

Norovirus Infectivity in Humans and Persistence in Water[∇]

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Received 10 June 2011/Accepted 8 August 2011

To examine the long-term infectivity of human norovirus in water, 13 study subjects were challenged at different time points with groundwater spiked with the prototype human norovirus, Norwalk virus. Norwalk virus spiked in groundwater remained infectious after storage at room temperature in the dark for 61 days (the last time point tested). The Norwalk virus-seeded groundwater was stored for 1,266 days and analyzed, after RNase treatment, by reverse transcription-quantitative PCR (RT-qPCR) to detect Norwalk virus RNA contained within intact capsids. Norwalk virus RNA within intact capsids was detected in groundwater for 1,266 days, with no significant log₁₀ reduction throughout 427 days and a significant 1.10-log₁₀ reduction by day 1266. Purified Norwalk virus RNA (extracted from Norwalk virus virions) persisted for 14 days in groundwater, tap water, and reagent-grade water. This study demonstrates that Norwalk virus in groundwater can remain detectable for over 3 years and can remain infectious for at least 61 days. (ClinicalTrials.gov identifier NCT00313404.)

Human noroviruses (HuNoV) are nonenveloped, single-stranded RNA viruses that spread primarily through the fecal-oral route (reviewed in references 11, 16, and 17). They are the primary cause of epidemic gastroenteritis worldwide (5, 11, 27) and are estimated to cause 218,000 deaths and 1.1 million hospitalizations among children in developing countries each year (27). Water is an important route of HuNoV transmission (11), and HuNoV are considered a significant cause of waterborne gastroenteritis worldwide (5, 37). To reduce the risk of waterborne HuNoV outbreaks, it is necessary to understand HuNoV infectivity and persistence in water.

HuNoV is highly resistant to environmental degradation in various water types. Due to the lack of a simple infectivity assay for HuNoV, previous studies have utilized molecular detection techniques to study HuNoV persistence in water. HuNoV was detectable in mineral water at 4 and 25°C up to 100 days by reverse transcription-quantitative PCR (RT-qPCR) (26) and in groundwater at 12°C for 728 days by conventional PCR (8). We previously calculated the nucleic acid reduction rate of the prototype HuNoV, Norwalk virus (NV), at 25°C to be 0.08 ± 0.02 log₁₀/day in surface water and 0.01 ± 0.05 log₁₀/day in groundwater (3). However, molecular detection of HuNoV

does not directly imply infectivity; for example, RT-PCR can detect both RNA released from viral capsids (presumably non-infectious) and RNA within viral capsids (potentially infectious) (35). Although infectivity assays with HuNoV surrogates (e.g., murine norovirus, feline calicivirus, and poliovirus) have been used to estimate HuNoV infectivity in water (3), the comparability of viral surrogate infectivity to HuNoV infectivity remains uncertain. Additional research has focused on the resistance of the HuNoV capsid to environmental degradation (29), yet little information exists on the persistence of purified or partially exposed HuNoV RNA in water.

In the present study, we conducted a blinded human clinical trial to measure the duration of the infectivity of NV in groundwater. Furthermore, we quantitatively analyzed the long-term persistence of potentially infectious NV in groundwater (in years) and purified NV RNA in various water types (in weeks).

MATERIALS AND METHODS

Study subjects. Study subjects were recruited from Atlanta, GA, from May 2006 to December 2006 and provided informed consent before the screening. Eligible volunteers were 18 to 50 years old, in good health (determined by a clinical evaluation), and secretor status positive (secreted carbohydrate H type 1, the putative NV receptor [19]). Exclusion criteria included having an enteric infection within 1 month of enrollment and pregnancy (determined by a pregnancy test). This clinical trial was approved by the Emory University Institutional Review Board and is registered on ClinicalTrials.gov (identifier NCT00313404).

Secretor status determination. Secretor status was assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (2). We modified the method by using polystyrene Costar enzyme immunoassay/radioimmunoassay (EIA/RIA) flat-well, medium binding plates (Corning, Lowell, MA), 0.1% unprocessed saliva in phosphate-buffered saline (PBS) (Lonza, Walkersville, MD) (incubated overnight at 4°C), and 1 mg/ml horseradish peroxidase (HRP)-conjugated rabbit anti-UEA-I antibody (EY Laboratories, San Mateo, CA) diluted to 0.2% in PBS (Lonza, Walkersville, MD) containing 5% nonfat milk (Blotto for

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[∇] Published ahead of print on 19 August 2011.

Western analysis/ELISA; Alpha Diagnostic Intl., San Antonio, TX). Reactions were developed with tetramethylbenzidine substrate (BioFX, Owings Mills, MD) and quenched with 0.18 M hydrochloric acid (Fisher Scientific, Pittsburgh, PA). The optical density at 450 nm (OD_{450}) of each well was read using the SmartSpec Plus spectrophotometer (BioTek, Winooski, VT) and KCJunior software (BioTek, Winooski, VT). Secretor-positive samples were defined as having an average OD_{450} value that was ≥ 4 times that of the negative control (a secretor-negative saliva sample).

Groundwater collection and spiking. Groundwater (1 liter) was collected from a residential well in Atlanta, GA (previously characterized in reference 3). An independent laboratory (Gel Laboratories, Cincinnati, OH) assayed a water sample from this untreated groundwater to determine whether it met U.S. Environmental Protection Agency (USEPA) drinking water standards and found that levels of chemical pesticide residue were slightly higher than USEPA drinking water guidelines. After we passed the groundwater samples through a Brita (The Brita Products Company, Oakland, CA) cartridge (combination of ion-exchange resin and activated carbon) twice, the independent laboratory confirmed the water sample met USEPA drinking water standards, and we were able to safely administer it to human subjects. The groundwater sample was frozen in 50-ml aliquots. For each human challenge, a 50-ml groundwater aliquot was thawed, and 9 ml of the groundwater was combined with 1 ml of 8FIIb NV inoculum (HuNoV genogroup GI.1) (34) to a final concentration of $\sim 6.5 \times 10^7$ NV genomic equivalent copies per milliliter (GEC/ml). This seeded groundwater was stored in the dark at room temperature for a set time.

Clinical trial. A total of 13 of 23 screened subjects were enrolled in the clinical trial. A total of 46% of enrolled subjects were male, 62% were Caucasian, and 38% were African-American. The only significant protocol deviation was one missed follow-up appointment, and this did not prevent assessment of the primary outcome of NV infection. There were no unexpected study-related adverse events.

Before challenge, 10 ml of the seeded groundwater ($\sim 6.5 \times 10^8$ NV GEC) was added to 90 ml of distilled water. Study subjects ($n = 13$) ingested the 100-ml water sample at Emory University Hospital's General Clinical Research Center. Subjects were blinded to the amount of time the NV-seeded groundwater was stored before challenge. Each subject ingested 100 ml of 2% sodium bicarbonate solution 2 min before and 5 min after challenge to neutralize gastric acidity (consistent with previous HuNoV challenge studies [9, 12, 18, 19]). Symptom information, stool, emesis, and blood samples were collected daily during a 5-day hospital stay and at outpatient visits on approximately 8, 14, 21, 28, and 35 days postchallenge. Blood samples were centrifuged (centrifuge 5810 R, rotor A-4-81; Eppendorf, Hamburg, Germany) at 25°C at $1,600 \times g$ for 20 min, and the sera were stored at -80°C . NV infection was defined as seroconversion (≥ 4 -fold increase in NV-specific serum IgG titer in any postchallenge versus prechallenge serum sample) (19) or the presence of NV RNA, detected by RT-PCR, in any postchallenge stool or emesis sample.

Seroconversion and viral shedding determination. NV-specific serum IgG was quantified by ELISA as previously described (19). For RT-PCR, 100 μl of stool or emesis samples was suspended in 400 μl of reagent-grade water (Cellgro, Manassas, VA), added to 500 μl of Vertrel XF (DuPont, Wilmington, DE), and centrifuged (centrifuge 5415 R, rotor F-45-24-11; Eppendorf, Hamburg, Germany) at 25°C at $13,000 \times g$ for 10 min. RNA was extracted from the supernatant by following the QIAamp viral RNA minikit vacuum protocol (Qiagen, Valencia, CA), except that 50 μl of reagent-grade water (Cellgro, Manassas, VA) was used for the elution step and emesis samples were concentrated five times through one filter. The Qiagen columns that we used have been shown to remove RT-PCR inhibitors from stool samples (1, 6). RT-PCR was performed with NV primer pair 51-3 as previously described (25, 36) with the following modifications: a 20- μl reverse transcriptase (RT) mixture was made with 4.5 U AMV reverse transcriptase (Promega, Madison, WI), 4.0 μl of $5\times$ Green GoTaq reaction buffer (Promega, Madison, WI), 20 U RNase inhibitor (Promega, Madison, WI), 0.25 mM deoxynucleoside triphosphate (dNTP) mixture (Qiagen, Valencia, CA), 5.63 μM primer 51 (Sigma, St. Louis, MO), 1.5 μl of Triton X-100 (Alfa Aesar, Ward Hill, MA), and 5 μl of stool- or emesis-extracted RNA. PCR modifications included an initial denaturation for 3 min and the addition of a 30- μl PCR mixture to create a final 50- μl reaction volume containing 1.25 U GoTaq DNA polymerase (Promega, Madison, WI), 6 μl of $5\times$ Green GoTaq reaction buffer (Promega, Madison, WI), and 2.25 μM primer 3 (Sigma, St. Louis, MO). Gel electrophoresis and sample analysis were performed as described previously (25).

Part of the dose used to challenge one pair of volunteers was stored at room temperature in the dark for 1,266 days. Specifically, 27 ml of the groundwater was combined with 3 ml of 8FIIb NV inoculum (HuNoV genogroup GI.1). Of this dose, 20 ml (10 ml per volunteer) was used to dose a pair of volunteers, and the remaining 10 ml was stored at room temperature in the dark for 1,266 days.

Fifty-microliter aliquots were removed at 0, 7, 14, 21, 35, 413, 441, 622, and 1,266 days and frozen at -80°C . Results from two to five 50- μl aliquots are presented. All of the aliquots were thawed and tested by RT-qPCR. To extract NV RNA, 3 μl of each aliquot was added to 26.4 μl of reagent-grade water (Cellgro, Manassas, VA) and 0.6 μl of RNase inhibitor (Promega, Madison, WI), heated to 99°C for 5 min, and chilled on ice for 2 min (22). Three or four additional 50- μl aliquots were removed at 0, 427, and 1,266 days and frozen at -80°C . These aliquots were treated with RNase before RNA extraction to degrade RNA released from damaged viral capsids (presumably noninfectious). For RNase treatment, 3 μl of each aliquot was incubated with 1 μl of RNase One (Promega, Madison, WI) diluted in 26.4 μl of reagent-grade water (Cellgro, Manassas, VA) at 37°C for 15 min. After addition of 0.6 μl of RNase inhibitor (Promega, Madison, WI), viral RNA was extracted by heating, as described above. To obtain the average concentration of NV GEC/ μl for each time point, all of the aliquots were analyzed in duplicate in several runs by RT-qPCR using DNA standards as previously described (23), except for an elongation temperature of 60°C for 30 s. The detection limit for the RT-qPCR assay is 20 genomic equivalent copies per reaction (22). This detection limit corresponds to a 2.57-log_{10} reduction or greater without RNase treatment and a 1.16-log_{10} reduction or greater with RNase treatment (determined by taking the \log_{10} of the detection limit [20 genomic equivalent copies per reaction] divided by the average concentration of genomic equivalent copies at day 0 for each experiment).

In the purified NV RNA persistence experiments, NV RNA was extracted from the stool of an 8FIIb NV-infected volunteer (34) as described above. A total of 150 μl of the purified NV RNA extracted from NV virions was added to 1.35 ml of Georgia groundwater (passed twice through a Brita [The Brita Products Company, Oakland, CA] cartridge), tap water (collected from laboratory faucets after running for 5 min; approximately 0.42 mg/liter of free chlorine, measured by a model 1200 single-test colorimeter [LaMotte, Chestertown, MD]), and reagent-grade water (Cellgro, Manassas, VA) to a final concentration of $\sim 8 \times 10^4$ NV GEC/ μl . The seeded water samples were stored in the dark at room temperature. To obtain the average concentration of NV GEC/ μl for each time point, two 37.5- μl aliquots at each time point (0, 7, and 14 days) were analyzed in duplicate in one single run by RT-qPCR as described above, except for the use of RNA standards (22). The results are representative of 5 separate experiments. The limit of detection corresponds to a 3.60-log_{10} reduction or greater.

The number of replicate results for each time point varied because some replicates failed quality control assays (e.g., threshold cycle [C_T] values for standards were not consistent, controls did not work, etc.), replicates could not be reused, and we were limited by the original number of replicates collected for each assay. However, for all of the assays, each aliquot per run was assayed in duplicate, resulting in at least 4 to 10 assays for each sample and time point to obtain the average concentrations in NV GEC/ μl .

Statistical analysis. Data were double entered into Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA), cleaned to resolve discrepancies, and analyzed in SAS 9.2 (SAS Institute, Cary, NC). To avoid underrepresentation and overrepresentation of RT-qPCR values, when values fell below the detection limit, values were assigned one-half of the RT-qPCR detection limit (22, 30). Significant differences between groups were analyzed by the Mann-Whitney test (2 comparisons) or the Kruskal Wallis test, followed by Dunnett's test (>2 comparisons). A P value of <0.05 was considered significant.

RESULTS

NV in groundwater can remain infectious for at least 61 days. After ingesting stored NV-seeded groundwater, 10 of 13 subjects became infected with NV and experienced a range of viral shedding and clinical symptoms (Table 1). Ingestion of NV-seeded groundwater stored for 61 days (the longest time point tested) caused infection in 2/2 study subjects (Fig. 1).

NV in groundwater can remain detectable for over 3 years. NV in groundwater was detected by RT-qPCR for 1,266 days postseeding (last sampling date). Compared to day 0, there was no significant NV \log_{10} reduction after 622 days of storage, but there was a significant 1.79-log_{10} reduction by day 1266 (Fig. 1). NV-seeded groundwater aliquots were treated with RNase before RT-qPCR to quantify only virions with intact capsids that were potentially infectious (RNase would degrade RNA

TABLE 1. Laboratory and clinical data for 13 volunteers challenged with groundwater seeded with 8FIIb NV inoculum

Storage time of groundwater (days)	No. infected/no. challenged	Seroconversion	Duration (range of days postchallenge) of:	
			Viral shedding ^a	Clinical symptoms ^b
0	2/2	Yes/yes	ND ^c /2–13	2–4/2–2
7	2/2	Yes/yes	2–15/5–13	1–4/2–3
14	2/2	Yes/yes	2–22/2–22	2–3/2–3
21	1/4	No/no/yes ^d /no	ND/ND/2–13/ND	NA ^e /NA/2–3/NA
27	1/1	Yes	2–34	2–3
61	2/2	Yes/yes	3–22/4–4	2–3/3–5

^a Viral shedding was defined as the presence of NV RNA, detected by RT-PCR, in any postchallenge stool sample.

^b Clinical symptoms included diarrhea (≥ 2 unformed stools/day), fever ($>38^{\circ}\text{C}$), and vomiting and self-reported nausea, abdominal cramping, headache, myalgia, and fatigue.

^c ND, not detected.

^d Corresponds to the 1 infected volunteer at 21 days of stored water.

^e NA, not applicable.

from presumably noninfectious virions with damaged capsids) (35). RNase-treated NV in groundwater was detected by RT-qPCR for 1,266 days. Compared to day 0, there was no significant \log_{10} reduction of NV in RNase-treated water after 427 days, but there was a significant $1.10\text{-}\log_{10}$ reduction of NV in RNase-treated water by day 1266 of storage (Fig. 1).

Purified NV RNA can remain detectable in groundwater, tap water, and reagent-grade water for at least 14 days. Purified NV RNA (extracted from NV virions) was detected by RT-qPCR in groundwater, tap water, and reagent-grade water for 14 days after seeding (last sampling date) (Fig. 2). Compared to day 0, there were significant \log_{10} reductions of purified NV RNA after storage in groundwater for 7 days ($0.08\text{-}\log_{10}$ reduction) and 14 days ($0.09\text{-}\log_{10}$ reduction). Similarly, there were significant \log_{10} reductions of purified NV RNA after storage in tap water for 7 days ($0.13\text{-}\log_{10}$ reduction) and 14 days ($0.25\text{-}\log_{10}$ reduction). Compared to day 0, there were no

significant \log_{10} reductions of purified NV RNA after 14 days of storage in reagent-grade water.

DISCUSSION

To our knowledge, this is the first study to examine the long-term infectivity of HuNoV in water. Our results indicate that NV remains infectious in groundwater for at least 61 days at room temperature in the dark. This result is supported by previous infectivity studies of other enteric viruses in water that have demonstrated the long-term infectivity of poliovirus 3 (at least 140 days), coxsackievirus B1 (70 days), adenovirus 2 (at least 364 days) (8), rhesus rotavirus (at least 210 days), and astrovirus Yuc8 (at least 120 days) (10). Additionally, previous reports have documented waterborne HuNoV outbreaks linked with detection of HuNoV in groundwater (4, 14). Our results are limited by the relatively high dose of NV used to

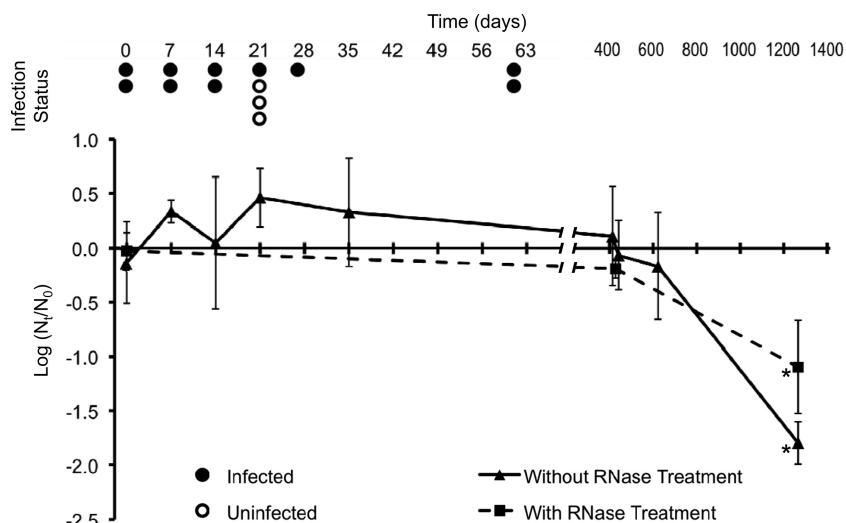


FIG. 1. Infectivity and persistence of NV in groundwater. Thirteen genetically susceptible volunteers ingested safety-tested groundwater that was seeded with 8FIIb NV inoculum and stored for the indicated time points. NV-infected (closed circles) and uninfected (open circles) subjects at each time point are indicated. Aliquots were analyzed by RT-qPCR, and NV \log_{10} reductions were calculated (number of genomic equivalent copies/ μl at each time point [N_t] divided by the average at day 0 [N_0]). The triangles (samples without RNase treatment) and squares (samples with RNase treatment) represent the means of 2 to 5 replicate samples analyzed in duplicate (i.e., 4 to 10 tubes per sample and time point). The break in the horizontal axis is indicated. Error bars indicate standard deviations. Asterisks correspond to a significant ($P < 0.05$) NV \log_{10} reduction compared to day 0.

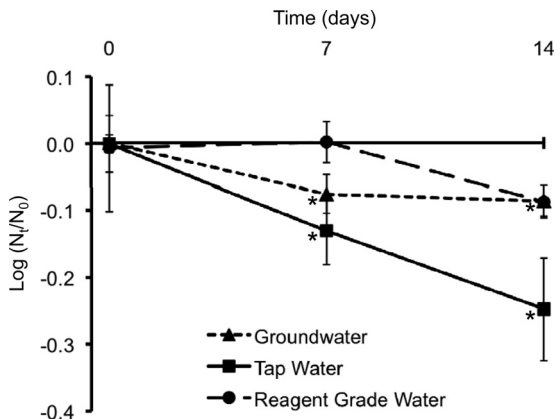


FIG. 2. Persistence of purified NV RNA in groundwater, tap water, and reagent-grade water. Extracted NV RNA was stored in groundwater, tap water, and reagent-grade water for 14 days. Aliquots were analyzed by RT-qPCR, and NV log₁₀ reductions were calculated (number of genomic equivalent copies/μl at each time point [N_t] divided by the average at day 0 [N₀]). The triangles (groundwater), squares (tap water), and circles (reagent-grade water) represent the means of 2 replicate samples analyzed in duplicate (i.e., 4 tubes per sample and time point). These results are representative of 5 separate experiments. Error bars indicate standard deviations. Asterisks correspond to a significant (P < 0.05) NV log₁₀ reduction compared to day 0 and are associated with groundwater and tap water at days 7 and 14.

challenge the subjects (~6.5 × 10⁸ NV GEC) since the inocula may have remained infectious despite significant NV reduction throughout storage. To measure the reduction of potentially infectious NV virions in groundwater over time, we treated NV-seeded groundwater samples with RNase before RT-qPCR analysis. This technique detects only NV RNA that is contained within intact capsids. We observed no significant NV reduction in RNase-treated samples (containing presumably infectious NV virions with intact capsids) after 427 days and only a significant 1.10-log₁₀ reduction after 1,266 days post-seeding. Because the original dose of NV-seeded groundwater (~6.5 × 10⁸ NV GEC) caused infection in the study subjects, the detection of NV in RNase-treated samples at the same concentration throughout 427 days and a slightly reduced concentration by day 1266 suggest that NV may remain infectious in water for years.

This study evaluated the persistence of HuNoV over the longest reported storage time to date. NV was detected by RT-qPCR after storage for 1,266 days in groundwater, with no significant log₁₀ reduction after 622 days and only a significant 1.79-log₁₀ reduction by day 1266 postseeding. This relative reduction is significantly larger than the relative reduction observed with RNase-treated samples. This result is expected since RNA released from damaged viral capsids (detected by RT-PCR without RNase treatment) is likely more susceptible to destruction than RNA contained within viral capsids (detected by RT-PCR with RNase treatment). The long-term NV reduction observed in this study is comparable to our previously calculated NV nucleic acid reduction rate in groundwater at 25°C (0.01 ± 0.05 log₁₀/day) (3). In a similar study that used different HuNoV strains and groundwater samples, Charles et al. (8) detected HuNoV genogroup I (one of the genetic HuNoV clusters that includes NV) after 588 days of storage in

groundwater and HuNoV genogroup II after 728 days of storage (their entire study period) with conventional RT-PCR. Those authors suggest that the long-term HuNoV persistence in water could result from a HuNoV capsid maturation process that forms a stable protein coat (demonstrated in murine norovirus but unknown in HuNoV) (8). HuNoV persistence could also be facilitated by the aggregation of viral particles, a phenomenon that is observed in natural environments (10). Furthermore, we hypothesize that the long-term detection of HuNoV in groundwater by RT-qPCR could partially result from the long-term persistence of HuNoV RNA. We found that there was no significant log₁₀ reduction of purified NV RNA (extracted from NV virions) over 14 days in reagent-grade water. After 14 days of storage in groundwater and tap water, there were significant yet relatively small log₁₀ reductions of purified NV RNA (0.09-log₁₀ reduction in groundwater and 0.25-log₁₀ reduction in tap water). Although several studies have examined the stability of HuNoV protein capsids in various environments (29), this is the first study to examine the persistence of purified HuNoV RNA in water.

Our specific results on the persistence and infectivity of HuNoV GI.1 in groundwater should not be generalized to all types of groundwater or strains and genogroups of HuNoV. As noted in Materials and Methods, our groundwater samples were passed through an activated carbon and ion-exchange resin cartridge to reduce pesticides, meet USEPA drinking water standards, and safely administer to human subjects. These water samples were also frozen in aliquots for dose-to-dose consistency. While it is likely that both treatments changed the quality of the water compared to an untreated groundwater sample, our results of persistence of HuNoV in our groundwater samples were consistent with those of other published reports (3, 8). As a separate issue, it is likely that different HuNoV genogroups will exhibit differing levels of environmental persistence (e.g., water [8] and surfaces [21]), infectivity (18–20), and resistance to inactivation (7, 13, 15, 28). Several groups have proposed that differences in environmental persistence, infectivity, and resistance to inactivation between the genogroups and strains may be due to differences in the stability of the HuNoV capsid coat (8), which influence the viral receptor binding and entry mechanisms of HuNoV (24, 33) (including the contribution of the histoblood group antigens (reviewed in references 31 and 32)). These hypotheses may also explain our unpublished analyses of a systematic review of 900 HuNoV outbreaks (between 1983 and 2010) in which we found that genogroup I strains, rather than genogroup II strains, were significantly more likely to be associated with waterborne outbreaks (data not shown) and that GII-associated outbreaks had a significantly lower prevalence of foodborne outbreaks (data not shown) (J. Matthews, B. Dickey, R. Miller, J. Felzer, B. Dawson, A. Lee, J. Rocks, J. Kiel, J. Sobolik, J. Eisenberg, C. Moe, and J. Leon, submitted for publication).

Our finding of the prolonged persistence of infectious HuNoV in groundwater and the previously reported low infectious dose (50% infectious dose [ID₅₀]) of HuNoV (34) make HuNoV an important concern for groundwater. Our study suggests that groundwater supplies contaminated with HuNoV need to be disinfected or abandoned until future testing indicates sufficient reduction of HuNoV. Due to the long-

term persistence of infectious HuNoV in groundwater, short-term quarantines of HuNoV-contaminated water supplies are likely to be insufficient for preventing waterborne HuNoV outbreaks.

ACKNOWLEDGMENTS

We acknowledge Stu Hooper for groundwater collection, Hui-Mien Hsiao and Pengbo Liu for technical assistance, and Peter Teunis for statistical advice. We thank the volunteers and the staff of Emory University Hospital's General Clinical Research Center.

This work was supported by grant 82911601-1 from the U.S. Environmental Protection Agency (subcontract 5-20900 to Emory University), grant PHS M01 RR0039 from the General Clinical Research Center program at the National Institutes of Health (NIH), grant PHS UL1 RR025008 from the Clinical and Translational Science Award program at the NIH, and grant AI056351 from the National Institute of Allergy and Infectious Diseases (NIAID) at the NIH. This work was partially supported by grant 1K01AI087724-01 from the NIAID at the NIH, grant 2010-85212-20608 from the National Institute of Food and Agriculture at the U.S. Department of Agriculture, and a grant from the Emory University Global Health Institute (to J.S.L.). We also acknowledge financial support from the Emory University Scholarly Inquiry and Research at Emory program (to S.R.S.).

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