact times of 0.25 and 1.1 min, respectively, was able to produce at least a 3-log reduction of FCV (45, 46). For other enteric viruses, such as human adenovirus type 2 (HAd-2), it has been reported that a 6-log reduction in viral infectivity was achieved using a ClO_2 solution at 100 ppm for 1 min (33). A 500-ppm ClO_2 solution was also shown to inactivate 4.3 log of hepatitis A virus after 5 min of exposure at 22°C (47). It appears that ClO_2 solution exhibits similar virucidal activity as the gaseous form. However, most of these studies were performed by directly mixing viral stock with ClO_2 solution, not by applying the ClO_2 solution to virus dried on surfaces or by adding any organic amendments. A recent study found that chlorine solution can remove/inactivate less than 1 log of MNV-1 from the surface of tableware and food preparation utensils, although it can achieve more than a 5-log reduction of *Escherichia coli* K-12 and *Listeria innocua* (24). This result demonstrated that ClO_2 solution may be ineffective against viruses on the food contact surfaces. In contrast, our results indicated that gaseous ClO_2 is highly effective in inactivating MNV-1 on the surface of SS coupons.

However, in the previously mentioned study, MNV-1 was mixed with a cream cheese or reduced-fat milk food matrix and applied to the utensils (24). It has been established that the presence of organic materials can affect the biocidal activity of ClO_2 solution and, potentially, gaseous ClO_2. Although not investigated in this study, it will be critical to evaluate the effect of organic matter on the inactivation of MNV-1 by gaseous ClO_2 for the application of this technology in industry. In addition, it has been shown that ClO_2 gas (5.0 mg/liter for 10 min) applied to lettuce

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and alfalfa sprouts leads to the presence of residual chlorination byproducts (chlorite and chlorate) on the food, as well as negative visual changes to the produce, such as discoloration (48). However, at the same level of ClO_2 gas application, minimal chlorination byproduct or visual changes were observed in cantaloupe and strawberries (48). Therefore, depending on the purpose of ClO_2 application (i.e., surface disinfection versus food disinfection), the concentration and treatment time must be considered to balance biocidal efficacy with consumer safety. Overall, these results suggest that gaseous ClO_2 may be more effective against viruses than aqueous ClO_2 on stainless steel surfaces. Furthermore, the superior penetration capability of ClO_2 gas has great potential to inactivate virus-contaminated surfaces to which liquid disinfectants cannot be applied.

The mechanism of viral inactivation by ClO_2. Our study also provides mechanistic insight into viral inactivation by ClO_2. For bacterial cells, it was reported that ClO_2 disrupted bacterial protein synthesis, caused the loss of membrane permeability control, and inhibited cell division (49–51). As for bacterial spores, ClO_2 caused membrane damage, subsequently inhibiting spore germination (52). Viruses are highly diverse in their structures, and virus inactivation mechanisms are often contradictory or equivocal. It has been suggested that the inactivation mechanism of hepatitis A virus (a picornavirus) by ClO_2 was due to the loss of the 5′ untranslated region of the genome and/or receptor-binding domain destruction on the capsid (53). It was also found that ClO_2 inactivated poliovirus, another picornavirus, primarily by disrupting a 40- to 80-nucleotide sequence in the 5′-noncoding region of the genome (54). On the other hand, working with bacteriophage MS2, it was found that the primary inactivation action of ClO_2 is viral protein degradation, not damage to the viral genome (55).

Caliciviruses are nonenveloped, single-stranded, positive-sense RNA viruses. The virion structure of caliciviruses is relatively simple. The outer shell of the virus particle is a highly stable protein capsid that protects the viral genomic RNA. We found that the mechanism of MNV-1 inactivation by ClO_2 involved damage to the integrity of the viral capsid structure and degradation of the viral capsid protein and viral genomic RNA. ClO_2 reacts with amino acids with electron-rich side chains, such as tryptophan, tyrosine, cysteine, and histidine (56–60), which leads to the disruption of primary and secondary structures, which ultimately results in the degradation of the viral capsid protein, VP1. Hauchman and others (61) found that naked viral RNA was more susceptible to degradation than RNA extracted from treated virus particles, suggesting that genomic RNA inside intact viral particles was partially protected from ClO_2 treatment. Similarly, Simonet and Gantzer (62) found that poliovirus genomic RNA was still detectable, even though the virus was completely inactivated. Hence, this evidence suggests that the primary mechanism of MNV-1 inactivation by ClO_2 is the degradation of the viral capsid protein and the disruption of the viral capsid structure, which lead to the leakage of genomic RNA and the subsequent degradation of RNA by ClO_2.

In conclusion, our study demonstrated that MNV-1, a human NoV surrogate, was effectively inactivated by ClO_2 gas at relatively low concentrations and short treatment times. ClO_2 gas is a promising intervention to minimize the risk of contact surface-related human NoV transmission. In addition, we found that ClO_2 damaged the viral particle structure and degraded viral proteins and RNA, providing insight into the mechanism underlying viral inactivation.

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