Human noroviruses are the leading cause of gastroenteritis in the United States, causing 58% of foodborne illnesses (30). Noroviruses typically cause self-limiting acute diarrheal disease lasting 2 to 3 days; however, more serious cases have been responsible for 26% of hospitalizations and 11% of deaths caused by foodborne illnesses (30). Noroviruses can be spread by water, aerosol vomitus droplets, person-to-person contact, and food. In a worst-case scenario, the low infectious dose, estimated average being 18 virus particles (35), and the high attack rate of 90 to 100% (20) means even a small amount of contamination has the potential to be a public health risk.

Viral survival and inactivation by disinfectants or processing treatments is assessed through infectivity assays, including the plaque assay, and molecular techniques. Despite the high sensitivity of traditional molecular detection methods, these methods cannot distinguish between infectious and noninfectious virus particles. Multiple efforts have been made to develop a cell culture model to propagate human noroviruses and assess infectivity (9, 33); however, these efforts have not yielded a reproducible model. Current research efforts have attempted to take advantage of attachment properties of human noroviruses, in combination with traditional molecular detection methods (7, 22, 23); however, viral detection after attachment does not necessarily reflect the infectivity of human noroviruses. While clinical trials can directly assess infectivity of noroviruses (21), these trials are expensive and inaccessible to many researchers. Because of these limitations in assessing human norovirus infectivity, current methods have been based on viral surrogates. Surrogates for human noroviruses have included murine norovirus (MNV), feline calicivirus (FCV), and the bacteriophage MS2 (2, 5, 13, 32). FCV was predominantly used before the discovery of MNV. In a study comparing MNV and FCV, Cannon et al. (5) showed MNV to be a more environmentally robust surrogate.

MNV was the first norovirus to be propagated in cell culture, and it shares similar genetic and structural features with human noroviruses (37). MNV is transmitted by the fecal-oral route, replicates in the intestine, and is shed in the stool of mice, similar to human norovirus infection (37). MNV causes lethal infection in mice, causing hepatitis, pneumonia, or inflammation of the nervous system (17). These symptoms are different from those of human noroviruses, but since MNV is transmitted via the fecal-oral route and shed in the feces, it is able to withstand lower pH levels. MNV is included in the genogroup GV of norovirus, which are closer in sequence comparison to the GI human noroviruses (15). The MNV model has allowed for further understanding of the relationship between basic mechanisms of norovirus replication in tissue culture and pathogenesis in a natural host (37).
A recently discovered calicivirus, the Tulane virus (TV), has been isolated from the stools of rhesus macaques (Macaca mulatta) and represents a new genus, Recovirus (11). Despite the fact that TV does not belong to the genus Norovirus as does MNV, sequence analysis has shown TV to be closely related to the GII noroviruses (10). TV has also been shown to bind to histo-blood group antigens, similar to how human noroviruses do (10). This characteristic could make TV structurally more similar than MNV to human noroviruses and ultimately, a good surrogate. Additionally, TV has the potential to be a useful animal model for understanding human norovirus biology; however, the disease in rhesus macaques has not yet been fully characterized.

This study aimed to compare the persistence of TV with the common human norovirus surrogate, MNV. The inactivation of these human norovirus surrogates was assessed after treatment in varying pH values, temperatures, and chlorine concentrations, and their survival in tap water at refrigeration and room temperatures was compared. Virus treatments were chosen based on common methods of controlling pathogens in food (pH, heat, survival at refrigeration temperatures) and disinfection strategies (chlorine).

MATERIALS AND METHODS

Virus propagation and infectivity assays. TV (generously provided by Dr. Xi Jiang, University of Cincinnati College of Medicine, Cincinnati, OH) was propagated in LLC-MK2 cells cultured in medium 199 (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Mediatech, Manassas, VA), 1% penicillin-streptomycin, and 1% sodium bicarbonate. MNV (generously provided H. Virgin, Washington University, St. Louis, MO) was propagated in the RAW 264.7 cell line cultured in Dulbecco’s modified Eagle’s medium (GIBCO-Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 1% penicillin-streptomycin, 1% sodium bicarbonate, and 1% glutamate. Viruses were purified from infected cells through three freeze-thaw cycles to lyse cells, and the supernatant containing the virus was recovered by centrifugation at 2,500 x g for 15 min and stored at −80 °C. Stock titers were approximately 6 log PFU/ml for MNV and TV. MNV and TV infectivity was determined by plaque assay as described by Hirneisen et al. (14) and Farkas et al. (11), respectively.

pH treatment. The pH stability of MNV and TV was determined according to the method of Cannon et al. (5), with few adaptations. MNV and TV (10 μl) were incubated with 90 μl of each buffer solution for 30 min at room temperature (20°C). Solutions of pH 2.0, 3.0, 4.0, and 5.0 were made from 100 mM citrate buffer, pH solutions of 6.0, 7.0, and 8.0 were made from 100 mM phosphate buffer, and pH solutions of 9.0 and 10.0 were made from 100 mM carbonate buffer. After incubation with MNV or TV, the pH was adjusted to 7.0 by the addition of 400 μl of MEM supplemented with 2% FBS and the addition of 1 M HCl or NaOH. Viral titers were determined by plaque assay.

Chlorine treatment. The efficacy of chlorine on MNV and TV was assessed by treating varying concentrations of chlorine solution (0.2, 2, 20, 200, and 2,000 ppm) for 5 min at room temperature (20°C). Chlorine solutions were prepared from sodium hypochlorite in household bleach in double-distilled water (ddH₂O). MNV or TV (10⁵ virus particles) was added to 900 μl of chlorine solution. Chlorine activity was quenched with 100 μl of sodium thiosulfate (5%) and 100 μl of FBS (10%). MNV and TV titers were determined by plaque assay and compared with control treatment, whereby ddH₂O (0 ppm of chlorine) was quenched with sodium thiosulfate and FBS.

Heat treatment. Heat treatment was performed in a Mastercycler at 20, 50, 55, 60, 65, 70, and 75°C for 2 min in 200-μl aliquots in 0.2-ml PCR tubes (Eppendorf, Düsseldorf, Germany). After treatment, samples were brought to 4°C. Viral titers were determined by plaque assay.

Virus survival in tap water. MNV and TV (10⁵ virus particles) in tap water were stored at room temperature (20°C) and refrigeration temperatures (4°C) for up to 30 days in the dark. Samples were analyzed on days 0, 1, 3, 5, 7, 10, 15, 20, 25, and 30 by plaque assay.

Statistical analysis. Experiments were performed in triplicate for treatment of each virus on different days. Results are reported as means and standard deviations. Viral reductions were determined by comparing viral titers after treatment with controls. Controls included MNV and TV at 20°C, pH 7.0, and a chlorine concentration of 0 ppm. The differences of mean of Student’s t tests were analyzed with Microsoft Excel 2010, and results were considered significant if P ≤ 0.05.

RESULTS AND DISCUSSION

Viral surrogates ideally should have a similar structure and size to the target virus, and be slightly more resistant to treatments, nonpathogenic, and easy to use in laboratory and industrial settings. As a member of the genus Norovirus, MNV is the most genetically related of all the cultivable human norovirus surrogates. However, a surrogate’s genetic relatedness to the target organisms should not be the only attribute used to validate an organism as a surrogate (32); the functional morphology, including receptor-binding properties, of the virus should also be taken into consideration. Considering other characteristics of the surrogate aside from genetics is vital; for example, bacteriophages MS2 and F2 are genetically very similar (16) but exhibit different resistances in various survival and disinfection scenarios (3). While both MNV and TV have been shown to be genetically related to human noroviruses (10, 37), TV shares similar receptor-binding properties to histo-blood group antigens with human noroviruses, making TV potentially structurally more related. This study aimed to compare the inactivation of MNV with the newly discovered TV through varying pH solutions, chlorine concentrations, heat treatment, and survival at refrigeration and room temperatures longer than 30 days.

MNV and TV reductions in infectivity in varying pH solutions ranging from pH 2.0 to 10.0 are shown in Figure 1. MNV reductions in varying pH solutions resulted in no significant difference (P > 0.05), with the exception of at pH 10.0. TV was not as stable in extreme pH solutions as MNV, whereby TV reductions at pH values 2.0, 9.0, and 10.0 were significant compared with that at pH 7.0 (P < 0.05). The difference between TV and MNV reductions at pH 2.0 was significant (P < 0.05). The stability of MNV in varying pH solutions was also observed by Cannon et al. (5), whereby MNV was able to survive at a pH range from 2.0 to 10.0, whereas FCV, a respiratory virus and
historically popular norovirus surrogate, only withstands a pH range of 3.0 to 9.0. It is hypothesized that the sensitivity of FCV to low pH is likely explained by the mechanism of uptake into host cells via clathrin-mediated endocytosis for infection (34), which is pH dependent as compared with MNV, which is internalized by a clathrin-independent pathway (29) that is independent of pH (28). In our study, MNV was observed to be more stable, resulting in less reduction of infectivity at both high and low pH values than TV. As the little is known about the pathology and virology of TV, it is difficult to hypothesize reasons for the pH instability. The stability of MNV to low pH values is important for its use as a surrogate, as human noroviruses were shown to cause infection in human volunteers after incubation at pH 2.7 for 3 h (8). This pH stability, especially in acidic conditions, is common for enteric viruses (19) and is crucial for viral infection, since the virus must survive the stomach pH to reach the target cells in the intestine.

Heat is the most common food processing technology used to reduce microbial populations. Reductions of infectivity of MNV and TV because of heat treatment are shown in Figure 2. Both MNV and TV were inactivated beyond the limit of detection at both 70 and 75°C. There was no significant difference between reductions of MNV and TV at all temperatures (P > 0.05), and MNV and TV remained infectious after treatment 60 and 65°C, which corresponds to the results of previous studies with surrogates. Human norovirus was found to remain infectious in human subjects after 30 min at 60°C (8), and Gibson and Schwab (12) observed no significant difference in reduction rates for MNV and FCV. Viral inactivation by heat is believed to be induced by conformational changes in the capsid structure (13). Norovirus-like particles were observed to undergo conformational changes in the secondary, tertiary, and quaternary structures at 60°C (1). The results of our study and previous studies indicate that there is likely no difference between norovirus surrogates during heat treatment in buffer.

Chlorine is the most widely used disinfectant because of its low cost, high effectiveness, and residual biocidal effect. Chlorine is used to disinfect water, as a wash for produce, and to sanitize food contact surfaces. Chlorine treatment levels for this study (0.2, 2, 20, 200, and 2,000 ppm) were chosen based on common uses in water treatment and in the food industry, whereby 2 ppm represents free-chlorine concentrations used for drinking water (36), 20 ppm for postcontamination water treatment (36), 200 ppm for produce dump tanks and washes (4), and 2,000 ppm for disinfection of hard, nonporous environmental surfaces (27). MNV and TV inactivation by chlorine concentrations in ddH₂O is shown in Figure 3. Both MNV and TV were inactivated beyond the limit of detection at 2,000 ppm of chlorine. TV was significantly inactivated at 2 ppm of chlorine compared with MNV (P < 0.05). Several studies have assessed the resistance of human noroviruses to chlorine treatment (as reviewed by Hirneisen et al. (13)), with varying results. It is believed that human noroviruses are resistant to those chlorine concentrations used for water treatment, as stool filtrates treated with 3.75 ppm of chlorine for 30 min caused illness in five of eight volunteers (18). The results of this study indicate that MNV is significantly more resistant to chlorine compared with TV at drinking water chlorination levels (2 ppm). Noroviruses are commonly transmitted by droplet contamination of fomites; therefore, survival in the environment is an important part of virus transmission (24). Studies assessing norovirus survival in water detected norovirus genome by reverse transcription PCR after 100 days in mineral water at 4 and 25°C (25) and after 728 days in groundwater at 12°C (6); however, the molecular detection of norovirus RNA does not imply
infectivity. In a recent study with volunteers, human noroviruses were shown to remain infectious after remaining in water for 2 months in the dark at room temperature, and intact capsids were detected for longer than 3 years when stored at room temperature in the dark (31). The norovirus nucleic acid reduction rate observed by Seitz et al. (31) is comparable to the reduction rate of nucleic acid in groundwater at 25°C of 0.01 ± 0.05 log/day (2).

In a study assessing norovirus surrogate survival in groundwater, MNV was shown to be a more environmentally robust surrogate than FCV (2). MNV and TV survival in tap water at 20 and 4°C is shown in Figure 4a and 4b, respectively. At 20°C, MNV and TV were both significantly reduced by day 25. There is no significant difference between MNV and TV survival at 20°C. Conversely, at 4°C, MNV survival in water does not significantly decrease over 30 days and remains between 4 and 5 log PFU/ml, whereas TV infectivity decreased significantly by day 10 from the initial inoculum. The D-values indicating the number of days needed for a 90% reduction in viral titer were determined from linear regression analysis of virus survival at 4°C (Fig. 4b). D-values were determined to be 19.04 ± 5.70 and 1.78 ± 0.21 days for MNV and TV, respectively, which were significantly different from each other (P < 0.05).

An ideal surrogate for human noroviruses would be associated with acute gastroenteritis and share similar environmental resistances. Based on RNA stability, clinical trials, and the epidemiological of norovirus outbreaks, noroviruses have been observed to be environmentally stable and resistant to many disinfectants and treatment technologies (21, 26, 31). Compared with TV, MNV is likely a better surrogate for human noroviruses, as MNV persisted over a wider range of pH values, at 2 ppm of chlorine, and without a loss of titer at 4°C. However, it is likely that one surrogate alone cannot fully mimic the characteristics of human norovirus stabilities in different treatments. In addition, the many different genogroups and genotypes of human noroviruses have slightly different environmental stabilities; therefore, several surrogates should be considered in the evaluation of disinfection and food processing technologies.

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REFERENCES


