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Viable Norovirus Persistence in Water Microcosms

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Abstract

Human noroviruses are one of the leading causes of acute gastroenteritis worldwide. Based on quantitative microbial risk assessments, norovirus contributes the greatest infectious risk of any pathogen from exposure to sewage-contaminated water; however, these estimates have been based upon molecular (i.e., RNA-based) data as human norovirus has remained largely unculturable in the laboratory. Current approaches to assess the environmental fate of noroviruses rely on the use of culturable surrogate viruses and molecular methods. Human intestinal enteroids (HIEs) are an emerging cell culture system capable of amplifying viable norovirus. Here, we applied the HIE assay to assess both viable norovirus and norovirus RNA persistence in surface, tap, and deionized water microcosms. Viable norovirus decreased to below the detection limit in tap and deionized water microcosms and was measured in a single replicate in the surface water microcosm at study conclusion (28 days). Conversely, the norovirus RNA signal remained constant over the duration of the study, even when viable norovirus was below the limit of detection. Our findings demonstrate the disconnect between current environmental norovirus detection via molecular methods and viability as assessed through the HIE assay. These results imply that molecular norovirus monitoring is not inherently representative of infectious norovirus.

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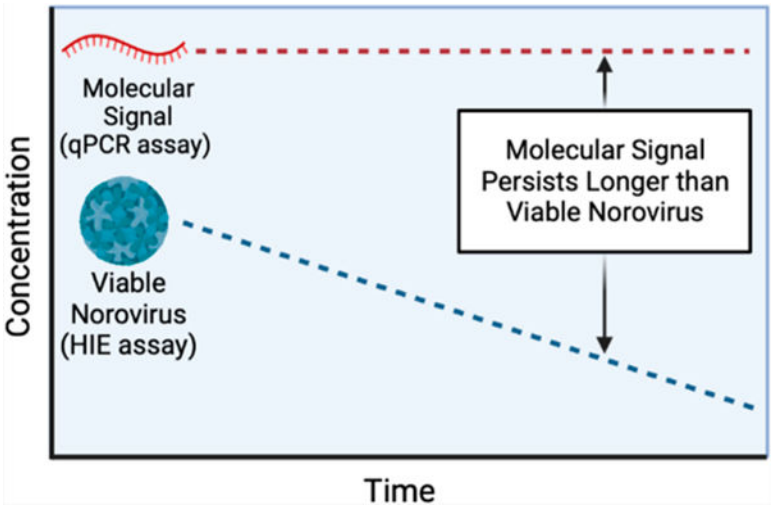
Graphical Abstract



Keywords

Norovirus; persistence; water quality; public health; molecular detection; culturing

INTRODUCTION

Noroviruses, nonenveloped single-stranded RNA viruses in the family *Caliciviridae*, are one of the leading causes of acute gastroenteritis among all age groups worldwide.[1] Noroviruses account for over 600 million illnesses annually, 50% of all acute gastroenteritis cases, and 90% of all nonbacterial epidemic outbreaks.[2,3] In addition to the significant disease burden, norovirus outbreaks result in substantial economic hardships from both health care costs and the loss of employee productivity; estimates from simulations predict the overall cost in the United States is $10.6 billion annually.[4] Additionally, with respect to environmental exposures and water quality, norovirus is the largest predicted contributor of infectious risk in sewage contaminated waters.[5,6] Norovirus RNA is regularly detected in wastewater and natural waters by molecular methods, and contaminated growing waters for bivalve mollusks frequently lead to norovirus outbreaks.[2,5]

Until recently, the lack of a robust cell culture for human norovirus has been a challenge to having a better understanding of the persistence of infectious norovirus in water.[7,8] Thus, estimates of viable norovirus persistence in environmental matrices have been primarily analyzed with surrogate viruses such as feline calicivirus, Tulane virus, murine norovirus, and MS2 bacteriophage.[9] Studies using surrogates show a reduction of surrogate viral infectivity is linked to a reduction in norovirus RNA and typically a larger reduction in surrogate RNA, via real-time quantitative polymerase chain reaction (RT-qPCR).[10] RT-qPCR is a molecular method used to detect the presence of genetic material in a sample; however, it does not distinguish between infectious and noninfectious virions, thus increasing the need for a culture method to assess norovirus viability.[11] Based on the use of these surrogate viruses, noroviruses are predicted to have high environmental stability, which

makes applying appropriate control measures in water systems crucial.[2,12] Beyond surrogate studies, environmental monitoring efforts for norovirus rely primarily on molecular (i.e., RNA-based) detection methods. These methods are inherently conservative in nature as they may detect damaged viral particles that are no longer infectious; however, this relationship has not yet been assessed with a norovirus cultivation method.

In 2016, a novel method to show replication of human norovirus was reported using human intestinal enteroids (HIEs).[13] HIEs are derived from Lgr5+ adult intestinal stem cells and contain multiple epithelial cell types including enterocytes, goblet cells, enteroendocrine cells, and Paneth cells which replicate the cellular diversity of the human gastrointestinal tract.[8,13] HIEs are grown as 3D cultures in an enriched media and monolayers are infected with fecal filtrate spiked water matrices or vomit samples to measure replication.[8,13,14] Notably, the quantitative relationship between replication in HIE assays and viral concentration has not yet been established; however, replication in the HIE assay confirms the presence of viable norovirus.

In this study, norovirus infectivity was analyzed by using HIE cultures and fecal filtrates spiked in laboratory water microcosms. In addition, RT-qPCR was used to evaluate the persistence of norovirus RNA. These data allow for the first time a comparison between viable human norovirus and RNA persistence in representative environmental water matrices. Ultimately, these data will help estimate the exposure risk to contaminated norovirus waters as well as the suitability of molecular methods for monitoring human norovirus in environmental waters.

MATERIALS AND METHODS

Sample Collection.

All water samples were collected at the University of Notre Dame on August 24, 2020. Ultrapure water and tap water were both aseptically sampled from the relevant laboratory water system. The Notre Dame campus supplies potable water via a small groundwater system without additional chemical addition or treatment, including chlorination. Surface water was aseptically sampled from the surface approximately one meter from the shore of St. Joseph's Lake; a small, spring-fed lake on the University Notre Dame campus. Triplicate readings of pH and turbidity of each sample were measured immediately after collection and the mean and standard deviation are shown in Table 1. Samples were stored in replicate 15 mL Falcon tubes at −70 °C and shipped overnight on ice to the Center for Disease Control and Prevention (Atlanta, GA, USA) and stored at −70 °C.

Ten Percent Stool Filtrate.

A 10% stool suspension was prepared by adding 0.5 g of GII.4 Sydney[P31] positive whole stool to 4.5 mL of PBS. The stool suspension was vortexed for 30 s, kept at room temperature for 5 min, and vortexed again. The sample was sonicated, and solids were removed by centrifugation for 10 min at 10 000*g*. The supernatant was sequentially filtered through 5, 1, 0.45, and 0.22 μm filters. The resulting 10% stool filtrate was aliquoted and stored at −70 °C.

Human Intestinal Enteroid Culture (HIE).

Adult secretor positive jejunal HIE cultures (J2 cell line) were grown at 37 °C and 5% CO_2 as undifferentiated 3D cultures as described previously with minor modifications.[8,13] Briefly, HIEs were recovered from liquid nitrogen (LN_2), suspended in 20 μL of Matrigel (Corning), plated in 24-well plates, and grown as 3D cultures in 500 μL of IntestiCult (INT) Organoid Growth Medium (Stem Cell Technologies) supplemented with 10 μM Y-27632 (Sigma-Aldrich).

Duplicate, 96-well-plate monolayers were prepared as previously described.[8] Briefly, HIE cultures were dissociated into a single-cell suspension in 100 μL of INT medium supplemented with 10 μM Y-27632 (Sigma-Aldrich) and plated as undifferentiated monolayers in collagen IV (Sigma-Aldrich) precoated 96-well plates. After 24 h, INT medium was replaced with differentiation medium to induce cell differentiation. Differentiation media was prepared by mixing equal volumes of component A and complete media without growth factors (CMGF-; Advanced DMEM/F12 medium supplemented with 1× Glutamax, 10 mM HEPES, and 100 U/ml penicillin-streptomycin). Monolayers were differentiated for 4 days at 37 °C and 5% CO_2, and differentiation media were refreshed every other day.

Water Infection Experiments.

Each microcosm consisted of 2 mL of water, and microcosms were tested in duplicated for each water type. The GII.4 Sydney[P31] 10% stool suspension was diluted in each microcosm to achieve 2.1×10^6 norovirus genomic copies/ml. Spiked water samples were stored at room temperature (18−22 °C) in the dark and sampled according to design. Samples were taken at 0-, 1-, 3-, 7-, 14-, 21- and 28-days post inoculation. Negative controls were nonspiked water. At each time point, 80 μL of spiked- and nonspiked-water was added to 720 μL of infection media [CMGF- supplemented with 500 μM glycochenodeoxycholic acid (GCDCA) plus 50 μM ceramide (C2)] and kept on ice. A second water aliquot of 200 μL was collected and frozen at −80 °C.

HIE Infections.

For each sample collected from each microcosm, infections were performed in triplicate wells on 100% confluent 4-day-old differentiated HIE monolayers. To determine viral infectivity, duplicate 96-well plates were inoculated with 100 μL of spiked and nonspiked water prediluted in infection media as described above. After a 1-h incubation at 37 °C and 5% CO_2, monolayers were washed twice with CMGF- and 100 μL differentiation medium containing 500 μM GCDCA and 50 μM C2 was added. For each set of infections, one plate was frozen immediately at −70 °C and a duplicate plate was incubated at 37 °C, 5% CO_2, for 72 h and frozen at −70 °C.

Viral RNA from spiked and nonspiked water aliquots, cells, and media at one hour and 72 h after infection was extracted using the MagMax-96 Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Norovirus genomic copies were quantified by reverse-transcription quantitative PCR as described previously.[15] A standard curve using 10-fold serial dilutions of Sydney GII.4

RNA transcripts was generated, and norovirus genomic copies from each sample were extrapolated from the curve. The real-time RT-qPCR limit of detection was 26.8 RNA copies per 1 μL (or 2.68×10^3 RNA copies/well). Measurements below the limit of detection were excluded unless all samples were below the limit of detection, in which case samples were arbitrarily assigned to half of the limit of detection (14.3 RNA copies per 1 μL of RNA or 1.43×10^3 RNA copies/well) for graphing.

Statistical Analysis.

All statistical analyses were completed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, U.S.A.). Data presented shows the mean and standard deviation from all the experimental runs. Significant differences in genomic copies in each water type over time were analyzed using a one-way ANOVA paired with a Dunnett's multiple comparisons test. The decay of viable norovirus was analyzed by using a monophasic decay model, which assumes first-order decay. 'The equation used to calculate the decay rates was:

$$\ln\left(\frac{N_t}{N_0}\right) = kt$$

where N_t is the genome copies at time t, N_0 is the genome copies at time 0, k is the decay rate, and t is the time in minutes.

RESULTS AND DISCUSSION

Norovirus Decay as Assessed by HIE.

The HIE assay showed a consistent decrease in amplified norovirus RNA signal over 28 days, at which point the HIE assay was below the limit of detection for 6/6 replicates of the tap water and ultrapure water samples and 4/6 replicates of surface water samples (Figure 1). There was a significant difference in norovirus genomic copies measured by the HIE assay between the initial spike in and the first experimental day for all water matrices, with p-values of 0.04, 0.03, and 0.001 for surface water, tap water, and ultrapure water, respectively. While the quantitative relationship between viable virus and RNA detected in the HIE has not yet been established and will require further experimentation, the expectation is that decreased viable norovirus results in decreased signal in the HIE assay. Here we determined presumptive first-order decay rates for viable norovirus via the HIE assays (Figures SI1−SI3). The presumptive decay rates for viable norovirus via the HIE assay were 0.11 ± 0.02 day^{-1}, 0.08 ± 0.02 day^{-1}, 0.10 ± 0.01 day^{-1} for surface water, tap water, and ultrapure water, respectively, and there was no significant difference between the k-values for different water types. The decay rates for surrogates have previously been analyzed for environmental waters within similar ranges, resulting in 0.18 ± 0.02 day^{-1}, 0.12 ± 0.01 day^{-1}, and 0.09 ± 0.01 day^{-1} for feline calicivirus, MS2, and murine norovirus, respectively.[9]

Norovirus Decay Measured by RT-qPCR.

No apparent RNA decay as measured by RT-qPCR was observed over the experimental time period (Figure 1). Based on a one-way ANOVA, decay was not significantly different

from zero for any of the water matrices ($p = 0.06$ to 0.37); i.e., there was no statistically observable decay in the norovirus RT-qPCR signal.

The molecular signal of norovirus has been used in previous studies to determine the presence of norovirus in samples.[9] Based on the data above, the molecular signal of human norovirus is stable over a 28-day time period which coincides with previous work.[9,11] Seitz et. al found that by RT-qPCR, norovirus RNA in fecal-filtrate-spiked groundwater was detected for 1266 days.[9] Bae et al. found that RNA may be detected for extended periods of time as RT-qPCR detects both free and capsid-bound RNA.[11]

Until the HIE system for human norovirus has become available, surrogate viruses such as MS2, feline calicivirus, and murine norovirus have been studied to infer the behavior of human norovirus.[10] Analyses using surrogate viruses with RT-qPCR determined the reduction rate of nucleic acids for MS2, murine norovirus, and feline calicivirus were 0.05 ± 0.03 log/day, 0.04 ± 0.03 log/day, and 0.08 ± 0.03 log/day, compared with human norovirus at 0.04 ± 0.01 log/day.[11]

Disconnect Between Norovirus Viability and Molecular Method Persistence.

The inability to determine the amount of infectious norovirus for environmental monitoring has to-date necessitated the application of molecular methods for quantifying human norovirus in environmental waters. Our data suggest that there is a clear disconnect between the persistence of viable norovirus and the persistence of molecular norovirus signal, as the molecular signal shows no decay, and viable norovirus shows decay throughout the experiment. Current literature indicates this disconnect is present for other enteric viruses, such as Poliovirus.[16] These findings have important implications for norovirus risk assessment applications as risk estimates based off molecular methods would lead to an overestimation of risk. Additional studies could be designed to better connect molecular and viable norovirus persistence in water. Finally, the results of our study may not be applicable under alternative inactivation scenarios or environments where molecular norovirus detections may more closely mirror viable norovirus measures; however, these conditions would need to be separately evaluated.

Implications and Future Work.

The results of this study imply that molecular norovirus monitoring is not inherently representative of infectious norovirus following decay in water matrices and thus is not inherently representative of infectious risk. Current molecular methods provide a conservative approach to norovirus detection; however, the results likely overestimate the inherent disease risk.

Additional work is necessary to confirm the relationship between molecular and viable norovirus detection methods, including improved method development and expanded conditions and inactivation scenarios to be considered. Furthermore, analyzing viability RT-qPCR assays have the potential to further our understanding of the disconnect between detection of norovirus RNA and viable norovirus.[17] The inclusion of a RNase pretreatment to eliminate free RNA and RNA from structurally compromised virions in the samples is a potential approach to demonstrate the disconnect between molecular detection methods

and viability. Further work is required to determine the suitability of various surrogates to represent viable human norovirus under the same experimental conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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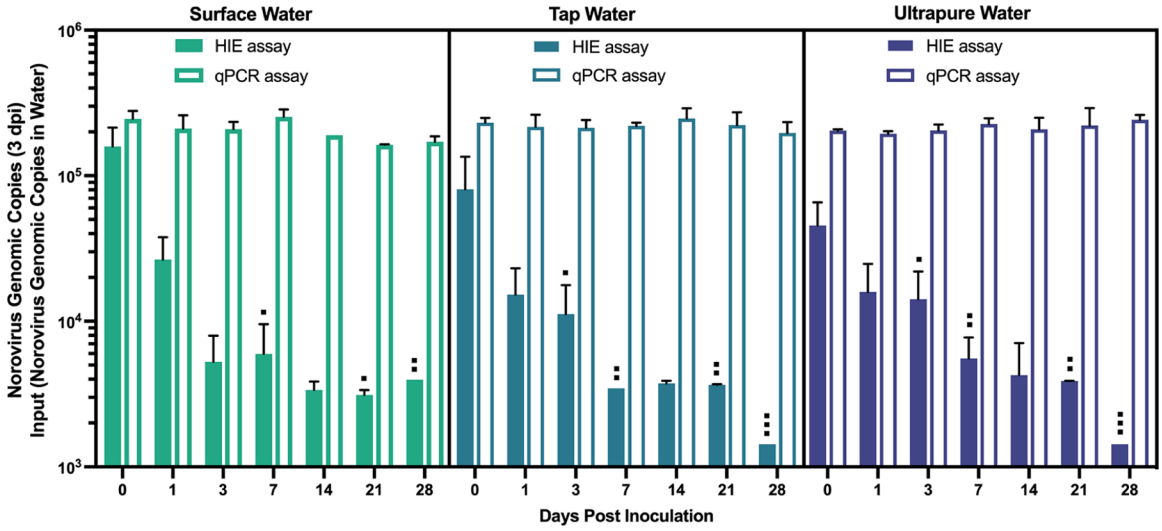

Figure 1.

RNA Persistence in HIE vs Water Matrix. The outlined bars represent the RT-qPCR assay results and show the input virus in the water matrix as genomic copies. The solid bars represent the HIE assay as norovirus genomic copies 3 days post inoculation. The data are shown as the mean and standard deviation for two experimental runs with three technical replicates per run. The boxes above the bars indicate the percentage of samples below the limit of detection. One box = 33% below LOD, 2 boxes = 67% below LOD, and 3 boxes = 100% below LOD.

Table 1.

pH and Turbidity of Water Samples

water type	pH	turbidity (NTU)
ultrapure water	6.82 ± 0.02	0.0 ± 0.0
tap water	7.47 ± 0.01	0.1 ± 0.0
surface water	9.59 ± 0.02	0.6 ± 0.1