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Evaluation of Murine Norovirus, Feline Calicivirus, Poliovirus, and MS2 as Surrogates for Human Norovirus in a Model of Viral Persistence in Surface Water and Groundwater[▽]

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Human noroviruses (NoVs) are a significant cause of nonbacterial gastroenteritis worldwide, with contaminated drinking water a potential transmission route. The absence of a cell culture infectivity model for NoV necessitates the use of molecular methods and/or viral surrogate models amenable to cell culture to predict NoV inactivation. The NoV surrogates murine NoV (MNV), feline calicivirus (FCV), poliovirus (PV), and male-specific coliphage MS2, in conjunction with Norwalk virus (NV), were spiked into surface water samples ($n = 9$) and groundwater samples ($n = 6$). Viral persistence was monitored at 25°C and 4°C by periodically analyzing virus infectivity (for all surrogate viruses) and nucleic acid (NA) for all tested viruses. FCV infectivity reduction rates were significantly higher than those of the other surrogate viruses. Infectivity reduction rates were significantly higher than NA reduction rates at 25°C (0.18 and 0.09 $\log_{10}$/day for FCV, 0.13 and 0.10 $\log_{10}$/day for PV, 0.12 and 0.06 $\log_{10}$/day for MS2, and 0.09 and 0.05 $\log_{10}$/day for MNV) but not significant at 4°C. According to a multiple linear regression model, the NV NA reduction rates (0.04 ± 0.01 $\log_{10}$/day) were not significantly different from the NA reduction rates of MS2 (0.05 ± 0.03 $\log_{10}$/day) and MNV (0.04 ± 0.03 $\log_{10}$/day) and were significantly different from those of FCV (0.08 ± 0.03 $\log_{10}$/day) and PV (0.09 ± 0.03 $\log_{10}$/day) at 25°C. In conclusion, MNV shows great promise as a human NoV surrogate due to its genetic similarity and environmental stability. FCV was much less stable and thus questionable as an adequate surrogate for human NoVs in surface water and groundwater.

Increasing human population and urbanization have placed burdens on source water used to provide potable water to most metropolitan areas (7). Human excreta present in these source waters have the potential to harbor hundreds of pathogenic microorganisms of public health concern (29). Of particular interest are human noroviruses (NoVs), which are one of the most frequent causes of nonbacterial gastroenteritis worldwide (5, 30). As members of the *Caliciviridae* family, NoVs (previously known as Norwalk-like viruses) are small (27 nm), icosahedral, nonenveloped human enteric viruses that cause acute gastroenteritis (20). Due to their nonenveloped structure, which is similar to those of other human enteric viruses, such as poliovirus (PV), coxsackievirus, and echovirus, NoVs are presumed to be as resistant to environmental degradation and chemical inactivation as the aforementioned viruses. Environmental degradation of viruses can result from extremes in pH, thermal inactivation, and sunlight (23, 56) and predation or release of virucidal agents from endogenous microorganisms in environmental water (14, 52). Chlorine, the most commonly used drinking water disinfectant, can also inactivate enteric viruses if sufficient doses and contact times are provided (13). However, due to the absence of an in vitro cell culture system or small animal model, detection of infectious NoV isolated from environmental waters has not been possible (12). The lack of a NoV infectivity assay has necessitated the use of viral surrogates to model the infectious nature of NoV in environmental samples. The selection of an appropriate surrogate is critical for assessing accurate NoV human health risks.

Historically, total coliform, fecal coliform, enterococcus, and *Escherichia coli* bacterial indicators have been the predominant microorganisms used to determine the microbiological quality of raw and finished drinking water in the United States (17). Numerous reports in the literature have documented the ineffectiveness of bacterial indicators in determining the health risks of human enteric viruses (6, 51). Currently, feline calicivirus (FCV), which is amenable to cell culture, has been considered one of the most appropriate surrogates for NoV, as this virus is located in the *Vesivirus* genus of the *Caliciviridae* family and thus is genetically similar to NoV (19). FCV has been widely utilized as a surrogate for NoV in a model of viral persistence during evaluation of water treatment efficiency (8, 18, 33, 44–47) and natural virus reduction in water (1, 26). However, FCV is a respiratory virus of felids (20), and unlike enteric viruses, it is susceptible to low pH and elevated temperature (10, 40). Attenuated vaccine strains of PV and the male-specific bacteriophage MS2 have also frequently been used as surrogates for human enteric viruses, and there is a large body of literature describing the survival of these viruses in water and during drinking water treatment processes (1, 2, 21, 34, 53).

Of particular interest is the recent reporting of a novel genogroup V murine NoV (MNV) that has been successfully propagated in cell culture (54). MNV is morphologically and genetically similar to human NoVs, and to date, this is the only

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NoV amenable to routine growth in cell culture and thus shows considerable promise as a human NoV surrogate (55).

The evaluation of appropriate viral surrogates for human enteric viruses of public health risk in source water used to produce potable water necessitates that the selected surrogates be evaluated by both viral infectivity and viral nucleic acid assays. Infectivity will facilitate determination of the health risk caused by infectious virions (15, 41), while nucleic acid detection will correlate with those viruses recalcitrant to replication in cell culture (36). The development of quantitative reverse transcription-PCR (qRT-PCR) has facilitated the enumeration of viral nucleic acid and thus substantially improved analysis, from a simple determination of presence/absence to a determination of viral nucleic acid concentration in a water sample (27, 48).

The goal of this study was to evaluate the applicability of selected enteric viral surrogates in predicting the persistence of human NoV seeded into surface water and groundwater used as source water for producing potable water. qRT-PCR assays were developed for each virus, and the levels of infectious virus and viral nucleic acid were monitored in spiked drinking source water over time.

MATERIALS AND METHODS

Sample sites. Two-liter grab water samples were collected approximately every 4 months for 1 year from five source waters used as raw water supplies at drinking water treatment plants. Three sites were surface waters supplying Baltimore, MD, Cincinnati, OH, or Atlanta, GA, and two sites were groundwater sources supplying Harford County, MD, and a community near Atlanta, GA. A total of 15 water samples were used to evaluate spiked virus infectious particles and nucleic acid persistence. Samples were placed on ice and shipped overnight to Johns Hopkins University for testing. Upon arrival at the laboratory and prior to initiation of virus seeding experiments, samples were analyzed for heterotrophic plate count (HPC) bacteria by using spread plate method 9215C (3), for fecal coliforms by using membrane filtration method 9222D (3), for *E. coli* by using USEPA filtration method 1603 (50), for enterococci by using USEPA filtration method 1600 (49), for pH by using a standard pH meter (Fisher Scientific, Auburn, AL), for conductivity by using portable conductivity meter (HACH, Loveland, CO), for alkalinity by using a digital titrator (HACH, Loveland, CO) following method 8023 in the manufacturer's manual, and for turbidity by using a calibrated turbidimeter (HACH, Loveland, CO).

Viral stock preparation. Mammalian viral stocks including MNV (kindly provided by Herbert Skip Virgin, Washington University, St. Louis, MO), PV, and FCV were generated by inoculation onto confluent monolayers of appropriate cell lines (RAW 267.4; buffalo green monkey kidney [BGMK] and feline renal [CrFK] cell lines, respectively) as previously described (4, 37, 54). Briefly, monolayers of cells were prepared in 150-cm^2 tissue culture flasks and were inoculated with virus stock by using a multiplicity of infection of 0.01. Following 1 hour of adsorption with periodic mixing, cell maintenance media were added and the flasks incubated at 37°C in 5% CO_2 until >90% of the cells were lysed and floating (3 to 5 days). Viral cell cultures were subsequently subjected to three rounds of freeze thawing to facilitate liberation of progeny virions from infected cells. Equal volumes of Vertrel XF (DuPont, Wilmington, DE) and virus-containing media from the flasks were subsequently homogenized (OMNI international, Inc., Marietta, GA) at 20,000 rpm for 3 min on ice. The emulsified mixture was then centrifuged for 15 min at 5,000 × *g* and 4°C, and recovered supernatant was filtered through 0.1-μm-pore-size low-protein-binding membrane filters (Millex PVDF, Millipore, Billerica, MA).

A diarrheal stool sample containing Norwalk virus (NV) GI-1 (Norwalk/1968/US), commonly denoted substrain 8fIIb (kindly provided by Christine Moe, Emory University, Atlanta, GA) was diluted 10-fold in Dulbecco's phosphate-buffered saline (D-PBS; pH 7.4, without calcium chloride or magnesium chloride; Invitrogen, Inc.) and subsequently emulsified with an equal volume of Vertrel XF by homogenization. Virus-containing supernatant was recovered by centrifugation at 5000 × *g* at 4°C for 15 min. Recovered supernatant was successively filtered thorough 0.45-μm- and 0.1-μm-pore-size low-protein-binding membrane filters.

Two hundred microliters of MS2 coliphage (ATCC 16696-B1) was inoculated into a 100-ml flask containing 10 ml of *E. coli* C3000 host cells at a ratio of approximately 5 × 10^7 PFU of MS2 coliphage per 10^{10} CFU of *E. coli* cells. The mixture was incubated for 20 min at 37°C, followed by the addition of 100 ml of sterile 3% tryptic soy broth and incubation at 37°C with vigorous shaking for 8 to 12 h until bacterial lysis occurred. Ten milliliters of chloroform was then added and incubated for a further 10 min. The culture was then centrifuged at 5,000 × *g* for 10 min to pellet the *E. coli* cells and cell debris, and the virus-containing supernatant was recovered. Recovered supernatant was filtered through 0.1-μm-pore-size low-protein-binding membrane filters.

All viral stocks were further concentrated and washed using a 100,000-Da ultra-membrane filter (Amicon Ultra; Millipore Corp., Bedford, MD) to increase the virus titers and remove soluble/low-molecular-weight components from the supernatant. Following initial concentration in the membrane (viruses are retained, and low-molecular-weight components, i.e., nutrients, salts, etc., are passed through the membrane), viral stocks were purified by repeatedly adding 14 ml of D-PBS into the 1 ml of virus-containing retentate and centrifuging the membrane (4,000 × *g*, 10 to 12 min) each time. Approximately 1 ml of virus-containing retentate remained on top of the membrane after each centrifugation. By repeating these steps three or four times, purified, dispersed virus particles were obtained.

Infectious virus plaque assays. Infective viral particles for viral stocks and subsequent experimental samples were assayed by standard 10-fold dilution plaque assays in duplicate (37). For MNV-1, the plaque assay descried by Wobus et al. (54) was followed with minor modifications. Briefly, RAW 264.7 cells were seeded into six-well plates (3.5-cm diameter) at a density of 2 × 10^6 viable cells per plate in complete Dulbecco's modified Eagle's medium. Plates were briefly rocked to evenly distribute cells and incubated 24 h until confluent. Tenfold dilutions of MNV-1 samples in complete Dulbecco's modified eagle's medium were prepared, and 0.5 ml was inoculated into each well following aspiration of media. Plates were incubated for an hour at room temperature, with rocking every 15 min, and subsequently overlaid with 2 ml of a 37°C 1:1 mixture of 1.5% SeaPlaque agarose and 2× minimum essential medium supplemented with 10% low-endotoxin fetal bovine serum, 2% L-glutamine, 2% penicillin-streptomycin, 1% HEPES. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. To visualize the plaques, cells were overlaid with an additional 2 ml of a 1:1 mixture of 1.5% SeaKem agarose and complete 2× minimum essential medium (supplemented with 2% of a 3.3-g/liter stock solution of neutral red) per plate. Plates were incubated for another 24 h, and plaques were counted and virus titers recorded as numbers of PFU/ml.

PV and FCV plaque assays were conducted using confluent BGMK cells and CrFK cells, respectively, in 60-mm dishes. Duplicate dishes were inoculated with 100 μl of a sample or a D-PBS-diluted sample after rinsing aspirated plates with D-PBS. Plates were incubated at room temperature (BGMK) or 37°C (CrFK) for 1 h, with gentle rocking every 15 min. Five milliliters of overlay agar containing medium and 2% of a 3.3-g/liter stock solution of neutral red was added to each dish after incubation. The plates were inverted, placed in a 37°C and 5% CO_2 atmosphere, and monitored for plaque formation for 2 to 4 days.

The double agar layer method was used for detection of MS2 coliphage (42). Briefly, log-phase *E. coli* C3000 host bacteria were prepared on the date of experiment from overnight cultures and kept on ice until use. Bottom agar (3% tryptic soy broth, 0.5% NaCl, 1.2% agar) was prepared and autoclaved at 121°C for 15 min and poured in 15- by 100-mm plates. Top agar (3% tryptic soy broth, 0.5% NaCl, 0.6% agar) was prepared and autoclaved at 121°C for 15 min, distributed at 5 ml into each tube, and kept at 48°C water bath until the samples were ready. One hundred microliters of 10-fold-D-PBS-diluted sample and 75 μl of log-phase host were added in each tube, mixed, and poured onto the bottom agar. The plates were allowed to solidify, inverted, and incubated overnight at 37°C and the resulting plaques enumerated.

Relative quantification of viral nucleic acids: qRT-PCR. qRT-PCR was performed using the SmartCycler system (Cepheid, Sunnyvale, CA) for detection of nucleic acids of all viruses seeded in environmental waters. For analysis of seeded virus in water, a heat release technique was used to liberate the viral RNA from capsids prior to qRT-PCR amplification (38). Virus-containing aqueous samples were incubated at 95°C for 5 min to denature viral capsids and release the viral nucleic acid, with subsequent chilling on ice for 2 min. Primers and fluorescent-dye-conjugated, viral-gene-specific probes were designed for each virus (Table 1). For each experiment, a tube containing diethyl pyrocarbonate-treated water (used for sample dilution) was used as a negative reagent control. A OneStep RT-PCR kit (Qiagen, Valencia, CA) was used for viral RNA amplification. The RT-PCR mixture contained final concentrations of 2.5 mM Mg^{2+}, 0.2 μM primers (Invitrogen, Carlsbad, CA), and a gene-specific probe (Biosearch Technologies, Inc., Novato, CA), 0.4 mM of deoxynucleoside triphosphates, 1 μl of

TABLE 1. qRT-PCR primer and gene-specific fluorescent-probe selection

Virus	GenBank accession no. (GenInfo identifier no.)	Primer or probe name	Probe label	Sequence	Product size (bp)	Product region	Source or reference
NV	M87661 (gi1061311)	NVKS1		5′ACAGCATGGGACTCAACACA3′	190	ORF1, nonstructural polyprotein region	This work
		NVKS2		5′GGGAAGTACATGGGAATCCA3′			
		NVKS3	FAM[a]	5′TCACCAGAATTGGCCGAGGTTGT3′			
MNV	AY228235 (gi29150715)	MNVKS1		5′AGGTCATGCGAGATCAGCTT3′	159	ORF1, protease, polymerase region	This work
		MNVKS2		5′CCAAGCTCTCACAAGCCTTC3′			
		MNVKS3	FAM	5′CAGTCTGCGACGCCATTGAGAA3′			
FCV	Z11536 (gi59260)	FCVKS1		5′CCAACATGGCTTGGAGTTTT3′	164	Nonstructural polyprotein	This work
		FCVKS2		5′CACTCGAGTCGATCTGGTCA3′			
		FCVKS3	FAM	5′CAACAGCCAGTTCAATGGCGTG3′			
PV	AJ293918 (gi9998764)	PanEn1_us		5′CCTCCGGCCCCTGAATG3′	197	5′ untranslated region	37
		PanEn2_ds		5′ACCGGATGGCCAATCCAA3′			
		PanEn3	FAM	5′TACTTTGGGTGTCCGTGTTTC3′			
Male-specific coliphage from ATCC 15597B1 (MS2)	V00642 (gi15081)	MS2KS1		5′CTCTCTGGCTACCGATCGTC3′	235	Replicase gene	This work
		MS2KS2		5′ACACTCCGTTCCCTCAACG3′			
		MS2KS3	CAL Red[b]	5′ACACGCGGTCCGCTATAACGAGT3′			

[a] The FAM (6-carboxyfluorescein) quencher is BHQ I.
[b] The CAL Red quencher is BHQ II.

enzyme mixture (Omniscript and Sensiscript reverse transcriptases and HotStarTaq DNA polymerase mixture), 5 U of GeneAmp RNase inhibitor (Applied Biosystems, Inc., Foster City, CA), and 10 μl of 10-fold-diluted virus-seeded sample in a total reaction volume of 25 μl. The RT-PCR thermocycling conditions for all virus tested were as follows: 50°C for 30 min, 95°C for 15 min (to denature RT enzymes and activate HotStarTaq DNA polymerase), and 60 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s. Cycle threshold (Ct) data corresponding to the viral RNA concentrations were obtained. To evaluate Ct variation among experiments, 10-fold dilutions of known virus stock concentration were include as positive controls in each experiment.

Experimental procedure. Twenty milliliters of each water sample was portioned into 50-ml polypropylene tubes, and test viruses were seeded to the final concentrations as follows: NV, ~10^5 RT-PCR units/ml; MNV, 10^6 to 10^7 PFU/ml; FCV, ~10^3 to 10^5 PFU/ml; PV, 10^6 to 10^7 PFU/ml; and MS2, 10^6 to 10^8 PFU/ml. MNV was seeded in only one of three samples from each site and seeded in a separate tube containing 10 ml environmental water due to potential cross-reactivity between NV and MNV in qRT-PCR. Sterile laboratory quality water seeded with the same concentration of viruses as the environmental water sample was used as a positive laboratory control for each environmental test water sample. This control was an important component of each environmental analysis because it facilitated differentiation between virus reduction by endogenous environmental water components and other extrinsic experimental conditions, such as incubation temperature and any potential variability in virus stocks. Prior to seeding into water samples, all virus stocks were evaluated by electron microscopy to confirm monodispersion (data not shown). Laboratory water positive controls and environmental test waters were incubated at 25°C or 4°C in the dark and continuously mixed by mounting on an ~40-cm-diameter rotating drum (~20 rpm).

Water samples were incubated for 3 to 5 weeks, depending on virus reduction rates. Approximately six to eight subsamples (1.5 ml each) were periodically removed during incubation and analyzed for virus infectivity (for surrogate viruses) and nucleic acid for all seeded viruses.

Data analysis. Surrogate virus infectivity levels were expressed as $\log_{10}$ numbers of PFU/ml. The decrease of infectious viral particles in each water sample was assumed to follow first-order kinetics, and reduction rates ($\log_{10}$ PFU/day) were calculated and R^2 values and P values obtained.

The reductions of NV and surrogate virus nucleic acid were determined by qRT-PCR. Log reduction of nucleic acids was determined based on Ct value changes over time and the slope values of 10-fold dilutions of standard virus stock, providing Ct differences per $\log_{10}$ (10-fold dilution). Viral nucleic acid reduction was also assumed to follow first-order kinetics, and viral nucleic acid reduction rates (nucleic acid reduction rates in $\log_{10}$/day) were calculated and the R^2 values and P values obtained. Statistical analysis was performed based on $\log_{10}$-transformed relative virus levels over incubation time. The Intercooled STATA 8.1 software package (Stata Corporation, College Station, TX) was used for the analysis. The significance of differences in reduction rates between methods, among tested viruses, and between types of water was tested using multiple linear regression models (Table 2).

RESULTS

Sample water characteristics. Nine surface water and six groundwater samples were collected and analyzed by biological (HPC, fecal coliform, *E. coli*, and enterococcus), chemical (pH, conductivity, and alkalinity), and physical (turbidity) methods. Table 3 provides the results of analysis of 15 water samples that are source waters for drinking water treatment facilities or community well water. The parameters for tested water from each region were relatively stable. Surface water pH values were slightly higher (pH >7) than those of groundwater (pH <7). Surface water had higher turbidity than groundwater, and bacterial indicators were detected only in surface water. All water samples contained HPC bacteria, ranging from 14 to 1.95×10^4 CFU/ml.

qRT-PCR sensitivity, cross-reactivity, and plaque assay cross-infections. Prior to the initiation of the seeding experiments, each environmental water sample was evaluated for potential qRT-PCR inhibition that could affect interpretation of nucleic acid results. Ten microliters of a 10-fold dilution of environmental water was not found to be inhibitory when directly compared to laboratory viral stock dilutions (data not shown). qRT-PCR amplification was at least as sensitive as the corresponding plaque assay in most cases. It was observed that qRT-PCR for PV, FCV, and MNV was more sensitive than the plaque assay, i.e., if 1 PFU was present in the test volume (10 μl) of qRT-PCR, a positive Ct value (Ct, 36.34 to 38.86) was obtained. For MS2, however, the average Ct value was higher than the other viruses (Ct, 50.96) when 1 PFU was in the test volume (10 μl), and only seven out of eight samples were positive. As the quantitative capability of qRT-PCR decreases when Ct values are >45, results with Ct values of >45 were excluded from reduction rate calculations.

TABLE 2. Multiple linear regression models for the comparison of test conditions[a]

Model	Multiple linear regression model	Remark
1	$V = \beta_0 + \beta_1 \cdot \text{day} + \beta_2 \cdot \text{method} + \beta_3 \cdot \text{method} \cdot \text{day} + \epsilon$, if vt = (−1, 0, 1, 2) $V = \beta_0 + \beta_1 \cdot \text{day} + \epsilon$, if vt = −2	*P* value of β_3 presented in Table 4
2	$V = \beta_0 + \beta_1 \cdot \text{day} + (\beta_2 \cdot \text{vt}_3 + \cdots + \beta_4 \cdot \text{vt}_5) + (\beta_5 \cdot \text{vt}_3 \cdot \text{day} + \cdots + \beta_7 \cdot \text{vt}_5 \cdot \text{day}) + (\beta_8 \text{Sp}_1 + \cdots + \beta_{21} \cdot \text{Sp}_{14}) + \epsilon$, if method = 0	
3	$V = \beta_0 + \beta_1 \cdot \text{day} + \beta_2 \cdot \text{method} + \beta_3 \cdot \text{method} \cdot \text{day} + (\beta_4 \text{Sp}_1 + \cdots + \beta_{17} \cdot \text{Sp}_{14}) + \epsilon$, if vt = (−1, 0, 1, 2) $V = \beta_0 + \beta_1 \cdot \text{day} + (\beta_4 \text{Sp}_1 + \cdots + \beta_{17} \cdot \text{Sp}_{14}) + \epsilon$, if vt = −2	*P* value of β_3 presented in Tables 5 and 6
4	$V = \beta_0 + \beta_1 \cdot \text{day} + (\beta_2 \cdot \text{vt}_2 + \cdots + \beta_5 \cdot \text{vt}_5) + (\beta_6 \cdot \text{vt}_2 \cdot \text{day} + \cdots + \beta_9 \cdot \text{vt}_5 \cdot \text{day}) + (\beta_{10} \text{Sp}_1 + \cdots + \beta_{23} \cdot \text{Sp}_{14}) + \epsilon$, if method = 1	
5	$V = \beta_0 + \beta_1 \cdot \text{day} + \beta_2 \cdot \text{wt} + \beta_3 \cdot \text{wt} \cdot \text{day} + \epsilon$, if method = 0 and vt = (−1, 0, 1, 2)	*P* value of β_3 presented in Tables 7 and 9
6	$V = \beta_0 + \beta_1 \cdot \text{day} + \beta_2 \cdot \text{wt} + \beta_3 \cdot \text{wt} \cdot \text{day} + \epsilon$, if method = 1 and vt = (−2, −1, 0, 1, 2)	*P* value of β_3 presented in Tables 8 and 10

[a] *V*, relative virus level in $\log_{10}$ numbers of PFU or nucleic acid; day, incubation time in days; Sp_1 to Sp_{14}, dummy variables (sample numbers designated from 1 to 14); ϵ, random error; method, test method (0, infectivity test; 1, nucleic acid detection by qRT-PCR); vt, virus type (−2, NV; −1, FCV; 0, MS2; 1, MNV; 2, PV); vt_1 to vt_5, dummy variables for virus type; wt, water type (0, surface water; 1, groundwater).

Primers and probes were carefully selected to detect only the nucleic acids of the specific viruses being analyzed, and none of the primer/probe combinations cross-reacted, with the exception of the NV primers cross-reacting with MNV nucleic acid, resulting in nonspecific PCR products, as determined by gel electrophoresis. However, the fluorescent-dye-conjugated probes for NV and MNV did not cross-react with the nonspecific products (data not shown).

There was no cross-infectivity in plaque assays among PV, FCV, and MS2 (data not shown). MNV was included in the later part of the virus seeding study, following a report in the literature (54). MNV did not grow in BGMK or CrFK cells, and PV and FCV did not form plaques on RAW 264.7 macrophage cells (data not shown).

Virus reductions in laboratory quality control waters incubated at 25°C. Based on our experimental analysis and previous reports in the literature (15, 24, 25, 56), first-order kinetics of viral infectivity reduction was assumed. Plaque assay-based infectivity reduction rates ($\log_{10}$ PFU/day) were obtained for all virus-seeded samples. PV, MS2, and MNV were similarly stable (reduction rate ± 95% confidence intervals, 0.02 ± 0.002 to 0.01 $\log_{10}$ PFU/day), and absolute values of variation were small when virus was seeded into reagent grade laboratory water (Table 4). FCV was less stable than the other viruses (0.08 ± 0.02 $\log_{10}$ PFU/day) (Table 4).

Nucleic acid reductions ($\log_{10}$/day) based on qRT-PCR Ct values were determined. From the multiple standard curves that were generated, a median slope value for each test virus was calculated: −3.51 Ct/$\log_{10}$ for NV (n = 103), −4.30 Ct/$\log_{10}$ for FCV (n = 77), −3.89 Ct/$\log_{10}$ for PV (n = 82), −4.41 Ct/$\log_{10}$ for MS2 (n = 74), and −4.23 Ct/$\log_{10}$ for MNV (n = 26). The changes of Ct over incubation time in days divided by the slope values calculated above provided the levels of nucleic acid reduction. Similar to infectivity reduction, nucleic acid reduction followed first-order kinetics.

For the laboratory positive control waters, minimal (no sig-

TABLE 3. Surface water and groundwater characteristics

Source	State	Date of sampling	pH	Turbidity (nephelometric turbidity units)	Conductivity (μS/cm)	Alkalinity (mg/liter as $CaCO_3$)	Fecal coliform count (no. of CFU/100 ml)	*E. coli* count (no. of CFU/100 ml)	Enterococcus count (no. of CFU/100 ml)	HPC (10^2 CFU/ml)
Groundwater	MD	8/17/2004	6.07	0.42	94.7	3.2	0	0	0	2.88
	MD	1/11/2005	5.74	0.60	98.6	3.9	0	0	0	0.14
	MD	4/26/2005	5.80	0.45	93.4	2.4	0	0	0	0.48
	GA	9/14/2004	6.52	0.72	82.3	33	0	0	0	4.4
	GA	12/1/2004	6.15	1.07	71.3	21.6	0	0	0	2.51
	GA	4/5/2005	6.26	1.21	68	15.6	0	0	0	150
Surface water	MD	9/28/2004	7.12	2.48	157	39.5	11.5	14	21	20.5
	MD	2/1/2005	7.57	3.62	160.8	34.4	2.5	4.5	9.5	0.37
	MD	5/17/2005	7.26	1.69	169.3	20.1	0	0	3	0.75
	GA	11/9/2004	6.86	4.53	36.8	10.2	1.2	3	0	1.66
	GA	3/16/2005	7.12	6.97	34.4	7.8	1.5	16	1	10.1
	GA	6/28/2005	6.97	2.94	33.9	11.2	9.5	8.5	22	27.7
	OH	10/19/2004	7.65	13.7	179.5	41.1	8,750	7,700	9,250	195
	OH	2/22/2005	7.62	50.9	238	42.2	235	200	105	66.5
	OH	6/14/2005	7.73	22.2	292	84.7	3,400	2,900	3,950	16.9

TABLE 4. Viral reduction rates in laboratory quality waters at 25°C

Virus	Mean rate ± 95% CI (log_{10}/day)[a] for reduction of:		*P* for comparison between rates
	Infectivity	Nucleic acid	
MS2	0.02 ± 0.01	0.00 ± 0.01	<0.001
MNV	0.02 ± 0.01	0.00 ± 0.02	<0.001
FCV	0.08 ± 0.02	0.03 ± 0.04	<0.001
PV	0.02 ± 0.002	0.01 ± 0.01	<0.001
NV	ND	0.00 ± 0.004	

[a] Estimated mean reduction rates and 95% confidence intervals as determined by multiple linear regression models. ND, not done.

TABLE 5. Virus reduction rates in environmental waters at 25°C

Virus	Mean rate ± 95% CI (log_{10}/day)[a] for reduction of:		*P* for comparison between rates
	Infectivity	Nucleic acid	
MS2	0.12 ± 0.02	0.06 ± 0.04	<0.001
MNV	0.09 ± 0.03	0.05 ± 0.08	0.074
FCV	0.18 ± 0.05	0.09 ± 0.10	0.010
PV	0.13 ± 0.02	0.10 ± 0.04	0.003
NV	ND	0.03 ± 0.01	

[a] Estimated mean reduction rates and 95% confidence intervals as determined by multiple linear regression models. ND, not done.

nificant) viral RNA losses (0.00 to 0.03 log_{10}/day) were observed over the sampling time for all viruses tested. Infectivity was significantly reduced over time (reduction rates were significantly different from 0) for all tested viruses. In the laboratory quality water incubated at 25°C, for all surrogate viruses tested, infectivity reduction rates were significantly higher than nucleic acid reduction rates ($P < 0.001$) (Table 4 and model 1 in Table 2). The 95% confidence intervals of the FCV reduction rates (both infectivity reduction and nucleic acid reduction) were larger than those of other viruses (Table 4).

Infectivity reduction of viral surrogates seeded into environmental water. Viral infectivity reduction at 25°C in each environmental water sample was measured for FCV, PV, MS2, and MNV. Infectivity reduction rate for each virus seeded into environmental waters was compared using a multiple linear regression model that is different from the model for comparison between infectivity reduction rate and nucleic reduction rate. The mean estimate and confidence intervals were only slightly different for the two models. FCV (0.18 ± 0.02 log_{10} PFU/day) was reduced significantly faster than PV (0.13 ± 0.01 log_{10} PFU/day), MS2 (0.12 ± 0.01 log_{10} PFU/day), and MNV (0.09 ± 0.02 log_{10} PFU/day) (model 2 in Table 2). The estimates of infectivity reduction rates among the other three surrogates were not significantly different.

Comparison between viral infectivity reduction and viral nucleic acid reduction. For surrogate viruses in environmental waters at 25°C (model 3 in Table 2), the estimated mean infectivity reduction rates were greater than the mean nucleic acid reduction rates (Table 5). The infectivity reductions for FCV, PV, and MS2 were all significantly larger than each virus's nucleic acid reduction after controlling for sample site ($P < 0.05$) (Table 5). For MNV, infectivity reduction was not statistically different from nucleic acid reduction ($P = 0.07$) (Table 5).

Infectivity reduction rates and nucleic acid reduction rates were not significantly different for any of the tested viruses, MS2 ($P = 0.658$), FCV ($P = 0.211$), and PV ($P = 0.070$), when environmental waters were incubated at 4°C (Table 6).

Comparison of viral nucleic acid reductions among surrogates and NV. The RNA reduction rate of NV was compared to surrogate virus RNA reduction rates by using a multiple linear regression model. When estimated nucleic acid reduction rates were compared for viruses in environmental water incubated at 25°C, the estimated NV nucleic acid reduction rate (0.04 ± 0.01 log_{10}/day) was not found to be statistically significantly different from the nucleic acid reductions of MS2 (0.05 ± 0.03 log_{10}/day) and MNV(0.04 ± 0.03 log_{10}/day). However, FCV (0.08 ± 0.03 log_{10}/day) and PV (0.09 ± 0.03 log_{10}/day) reduced at significantly higher rates than NV and the other two surrogates (model 4 in Table 2).

In laboratory quality water at 25°C, FCV nucleic acid loss was significantly faster than NV loss ($P < 0.001$), while the other surrogate viruses were similar to NV in nucleic acid reduction rates.

In environmental water incubated at 4°C, FCV (0.08 ± 0.05 log_{10}/day) and PV (0.06 ± 0.04 log_{10}/day) nucleic acid reductions were significantly faster than NV (0.02 ± 0.02 log_{10}/day) nucleic acid reductions. MS2 (0.03 ± 0.04 log_{10}/day) nucleic acid reduction rates remained the same as those of NV at 4°C.

Comparison between surface water and groundwater sources. MNV, FCV, and PV infectivity decreased significantly faster ($P = 0.003$ for MNV and $P < 0.001$ for FCV and PV) in surface water than in groundwater (Table 7). However, there was no statistical difference in MS2 infectivity ($P = 0.127$) between surface water and groundwater (Table 7).

When viral nucleic acid reduction rates were compared for surface water and groundwater incubated at 25°C, MS2, MNV, PV, and NV nucleic acid were found to decrease significantly faster in surface water than in groundwater ($P < 0.01$) (Table 8). However, for FCV, there was no statistical difference in nucleic acid reduction rates between surface water and groundwater ($P = 0.08$) (Table 8).

When environmental waters were incubated at 4°C, almost all virus infectivity reductions (Table 9) and nucleic acid reductions (Table 10) were significantly different for surface waters and groundwaters. Only MS2 nucleic acid reduction rates were not significantly different for the two water sources ($P = 0.158$) (Table 10). It was observed that the reduction rate estimates were lower at 4°C than at 25°C (Tables7 to 10 and models 5 and 6 in Table 2). The infectivity and viral RNA reduction rate mean values of six water samples incubated at

TABLE 6. Virus reduction rates in environmental waters at 4°C

Virus	Mean rate ± 95% CI (log_{10}/day)[a] for reduction of:		*P* for comparison between rates
	Infectivity	Nucleic acid	
MS2	0.03 ± 0.01	0.03 ± 0.03	0.658
MNV	ND	ND	
FCV	0.12 ± 0.04	0.08 ± 0.10	0.211
PV	0.09 ± 0.02	0.06 ± 0.05	0.070
NV	ND	0.02 ± 0.01	

[a] Estimated mean reduction rates and 95% confidence intervals as determined by multiple linear regression models. ND, not done.

TABLE 7. Comparison in viral infectivity reductions in surface waters and groundwaters incubated at 25°C

Virus	Mean infectivity reduction rate ± 95% CI (log_{10} PFU/day)[a] for:		*P* for comparison between rates
	Surface water	Groundwater	
MS2	0.13 ± 0.04	0.09 ± 0.09	0.127
MNV	0.16 ± 0.06	0.04 ± 0.13	0.003
FCV	0.40 ± 0.14	0.12 ± 0.28	<0.001
PV	0.19 ± 0.03	0.09 ± 0.07	<0.001
NV	ND	ND	

[a] Estimated mean reduction rates and 95% confidence intervals as determined by multiple linear regression models. ND, not done.

TABLE 9. Comparison in viral infectivity reductions in surface waters and groundwaters incubated at 4°C

Virus	Mean infectivity reduction rate ± 95% CI (log_{10} PFU/day)[a] for:		*P* for comparison between rates
	Surface water	Groundwater	
MS2	0.05 ± 0.02	0.00 ± 0.05	0.002
MNV	ND	ND	
FCV	0.19 ± 0.05	0.06 ± 0.13	0.001
PV	0.14 ± 0.02	0.02 ± 0.06	<0.001
NV	ND	ND	

[a] Estimated mean reduction rates and 95% confidence intervals as determined by multiple linear regression models. ND, not done.

4°C were 0.12 and 0.09 log_{10}/day for FCV, 0.09 and 0.06 log_{10}/day for PV, and 0.02 and 0.03 log_{10}/day for MS2. The viral RNA reduction rate for NV was 0.02 log_{10}/day. These mean values are generally considerably lower than the mean reduction rate values of the corresponding six samples incubated at 25°C: 0.57 and 0.20 log_{10}/day for FCV, 0.28 and 0.12 log_{10}/day for PV, and 0.14 and 0.16 log_{10}/day for MS2, with a viral RNA reduction rate of NV 0.20 log_{10}/day.

DISCUSSION

NoVs are known to be one of the most frequent causal agents of viral gastroenteritis worldwide, and waterborne transmission is a significant route of exposure (5). This study describes an evaluation of the persistence of selected NoV surrogates and human NV in surface waters and groundwaters.

Due to the absence of a routine in vitro NoV infectivity assay (12), the prevalence of NoV in clinical samples and environmental media is most often measured with molecular detection methods, such as qRT-PCR (27, 48). Molecular methods provide information on the presence of viral genetic material but usually cannot differentiate between infectious and noninfectious virions (41). Thus, surrogate viruses that resemble NoV in prevalence and inactivation characteristics have been used to estimate levels of infectious NoV (4, 39, 40). In this study, FCV, PV, MS2, and MNV were selected as surrogate viruses for NoV to indirectly estimate NoV reductions in water. Infectivity reductions of these surrogate viruses were monitored in different types of source waters used for drinking water from different regions of the United States.

To our knowledge, this is the first published study evaluating MNV as a surrogate for human NoV in a model of viral persistence in environmental waters. Because MNV became available in our laboratory near the end of the environmental water testing, MNV was included only in the last water sample from each site. Although the number of MNV survivability tests was fewer than that for the other surrogate viruses, MNV has the potential to be superior to any other surrogate virus tested in this study. Comparison of the mean value reduction rates revealed that MNV was one of the most persistent viruses in environmental waters (Tables 5 to 8) and was very stable over time in the laboratory quality control waters at 25°C (Table 4).

MS2 coliphage has been reported to be a better indicator for enteroviruses than traditional bacterial indicators (18, 36). MS2 was found to be removed at rates comparable to those of enteroviruses during drinking water treatment and exhibited seasonal variation and association with incidence of disease similar to those exhibited by enteroviruses (9, 32, 56). Many researchers have attempted to use MS2 and enteric viruses as surrogates for NoV by estimating the reduction of viral nucleic acid by using RT-PCR amplification, with subsequent comparison of nucleic acid reduction to the reduction of infectious viruses by infectivity assays (31, 36, 39, 41). However, conventional RT-PCR estimates virus levels by end point dilution, and RT-PCR (i.e., detection of log_{10}-fold reductions) does not achieve the same precision as infectivity tests, and thus, meaningful comparison between genomic RNA and infectivity was not possible. To overcome this problem, Rose et al. (36) used triplicate most-probable-number PCR. However, this method involves multiple tubes per sample per dilution and is costly. With the advent of qRT-PCR, which provides a numeric value

TABLE 8. Comparison in viral nucleic acid reductions in surface waters and groundwaters incubated at 25°C

Virus	Mean nucleic acid reduction rate ± 95% CI (log_{10}/day)[a] for:		*P* for comparison between rates
	Surface water	Groundwater	
MS2	0.08 ± 0.03	0.02 ± 0.08	0.007
MNV	0.09 ± 0.04	0.00 ± 0.09	0.002
FCV	0.11 ± 0.04	0.06 ± 0.09	0.080
PV	0.14 ± 0.02	0.05 ± 0.05	<0.001
NV	0.08 ± 0.02	0.01 ± 0.05	0.001

[a] Estimated mean reduction rates and 95% confidence intervals as determined by multiple linear regression models.

TABLE 10. Comparison in viral nucleic acid reductions in surface waters and groundwaters incubated at 4°C

Virus	Mean nucleic acid reduction rate ± 95% CI (log_{10}/day)[a] for:		*P* for comparison between rates
	Surface water	Groundwater	
MS2	0.04 ± 0.01	0.02 ± 0.04	0.158
MNV	ND	ND	
FCV	0.15 ± 0.11	0.02 ± 0.11	0.001
PV	0.10 ± 0.02	0.02 ± 0.05	<0.001
NV	0.04 ± 0.02	0.01 ± 0.05	<0.001

[a] Estimated mean reduction rates and 95% confidence intervals as determined by multiple linear regression models. ND, not done.

(called a Ct value) corresponding to the amount of template genomic RNA, a more precise estimation of viral RNA level is possible. Thus, statistical comparisons between infectivity reduction rates and genomic RNA reduction rates are now possible. In a recent report by O'Connell et al. (35), different sets of qRT-PCR primers/probes optimized for different strains of MS2 detection were described. We have expanded on these reports and have applied qRT-PCR to detection of MS2 as a surrogate for NoV in a model of viral reduction in environmental waters. Our qRT-PCR results (Tables 4 to 6) for MS2 indicate that MS2 is a conservative surrogate for mammalian nonenveloped viruses that closely reflects the nucleic acid reductions of PV and NV.

FCV has been widely reported in the literature as an appropriate surrogate for NoV, in large part due to the recognition that the FCV genome is more similar to NoV than any other surrogate viruses and the availability of an FCV cell culture infectivity assay (4). However, when the infectivity reduction of FCV was compared to those of the other surrogates in this study, FCV was found to be unstable even in laboratory quality control waters at 25°C (infectivity reduction rate, 0.08 $\log_{10}$ PFU/day) compared to other surrogate viruses, all of which have infectivity reduction rates of 0.02 $\log_{10}$ PFU/day (Table 4) as well as in environmental waters at 25°C, where the infectivity reduction rate of FCV (0.18 $\log_{10}$ PFU/day) was greater than those of the other surrogates (0.09 to 0.13 $\log_{10}$ PFU/day) (Table 5). The infectivity of FCV was also less stable in environmental water incubated at 4°C (0.12 $\log_{10}$ PFU/day) than those of PV (0.09 $\log_{10}$ PFU/day) and MS2 (0.03 $\log_{10}$ PFU/day) (Table 6).

When FCV was evaluated as a surrogate for NoV in a model of viral reduction in natural waters by using plaque assays, several researchers observed significant FCV reduction compared to those for the other viruses (1, 26). FCV has also been used as a surrogate for NoV in a model of viral removal efficiency in various water treatment processes with inactivation of FCV by chlorine (46), chlorine dioxide (45), ozone (44), UV radiation (8, 33), ionizing radiation (8), heating (40), or conventional drinking water treatment systems (18) and wastewater treatment (47). Some studies evaluated FCV inactivation as a surrogate for NoV control in certain settings, like hospitals, nursing homes, and cruise ships, by use of chemicals such as alcohol (16), various ethanol-based hand rubs (28), and other chemical disinfectants (43).

However, there is some concern regarding the applicability of FCV as an adequate surrogate for NoV due to the rapid reductions of FCV infectivity, especially at 25°C. For example, similar to our findings (Tables 5 and 6), Allwood et al. (1) reported that FCV decreased at rates of 0.14 $\log_{10}$ PFU/day at 4°C and 0.19 $\log_{10}$ PFU/day at 25°C, whereas MS2 decreased at rates of 0.04 $\log_{10}$ PFU/day at 4°C and 0.05 $\log_{10}$ PFU/day at 25°C. Other researchers have reported similar ranges of FCV reductions in relatively clean water. Duizer et al. (11) reported a 3-$\log_{10}$ infectivity reduction of undiluted FCV stock over 1 week (0.43 $\log_{10}$ PFU/day) at 20°C. Hewitt et al. (22) reported how a field-isolated FCV strain stored in D-PBS at 4°C had decreases of about 2.5 $\log_{10}$ PFU (0.36 $\log_{10}$/day) for infectivity and 4 $\log_{10}$ (0.57 $\log_{10}$/day) for nucleic acid, while there was no significant NV nucleic acid reduction observed under the same conditions.

It is still unclear which surrogate virus model best represents NoV infectivity reduction, but evidence indicates that FCV is less appropriate than other nonenveloped surrogate viruses because the reduction in FCV infectivity is significantly faster than reductions of other enteric viruses in water at a higher temperature. In addition, there were larger disparities between infectivity reduction rates and genomic RNA reduction rates for FCV in both laboratory quality water (Table 4) (where the absolute reduction rate difference for FCV was 0.05 $\log_{10}$/day, compared to 0.01 to 0.02 $\log_{10}$/day for the other surrogates) and environmental water (Table 5) (where the absolute reduction rate difference for FCV was 0.09 $\log_{10}$/day, compared to 0.03 to 0.06 $\log_{10}$/day for the other surrogates), thus challenging the ability of FCV to be a suitable surrogate for NV.

Duizer et al. (11) reported Ct changes in qRT-PCR of FCV and compared these changes to differences in conventional RT-PCR results. However, the authors conducted only a small number of tests per condition ($n = 2$), and thus, the changes of Ct values could not be translated into log reductions of viral nucleic acid. In our study, however, changes in qRT-PCR Ct values were translated into reductions of viral nucleic acid, which were then statistically compared among viruses. Viral nucleic acid reductions were also compared to reductions in infectivity. These comparisons indicate that MNV, MS2, and PV all have potential to be useful surrogates for human NoVs, whereas FCV is questionable regarding its applicability as an adequate NoV surrogate.

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