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Characterization of Ozone Disinfection of Murine Norovirus▽

Mi Young Lim,[1] Ju-Mi Kim,[1] Jung Eun Lee,[1] and GwangPyo Ko[1,2]*

Department of Environmental Health and Institute of Health and Environment, School of Public Health, Seoul National University, Seoul, South Korea,[1] and Institute of Microbiology, School of Biological Sciences, Seoul National University, Seoul, South Korea[2]

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Despite the importance of human noroviruses (NoVs) in public health, little information concerning the effectiveness of ozone against NoVs is available. We determined the efficacy of ozone disinfection using murine norovirus (MNV) as a surrogate of human NoV. MNV in ozone demand-free buffer was exposed to a predetermined dose of ozone at two different pHs and temperatures. The virus remaining in the solution was analyzed by plaque assay, real-time TaqMan reverse transcriptase PCR (RT-PCR) (short template), and long-template conventional RT-PCR. Under all conditions, more than 99% of the MNV was inactivated by ozone at 1 mg/liter within 2 min. Both RT-PCR assays significantly underestimated the inactivation of MNV, compared with that measured by plaque assay. Our results indicate that NoV may be more resistant to ozone than has been previously reported. Nevertheless, proper ozone disinfection practices can be used to easily control its transmission in water.

Noroviruses (NoVs) are the major etiological agent of gastroenteritis in all age groups worldwide and are transmitted via a fecal-oral route (3, 18, 22, 27, 35). As important waterborne and food-borne pathogens, they are included on the U.S. EPA Contaminant Candidate List 3 (CCL 3) (7, 11). Water can be contaminated by NoV-containing feces released from either symptomatic or asymptomatic patients (26). High concentrations of the virus in fecal matter of infected people ($\sim 10^9$ viral particles/g of feces) (25), low infectious doses (<10 viral particles) (32), and high resistance to various environmental stresses (9, 21) contribute to the high prevalence of outbreaks caused by NoV. Proper disinfection of drinking and recreational water is necessary to prevent waterborne outbreaks (33).

Among currently available analytical methods of virus cultivation, the plaque assay is the most convenient and commonly applied analytical method for determining virus inactivation by a disinfectant (6, 12). However, no conventional cultivation assays or convenient laboratory animal models have been available for human NoV. Thus, despite the importance of NoV in public health, limited information is available regarding the effectiveness of various disinfectants against NoV.

Ozone (O_3), a strong oxidizing agent, has been widely used as a disinfectant in drinking water treatment plants in many European countries, receiving more attention after the discovery of potentially harmful chlorine by-products (37, 38). Owing to its high effectiveness and lack of residue after disinfection, ozone can be used for both surface and groundwater disinfection. Previously, a number of ozone disinfection studies of enteric viruses such as adenoviruses and enteroviruses have been reported (29, 33). In all of these studies, ozone disinfection was determined by either plaque assay of feline calicivirus (FCV) or long-template (LT) reverse transcriptase PCR (RT-PCR) assay of human NoV. FCV, a member of the *Caliciviridae* family, is easily cultivated *in vitro* and has been commonly used as a surrogate of NoV (10, 24, 33). However, FCV is considered a respiratory pathogen and has characteristics very different from those of enteric viruses (33). FCV is very susceptible to low pH and elevated temperature (8, 30), whereas murine norovirus (MNV), which has recently been shown to be transmitted through a fecal-oral route, is more resistant to pH extremes (19). Therefore, MNV is considered to be the most suitable surrogate in determining the survival and inactivation characteristics of NoVs that cause viral gastroenteritis (4). Additionally, it is unclear whether the LT RT-PCR assay accurately determines the inactivation rate of NoV, as previously suggested (29).

The objectives of this study were (i) to determine the kinetics of viral inactivation by ozone under various pH and temperature conditions, using an MNV surrogate, and (ii) to characterize and compare the MNV inactivation rates measured by short-template (ST) real-time TaqMan RT-PCR, LT RT-PCR, and plaque assays.

MATERIALS AND METHODS

Preparation of MNV stock. MNV was obtained from Herbert W. Virgin (Washington University School of Medicine, St. Louis, MO) and was propagated in RAW 264.7 cells. MNV stock was treated following a previously reported procedure (29). Briefly, lysates of infected cells were frozen and thawed three times to release the virus particles, which were extracted with an equal volume of chloroform. The supernatant was recovered by low-speed (4,000 × *g*) centrifugation for 10 min at 4°C. This virus extract was further purified by centrifugal ultrafiltration (Amicon Ultra-15; Millipore, Billerica, MA) and was dispersed by serial filtration through polycarbonate filters with 0.2- and 0.08-μm pores, pretreated with 0.1% Tween 80 solution in water. The MNV stock was stored at −80°C until use.

Ozone production and analysis. Ozone was generated from ultrapure oxygen by an ozone generator (LAB 2B; Ozonia North America, Elmwood Park, NJ) in a biohazard hood. A concentrated ozone stock solution (>40 mg/liter) was prepared by bubbling ozone gas through a 500-ml amber bottle containing distilled water. Ozone concentrations were determined using the indigo colorimetric method (1). The A_{600} of indigo trisulfonate was measured using a Biomate 3 UV-VIS spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). All glassware was cleaned by soaking in an ozone solution (>1 mg/liter) for 1 h, rinsing with ozone demand-free (ODF) water, and baking overnight in an oven at 80°C. All solutions used in these experiments were

* Corresponding author. Mailing address: Department of Environmental Health, School of Public Health, Seoul National University, 28 Yunkeun-Dong, Chongro-Ku, Seoul 110-799, South Korea. Phone: 82-2-3668-7881. Fax: 82-2-745-9104. E-mail: gko@snu.ac.kr.

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prepared using distilled/deionized water from a Milli-Q ultrapure water purification system (Millipore).

Experimental conditions for ozone disinfection. Ozone disinfection was performed at two different pHs (5.6 and 7) and two different temperatures (5 and 20°C) in a headspace-free Pyrex piston-type reactor. Immediately before the ozone stock solution was injected into the reactor, a control sample (time = 0) was taken and used as N_0 for determining the kinetics of ozone disinfection. To generate an initial ozone target concentration of 1 mg/liter for MNV inactivation, an appropriate volume of ozone stock solution was transferred into a reactor containing ODF buffer and target virus to a final concentration of 10^4 PFU/ml. The mixture was immediately stirred. Every 30 s, samples were withdrawn via the sampling outlet of the reactor and were used to measure both the residual ozone concentration and virus inactivation. Samples for virus inactivation measurements were collected into tubes containing 0.1% sterile sodium thiosulfate solution to quench any residual disinfectant activity. MNV was assayed by plaque assay, ST real-time TaqMan RT-PCR assay, and LT RT-PCR assay. Each experiment was performed in triplicate.

Plaque assay of MNV. MNV was assayed by the conventional plaque technique on confluent layers of RAW 264.7 cell cultures grown in six-well plates. Cells were infected with 0.5 ml of a 10-fold serial dilution of MNV virus suspension for 1 h at 37°C in the presence of 5% CO_2. After the inoculum was aspirated, the cells were overlaid with 3 ml of a solution containing one part 1.5% SeaPlaque agarose and two parts supplemented minimal essential medium (MEM), which was allowed to solidify. The cells were then incubated at 37°C for 36 to 48 h, until plaques appeared. A second agarose overlay (3 ml) containing 0.1% neutral red solution was added, and after 6 to 8 h, the plaques were counted. Plaques from plates containing 5 to 50 plaques were used to determine the virus titer in PFU per milliliter.

RNA extraction and LT RT-PCR of MNV. Viral RNA was extracted from sample suspensions of MNV, using a QIAamp viral RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Purified RNA samples were stored at −80°C until being used for LT RT-PCR and ST real-time TaqMan RT-PCR. The oligonucleotide primers used for LT RT-PCR generated an 880-bp PCR product and were previously described (5′ primer, 5′-ATG GTC CTG GAG AAT GGG TG-3′; 3′ primer, 5′-TCC CGT AGA TCT TGT CTG GC-3′) (17). A one-step reverse transcriptase PCR (RT-PCR) kit (Qiagen) was used for the LT RT-PCR assay. Viral RNA was reverse transcribed for 30 min at 42°C and then for 15 min at 95°C to activate the *Taq* polymerase. Thermocycling conditions consisted of 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. Each amplified product (15 μl) was analyzed on ethidium bromide-stained 1% agarose gels and visualized by UV light using a transilluminator.

ST real-time TaqMan RT-PCR assay of MNV. The oligonucleotide primers and probe for real-time RT-PCR were designed in a previous study (5′ primer, 5′-ACG CCA CTC CGC ACA AA-3′; 3′ primer, 5′-GCG GCC AGA GAC CAC AAA-3′, probe, 5′-VIC-AGC CCG GGT GAT GAG-MGB-3′ [21]). Real-time RT-PCR was performed using an ABI 7300 real-time PCR machine (Applied Biosystems, Foster City, CA). The final reaction volume of 25 μl contained 2.5 μl of MNV viral RNA, 0.4 M each primer, and 0.1 μM fluorescently labeled probe, plus the nucleotides, RT-PCR enzyme mix, and buffer provided in an AgPath-ID one-step RT-PCR kit (Ambion, Austin, TX). The reactions were performed at 42°C for 10 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s.

Data analysis. The ozone disinfection efficacy was characterized by three different well-known disinfection kinetic models: the Chick-Watson model (16), the modified Chick-Watson model (16), and the efficiency factor Hom (EFH) model (14). The latter two models account for residual decay. Residual concentrations in each experiment were fit to a first-order kinetic equation, equation 1 below, separately by least-squares regression analysis using the Solver function in Excel 2007 (Microsoft Corp.), to give independent values of k' and C_0:

$$C_t = C_0 e^{-k't} \quad (1)$$

where C_t is residual ozone (mg/liter) at time t, C_0 is the initial ozone concentration, t is time (min), and k' is the first-order ozone decay rate constant (min^{-1}). C_0 and k' determined from equation 1 were used in equations 2, 3, and 4 below to model the disinfection kinetics (14).

Chick-Watson model:

$$\text{Ln } N/N_0 = -kCt \quad (2)$$

Modified Chick-Watson model:

$$\text{Ln } N/N_0 = -\frac{kC_0^n}{nk'}[1 - \exp(-nk't)] \quad (3)$$

EFH model:

$$\text{Ln } N/N_0 = -kC_0^n t^m([1 - e^{\{-nk't/m\}}]/[-nk't/m])^m \quad (4)$$

k is the inactivation rate constant, n is the coefficient of dilution, and m is Hom's exponent (13). $\ln(N/N_0)$ is the natural log of the survival ratio (number of infective organisms remaining at time t divided by the number at time zero). To determine the parameter values for each model (k in Chick-Watson, k and n in modified Chick-Watson, n, m, and k in EFH), Microsoft Excel Solver (Microsoft Excel 2007, Microsoft Corp.) was used to minimize the error sum of squares (ESS) between the observed and predicted $\ln(N/N_0)$ for viral disinfection experiments based on the cultivation assay.

The model equations with determined parameter values were used with the arithmetic mean of ozone decay rate constants (k') and C_0 estimates to calculate the times when a specific log inactivation occurred in the average disinfection reaction at that condition. Then, Ct values were calculated by multiplying these times by the average ozone residual determined from the mean of the initial residual and the predicted residual at these times.

RESULTS

Figure 1 shows the inactivation rate of MNV by ozone disinfection (1 mg/liter) as measured by three different analytical methods (ST RT-PCR, LT RT-PCR, and plaque assay). MNV suspended in ODF buffer was rapidly inactivated by ozone. In all of our experiments, the MNV inactivation curve was steeply rising during the initial period (until 30 s) and then flattened out as the exposure time proceeded. At both 20°C and 5°C, the inactivation rate was higher at pH 5.6 than at pH 7. This difference was significant based on the EFH model ($P < 0.05$ by F test). MNV was inactivated by ozone more rapidly at lower temperatures than at higher temperatures, but the difference was not significant. Both ST RT-PCR and LT RT-PCR significantly underestimated the inactivation rate of MNV compared with that measured by plaque assay. Real-time TaqMan RT-PCR with a 54-bp amplicon did not show any significant inactivation in the majority of the experimental conditions. On the other hand, LT RT-PCR revealed a significant inactivation rate, although it was still less, by up to a 1-log reduction, than that determined with the plaque assay.

Table 1 summarizes the results of the three mathematical models of ozone disinfection based on plaque assays. Overall, the Chick-Watson model had higher ESS values and much lower correlation coefficients compared with the Chick-Watson and EFH models, suggesting that one of these two may be a more appropriate model. The best-fit kinetic parameters for the three disinfection models are presented in Table 1. As indicated by a previous study (15), models having more parameters have a tendency to present a relatively better fit of the data ($P < 0.05$ by F test). Based on ESS and R^2 values, the EFH model appears to be the best of the three applied models.

Table 2 shows the Ct values estimated by both the modified Chick-Watson model and EFH model, using the results of the plaque assays. In general, the two models gave similar results, except for the results using a short contact time, particularly at pH 5.6. During this period, the curve was steeper with the EFH model than with the modified Chick-Watson model. For example, for a 2-log reduction of MNV, the EFH model predicted a Ct value of <0.01 mg/liter · min at pH 5.6 and 20°C, whereas the modified Chick-Watson model predicted 0.16 mg/liter · min under the same conditions. Overall, a 2-log reduction of MNV was achievable with ozone concentrations of less

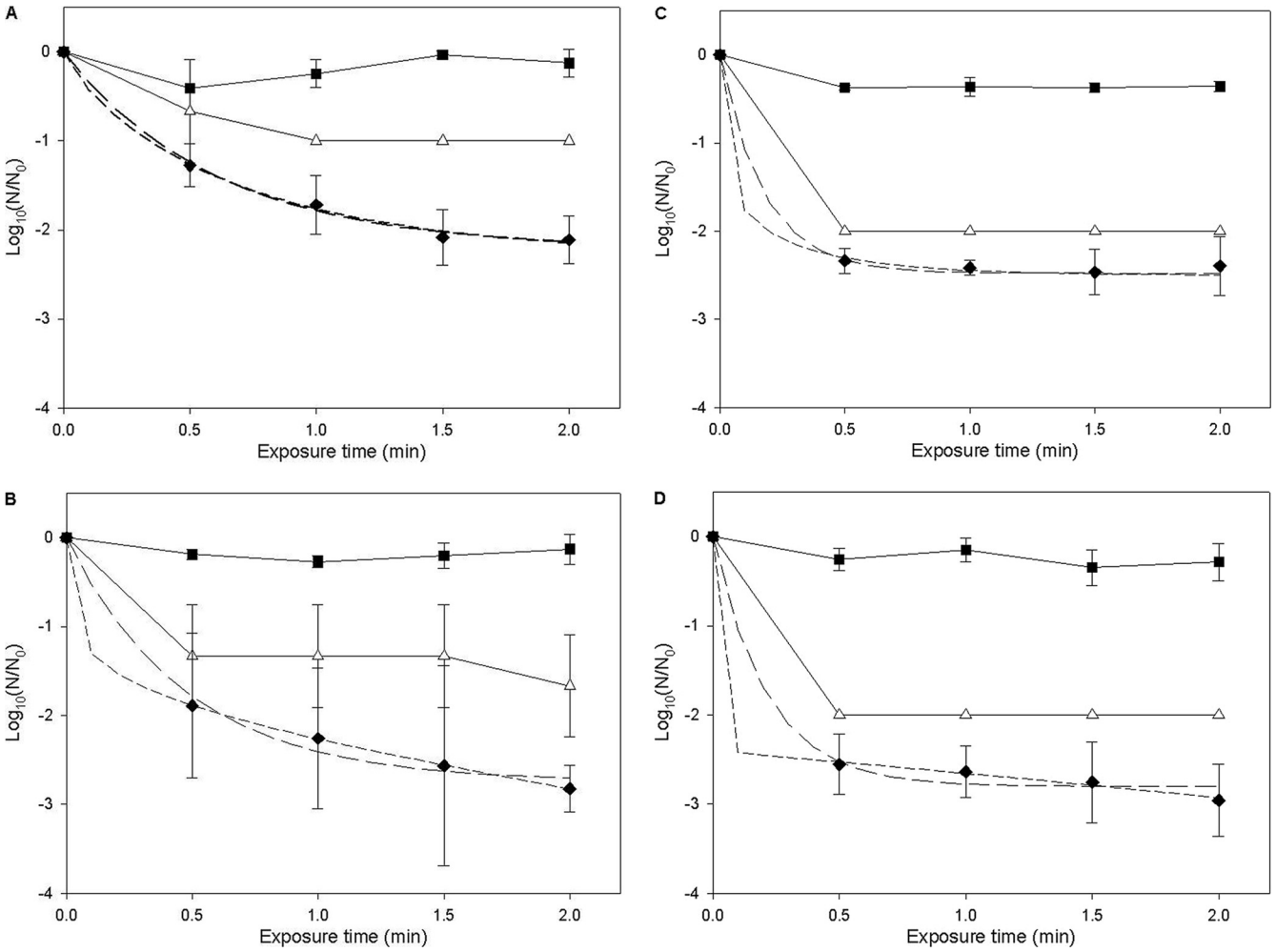

FIG. 1. Kinetics of MNV inactivation by ozone (initial concentration, 1 mg/liter) at (A) pH 7 and 20°C, (B) pH 7 and 5°C, (C) pH 5.6 and 20°C, and (D) pH 5.6 and 5°C, as measured by ST real-time RT-PCR (■), LT RT-PCR (△), and plaque assay (◆). Short-dashed and long-dashed lines represent the best fits using the EFH and MCW models, respectively.

than 0.72 mg/liter · min under all experimental conditions (i.e., pH 5.6 or 7 at 5°C or 20°C).

DISCUSSION

Our study demonstrates that ozone may be an effective disinfectant to control the transmission of NoV in water. We quantitatively determined the efficacy of ozone for inactivating MNV in ODF buffer and characterized the inactivation rate using three different models. Among them, the modified Chick-Watson model and EFH model provided better fits than the Chick-Watson model. The simpler Chick-Watson model, although easier to apply, did not account for the residual decay of ozone and was thus insufficient to explain the shoulder and tailing of our microbial inactivation curves (16). On the other hand, the modified Chick-Watson model and EFH model were able to explain the inactivation kinetics, including the disinfectant decay. The EFH model incorporates both the Chick-Watson model and the modified Chick-Watson model to eliminate the linearity of disinfection kinetics and to account for factors such as substrate concentration, temperature, and disinfectant concentrations (2, 14).

According to our experimental data and subsequent model fitting, the *Ct* values for achieving 2- and 3-log reductions were approximately 0.3 and 1.2 mg/liter · min, respectively. The U.S. EPA guidance manual for virus disinfection by ozone in surface water at 5°C recommends *Ct* values of 0.6 and 0.9 mg/liter · min for 2- and 3-log reductions, respectively (28, 34). Our results confirm that the EPA-recommended $Ct_{99\%}$ value for a 2-log reduction of MNV at pH 7 and 5°C is sufficient; however, our results indicate that a *Ct* value higher than the EPA-recommended value is required for a 3-log reduction of MNV. A previous study reported $Ct_{99.9\%}$ values ranging from 0.04 to 0.10 mg/liter · min for the inactivation of enteric adenovirus (33). In addition, an ozone concentration of 0.37 mg/liter inactivated poliovirus by 3 log units within 10 s (29), and ozone at 1 mg/liter reduced hepatitis A virus by 5 log units within 60 s (36). In our experiment, the EFH model predicted a $Ct_{99.9\%}$ value of 1.18 mg/liter · min for MNV inactivation at pH 7 and 5°C. These results suggest that compared with other enteric viruses, NoVs require a higher dose of ozone to achieve greater than a 3-log reduction. However, our data should be carefully interpreted because differences in ozone exposure

TABLE 1. Three kinetic models and the best-fit kinetic parameters for MNV inactivation by ozone

Conditions (pH, °C)	k'[a]	Model[b]	Result for parameter			ESS[f]	R^2
			k[c]	n[d]	m[e]		
7, 20	5.005	CW	1.294			0.822	0.832
		MCW	8.414	0.331		0.050	0.997
		EFH	6.374	0.201	0.788	0.043	0.997
7, 5	5.139	CW	1.693			1.725	0.794
		MCW	13.275	0.409		0.275	0.990
		EFH	4.871	−0.025	0.212	<0.001	1.000
5.6, 20	5.849	CW	1.647			3.453	0.566
		MCW	32.243	0.967		0.035	0.999
		EFH	6.932	0.087	0.220	0.021	0.999
5.6, 5	6.354	CW	1.861			3.821	0.620
		MCW	29.876	0.729		0.241	0.992
		EFH	5.661	−0.015	0.009	0.015	1.000

[a] Average disinfectant decay constant for replicate experiments.
[b] CW, Chick-Watson model; MCW, modified Chick-Watson model; EFH, efficiency factor Hom model.
[c] Inactivation rate constant.
[d] Coefficient of dilution.
[e] Constant for the inactivation law (describes deviation from Chick-Watson kinetics).
[f] Error sum of squares.

and the reaction conditions of disinfection may influence the efficacy of ozone disinfection (29). In addition, our experiments were performed in ODF buffer. It has been reported that ozone disinfection is affected by factors such as dissolved organic matter (DOM) and the pH (5, 37). Therefore, the amount of ozone production required to inactivate the virus should be higher in natural conditions, which include dissolved organic matter other than that in ODF.

Our experimental data did not demonstrate MNV inactivation greater than 3 log units because of the low concentration of the MNV stock (~10^4 PFU/ml). The viral stock solution was prepared at this low concentration in order to reduce nonviral organics and thereby minimize the ozone demand in the viral stock solution. As a strong oxidant, ozone would have decayed rapidly if the viral stock solution contained other organic chemicals; thus, an ozone demand-free stock solution is ideal. During the preparation of the stock solution, centrifugation, ultrafiltration, and a series of filtrations probably resulted in a high loss of viral titer.

TABLE 2. *Ct* values predicted by the MCW and EFH models for MNV inactivation by ozone

Conditions (pH, °C)	Model	Estimated *Ct* values (mg/liter · min)		
		1 $\log_{10}$ (90%) reduction	2 $\log_{10}$ (99%) reduction	3 $\log_{10}$ (99.9%) reduction
7, 20	MCW[a]	0.21	0.72	
	EFH[b]	0.18	0.70	
7, 5	MCW	0.14	0.33	
	EFH	0.03	0.36	1.18
5.6, 20	MCW	0.07	0.17	
	EFH	<0.01	0.13	
5.6, 5	MCW	0.07	0.16	
	EFH	<0.01	<0.01	1.12

[a] MCW, modified Chick-Watson model.
[b] EFH, efficiency factor Hom model.

Although the concentration of ozone significantly declined during the disinfection process, residual ozone remained after disinfection, despite its high reactivity. This residual ozone might have produced the tailing of our inactivation curves.

In this study, MNV was assayed using three different analytical methods: plaque assay, real-time TaqMan RT-PCR (ST RT-PCR), and LT RT-PCR. The two molecular assays (real-time TaqMan RT-PCR and LT RT-PCR) used in this study are likely to have different sensitivities for detecting virus. However, since only the log reduction was measured, in comparison with the initial concentration of the viral suspension following the same experimental procedure, the sensitivity of the molecular assays would not affect the *Ct* value for the log reduction.

For estimating the inactivation rate of MNV by ozone, cultivation assays such as the plaque assay are more appropriate than molecular assays, which cannot represent viral infectivity because nucleic acids of inactivated viruses are also detected. As expected, the real-time TaqMan RT-PCR assay (ST RT-PCR) tremendously underestimated viral inactivation, as the short PCR template (54 bp) used in this assay would have easily detected short nucleic acid fragments from inactivated viruses. These results are consistent with previous studies (21, 31). However, LT RT-PCR may be applicable for noncultivatable viruses, because the nucleic acids detected by the longer template would likely be associated with infective viruses (29). Thus, LT RT-PCR could be used to detect active viruses as a surrogate of viral inactivation. A previous study by Shin et al. has suggested that LT RT-PCR can serve as a surrogate for infectivity assays to determine ozone disinfection of NoV (29). However, without a direct comparison between LT RT-PCR and infectivity assays, these data were difficult to interpret. We used the plaque assay as well as LT RT-PCR with a template that covers approximately 11% of the entire MNV genome. Our results with LT RT-PCR were somewhat similar to those with the plaque assay, but LT RT-PCR still significantly underestimated the inactivation rate of MNV. These results suggest that LT RT-PCR of MNV could be used as a conservative

surrogate of NoV inactivation by oxidizing chemical disinfectants. A previous study of human NoV using LT RT-PCR showed that at least a 3-log reduction of the human virus occurred with the administration of ozone at 0.37 mg/liter for 10 s (29). Compared with this previous result, our results using both the plaque assay and LT RT-PCR showed a higher resistance of MNV to ozone. It is not clear whether there is a true difference between human NoV and MNV, or whether this difference is attributable to differences between the analytical methods. Further study is warranted.

We investigated the efficacy of ozone for inactivating MNV at different pHs and temperatures. We chose the temperature conditions (5 and 20°C) in order to represent water disinfection in typical and cold weather. In addition, since ozone is known to inactivate bacteria and viruses over a pH range of 5.6 to 9.8 (23), we chose two different pH conditions (pH 5.6 and 7). We speculated that temperature may affect the efficacy of ozone, as temperature can influence the solubility and decomposition rate of ozone. However, our results showed that temperature (5°C and 20°C) did not have a significant effect on the efficacy of ozone for inactivating MNV, as has been shown in previous studies with *Escherichia coli* (20, 39). On the other hand, the pH of the solution had a significant effect on the efficacy of ozone. MNV was inactivated more rapidly at pH 5.6 than at pH 7. Rapid inactivation at low pH was consistent with the results of a previous study with *E. coli* (39). Low pH may affect the solubility and oxidant characteristics of ozone.

In conclusion, ozone may be useful as a disinfectant in water. Our results suggest that NoV is more resistant to ozone than has been previously reported (29). However, a careful comparison should be made based on the type of virus, water conditions, and disinfection systems. Further studies concerning field applications are needed.

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