Research Note

Surrogates for the Study of Norovirus Stability and Inactivation in the Environment: A Comparison of Murine Norovirus and Feline Calicivirus

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ABSTRACT

Human noroviruses (NoVs) are the leading cause of food- and waterborne outbreaks of acute nonbacterial gastroenteritis worldwide. As a result of the lack of a mammalian cell culture model for these viruses, studies on persistence, inactivation, and transmission have been limited to cultivable viruses, including feline calicivirus (FCV). Recently, reports of the successful cell culture of murine norovirus 1 (MNV-1) have provided investigators with an alternative surrogate for human NoVs. In this study, we compared the inactivation profiles of MNV-1 to FCV in an effort to establish the relevance of MNV-1 as a surrogate virus. Specifically, we evaluated (i) stability upon exposure to pH extremes; (ii) stability upon exposure to organic solvents; (iii) thermal inactivation; and (iv) surface persistence under wet and dry conditions. MNV-1 was stable across the entire pH range tested (pH 2 to 10) with less than 1 log reduction in infectivity at pH 2, whereas FCV was inactivated rapidly at pH values <3 and >9. FCV was more stable than MNV-1 at 56°C, but both viruses exhibited similar inactivation at 63 and 72°C. Long-term persistence of both viruses suspended in a fecal matrix and inoculated onto stainless steel coupons were similar at 4°C, but at room temperature in solution, MNV-1 was more stable than FCV. The genetic relatedness of MNV-1 to human NoVs combined with its ability to survive under gastric pH levels makes this virus a promising and relevant surrogate for studying environmental survival of human NoVs.

Human noroviruses (NoVs), members of the family Caliciviridae, are now recognized as the leading cause of outbreaks of acute nonbacterial gastroenteritis (5, 13). Epidemiological investigation of outbreaks has shown that the most important modes of transmission are person-to-person contact and consumption of contaminated food (13). The so-called ready-to-eat foods are most often associated with NoV outbreaks and include items such as salad bars, deli meats, fresh produce, raw and undercooked molluscan shellfish, and baked desserts (1, 2, 22). NoV outbreaks have also occurred in highly acidic foods such as orange juice (pH 3.3 to 4.19) and frozen raspberries (pH 3.18 to 3.26) (12, 22). Recent estimates indicate that as much as 50% of all foodborne disease outbreaks in the United States would be caused by NoVs if specimens from all infected individuals were screened (25).

To date, human NoV cannot be grown in cell culture, which not only hampers the study of the basic virology of the NoVs, but also hinders studies on the environmental persistence of these viruses and the efficacy of various control measures such as disinfection and cooking. Such studies are important because NoVs have a low infectious dose (10 to 100 virus particles) (20), meaning that only a few infectious virus particles can cause infection, and environmental contamination may prolong outbreaks (6). Surfaces, serving dishes or containers, utensils, and food handled by ill persons who are not practicing adequate personal hygiene before preparing food may also contribute to illness. Because feline calicivirus (FCV), from the genus Vesivirus, can be propagated in cell culture, it has been extensively studied as a surrogate for human NoVs in environmental survival and inactivation studies (9, 10). However, FCV is transmitted by the respiratory route and is inactivated at a relatively low pH, and hence, it may not predict human NoV environmental stability or inactivation.

Recently, the first NoV to be propagated in cell culture was reported (26). This virus, designated mouse norovirus 1 (MNV-1), causes a lethal infection in mice that presents as hepatitis, pneumonia, or inflammation of the nervous system and is therefore very different from the clinical presentation of the human NoVs; however, MNV-1 is shed in mouse feces and is commonly transmitted by the fecal-oral route (17). In the present study, we compared inactivation profiles of MNV-1 to FCV in an effort to examine the relevance of MNV-1 as a surrogate for human NoVs. Specifically, we evaluated (i) stability upon exposure to pH extremes; (ii) stability upon exposure to organic solvents; (iii)
thermal inactivation; and (iv) surface persistence under wet and dry conditions.

**MATERIALS AND METHODS**

**Viruses, cells, and plaque assays.** RAW 264.7 cells (ATCC TIB-71) were cultured in complete minimum essential medium (MEM) containing 10% low endotoxin fetal bovine serum (HyClone, Logan, Utah). Murine norovirus (MNV-1), strain P3 (a gift of Dr. Skip Virgin, Washington University School of Medicine, St. Louis, Mo.) was cultured by infecting 80 to 90% confluent monolayers of RAW 264.7 cells in complete MEM containing 3% low endotoxin fetal bovine serum. FCV strain F9 (ATCC VR-782) was propagated in CRFK (Crandell Reese Feline Kidney) cells (a gift of Dr. James Guy, North Carolina State University) using complete Dulbecco modified Eagle medium (Sigma-Aldrich, St. Louis, Mo.) supplemented with 10% fetal calf serum (Gibco-Invitrogen, Carlsbad, Calif.). Both viruses were harvested after complete cytopathic effect was apparent by repeated cycles of freeze-thaw, followed by centrifugation and storage at -80°C until use. In an effort to mimic the natural state of enteric viruses as they might be found in the environment, we chose not to monodisperse or otherwise further purify the virus stocks.

To determine the infective titer of MNV-1 and FCV, standard plaque assay techniques were used as previously reported (4). Briefly, cells (RAW 264.7 for MNV-1 and CRFK for FCV) were dispensed in 60-mm-diameter cell culture plates at a density of 2 x 10^6 cells per plate and grown to 80 to 90% confluence in 5 ml of complete MEM at 37°C. Cell monolayers were infected with 10-fold serial dilutions of the virus for 1 h at 37°C, and after removal of the inoculum, cells were overlaid with 5 ml of overlay medium containing 0.5% agarose and incubated for 48 h. Plaques were subsequently counted 5 to 8 h after a second agarase overlay (3 ml) including 0.75% neutral red solution (Sigma-Aldrich) was added. Plates with 5 to 50 plaques were used to determine the virus titer in PFU.

**Virus inactivation studies: pH stability.** The pH stability of MNV-1 and FCV was determined by mixing 10 μl of 10-fold serial dilutions of virus solution with 90 μl of each buffer (pH 2, 3, 4, 5 [100 mM citrate buffer], 6, 7, 8 [100 mM phosphate buffer], and 9 and 10 [100 mM carbonate buffer]) and incubating at 37°C for 30 min and 2 h. The pH was subsequently adjusted to 7 by the addition of 400 μl of complete MEM supplemented with 3% fetal bovine serum and the dropwise addition of 0.5 M NaOH or 0.5 M HCl.

**Organic solvent extraction.** Three different organic solvents—chloroform (Fisher Scientific, Atlanta, Ga.), trichlorofluoromethane (Freon, Fisher Scientific), and Vertrel XF (DuPont, Wilmington, Del.)—were tested. A 500-μl volume of organic solvent was mixed with an equal volume of virus stock (1 x 10^6 PFU to 2.3 x 10^8 PFU), mixed by vortexing for 1 min, and centrifuged for 5 min at 2,000 x g to separate the aqueous from the organic phase. The aqueous phase was recovered, serially diluted in phosphate-buffered saline (PBS), and immediately assayed.

**Thermal inactivation.** The capillary tube method of Fairchild and Foegeding (11) was used for the thermal inactivation experiments. Briefly, capillary tubes (Kimble Products, Vineland, N.J.) were filled with 50 μl of each virus stock (approximately 5 x 10^6 to 1 x 10^8 PFU), heat sealed, submerged in a water bath, and held at various temperatures (56, 63, and 72°C) for times ranging from 5 s (at 72°C) to 20 s (at 56°C). At each time point, three capillary tubes were removed and cooled on ice. Each tube was then sanitized by immersion in 10% sodium hypochlorite followed by 70% ethanol, crushed, serially diluted in PBS, and assayed by plaque assay. Consistent with published literature (23), virus inactivation was calculated as the decimal reduction time (D-value), which is defined as the time needed to achieve a 1-log reduction in infectious virus titer at a given temperature.

**Stability on environmental surfaces.** Virus stocks were mixed with reconstituted artificial feces (Feclone, SiliClone Laboratories, Valley Forge, Pa.), and aliquots of 50 μl (representing a total of 5 x 10^5 PFU of MNV-1 or FCV) were placed on the center of individual sterilized stainless steel coupons (5 by 5 cm). At various time points (1, 2, 3, 4, 5, 6, and 7 days), viruses were eluted from the coupon surfaces by repeated pipetting (25 times) of the contaminated area with 450 μl of 10 mM glycine–150 mM NaCl buffer, pH 7.0. To assess the effect of desiccation on virus survival, spiked artificial feces suspensions (20%) were also evaluated. All experiments were done at 4°C (54% relative humidity) and at room temperature (22°C) (75 to 85% relative humidity).

**Statistical analysis.** All virus inactivation experiments were done in triplicate. For thermal inactivation, linear regressions were plotted as the negative reciprocal of the slope of the regression line. Analysis of variance (SAS 9.1, SAS Institute, Cary, N.C.) was used to identify significant differences in virus recovery between treatments and/or viruses.

**RESULTS**

**pH stability.** At low pH values of 2, 3, and 4, MNV-1 infectivity was reduced by less than 1 log (0.6, 0.6, and 0.5, respectively), while the FCV titer was reduced by 2 to 4 log (4.4, 3.7, and 2.3, respectively) (Fig. 1A). At higher pH values (pH 5 to 9) the MNV-1 titer dropped less than 1 log, whereas the FCV titer dropped by approximately 2 log. At pH 10, the MNV-1 titer fell by 1.8 log, whereas the infectivity of FCV was reduced by 5.1 log. At all pH values tested, the differences in log reductions between MNV-1 and FCV were statistically significant (P < 0.05). Increasing the incubation time from 30 min to 2 h did not result in statistically significant changes in virus stability.

**Organic solvent stability.** To compare the sensitivity of MNV-1 and FCV to inactivation by means of organic solvents, aliquots of virus were extracted with chloroform, Freon, and Vertrel and assayed for infectivity. Both viruses were relatively resistant to all three organic solvents, with recoveries ranging from 95 to 104% for MNV-1 and 85 to 91% for FCV (Fig. 1B). Regardless of which organic solvent was used, the recovery of MNV-1 was better than that for FCV (P < 0.05).

**Thermal inactivation.** Virus inactivation was measured at three different temperatures (56, 63, and 72°C). At 56°C (which corresponds to the lower temperature limit for “hot bars”), a 1-log inactivation was achieved at 3.5 and 6.7 min for MNV-1 and FCV, respectively (Table 1). At 63°C (consistent with low-temperature, long-time pasteurization), both viruses were inactivated by 1 log in about 25 s and at 72°C (consistent with high-temperature, short-time pasteurization), and both MNV-1 and FCV were inactivated by 1 log in less than 10 s (MNV-1 = 9.9 s and FCV = 7 s) (Table 1). Survival of MNV-1 and FCV at 56°C differed
significant (P < 0.05), but at 63 and 72°C, virus infectivity after temperature treatment was not significantly different (P > 0.05).

Environmental stability. To study the environmental persistence of MNV-1 and FCV, we tested the infectivity of both viruses in suspension as well as dried onto stainless steel surfaces for up to 7 days. At 4°C, both viruses were inactivated at a similar rate (Fig. 2A). At room temperature, the difference in inactivation pattern was more profound (Fig. 2B). The rates of virus reduction over time for FCV and MNV-1 were similar from day 1 to day 7 for all conditions except when wet at room temperature, in which case differences were significant (P < 0.05).

**DISCUSSION**

MNV-1, the first norovirus to be routinely propagated in cell culture, is genetically related to the noncultivable human NoVs and is spread by the fecal-oral route, although it causes a different disease syndrome in infected mice (17).

To date, several cultivable enteric caliciviruses belonging to other genera within the family Caliciviridae have been used as surrogates for the human NoVs, including porcine enteric calicivirus (a sapovirus) (14), canine calicivirus (a vesivirus) (19), and FCV (also a vesivirus (15)). Of these, FCV has been the most commonly used surrogate to model survival, persistence, and inactivation of human NoVs (4, 10, 24). In the present study, we compared the survival of infectious FCV and MNV-1 under a variety of environmental conditions, including extremes of pH, increased temperature, and desiccation. Although virus inactivation kinetics associated with the use of disinfectants in water has historically been evaluated by means of monodispersed viruses, we and others (9, 10) have used a more environmentally relevant experimental design by using cell culture lysates as virus inoculum. This approach yields a comparatively more conservative prediction of the behavior of viruses in the environment.

We demonstrated that MNV-1 is considerably more resistant to both high and low pH values, with only a minimal loss of infectivity at pH 2. By contrast, and consistent with the findings of other investigators (10, 15), FCV is quite unstable at lower pH values. Such acid resistance is probably crucial for successful infection because enteric viruses must survive the stomach to reach their target cells in the small intestine. Norwalk virus, the prototype human NoV, was reported to cause infection in human volunteers after incubation for 3 h at pH 2.7 (8). Most enteric viruses demonstrate resistance to low pH; for example, cell culture–adapted hepatitis A virus can remain infectious after being held at pH 1 for 5 h, and human enteroviruses retain stability at pH 3 (21). An ideal surrogate for the human NoVs

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**FIGURE 1.** Survival of MNV-1 and FCV (A) across a range of pH values (pH 2 to 10) after 30 min at 37°C and (B) after extraction with Vertrel, Freon, and chloroform. Light shading, MNV-1; dark shading, FCV. Error bars indicate standard deviation (n = 3).

**TABLE 1.** D-values for thermal inactivation of MNV-1 and FCV at different temperatures

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>MNV-1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>FCV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>F test</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>σ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>D</td>
</tr>
<tr>
<td>56</td>
<td>3.473</td>
<td>0.092</td>
<td>6.715</td>
</tr>
<tr>
<td>63</td>
<td>0.435</td>
<td>0.005</td>
<td>0.406</td>
</tr>
<tr>
<td>72</td>
<td>0.166</td>
<td>0.016</td>
<td>0.118</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as D-values (min). Values are the mean of three experiments.

<sup>b</sup> Standard deviation.
should have the capability of surviving at low pH, and our results demonstrate that MNV-1 is superior in acid tolerance when compared with FCV.

Because organic solvent extraction is often an essential step in concentrating enteric viruses from environmental, food, and water samples (7), we evaluated the effect of three commonly used organic solvents on MNV-1 and FCV infectivity. None of the organic solvents, including Vertrel, a Freon substitute not implicated in ozone depletion (18), substantially impacted or otherwise reduced virus infectivity.

Overall, our findings on the thermal resistance of FCV are consistent with or less conservative than the results of others; this might be explained by the use of the capillary tube method, which offers more efficient heat transfer than test tube methods (10, 11). Although the somewhat minimal differences between MNV-1 and FCV survival after exposure to high temperatures probably reflects differences in capsid structure, pasteurization by the batch method (63°C for 30 min), classical method (72°C for 2 min), or continuous method (72°C for 15 s) would most likely result in complete inactivation, unless the initial virus contamination level was high (>4 log). However, it is conceivable that the D-values could be higher in the presence of certain food components that might have a protective effect on the thermal stability of viruses (3).

Our environmental persistence studies were unique in that we suspended virus stocks in artificial feces to mimic fecally contaminated surfaces, because it has been suggested that the fecal matrix can have protective effects on virus persistence on surfaces (16). We found that both viruses were inactivated at similar rates under all conditions except when wet at room temperature, a condition under which MNV-1 was more stable than FCV. Overall, our data for environmental survival of FCV are in agreement with results obtained in previous studies (9).

The results of this study clearly demonstrate that important differences exist in the stability of MNV-1 and FCV under environmental conditions, such as exposure to low pH, high temperature, and desiccation. The most dramatic finding was that MNV-1 is more stable to extremes of pH than FCV and in this regard is more characteristic of the human NoVs. Until an in vitro model for the evaluation of human NoV infectivity becomes available, the genetically related MNV-1, which is easy to grow in the laboratory and is stable at low pH, may be a more relevant surrogate for studying the environmental persistence of human NoVs.

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REFERENCES


FIGURE 2. Survival of MNV-1 and FCV over a 7-day period at (A) 4°C and (B) room temperature in suspension (wet) and on a stainless steel coupon (dry). MNV-1 is indicated with closed circles and triangles; FCV is indicated with open circles and triangles. Error bars indicate standard deviation (n = 3). (MNV-1 was inactivated beyond the detection limit of the plaque assay after day 5 under dry conditions at room temperature; therefore, data for day 6 and 7 are not included.)