Effect of Temperature, pH, and NaCl on the Inactivation Kinetics of Murine Norovirus

KYEONGJIN SEO, JUNG EUN LEE, MI YOUNG LIM, AND GWANGPYO KO*

Institute of Health and Environment, Department of Environmental Health, School of Public Health, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-742, Korea

MS 11-199: Received 22 April 2011/Accepted 15 August 2011

ABSTRACT

We investigated the resistance of murine norovirus (MNV) and coliphage MS2, a culturable human norovirus surrogate, to temperature, salt, and pH. Virus inactivation was measured by plaque, real-time TaqMan reverse transcription (RT) PCR, and long-template RT-PCR assays. Both MNV and MS2 were rapidly inactivated at temperatures above 60°C. Similarly, MNV tolerated low salt concentrations (0.3% NaCl) to a greater degree than high salt concentrations (3.3 to 6.3% NaCl). MNV was relatively resistant to strong acidic conditions (pH 2) and was more tolerant of slightly acidic (pH 4) or neutral (pH 7) conditions. In contrast, MS2 was resistant to high salinity. Overall, temperature had a greater effect on infectivity than salt or low pH. Additionally, temperature and low pH had a synergistic effect on MNV infectivity. Both real-time and long-template RT-PCR assays significantly underestimated the inactivation by temperature, salt, and pH. The inactivation kinetics of both MNV and MS2 under various environmental conditions gave a good fit by the Weibull model ($R^2 > 0.9$). This study suggests both the capacity of infectious human norovirus to persist in the face of various environmental conditions and its sensitivity to high temperatures, which may provide a mechanism of protection against this virus.

Human noroviruses (HuNoVs) are single-stranded RNA viruses belonging to the family Caliciviridae. HuNoVs are the major etiological agent of acute viral gastroenteritis. HuNoV infections are usually transmitted through fecally contaminated food, water, or fomites; person-to-person contact; or aerosolized vomitus. HuNoVs have a low infectious dose of 10 to 100 viral particles and are responsible for 80 to 94% of all outbreaks of gastroenteritis in crowded locations, such as restaurants, tourist resorts, hospitals, schools, and nursing homes.

Despite the significance of HuNoVs to public health, many aspects of HuNoV biology are not well understood. This is due primarily to the absence of a cell culture system or small animal model. In 2003, murine norovirus (MNV), which can be cultivated using RAW 264.7 cells, was reported. MNV may be a suitable surrogate because of its taxonomic closeness to HuNoV, ease of cultivation, and strong resistance to various environmental stresses. Thus, MNV may be a more suitable surrogate for determining the inactivation characteristics of HuNoV. Molecular assays, such as reverse transcription (RT) PCR, are used to detect HuNoV. Several previous reports have suggested that RT-PCR assays detect inactivated viral nucleic acid and, thus, significantly underestimate HuNoV inactivation. To characterize HuNoV infectiousness, enzyme pretreatment and the subsequent molecular detection of long-template (LT) viral RNA have been used to distinguish infectious from noninfectious virus. For example, treatment with proteinase K and RNase A and greater amplification of the viral genome have enabled the partial differentiation of infectious and noninfectious viruses. However, whether this technique can be applied to MnV remains unclear.

HuNoVs are resistant to various environmental stressors, including low pH and high salt concentrations. HuNoV maintains viability in the acidic environment of the stomach, and in acidic, salted, pickled, and lightly cooked foods. In a previous study of human volunteers, HuNoV maintained infectivity after exposure to 60°C for 30 min, suggesting that HuNoV is highly thermostable. However, the inactivation kinetics of HuNoVs have not yet been fully evaluated under different environmental conditions. The aims of this study were to characterize the effects of temperature, pH, and NaCl on MNV inactivation kinetics. Additionally, we investigated the efficacy of (i) a real-time TaqMan RT-PCR assay with enzyme treatment and (ii) an LT RT-PCR assay for the estimation of viral inactivation.

MATERIALS AND METHODS

Viral culture and preparation. MNV was kindly supplied by Dr. Herbert W. Virgin (Washington University School of Medicine, St. Louis, MO) and cultured in RAW 264.7 cells in Dulbecco’s modified Eagle’s medium supplemented with 10% ...
fetal bovine serum, 10 mM HEPES, 10 mM sodium bicarbonate, 50 μg/μl gentamicin, and 10 mM Earle’s minimal essential-nonessential amino acid medium (all from Gibco, Grand Island, NY). RAW 266.7 cells (~5 × 10^6) were cultured to 80 to 90% confluence (usually 3 to 4 days) and then were inoculated with MNV and incubated at 37°C until cytopathogenic effects were observed (usually 3 to 4 days). Virus-infected cells were frozen and thawed three times and centrifuged (2,000 × g, 10 min, 4°C) with an equal volume of chloroform for the extraction of MNV. Supernatants were ultrafiltered using an Amicon Ultra-15 (Millipore, Billerica, MA; 4,000 × g, 20 min, 4°C) and stored at −80°C. The MNV titer was estimated by plaque assay on RAW 266.7 cell monolayers to be 6 × 10^8 PFU/ml. MS2 bacteriophage (ATCC 15597B1) was propagated using E. coli C3000 (ATCC 15597) as a host on tryptic soy agar (TSA) plates using the single agar layer method (53). The surface of the culture plates was washed using phosphate-buffered saline (PBS), and an equal volume of chloroform was added to the resulting fluid. This mixture was then centrifuged (4,000 × g, 30 min, 4°C). Supernatants were stored at −80°C and used as the MS2 stock. The concentration of the viral stock was −1.5 × 10^11 PFU/ml; it was used as described previously (37).

Heat inactivation and plaque assay. The MNV and MS2 stock solutions (0.1 ml) were diluted 1:100 in Dulbecco’s modified Eagle’s medium and PBS to a final concentration of ~6 × 10^6 and ~1.5 × 10^11 PFU/ml, respectively. Viral suspensions (500 μl) were transferred to 1.5-ml preheated microcentrifuge tubes and then incubated in a water bath at 24, 37, 50, 60, 70, and 85°C. Heat treatment of MS2 was continued for 20 days at 24°C. After a predetermined time, samples were chilled immediately on ice. The effect of NaCl concentration (0.3, 1.3, 3.3, and 6.3% [wt/vol]) on MNV and MS2 inactivation at 24, 37, and 50°C was also determined. Additionally, the pH of the viral stock suspensions was adjusted to 2, 4 (0.1 M citrate buffer), and 7 (0.1 M phosphate buffer) to evaluate the effect on virus infectivity at 24, 37, and 50°C (8). After NaCl and pH inactivation for the designated periods, all samples were immediately chilled on ice.

For the MNV plaque assay, RAW 266.7 cells were seeded into six-well plates (3.5 cm in diameter) and cultivated until 80 to 90% confluence at 37°C under 5% CO2. Inactivated virus was serially diluted 10-fold with Dulbecco’s modified Eagle’s medium to give the desired MNV PFU number and kept on ice. After the cells had reached confluence, the culture medium was aspirated and 0.5 ml of diluted virus was added to the wells. The culture plates were gently rocked every 15 min for 1 h at 37°C with 5% CO2. After 1 h, the supernatants were aspirated, and the cells were overlaid with 3 ml of overlay medium (1.5% sea plaque agarose, Lonza, Rockland, ME) supplemented with minimal essential medium and incubated until plaques became visible (usually 2 to 3 days). To visualize the plaques, the cells were stained with 3 ml of sea plaque agarose (1.5%) supplemented with minimal essential medium containing 1% neutral red solution for 6 to 8 h at 37°C under 5% CO2. Plaque counts of 5 to 50 PFU per well were recorded and expressed as PFU per milliliter. For the MS2 plaque assay, virus suspensions exposed to predetermined experimental conditions were serially diluted 10-fold with PBS and stored on ice. Aliquots (0.1 ml) of the virus suspensions were then mixed with 0.3 ml of E. coli C3000 suspension and cultivated on TSA plates using the single agar layer method (53). Each experiment was performed in triplicate.

Nucleic acid extraction. Viral RNA was extracted using a QIAamp Viral RNA kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. A 140-μl volume of each sample was incubated with 560 μl of QIAamp lysis buffer (AVL) and carrier RNA for 10 min. After viral particle lysis, the samples were applied to spin columns and washed twice with 500 μl of buffer AW (containing 96 to 100% ethanol). Viral RNA was eluted using 60 μl of buffer AVE and stored at −80°C until required.

Quantitative real-time TaqMan RT-PCR assay. Quantitative real-time TaqMan RT-PCR assays were performed using the Applied Biosystems 7300 real-time PCR system (Foster City, CA). To prepare quantitation standards, the target gene was amplified by RT-PCR and cloned using a pGEM-T Easy TA Cloning kit (Promega Biotech, Madison, WI). Plasmids were purified with a Lapobase plasmid DNA purification kit (Cosmo Genetech, Seoul, Korea) and quantified using a Nanodrop Technologies ND-1000 spectrophotometer (Wilmington, DE). Standard curves were generated from serial dilutions of plasmid DNA.

Additionally, the effect of proteinase K and RNase A treatment after heat inactivation was evaluated. Enzyme treatment was performed by incubation with both proteinase K (20 U/100 μl) and RNase A (1 μg/100 μl) at 37°C for 90 min (3). The initial quantitative real-time TaqMan RT-PCR titer in the enzyme-treated samples was the same as in the untreated samples. Real-time RT-PCR was performed in a 25-μl volume consisting of 2.5 μl of template RNA, 12.5 μl of 2 × reaction buffer (AgPath-ID OneStep RT-PCR kit, Ambion, Austin, TX), 0.2 μl of primer (50 pmol), 0.05 μl of probe (100 mM), and distilled water. The amplification profile consisted of an initial cDNA step for 30 min at 48°C followed by 15 min at 95°C and 45 cycles of denaturation for 10 s at 95°C, annealing for 20 s at 50°C, and elongation for 30 s at 72°C. The TaqMan probe (VIC 5′-AGC CCG GGT GAT GAG-3′ MGB) and primers (forward, 5′-ACG CCA CTC CGC ACA AA-3′, and reverse, 5′-GGC GCC AGA GAC CAA AAA-3′) used to amplify the MNV capsid region sequence were described previously (37). Each experiment was performed in triplicate.

LT RT-PCR assay. In addition to real-time TaqMan RT-PCR, MNV inactivation was assayed by LT RT-PCR using a Qiagen OneStep RT-PCR kit. The virus suspension was serially diluted, and its concentration was determined by an LT RT-PCR assay. Viral titers were expressed as the last dilution giving a visible band on a 1% agarose gel stained with ethidium bromide and viewed under UV light. Titers were estimated by end-point dilutions. RT-PCR was performed in a 25-μl reaction mixture consisting of 2.5 μl of template RNA, 5 μl of 5 × OneStep RT-PCR buffer, 1 μl of deoxynucleoside triphosphates mix, 1 μl of RT-PCR enzyme mix, 0.25 μl of primer (50 pmol), 0.5 μl of RNase inhibitor (40 U; Takara, Shiga, Japan), and distilled water. The amplification profile consisted of an initial cDNA step for 30 min at 42°C, followed by 15 min at 95°C and 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 1 min at 72°C. Amplification was completed by a final elongation step at 72°C for 10 min. Two primers (forward, 5′-ATG GTG GAG GAA GAG AAT GAG AAT GGG TG-3′, and reverse, 5′-TCC GGT AGA TCT TGT CTG GC-3′) were used to amplify an 880-bp fragment as reported previously (28). Each experiment was performed in triplicate.

Data analysis and model fitting of the inactivation curves. The exponential decay linear model was used as described previously (24, 34). The decimal reduction time (D-value), which is the time required for a 1-log reduction (90% inactivation), was estimated using a linear exponential decay model. The D-values of the logarithms of the surviving fraction (log[N/N0]), where N,
equals the PFU per milliliter after an exposure time $t$ and $N_0$ equals the initial PFU per milliliter, were plotted. In addition, a nonlinear model based on the Weibull distribution was applied to the survival kinetics curves (45). The Weibull model is

$$\log \frac{N_t}{N_0} = -(t/b)^n$$

where $t$ is the exposure time and $b$ and $n$ are distribution parameters ($b =$ scale, $n =$ shape).

The Weibull distribution corresponds to a concave upward survival curve if $n < 1$, concave downward if $n > 1$, and linear if $n = 1$. Survival curves were fitted using the nonlinear regression procedure in Number Cruncher Statistical Systems 2007 (Kaysville, UT).

To compare these two models, the mean square error (MSE) and correlation coefficient ($R^2$) were calculated. The smaller the MSE value, the better the model fits the data. The higher the $R^2$ value, the more adequately the model describes the data (42). MSE is defined as

$$\text{MSE} = \frac{\sum (\text{predicted} - \text{observed})^2}{n - p}$$

where $n$ is the number of observations and $p$ is the number of parameters to be estimated. $R^2$ and MSE values were estimated using Microsoft Excel software (Microsoft Corp., Redmond, WA).

Differences between treatments or environmental conditions were analyzed using a one-sided unequal variance Student’s $t$ test with Microsoft Excel software. One-sided $P$ values $< 0.025$ were considered to be statistically significant.

**RESULTS**

**Heat inactivation.** A 2-log reduction in MNV (99% inactivation) occurred after 12 days at 24°C (Fig. 1). Additionally, 4 days of incubation at 37°C and 3 h at 50°C resulted in 4.5- and 3-log reductions in viable MNV, respectively. MNV was rapidly inactivated at temperatures $>60°C$. A 4-log reduction occurred after exposure to 60°C for 10 min, and a greater than 3-log reduction after 2 min at 70°C. At 85°C, MNV was inactivated within 1 min. The observed reductions in MS2 infectivity were lower than was the case for MNV. After incubation at 24 and 37°C, the viable MS2 numbers were around fourfold lower than in the case of MNV, while at 50 and 60°C the numbers were about threefold lower. MS2 was also rapidly inactivated by temperatures $>60°C$.

Real-time TaqMan and LT RT-PCR assays showed a less marked decrease in viral number compared with the plaque assays. Proteinase K and RNase A pretreatment, which was used to remove the nucleic acids of the inactivated viruses, resulted in a slightly greater reduction than in the untreated samples; however, this difference was not statistically significant ($P = 0.1575$). Thus, enzyme pretreatment followed by real-time RT-PCR greatly underestimated infectivity compared with the plaque assays; this was also true of LT RT-PCR. However, exposure to $>70°C$ resulted in a narrowing of the gap between the molecular and plaque infectivity assays.

**Effect of NaCl on thermal inactivation.** MNV infectivity decreased with increasing NaCl (Table 1). At 50°C, the inactivation of MNV in 1.3% NaCl was similar to that in PBS (0.3% NaCl). An approximately twofold greater inactivation at higher NaCl concentrations (3.3 and 6.3%) was detected. MS2 appeared to be more resistant to higher NaCl concentrations (Table 1). The difference in $D$-values at different NaCl concentrations was less than 0.2 log. In comparison with the plaque assays, real-time TaqMan RT-PCR significantly underestimated the log reduction in MNV under all conditions. The maximum inactivation of MNV was a $<-0.5$-log reduction regardless of the NaCl concentration. Like the real-time RT-PCR assay, LT RT-PCR did not show any correlation with the plaque assay, suggesting that LT RT-PCR did not reflect the inactivation of MNV.

**Effect of pH on thermal inactivation.** MS2 was sensitive to low pH (pH 2); immediate inactivation (1-log reduction) occurred after exposure for less than 1 min. In contrast, MNV was more resistant to a low pH (Table 2). MNV inactivation at 24°C and a pH of 2 or 4 was not significantly different from that at a neutral pH. However, higher temperatures (37 and 50°C), in conjunction with a low pH, caused a marked increase in viral inactivation. On the other hand, MNV inactivation at pH 4 was not significantly different from that at a neutral pH. Both real-time TaqMan RT-PCR and LT RT-PCR significantly underestimated the inactivation rate. The maximum reduction in MNV infectivity, as assayed by TaqMan RT-PCR, was approximately 0.2 log, regardless of pH. However, a 1-log reduction in infectivity for 2 days was detected by LT RT-PCR at 37°C after exposure to pH 2.

**Combined effects of NaCl and pH on thermal inactivation.** Table 1 shows the relationship between the $D$-values estimated from a linear model describing MNV and MS2 survival curves at different temperatures and NaCl concentrations. The relationship between $D$-value, temperature, and pH is shown in Table 2. The $D$-values for both MNV and MS2 were not significantly affected by the NaCl concentration. However, the $D$-values for MNV decreased gradually with pH. Additionally, the $D$-values for MS2 were significantly lower at a pH of 2 or 4 than at pH 7.

**Modeling of heat inactivation curves.** Survival curves were fitted using linear and Weibull models (Table 3). We found that a nonlinear, rather than a linear, model provided the best fit to the data. Higher $R^2$ values and lower MSE values were obtained using the nonlinear model. However, a linear model using the data for inactivation by low temperatures (<60°C) also provided a good fit. Table 3 shows the results of goodness-of-fit analyses using the MSE and $R^2$ values produced by comparing the linear and nonlinear models. When the linear and Weibull models were compared, the Weibull model showed a markedly higher $R^2$ and lower MSE value, indicating a better fit to the inactivation curves. The mean $R^2$ values for the linear and Weibull models were 0.940 (MNV = 0.965, MS2 = 0.914) and 0.981 (MNV = 0.988, MS2 = 0.973), while the mean MSE values were 0.692 (MNV = 1.106, MS2 = 0.277) and 0.059 (MNV = 0.050, MS2 = 0.067), respectively (Table 3). The Weibull model conformed to the inactivation curves of MNV better than it did to those of MS2.
HuNoV is known to be resistant to various environmental stresses. Based on data from a recent human challenge study, HuNoV can survive for more than 2 months in water \(^{(38)}\). The data presented here support those of a previous study that showed a 2-log reduction in infectious MNV particles after 12 days at 24°C \(^{(37)}\). Our study also indicates that MNV is highly stable at temperatures <50°C, but that it is susceptible to elevated temperatures. For example, an approximately 4-log reduction occurred after 4 days and 2.5 min at 37 and 70°C, respectively. These results are consistent with those from a study of feline calicivirus, a previously commonly used surrogate for HuNoV \(^{(16, 17)}\). A 3-log reduction in feline calicivirus was achieved at 71.3°C following 1 min at 37°C. These data suggest that HuNoV transmission may be prevented by exposing the virus to a high temperature. However, considering both the low infectious dose (10 to 100 virions) of HuNoV \(^{(29, 50)}\) and its typically high concentration in specimens from infected patients \(^{(1)}\), careful management...
and good hygiene are still necessary under typical environmental conditions if outbreaks are to be prevented.

MNV was resistant to salt and a low pH. At 24°C, less than 2- and 3-log reductions in infectivity were caused by exposure to pH 2 and 6.3% NaCl, respectively, for 2 days. These data are generally consistent with those of previous studies (8, 37). Hepatitis A virus remained infectious for 90 min after exposure to pH 1 at 38°C and was stable when the solution was supplemented with up to 6% NaCl (33, 48). Many other enteric viruses can survive longer in natural seawater than in freshwater and can retain stability at pH 3 (32, 48). However, in contrast to the results with heat, a previous study indicated that feline calicivirus is more sensitive than MNV to a low pH (8). An approximately 4-log reduction in feline calicivirus infectivity was reported after 30 min at pH 2 and 37°C. HuNoV is thought to be resistant to a low pH because a human challenge study with volunteers indicated that Norwalk virus was still infectious after incubation for 3 h at pH 2.7 (14). These results demonstrate that MNV is a highly versatile surrogate for HuNoV in studies involving survival in acidic, fermented, or salted foods, or in the environment. Despite its resistance to low pH and salt, the susceptibility of MNV to low pH or high salt concentrations increased markedly at elevated temperatures. MNV inactivation significantly increased at temperatures >50°C and at pH 2 in comparison with pH 7 (P = 0.0019). Salt concentration and elevated temperature also showed a synergistic effect; however, this was statistically insignificant at temperatures >50°C and at 6.3% compared with 0.3% NaCl (P = 0.0602).

We attempted to reproduce various environmental conditions. For example, 25, 37, and 50 to 85°C are representative of room, body, and pasteurization temperatures, respectively. The salt concentrations used ranged from 0.3 to 6.3%, which correspond to seawater (3.3% NaCl) and salted foods (1 to 6% NaCl). The pH values used were selected to mimic fermented foods (pH 4) and the human stomach (pH 2) and duodenum (pH 4). These data provide useful information regarding the survival time of HuNoV under environmental conditions.

MS2 is the most commonly used surrogate for enteric viruses (2, 12) because it is highly resistant to environmental

<table>
<thead>
<tr>
<th>Conditions</th>
<th>MNV</th>
<th>MS2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D-value (min)</td>
<td>Resistance relative to 0.3% NaCl</td>
</tr>
<tr>
<td>24°C 7 pH</td>
<td>4,046.8 ± 1,324.5</td>
<td>1.00</td>
</tr>
<tr>
<td>37°C 7 pH</td>
<td>1,305.6 ± 218.1</td>
<td>1.00</td>
</tr>
<tr>
<td>50°C 7 pH</td>
<td>28.0 ± 1.4</td>
<td>0.51</td>
</tr>
</tbody>
</table>

*The D-values are the means of three independent experiments ± standard deviation. Resistance values are the ratio of the mean D-value for 0.3% NaCl to the mean D-value at different NaCl concentrations.*

**TABLE 2. Summary of MNV and MS2 D-values at various pH values and temperatures**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>MNV</th>
<th>MS2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D-value (min)</td>
<td>Resistance relative to pH 7</td>
</tr>
<tr>
<td>24°C 7 pH</td>
<td>4,046.8 ± 1,324.5</td>
<td>1.00</td>
</tr>
<tr>
<td>37°C 7 pH</td>
<td>1,305.6 ± 218.1</td>
<td>1.00</td>
</tr>
<tr>
<td>50°C 7 pH</td>
<td>28.0 ± 1.4</td>
<td>0.51</td>
</tr>
</tbody>
</table>

*The D-values are the means of three independent experiments ± standard deviation. Resistance values are the ratio of the mean D-value at pH 7 to the mean D-value at pH 4 or 2.*
TABLE 3. Summary of the linear and Weibull models for the inactivation of MNV and MS2, as determined by plaque assay

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Temp (°C)</th>
<th>Model</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>−k</td>
</tr>
<tr>
<td>MNV</td>
<td>24</td>
<td>Linear</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>Linear</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Linear</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>Linear</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>Linear</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td></td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>Linear</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td></td>
</tr>
</tbody>
</table>

a: k, slope of the linear model; b and n, scale and shape, respectively, of the Weibull model (95% confidence interval).

b: \( R^2 \), correlation coefficient.

MSE: mean square error.

Stresses, nonpathogenic, and easy to propagate and cultivate in the laboratory (5, 15). We used MS2 in this study because it has been well characterized and can be used as a model organism for quality assurance and quality control. Overall, our MS2 data are similar to those reported previously (20, 41). Our results also indicate that MS2 is more resistant than MNV to heat and NaCl. MS2 was even more stable at higher NaCl concentrations at 50°C (Table 1). Previous studies have shown that enteroviruses are also more stable at 50°C in the presence of 1 to 2 M salt (6, 47). A high salt concentration may protect against thermally induced capsid opening or stabilize the viral protein-RNA complex (6, 55). These possibilities should be investigated further in future. However, MS2 was much more susceptible to a low pH.

Our data suggest that MS2 could be used as a conservative surrogate for HuNoV, except at a low pH.

In this study, MNV was used instead of HuNoV because it is currently not possible to determine HuNoV infectivity (7, 26). However, as revealed by RT-PCR, MNV does not always show identical inactivation properties to HuNoV (26). Nevertheless, MNV remains the agent of choice due to the noncultivability of HuNoV and difficulties in performing HuNoV human challenge studies.

To estimate the inactivation rate of MNV under various conditions, three different analytical methods (plaque, real-time RT-PCR, and LT RT-PCR assays) were applied. Of these, the plaque assay is known to best represent viral infectivity. As expected, real-time RT-PCR significantly underestimated MNV inactivation (<0.5-log reduction regardless of the conditions); this is consistent with data from previous studies (20, 41). LT RT-PCR requires that the larger fragment of the viral genome be intact for successful amplification; thus, LT RT-PCR better represented MNV inactivation after exposure to 70 and 80°C. Indeed, our LT RT-PCR results were closer to those from the plaque assay (maximum 3-log reduction at 70 and 80°C). However, LT RT-PCR significantly underestimated MNV infectivity.

We used proteinase K and RNase A pretreatment to overcome the limitations of real-time and LT RT-PCR. When MNV is inactivated by heat, the viral capsid proteins denature, creating gaps in the viral particles (3, 23); thus, the particles are more likely to be susceptible to proteinase K and RNase A treatment. Therefore, enzyme treatment with a subsequent real-time RT-PCR assay is more likely to detect intact virus and better represent viral infectivity. A previous study showed that enzyme treatment, when combined with real-time RT-PCR, gave a good representation of viral inactivation at 80°C (3). Our data also showed approximately 2-log reductions at 70 and 85°C, which is significantly higher than with real-time RT-PCR alone. However, no reduction in infectivity was detected at <70°C. Therefore, enzyme treatment with real-time RT-PCR should be used at >70°C to conservatively represent viral infectivity.

The inactivation curves of MNV and MS2 could be fitted to each model. The simplest was the exponential...
decay model, which assumes equal susceptibility of the viruses to heat and first-order kinetics (13, 27). The D-value could be estimated from the linear model (24, 34). By comparing the D-values of different microorganisms under diverse conditions, their relative resistance to thermal and other environmental stresses can be evaluated. For example, the synergistic effect of heat and a low pH on MNV and MS2 inactivation was apparent from their D-values.

However, in our study, most MNV and MS2 inactivation curves appeared to better follow the Weibull model, which is a statistical distribution model that provides more flexibility to fit inactivation of microorganisms over time. Despite its empirical nature, biological factors related to thermal inactivation were also taken into account (4, 40). The values of model parameters could be linked to any physiological effect (40). For example, a scale >1 indicates that the microorganisms become increasingly susceptible to heat damage with prolonged exposure to heat (54). In addition, different susceptibilities to heat may be possible due to aggregation with other materials and viral clumping. Thus, in addition to the linear exponential decay model, the Weibull model was adequately fitted to the MNV and MS2 survival curves (Table 1) as in previous studies (9, 34, 45). When a linear exponential model did not provide a good fit ($R^2 < 0.9$), the Weibull model appeared to better fit the experimental data based on both the $R^2$ and MSE.

In conclusion, MNV is resistant to various environmental conditions. However, of the various conditions tested, exposure to temperatures >70°C required only a few minutes to inactivate MNV and proved to be the most effective method of inactivation. The methods described in this study may be applied to studies of the persistence of other enteric viruses of major public health concern and may assist in efforts to prevent outbreaks of such viruses.

ACKNOWLEDGMENTS

This study was supported by a research grant (S-10-04-2-FAW-995-0-H) from the Korean Food and Drug Administration and National Research Foundation of Education, Science, and Technology (MEST no. 2011-0001777).

REFERENCES


