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Field-based evaluation of a male-specific (F+) RNA coliphage concentration method

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A B S T R A C T

Fecal contamination of water poses a significant risk to public health due to the potential presence of pathogens, including enteric viruses. Therefore, sensitive, reliable and easy to use methods for the concentration, detection and quantification of microorganisms associated with the safety and quality of water are needed. In this study, we performed a field evaluation of an anion exchange resin-based method to concentrate male-specific (F+) RNA coliphages (FRNA), fecal indicator organisms, from diverse environmental waters that were suspected to be contaminated with feces. In this system, FRNA coliphages are adsorbed to anion exchange resin and direct nucleic acid isolation is performed, yielding a sample amenable to real-time reverse transcriptase (RT)-PCR detection. Matrix-dependent inhibition of this method was evaluated using known quantities of spiked FRNA coliphages belonging to four genogroups (GI, GII, GII and GIV). RT-PCR-based detection was successful in 97%, 72%, 85% and 98% of the samples spiked (10^6 pfu/l) with GI, GII, GIII and GIV, respectively. Differential FRNA coliphage genogroup detection was linked to inhibitors that altered RT-PCR assay efficiency. No association between inhibition and the physicochemical properties of the water samples was apparent. Additionally, the anion exchange resin method facilitated detection of naturally present FRNA coliphages in 40 of 65 environmental water samples (61.5%), demonstrating the viability of this system to concentrate FRNA coliphages from water.
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1. Introduction

Fecal contamination is the primary source of microbiological hazard in both fresh and marine waters due to the possible content of pathogenic microorganisms (Figueras and Borrego, 2010). Hundreds of species of enteric bacterial, viral and parasitic pathogens can be found in water which require a variety of strategies for their detection (Figueras and Borrego, 2010). For routine water quality monitoring, determining the presence of all pathogenic microorganisms is not practical at this time. Thus, fecal indicator organisms are used for this purpose. One such group of fecal indicator organisms are male-specific (F+) RNA coliphages (FRNA) (family *Leviviridae*, icosahedral capsid, single stranded positive sense RNA). FRNA coliphages proliferate in the gastrointestinal tract of warm-blooded animals, are shed exclusively in feces and loosely display similar sensitivities to several chemical and environmental stresses in comparison to enteric viruses (Grabow, 2001; Havelaar et al., 1993). It has also been demonstrated that FRNA coliphages are indicative of the presence and persistence of human enteric viruses in various water types and shellfish (Bae and Schwab, 2008; Doré et al., 2000; Havelaar et al., 1993). Additionally, specific FRNA coliphages have been associated with the presence of several enteric viruses in freshwater (Vergara et al., 2015).

The FRNA coliphage family *Leviviridae* consists of two genera, *Levivirus* and *Allolevivirus*. Genetic and serological characterizations supported the subdivision of these genera into four genogroups: with *Levivirus* divided into genogroup I (GI) and genogroup II (GII) and *Allolevivirus* into genogroup III (GIII) and genogroup IV (GIV). Although not always absolute, studies suggest that different FRNA coliphage genogroups are preferentially associated with specific animal hosts and humans, making them useful for source track-

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ing applications (Jofre et al., 2011; Noble et al., 2003; Scott et al., 2002). GI and GIV have been found predominantly associated with animal feces and GII and GIII in human sources of fecal contamination (Osawa et al., 1981; Schaper et al., 2002). Fecal origin has been differentiated on the basis of human and non-human sources using principle coordinate analysis of the FRNA coliphage genotypes present in a particular sample (Lee et al., 2011).

Dispersal within water, and the associated dilution effect, is a complicating factor in the detection of FRNA coliphages and other waterborne viruses. Multiple strategies have been developed to concentrate viruses from water, including electronegative and electropositive filters (Ikner et al., 2012). However, these filters are prone to clogging, viral elution is achieved in relatively large volumes (10 ml to 300 ml) adding undesirable dilution effects and elution buffers are often of extreme pH or protein content which may limit downstream detection (Grabow, 2001; Ikner et al., 2012; Paar et al., 2015).

In efforts to circumvent the issues associated with the above charge-based filters, we recently developed an anion exchange resin-based system to concentrate FRNA coliphages and enteric viruses (Perez-Mendez et al., 2014a,b). In this method, anion exchange resin is dispersed in water samples to adsorb negatively charged viruses, followed by direct isolation of RNA from the recovered resin (final eluate volume of 60 μl) and real-time reverse transcription PCR-based (RT-PCR) detection. Under ideal laboratory conditions, the anion exchange resin method enabled equal or better concentration of the tested viruses compared to previously developed strategies (Perez-Mendez et al., 2014a,b). This method had the added benefits of low sample concentration costs ($0.24/sample for the anion exchange resin), ease of use and the ability to process large volumes of water (up to 10 l tested). However, the aforementioned studies utilized water samples that are not entirely representative of the dynamic nature of water in the environment which has a range of PCR inhibitors, particulate, salinity, etc. To this end, the objective of this study was to field test the performance of the anion exchange resin-based methodology for concentrating FRNA coliphages in diverse environmental water samples.

2. Materials and methods

2.1. FRNA coliphages and bacterial strains

Bacterial host *Escherichia coli* HS (pFamp)R (ATCC 700891), FRNA coliphages MS2 (GI, ATCC 15597-B1) and Qβ (GIII, ATCC 23631-B1) were obtained from the American Type Culture Collection (Manassas, VA, USA). FRNA coliphages GA (GII), MX-1 (GIII) and BR-1 (GIV) were provided by Stephanie Friedman (U.S. EPA, Gulf Breeze, FL, USA). Host bacteria and FRNA coliphage stocks were prepared as described in the U.S. EPA method 1602 (U.S. EPA, 2001), using tryptic soy broth (TSB) (Difco laboratories, Detroit, MI, USA) supplemented with 5 mM magnesium chloride and 50 μg/ml of ampicillin and streptomycin (Sigma-Aldrich, Saint Louis, MO, USA). The coliphage stocks were filtered through a 0.22 μm low protein-binding filter (PALL Life Sciences, Ann Arbor, MI, USA) and stored at −80 °C in 20% glycerol. The phages were enumerated using the double agar overlay plaque assay (Hershey et al., 1943) as modified by Kropinski et al. (Kropinski et al., 2008).

2.2. Water samples

A total of 65 environmental water samples (4 l each), suspected to be fecally contaminated, were collected from 39 different sites within the greater Boston area (MA, USA) (Table 1) in accordance with standard operating procedures established by the U.S. EPA New England Regional Laboratory, Chelmsford, MA, USA (Paar et al., 2015). Samples included water from brooks (n = 24), canals (n = 2), drains (n = 3), rivers (n = 8), storm drains (n = 5), storm outfalls (n = 21) and tidal creeks (n = 2). A total of 49 samples were fresh water and 16 samples were marine water. Twelve sites were visited on three consecutive days. Samples were collected in sterile polypropylene bottles containing 50 mg/l of sodium thiosulphate pentahydrate (Mallinckrodt Baker Inc. Phillipsburg, NJ, USA) for dechlorination and immediately placed in coolers with ice. Upon arrival at the U.S. EPA New England Regional Laboratory, a 50 ml aliquot of each water sample was transferred to polypropylene tubes and stored at −80 °C until total suspended solids (TSS), total disolved solids (TDS), total organic carbon (TOC) and pH analyses could be performed at the Soil, Water & Plant Testing Laboratory at Colorado State University (Fort Collins, CO, USA). The remaining samples were maintained at 4 °C for up to 12 h until viral adsorption to resin could be performed.

2.3. Spiked water samples and laboratory spiked controls

From each water sample, two 1 l aliquots were transferred to sterile polypropylene bottles. One of these aliquots (spiked sample) was inoculated with a cocktail containing 10^6 pfu (each) of GI, GII, GIII and GIV and then mixed. Both aliquots (spiked and unspiked) were kept at 4 °C until further processing (< 2 h). Additionally, a spiked control (S-PBS) was included on each sampling day, consisting of 1 l of 1:4 diluted phosphate buffered saline, pH 7.4 (PBS) spiked as described above. Method blank negative controls consisted only of PBS (all method blank samples tested negative for FRNA coliphages by RT-PCR). To avoid cross contamination, unspiked samples were processed prior to spiked samples.

2.4. Anion exchange resin-based viral concentration

Prior to viral adsorption to Amberlite IRA-900 anion exchange resin (Polysciences Inc., Warrington, PA, USA), water samples were filtered through DIF-IN30 disposable inline filters (United filtration, Sterling Heights, MI) and transferred to sterile glass bottles using a peristaltic pump (0.33 l/min) to remove particulates larger than 0.3 μm. Anion exchange resin (0.5 g/l) was then added to each of the samples and incubated for 120 min at room temperature with continuous mixing using a stir bar and stir plate. Following incubation, the anion exchange resin was allowed to settle for approximately one minute. Most of the water was decanted and resin with the remaining water (approx. 30 ml) was transferred to a 50 ml conical tube. The anion exchange resin was allowed to settle and the remaining liquid was removed. RNA was immediately isolated from the anion exchange resin as described below.

2.5. RNA isolation

Nucleic acid isolation was performed directly from the anion exchange resin via the addition of 560 μl of AVL buffer from the QIAamp viral RNA kit (Qiagen, Valencia, CA, USA). After a 10 min incubation with occasional agitation, the supernatant containing released RNA was transferred to 1.5 ml Eppendorf tubes and RNA isolation was performed using the Qiagen QIAamp viral RNA kit according to the manufacturer's instructions. To maximize RNA yield, a two-step elution of RNA was performed in which 40 μl of AVE buffer was added to spin columns and incubated for five min before elution by centrifugation. This was repeated with an additional 40 μl of AVE buffer. The resulting eluates were pooled to yield a final volume of 80 μl. RNA was stored at −80 °C until use.

Table 1
Water sampling locations and the associated physicochemial properties of each sample.

Sample #	Site	Water source	Location and description	Water type	Sampling day collected	pH	TSS mg/L	TDS mg/L	TOC mg/L
1	–	River	Merrimack River at Pawtucket St., Lowell, MA	Fresh	1	7.77	0	183	75
2	–	River	Merrimack River near Lowell Hospital, Lowell, MA	Fresh	1	7.64	0	183	68
3	–	River	Merrimack River at boat club, Lowell, MA	Fresh	1	7.37	0	173	64
4	–	River	Merrimack River at boat ramp, Lowell, MA	Fresh	1	7.33	0	51	61
5	–	River	Merrimack River upstream from beach, Lowell, MA	Fresh	1	7.34	0	265	61
6	–	Brook	Beaver Brook at Lakeview Dracut, MA	Fresh	1	7.78	0	303	39
7	–	Brook	Beaver Brook at Pleasant St Dracut, MA	Fresh	1	7.34	0	328	104
8	–	Canal	Umass/Lowell	Fresh	1	7.84	0	178	39
9	–	Canal	DCR Park	Fresh	1	7.47	0	211	39
10	–	Storm drain	Teal Pond-Field, Quincy, MA	Fresh	2	7.47	15	2324	207
11	–	Storm drain	Billings Road, Quincy, MA	Fresh	2	7.18	0	2058	182
12	–	Tidal creek	Blacks Creeek Inlet, Quincy, MA	Fresh	2	7.99	0	21980	1835
13	–	Tidal creek	Blacks Creek Near Armory, Quincy, MA	Fresh	2	7.7	0	15820	1417
14	–	Brook	Furnace Brook at Cunningham MBTA Station, Quincy, MA	Fresh	2	8.34	0	707	54
15	–	Brook	Furnace Brook at Golf Course, Quincy, MA	Fresh	2	7.63	0	622	50
16	–	Storm drain	The Strand Pump Station, Quincy, MA	Fresh	2	7.53	3	9380	646
17	–	Storm drain	Woodbine MBTA Station, Quincy, MA	Fresh	2	8.25	1	421	36
18	–	Storm drain	Willet/Oxenbridge, Quincy, MA	Fresh	2	7.97	1	385	50
19	–	Stormwater outfall	SWO 06, Quincy, MA	Marine	2	7.4	1	18410	1810
20	–	Stormwater outfall	SWO 08, Quincy, MA	Marine	2	7.64	0	20090	2238
21	–	Stormwater outfall	SWO 07, Quincy, MA	Marine	2	7.5	0	13300	1378
22	–	Stormwater outfall	SWO 04, Quincy, MA	Marine	2	7.61	0	18970	1839
23	–	Stormwater outfall	SWO 05, Quincy, MA	Marine	2	7.33	1	15960	1289
24	–	Stormwater outfall	BOS 10L094 at Hallet St. Dorchester, MA	Fresh	3	7.42	1	10360	985
25	–	Brook	Aberjona River at Mill Pond, Winchester, MA	Fresh	3	7.66	0	596	54
26	–	Brook	Aberjona River at Winthrop/Beacon St., Winchester, MA	Fresh	3	7.53	1	442	18
27	–	Stormwater outfall	Fort Point Channel, Boston, MA	Marine	4	7.65	0	22120	2513
28	–	Stormwater outfall	BOS 10L094 at Hallet St., Dorchester, MA	Fresh	5	7.67	50	7980	507
29	–	Stormwater outfall	Fort Point Channel, Boston, MA	Marine	5	7.32	3	19670	2142
30	A	Stormwater outfall	SWO BOS 090 at Victory Park, Dorchester, MA	Marine	3	7.67	1	20020	1974
31					4	7.11	0	17640	1382
32					5	7.59	2	14350	1389
33	B	River	Mystic River at High Starlington, MA	Fresh	3	8.26	0	742	57
34					4	8.36	0	564	107
35					5	7.74	0	557	96
36	C	Brook	Alewife Brook at Mass. Ave, Arlington, MA	Fresh	3	7.5	0	645	46
37					4	7.6	0	655	250
38					5	7.63	0	613	118
39	D	Stormwater outfall	Island End River, Outfalls, Everett, MA	Marine	3	7.72	0	19110	1639
40					4	7.55	0	19740	2185
41					5	7.45	2	17150	1175
42	E	Brook	Aberjona River Culvert at Skillings Road, Winchester, MA	Fresh	3	7.29	0	656	64
43					4	7.72	0	692	104
44					5	7.42	4	607	46
45	F	Brook	Alewife Brook, behind Dilboy Stadium, Arlington, MA	Fresh	3	7.78	0	594	57
46					4	7.49	0	662	132
47					5	7.4	0	581	150
48	G	Brook	Wellington Brook Outlet at Clay Pit Pond, Arlington, MA	Fresh	3	7.63	0	749	43
49					4	7.83	1	707	86
50					5	7.55	3	560	93
51	H	Stormwater outfall	Island End River, Outfall, Admiral's Hill, Chelsea, MA	Fresh	3	7.66	0	18970	2242
52					4	7.83	0	18060	1985
53					5	7.18	0	19040	182
54	I	Brook	Wellington Brook at Grove St, Arlington, MA	Fresh	3	7.65	0	425	11
55					4	7.75	0	369	82
56					5	7.56	1	532	111
57	J	Brook	Alewife Brook, at Broadway near cemetary, Arlington, MA	Fresh	3	7.57	0	612	154
58					4	ND	ND	ND	ND
59					5	7.6	1	676	96
60	K	Stormwater outfall	Mystic River, at Amelia Aerhart Dam, BOS 205, Somerville, MA	Marine	3	7.75	0	18060	2103
61					4	7.56	2	16380	878
62					5	7.26	0	8470	818
63	L	Drain	Island End River, Storm Drain, Admiral's Hill, Chelsea, MA	Fresh	3	7.95	0	10640	939
64					4	7.66	0	19180	1960
65					5	7.29	1	2170	182

2.6. Real-time RT-PCR assays

RT-PCR for the detection of GI, GII and GIV was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad laboratories, Hercules, CA, USA), with primers and hydrolysis probes (Tib Molbiol, Adelphia, NJ, USA) designed by Friedman et al. (Friedman et al., 2011) (Table S1). Probes were labeled with 5′ 6-FAM and 3′ BlackBerry Quencher (BBQ). The one-step RT-PCR was conducted in a 15 μl volume using the QuantiTect Probe RT-PCR Kit (Qiagen) containing 1 U of SUPERase• In (Life Technologies Corporation, Carlsbad, CA, USA) and 5 μl of sample RNA. Primer concentrations (both forward and reverse) were 600 nM for GI, 1600 nM for GII and 800 nM for GIV. For these three FRNA coliphage genogroups, the probe concentration was 266 nM. Additionally, an internal amplification control (IAC) RT-PCR utilizing a synthetic RNA (BioGx, Birmingham, AL) was used in probe-based FRNA coliphage RT-PCR assays as described previously (Friedman et al., 2011). The IAC reaction was included in duplex with each FRNA genogroup RT-PCR and consisted of 200 nM of each IAC forward and reverse primer and 100 nM of IAC probe (Tib Molbiol) labeled with 5′ Cy5 and 3′ BBQ and 0.3 ng of IAC RNA. Thermocycling conditions were: 30 min at 50 °C, 15 min at 95 °C and 40 cycles of 1 min at 95 °C, 30 s at 60 °C and 1 min at 72 °C.

For detection of GIII, the RT-PCR assay developed by Kirs and Smith was used with minor modifications that allowed for enhanced detection (Kirs and Smith, 2007; Perez-Mendez et al., 2014b). Briefly, RT-PCR was performed in a StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using the OneStep RT-PCR kit (Qiagen). RT-PCR (15 μl) contained 5 μl of sample RNA, 0.6 μl of enzyme mix, 3 μl of 5 × buffer, 0.4 mM (each) deoxynucleotide triphosphates, 1.2 U RNAse inhibitor (Qiagen), 100 nM of each forward and reverse primer (Tib Molbiol) and 0.3 μl of 10 × SYBR Green solution (Sigma-Aldrich). Thermocycling conditions were the same as described above and a melting curve analysis was included at the end of the RT-PCR assay. Only reactions with amplicons having the same melting temperature as FRNA coliphage Qβ (GIII) (84.6 °C to 85.2 °C) were considered positive.

Standard curves for each RT-PCR were generated using ten-fold dilutions (5 μl/reaction) of RNA purified from FRNA coliphage stocks with known titers. The range of titers (pfu) tested for each FRNA coliphage genogroup were: GI, 1.75×10^0 to 1.75×10^8; GII, 5.60×10^{-3} to 5.6×10^5; GIII, 3.06×10^{-3} to 3.06×10^6; and GIV, 1.66×10^{-2} to 1.66×10^6). Cycle thresholds (Ct) were plotted against the logarithm of pfu/reaction using StatPlus:mac LE 2009 to conduct linear regression analysis to determine the correlation coefficient (R^2) and slope. Amplification efficiency (E) of the reaction was calculated using the formula $E = 10^{(-1/m)} - 1$, where m = slope of the regression analyses.

2.7. Interpretation of RT-PCR data and inhibitory properties of the water matrices

To quantify the degree of inhibition due to each specific water matrix on RT-PCR detection, the Ct obtained for each spiked sample was compared to the Ct obtained for the S-PBS control, which in this study was considered free of RT-PCR inhibitors. This shift in Ct was referred to as inhibition delta Ct (iΔCt) and was calculated according to the formula iΔCt = Ct of spiked sample − Ct of S-PBS. For RT-PCR that is 100% efficient, a shift of 3.32 cycles in Ct is obtained when a 1-log difference in target concentration is observed. Thus, iΔCts of 3.32, 6.64 and 9.96 cycles are interpreted as a decrease in RT-PCR sensitivity by 1, 2 and 3-logs, respectively. When the target within specific samples was not detected within 40 cycles, the Ct was automatically assigned as 40 for numerical calculations.

To investigate correlations between the physicochemical properties (TSS, TDS, TOC and pH) in each water sample and the observed degree of inhibition of the RT-PCR assays (iΔCt), the Pearson product-moment correlation coefficient (Pearson's "r") was calculated for each parameter and each FRNA coliphage genogroup-specific RT-PCR assay. To assess the potential effects of salinity on adsorption of FRNA coliphages to the anion exchange resin, nucleic acid isolation efficiency, and/or RT-PCR inhibitory effects, a Fisher's Exact Test was performed to compare successful detection of each FRNA coliphage genogroup in spiked fresh vs. marine water samples. Statistical analyses were performed using StatPlus LE.2009.

3. Results

3.1. Standard curves and IAC reactions

The amplification efficiencies of the RT-PCR assays were 93.5%, 96.6%, 85.5% and 89.7% for GI, GII, GIII and GIV, respectively. R^2 values were ≥0.994 for all reactions. The performance of the IAC reaction in duplex with each FRNA coliphage genogroup-specific RT-PCR was tested in S-PBS and produced sigmoidal amplification curves with an average Ct of 29.2 for GI, 25.4 for GII and 27.6 for GIV. Under ideal laboratory conditions, the Ct of the IAC reaction remained stable in the presence of working concentrations of each FRNA coliphage genogroup. An IAC was not included for the GIII reaction, as this reaction utilized SYBR Green in lieu of an oligonucleotide probe.

3.2. Evaluation of matrix effects on spiked FRNA coliphage concentration and detection

To assess the impact of each water matrix on FRNA coliphage concentration and detection, an aliquot of each water sample was spiked with a defined FRNA coliphage cocktail and tested using the anion exchange resin-based method. An additional buffer control, S-PBS, was spiked with the FRNA coliphage cocktail and processed with each batch (total of 5 batches) of samples to simulate optimal conditions for viral detection. In the S-PBS controls, analysis of the obtained RNA allowed for all FRNA coliphage genogroups to be detected with average Cts of 22.6 for GI, 27.9 for GII, 16.7 for GIII, and 15.0 for GIV. Analyses of RNA from environmental waters spiked with FRNA coliphages allowed for detection of 94% (GI), 57% (GII), 77% (GIII) and 95% (GIV) of the water samples (Table S2). The observed Cts in the water samples spiked with FRNA coliphages were typically higher compared to the S-PBS controls. This shift in Ct (iΔCt) ranged from less than one cycle to more than 10 cycles, and did not uniformly alter detection of the different FRNA coliphage genogroups in 53 of the 65 samples (Table S3). For example, inhibition of RT-PCR detection of up to one log was observed in 21, 22, 8 and 24 of FRNA coliphage spiked samples for GI, GII, GIII and GIV, respectively. In samples in which spiked FRNA coliphage detection was not possible for one or more genogroup, the inhibitory effect was most frequently observed for GII and GIII (Table S2). The detection of spiked FRNA coliphages was not possible in three water samples (samples 50, 56 and 65).

The inhibitory effects of the specific water samples on FRNA coliphage detection could be a result of differential viral adsorption to the anion exchange resin, nucleic acid extraction efficiency and/or RT-PCR inhibition. For the latter, a number of strategies have been employed to overcome inhibitory effects, including the dilution of RNA samples which also dilutes inhibitors (Bosch et al., 2011). Thus, analyses of diluted RNA from the FRNA coliphage spiked samples were performed to gauge the extent of RT-PCR inhibition (Fig. 1). In RT-PCR that is 100% efficient, diluting a RNA sample 1:5 would result in a Ct increase of 2.3. This is exemplified by dilution of the S-PBS control RNA with all four FRNA coliphage RT-PCR assays, resulting in an average Ct increase of 2.4. In contrast, dilution of the

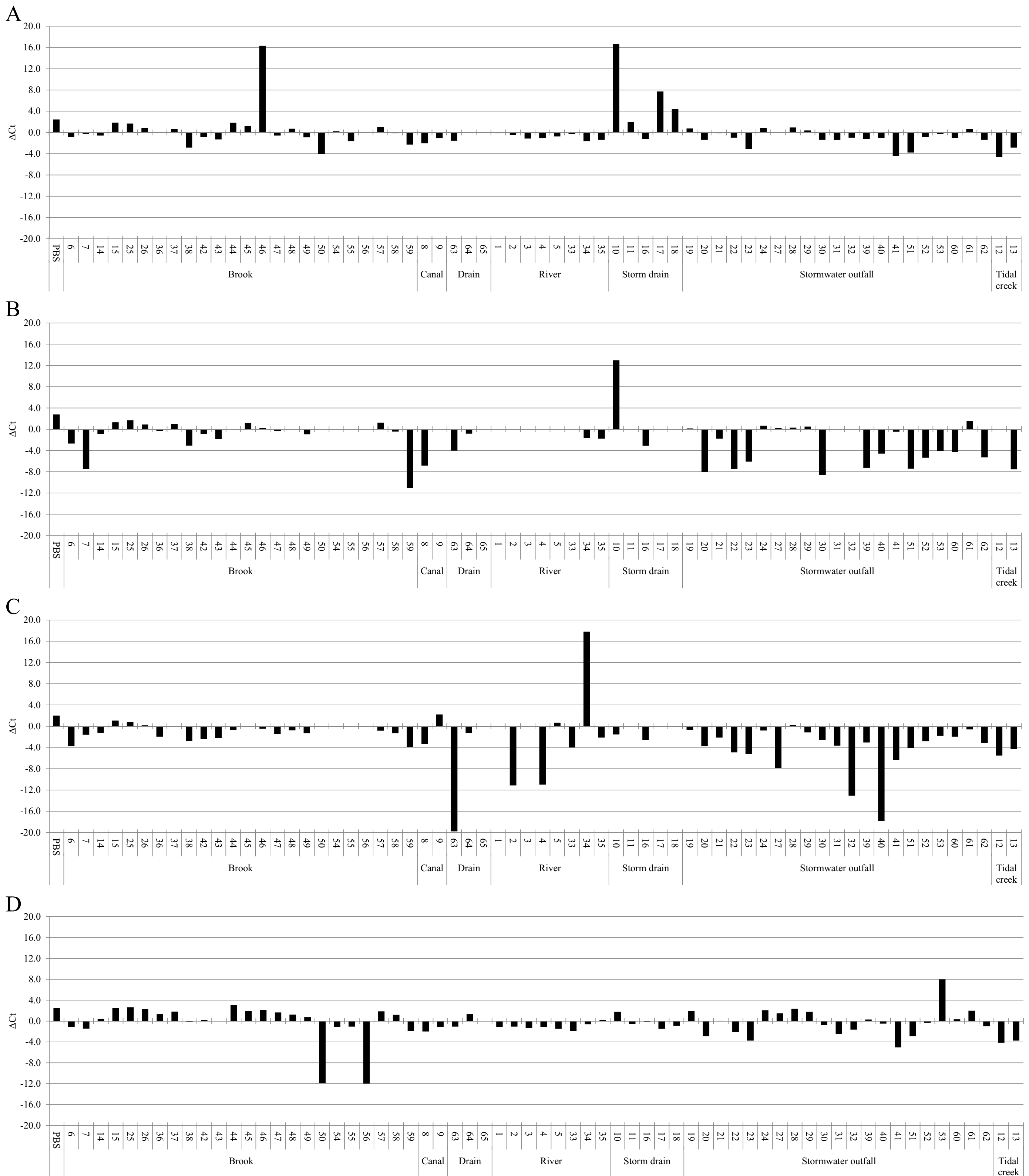

Fig. 1. Dilution (1:5) of RNA obtained from FRNA coliphage spiked samples reduces the inhibitory impacts of the matrix on RT-PCR detection. Dilution of an RNA sample that is free of inhibitors will typically result in an increased Ct compared to undiluted samples. However, when RNA collected from the FRNA coliphage spiked samples was diluted, the majority of samples had a decreased Ct. Panels (A), (B), (C), and (D) are RT-PCR data from GI, GII, GIII, and GIV assays, respectively.

RNA from the FRNA coliphage spiked samples resulted in an average Ct decrease of 1.2 Ct. This indicated that RT-PCR inhibition was a principle factor affecting FRNA coliphage detection. For spiked samples in which FRNA coliphages were not detected in undiluted RNA, 1:5 RNA dilutions enabled detection of an additional two, ten, five and two samples for GI, GII, GIII and GIV, respectively (Table S2). For spiked samples that were initially positive for FRNA coliphages, dilution of the RNA caused non-detection of four, one and

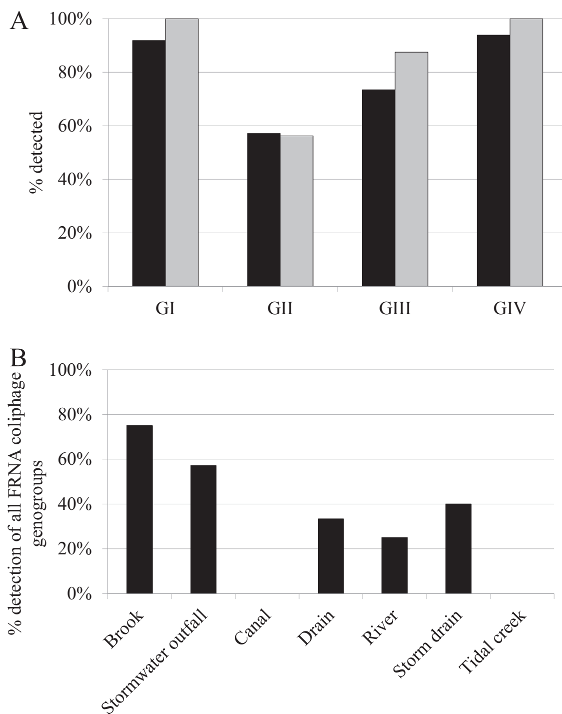

Fig. 2. The impact of water type and source on the detection of spiked FRNA coliphages in environmental waters. (A) the percentage of fresh water samples (light gray bars, n = 59) or marine water samples (dark gray bars, n = 16) in which genogroup-specific detection of spiked FRNA coliphages was possible. A Fisher's Exact Test was performed to compare successful detection of each FRNA coliphage genogroup in spiked fresh vs. marine water samples, these differences were not statistically significant (p-value > 0.05). (B) the influence of water source on the detection of all four spiked FRNA coliphage genogroups.

three samples for GI, GII and GIII, respectively. Considering both diluted and undiluted RNA, the overall detection of spiked FRNA coliphages in the water samples was increased to 97% for GI, 72% for GII, 85% for GIII and 98% for GIV.

An additional strategy to assess RT-PCR-associated inhibition of FRNA coliphage detection utilized a previously described non-competitive IAC reaction (Friedman et al., 2011). This IAC reaction was duplexed with all three probe-based RT-PCR assays (for detection of GI, GII and GIV) used in this study and was tested in undiluted RNA samples. The inhibition of the IAC reaction was not in agreement with the inhibitory properties observed with RT-PCR detection of the spiked FRNA coliphage samples (Table S4). Specifically, the IAC reaction was inhibited in 75% (3 of 4) of the samples in which the spiked GI was not detected due to inhibition. The IAC reaction was inhibited in only 3.6% (1 of 28) of the spiked samples in which GII was non-detectable and was inhibited in none (0%) of the spiked samples (0 of 3) in which GIV were non-detectable. Furthermore, in the presence of the inhibitors innate to each water sample, the expected behavior of the IAC reaction differed when duplexed with the FRNA coliphage specific RT-PCR assays (Table S4). Thus, the robustness of the RT-PCR assays could not be adequately assessed using this IAC reaction.

3.3. Relationships of water quality and origin on concentration and detection of spiked FRNA coliphages

The relationships of water quality metrics including pH, TDS, TOC and TSS (Table 1) to the degree of RT-PCR inhibition (iΔCt) observed in spiked samples were investigated. Pearson's r correlation was calculated for each FRNA coliphage genogroup-specific RT-PCR. None of these physicochemical metrics showed a significant correlation (r > 0.7) with RT-PCR inhibition.

Due to the potential impact of salinity on competitive adsorption of FRNA coliphages to the anion exchange resin, differences in detection behavior between fresh and marine waters were examined using spiked samples (Fig. 2A). Of the 65 water samples tested, 49 samples were collected from fresh waters and 16 samples from marine waters. The percentage of spiked FRNA coliphages belonging to GI, GIII and GIV detected in marine waters was slightly higher compared to fresh waters, but this difference was not statistically significant (p-value > 0.05). For GII, the percentage of RT-PCR positive samples was nearly identical between fresh waters (57% of spiked samples detected) and marine waters (56% of spiked samples detected).

A comparison of the different sources of water (brook, canal, drain, river, storm drain, storm water outfall and tidal creek) revealed that the detection of spiked FRNA coliphages varied between these sources (Fig. 2B). Detection of the four FRNA coliphage genogroups was more readily achieved in brook and storm water outfall samples, in which detection was possible in 75% and 57% of the samples, respectively. In contrast, detection of all four spiked coliphages was possible in 0% of canal, 33% of drain, 25% of river, 40% of storm drain and 0% of tidal creek samples. Nevertheless, one or more of the FRNA coliphage genogroups were detected in 91% of brook, 100% of canal, 66% of drain, 100% of river, 100% of storm drain, 100% storm water outfall and 100% of tidal creek waters.

Twelve sites (Table 1) were sampled on consecutive days which allowed for temporal assessments of water variability related to the inhibition of FRNA coliphage detection. In general, the extent of inhibition was temporally variable and did not uniformly alter the concentration or detection of the four FRNA coliphage genogroups using the anion exchange resin method (Table S3).

Sample	GII	GIII	GIV	Sample	GII	GIII	GIV
6		†		40		‡	‡
7		+		41			†
12		‡		42		‡	‡
13			†	43		†	+
15		+	+	44			‡
19		‡	‡	45		†	
20		†	‡	46		†	
21			+	47	†		+
22			+	48	‡	‡	
24	+	‡	+	49	‡	‡	
26			+	52		†	‡
27		‡	‡	53		†	‡
28	‡	‡	‡	54	†		
29			‡	56	†		
34			‡	57		†	
35		+		58		†	+
36		†	+	59	†	†	
37			+	61		‡	‡
38			†	63		†	
39	†	+		64		‡	‡

Fig. 3. Detection of naturally occurring FRNA coliphages (GII, GIII, and GIV) in unspiked waters. Darkening shades of gray indicate increasing inhibitory levels as qualified through evaluation of the FRNA coliphage spiked samples (Table S3). A "+" indicates that the FRNA coliphages were detected only in undiluted RNA. † indicates that the FRNA coliphages were only detectable using diluted RNA. ‡ indicates that FRNA coliphages were detected in both diluted and undiluted RNA.

3.4. Concentration and detection of FRNA coliphages in naturally contaminated water samples

The ability of the anion exchange resin method to concentrate and detect naturally occurring FRNA coliphages was assessed in unspiked water samples (Fig. 3). Utilizing undiluted RNA, GI was not detected in any of the unspiked samples, GII was detected in four samples, 15 samples were positive for GIII and 23 samples were positive for GIV. Analyses of diluted RNA enabled the detection of additional positive samples for GII, GIII and GIV. Considering both undiluted and diluted RNA samples, a total of nine, 27 and 26 samples were positive for GII, GIII and GIV, respectively. Dilution of the RNA samples prevented FRNA coliphage detection in some samples (one sample for GII, four samples for GIII and 10 samples for GIV), indicating a low natural abundance of these coliphages. In four samples, in which spiked FRNA coliphage detection was totally inhibited in undiluted RNA (samples 40, 54, 56 and 63), detection of naturally present coliphages was possible. Overall, 40/65 of the water samples tested positive for one or more FRNA coliphage genogroup. Of the fresh water and marine water samples, 16% and 6% tested positive for GII, respectively. For GIII, 43% of the fresh water and 38% of the marine water samples were positive and for GIV. 35% of the fresh water and 56% of the marine water samples were positive for GIV. Considering all FRNA coliphage genogroups, there were no statistically significant ($p < 0.05$) differences in frequency of detection between fresh and marine waters using the anion exchange resin method.

4. Discussion

Although initially shed in high numbers, viruses including FRNA coliphages are often difficult to detect in environmental waters due to the dilution effect and inhibitory properties of the matrix (Perez-Mendez et al., 2014b; Wong et al., 2012). In this study, a field evaluation of an anion exchange resin-based virus concentration method was performed utilizing 65 water samples collected in the greater Boston area (MA, USA). The multiple water types (brooks, canals, drains, rivers, storm drains, storm outfalls and tidal creeks) tested were expected to be impacted by both human and animal feces, and 27 of these sites previously tested positive for FRNA coliphages and/or fecal indicator bacteria using established methods (Paar et al., 2015). The anion exchange resin method was vetted by detecting both spiked and naturally occurring FRNA coliphages in replicate volumes of each sample, with the data from the spiked samples allowing for detailed assessments of matrix-dependent inhibitory effects. In 95% of the spiked samples, at least one FRNA coliphage genogroup was detected, suggesting that the anion exchange resin-based concentration of these coliphages was amenable to a wide variety of water types and sources.

Naturally occurring FRNA coliphages were detected in 60% of the unspiked water samples using the anion exchange resin method. In this study, we expected the majority of fecal contamination to be of human origin due to the sheer predominance of their population in the Greater Boston metropolitan area. It is also recognized that untreated sewage directly enters several of the water bodies sampled and analyzed in this study. Our hypothesis was supported in that both GII and GIII (these genogroups are associated with human feces) were detected, and that GIII was the most frequently detected FRNA coliphage genogroup in the naturally contaminated samples. Nevertheless, wildlife such as birds (especially waterfowl), rats, possums, raccoons, skunks, rabbits, squirrels, muskrats, beavers and deer, along with a large number of pets, contribute fecal matter to the sampled watersheds. Thus, the detection of FRNA coliphages genogroups associated with animal sources of fecal contamination were expected.

Matrix-dependent inhibitory effects differentially altered FRNA coliphage detection with the anion exchange resin method. Dilution of the RNA from most of the FRNA coliphage spiked samples improved the Ct of the RT-PCR assays, and allowed for the detection of FRNA coliphages even in samples in which the IAC was positive in undiluted RNA. This finding was consistent with numerous reports indicating that PCR inhibitors are co-purified with nucleic acid isolation procedures, and these inhibitors may differentially impact the molecular detection outcome for various targets (Abbaszadegan et al., 1999; Ikner et al., 2011, 2012; Jones et al., 2009; Lambertini et al., 2008). Our previous studies suggest that the altered detection of FRNA coliphages are not related to differential adsorption to the anion exchange resin or RNA extraction efficiency (Perez-Mendez et al., 2014b). Specifically, in water we have shown that FRNA coliphages adsorb to the anion exchange resin with over 96% efficiency and that RNA recovery from GII and GIII (the FRNA coliphage genogroups in which detection was most inhibited in this study) was higher than for GI and GII (Perez-Mendez et al., 2014b). Thus, additional optimization of the genogroup-specific FRNA coliphage RT-PCR assays and/or RNA extraction procedures in the presence of inhibitors will be required to realize the full capability of this method. Alternatively, other molecular diagnostic methods such as next-generation sequencing and isothermal nucleic acid amplification offer potential avenues to overcome RT-PCR associated inhibition. Next-generation sequencing has the added benefit of detecting additional targets (e.g. pathogens and other indicators) within each sample's concentrated nucleic acid extract.

The efficiency of the anion exchange resin method was hypothesized to be impacted by the physicochemical properties of the water analyzed, and in particular by salinity, given that viral adsorption to the anion exchange resin is expected to be largely charge-based (Gerba, 1984). It was reported that electropositive filters showed low recovery of the GI FRNA coliphage MS2 spiked to a concentration of 10^6 pfu/l in water containing NaCl (Lukasik et al., 2000; Wong et al., 2012). In contrast, excellent adsorption of 10^{11} pfu/l of the GIII FRNA coliphage Qβ to electropositive NanoCeram filters (Argonide, Sanford, FL) in marine water has been reported (Gibbons et al., 2010). The results of the current study show that successful detection of the different spiked FRNA coliphages follows the same trend (FRNA coliphage detection: GIV > GI > GIII > GII) in both fresh and marine waters, and no significant differences in detection were identified between these water groups. The source of the sample (brook, canal, drains, river, storm drain, storm outfall and tidal creek) was associated with FRNA coliphage detection variability, suggesting that characteristics of the water sample other than salinity have more impact on this method. Other studies indicate that solids suspended in the water sample, organic matter and other non-viral constituents of the sample interfere with virus concentration and detection methods (De Keuckelaere et al., 2013; Gerba, 1984; Ikner et al., 2012; Logan et al., 1980; Sobsey et al., 1990; Wu et al., 2011; Wyn-Jones and Sellwood, 2001). However, in this study, none of the physicochemical characteristics measured showed a significant association with inhibition of concentration and/or detection of the FRNA coliphages using the anion exchange resin system, highlighting the ability of this method to concentrate viruses from diverse water types.

5. Conclusions

The anion exchange resin method described in this study provides a rapid (sampling and detection in approximately 6 h), simple, reliable and inexpensive strategy for the concentration of FRNA coliphages from diverse waters. Such a tool may be particularly useful for source tracking applications in which there is need for frequent or continuous sampling to establish the sanitary status of

a water body and to identify sources of contamination. The results of this study also highlight the variable properties of environmental waters that suggest controls using closely related model organisms and/or nucleic acids are needed to assess matrix-dependent inhibitory effects that potentially impact viral concentration and molecular detection.

Disclaimer

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2016.10.007.

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