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Viral Multiplex Quantitative PCR Assays for Tracking Sources of Fecal Contamination▽

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Human and animal fecal pollution of the environment presents a risk to human health because of the presence of pathogenic viruses and bacteria. To distinguish between human and animal sources of pollution, we designed specific real-time reverse transcription (RT)-PCR assays for human and animal enteric viruses, including norovirus genogroups I, II, and III; porcine adenovirus types 3 and 5; ovine adenovirus; atadenovirus; and human adenovirus species C and F, which are excreted by infected humans, pigs, cattle, sheep, deer, and goats, and for the detection of F+ RNA bacteriophage genogroups I to IV, which are associated with human and animal wastes. The sensitivity of this viral toolbox (VTB) was tested against 10-fold dilution series of DNA plasmids that carry the target sequences of the respective viruses and was shown to detect at least 10 plasmid copies for each assay. A panel of human and animal enteric and respiratory viruses showed these assays to be highly sensitive and specific to their respective targets. The VTB was used to detect viruses in fecal and environmental samples, including raw sewage and biosolids from municipal sewage treatment plants, abattoir sewage, and fecally contaminated shellfish and river water, which were likely to contain animal or human viruses.

It is important that sources of fecal pollution are rapidly and accurately identified so that environmental managers can develop strategies to eliminate the source of the pollution in an efficient and cost-effective manner. The identification of fecal sources also allows the potential risk to human health following the consumption of or exposure to contaminated food or water to be estimated. For this purpose, numerous fecal source tracking methods have been developed. Among these are library-dependent methods such as antibiotic resistance testing or molecular fingerprinting (e.g., terminal restriction length fragment polymorphism [T-RFLP] or ribotyping) and library-independent approaches, including the detection of human-specific bacteriophages by plaque assay (e.g., *Bacteroides fragilis* bacteriophages), the detection of certain chemicals associated with human and nonhuman wastes (e.g., caffeine, laundry brighteners, fecal sterols, and stanols), and the detection of specific genetic markers by PCR. The molecular detection of human and animal viruses and bacteriophages falls into the latter category. Each method has advantages and disadvantages depending on the circumstances and the questions at hand. To date, none has been proposed as a standard method (9, 34, 40, 45).

The detection and genotyping of F+ RNA bacteriophages have been widely used as a tool for microbial source tracking (MST) (5, 8, 17, 18, 31, 44). F+ RNA bacteriophage genogroup I (GI) and GIV have been associated with animal contamination, and genogroups II and III indicate predominantly human fecal contamination or domestic sewage-associated inputs (20, 42). In contrast, human and animal viruses, including adenoviruses (AdVs) and norovirus (NoV), generally appear to be highly host specific even if some of them are genetically closely related. For instance, NoV GII consists of human and porcine strains, but they cluster in separate genetic groups or genotypes, and neither of them is known to naturally cross the species barrier.

This characteristic has been exploited in numerous MST studies by specifically targeting animal and human viruses using PCR or quantitative PCR (qPCR), e.g., in assays for bovine enteroviruses (10, 30), bovine AdV (BAdV) and porcine AdV (PAdV) (22, 32), human AdV (HAdV) and enteroviruses (10, 21, 22), porcine teschoviruses (24), and bovine polyomaviruses (21, 22).

Choosing an appropriate PCR assay for MST studies can be challenging. Many existing PCR and qPCR assays for "human viruses" were designed primarily to detect the virus in clinical specimens. For these PCR/qPCR assays, the deliberate exclusion of animal virus sequences was possibly not one of the design criteria. Similarly, this may also apply for animal virus-specific assays, which may have been designed without the need to exclude closely related human strains. However, for MST studies, it is essential to distinguish between closely related human and animal viruses. Therefore, special attention is required for the design of the assay and during the evaluation process to ensure that the primers and probes are specific for either animal or human viruses.

A viral toolbox (VTB) consisting of three multiplex reverse transcription (RT)-qPCR assays (VTB-1 to VTB-3) for the detection of human and animal AdV and NoV, including viruses found in pigs, cattle, sheep, deer, and goats, is described. In addition, the VTB is complemented by a qRT-PCR assay for the detection and genotyping of F+ RNA bacteriophages (VTB-4). Although less specific for either animal or human

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sources, the detection of F+ RNA bacteriophages provides additional information about the level of viral contamination. For validation, human and animal enteric and respiratory viruses and samples of influent and effluent wastewaters, river water, shellfish, and human and animal fecal specimens were tested.

MATERIALS AND METHODS

Samples and specimens. To evaluate the specificity of the multiplex RT-qPCR assays, New Zealand environmental and animal and human fecal samples were collected between December 2003 and July 2008. Fifteen shellfish samples (cockles, clams, and oysters) were collected from various sites between March and October 2008; 11 sewage influent samples were collected between December 2003 and March 2004 from small, medium, and large sewage treatment plants (population sizes, <10,000, 10,000 to 100,000, and >100,000, respectively). Four biosolid samples (collected in 2006) and six river water samples (collected from June 2007 to August 2008) impacted by mixed urban and agricultural land use were selected for testing. Two sewage effluent samples from sheep- and calf-processing abattoirs were also collected. In addition, 10 fecal specimens from New Zealand domestic livestock (sheep, cattle, pigs, and deer); 48 bird fecal specimens from Canada geese (*Branta canadensis*), ducks (*Anas platyrhynchos*), and black swans (*Cygnus atratus*); and 15 human fecal specimens collected from NoV outbreak cases between 2006 and 2008 were selected for testing. Several target and nontarget viruses were also used for the evaluation, including F+ RNA bacteriophages GI to GIV (kindly provided by D. Love and M. Sobsey, University of North Carolina, Chapel Hill); hepatitis A virus (kindly provided by M. Sobsey); a New Zealand field isolate of feline calicivirus (Investigation and Diagnostic center [IDC], Upper Hutt, New Zealand); bovine AdV (BAdV) type 10 (IDC); canine AdV (IDC); HAdV types 1, 3, 4, 5, 8, 9, 12, 13, 15, 17, 19, 21, 22, 29, 31, and 37 (National Centre for Biosecurity and Infectious Disease [NCBID], Upper Hutt, New Zealand); HAdV types 2 and 41 (American Type Culture Collection, Manassas, VA); and poliovirus type 2 (NCBID, New Zealand).

Sample processing. For the fecal samples, 10% (wt/vol) suspensions were prepared in viral transport medium, clarified by the addition of chloroform (10%, wt/vol), and then centrifuged at 13,000 × *g* for 10 min. Viruses from the water samples were concentrated by hollow-fiber ultrafiltration using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Bad Homberg, Germany) as previously described (19) but with minor modifications to elute the viruses from the solid fraction using a 3% (wt/vol) beef extract–0.05 M glycine solution (pH 9.0). Viruses were recovered from shellfish by using a protease digestion method described elsewhere previously (15). Viruses from sewage and biosolids were concentrated by polyethylene glycol (PEG) 6000 precipitation following virus elution as described previously for water samples (modified from procedures described in references 14, 29, and 48). Viral RNA was extracted from the concentrates (200 μl) by using a High Pure viral nucleic acid kit (Roche Molecular Biochemicals Ltd., Mannheim, Germany) according to the manufacturer's instructions.

Design of multiplex qPCR and RT-qPCR assays. Both NoV and F+ RNA bacteriophage assays are based on previously reported assays (49, 50). However, modifications were necessary after the sequencing of environmental isolates revealed mismatches of the viral genome toward some of the primer/probe sequences of the original assay or to further optimize the assays for sequences reported in the GenBank database (data not shown). Sequences from several AdV types from the genera *Mastadenovirus* and *Atadenovirus*, including HAdV, BAdV, PAdV, ovine AdV (OAdV), goat AdV (GAdV), and deer AdV (OdAdV) and sequences from human, porcine, bovine, and ovine NoV and F+ RNA bacteriophages were retrieved from GenBank and were aligned in separate alignments by using ClustalW, version 1.83, implemented in Geneious, version, 4.5.4 (Biomatters Ltd.). Alignments were imported into GeneDoc, version 2.6.002 (K. B. Nicholas and H. B. Nicholas, Jr.). Primers and probes were designed manually with the aim of (i) high specificity through a high degree of primer/probe mismatches toward nontarget viruses, (ii) high sensitivity through a low degree of primer/probe mismatches toward target virus sequences (generally ≤1 mismatch), (iii) broad reactivity by targeting conserved regions, and (iv) high sensitivity and specificity through short amplicons (<150 bp), minimal primer/probe interactions, and optimal annealing temperatures for primers and probes (approximately 60°C and 72°C, respectively). Primers and probes were tested for their melting temperatures, potential hairpins, self-annealing sites, and primer-primer/primer-probe interactions by using the Oligonucleotide Properties Calculator, version 1.0 04/15/97 (www.basic.northwestern.edu/biotools/oligocalc.html), and OligoAnalyzer 3.0 (Integrated DNA Technologies; http://www.idtdna.com/ANALYZER/Applications/OligoAnalyzer/). Target viruses and associated hosts for each VTB assay with primer and probe sequences are listed in Table 1.

RT-qPCR and qPCR. Reverse transcription for NoV and F+ RNA bacteriophages was carried out by using a SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, CA) in separate reactions for VTB assays 3 and 4. The 10-μl RT reaction mixture was comprised of 100 units SuperScript III reverse transcriptase, 10 units RNase inhibitor (RNaseOUT; Invitrogen), 100 nM each reverse primer, 1 mM each deoxynucleoside triphosphate (dNTP) (dATP, dCTP, dGTP, and dTTP), 1× first-strand RT buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$), and 5 μl viral RNA. RT was carried out at 50°C for 30 min followed by 95°C for 4 min and then held at 4°C until qPCR amplification was performed.

For qPCR, each 25-μl reaction mixture contained 5 μl of DNA or cDNA; 1× qPCR Supermix-UDG (Invitrogen); 0.2 μM (VTB-1), 0.3 μM (VTB-2 and VTB-4), or 0.4 μM (VTB-3) each primer; and 0.15 μM (VTB-4) or 0.2 μM (VTB-1, -2, and -3) each probe. The initial activation of the HotStar polymerase at 95°C for 5 min was followed by a two-step cycling protocol comprised of a denaturation step at 95°C for 15 s and an annealing/extension step at 57°C (VTB-3) or 59°C (VTB-1, -2, and -4) for 60 s over 45 cycles. All qPCR assays were carried out with a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia). Quantification of viral copy numbers was carried out by using specific standard curves generated from DNA plasmids that carry the specific virus target sequences described below.

Nuclease-free water was used as a negative extraction control and as a reagent blank for each RT-qPCR. Procedures to prevent false-positive or false-negative results were followed, including the use of separate areas for qPCR reagent preparation and viral nucleic acid extractions/RT and to carry out qPCR assays.

DNA plasmids. Fragments of target virus PCR products cloned into DNA plasmids were used to determine the sensitivity of each VTB assay and to evaluate any possible competition between individual assays in the multiplex assays. The RT-qPCR products were cloned into TOPO vectors and transformed into One Shot TOP10 cells (Invitrogen). Plasmids were purified with a PureLink Quick plasmid miniprep kit (Invitrogen), and after plasmid linearization with EcoRI for 1 h at 37°C (1 U/10 μl), the concentrations were determined with the Quant-iT dsDNA HS assay kit by using a Qubit fluorometer (Invitrogen).

Sensitivity and evaluation of competitive effects. To determine the sensitivity of the VTB, all assays were evaluated against 10-fold dilution series of DNA plasmids. Possible competitive effects between individual qPCR reactions in the multiplex VTB assays were assessed by preparing mock multiple-source samples containing low copy numbers of one virus plasmid (PAdV type 5 [PAdV-5], with 100 copies per reaction) and different quantities of a second virus plasmid (HAdV type 5 [HAdV-5], with 0, 10^2, 10^3, 10^4, and 10^5 copies per reaction). Each combination was then analyzed in four replicates by using the VTB-2 assay (Table 2).

Confirmation of qPCR-positive samples in environmental samples. To confirm positive results for a selection of samples, RNA or DNA was again analyzed by using the respective single qPCR. PCR products were subsequently electrophoresed on a 2% (wt/vol) agarose gel containing SYBR Safe (Invitrogen). PCR products or excised bands were purified by using a QIAquick PCR purification kit (Qiagen, Inc., Germany) according to the manufacturer's instructions. DNA sequencing reactions were performed as described below.

Nucleotide sequencing. DNA sequencing was carried out in both directions by using Big Dye terminator cycling methodology (Applied Biosystems Corp., CA) and an automated ABI 3130XL sequencer (Applied Biosystems). DNA sequences were assembled by using BioNumerics, version 4.6 (Applied Maths, Kortrijk, Belgium).

Statistical analysis. To identify statistical significance, a Student's *t* test analysis using Microsoft Excel (Microsoft, Redmond, WA) was performed. Results with *P* values of <0.05 were considered to be significant.

RESULTS

Sensitivity. Four multiplex RT-qPCR assays were developed for the detection of human and animal AdV and NoV strains and for the genogrouping of F+ RNA bacteriophages. Fewer than 10 DNA plasmid copies per reaction could reproducibly be detected for any given assay. No cross-reactivity with nontarget DNA plasmids was observed for any assay, nor was there any detectable cross talk between neighboring channels of the qPCR cycler.

TABLE 1. Target viruses and associated hosts for each assay in the viral tool box with primer and probe sequences

Assay	Target virus	Host(s)	Product length (bp)	Primer or probe	Sequence	Sense	Position
VTB-1	PAdV-3	Pig	144	VTB1-PoAdV3f	CCTCAACAACCTCAT TGATACC	+	20574–20595[e]
				VTB1-PoAdV3r	CTTGCAGTAGCGG CCGT	−	20702–20718[e]
				VTB1-PoAdV3probe	TACGGCCTGCGCTAC CGCTCCCA[a]	+	20668–20690[e]
	OAdV (OAdV-2/3/4/5, BAdV-2)	Sheep, cattle	102	VTB1-OvAdVf	GCTATGGATGAACCT ACATTGC	+	19226–19247[f]
				VTB1-OvAdVr	AGTTCTCAGGTACAC GGTTTCA	−	19308–19328[f]
				VTB1-OvAdVprobe	TGTGTGGTTGATGCA CGCGCACAACA[b]	−	19273–19298[f]
	HAdV-F (HAdV types 40, 41)	Human	137	VTB1-HAdVFf	GCCTGGGGAACAAG TTCAGA	+	336–355[g]
				VTB1-HAdVFr	GCGTAAAGCGCACTT TGTAAG	−	453–473[g]
				VTB1-HAdVFprobe	CAGTCGCTGYGACCT GTCTGTGGTT[c]	−	385–409[g]
VTB-2	HAdV-C (HAdV types 1, 2, 5, 6)	Human	101	VTB2-HAdVCf	GAGACGTACTTCAGC CTGAAT	+	106–126[h]
				VTB2-HAdVCr	GATGAACCGCAGCG TCAA	−	190–207[h]
				VTB2-HAdVCprobe	CCTACGCACGACGTG ACCACAGA[a]	+	154–176[h]
	AtAdV (OAdV-7, BAdV-4/5/6/7/8, OdAdV, CaAdV-1)	Sheep, cattle, deer, goats	81	VTB2-AtAdVf	CAGATGGAGCCMC AGCG	+	14668–14684[i]
				VTB2-AtAdVr	TGAAYTGYACSAGAT TTTCAGACA	−	14726–14749[i]
				VTB2-AtAdVprobe	TCCCTYGCATTTCTA CCCGCAATGTG[d]	−	14695–14720[i]
	PAdV-5	Pig	133	VTB2-PoAdV5f	CGGCGCTTCCATCAG AATAG	+	18130–18149[j]
				VTB2-PoAdV5r	ACGWCAGGTAATCA ATAAAGGAC	−	18241–18263[j]
				VTB2-PoAdV5probe	CTACTCTGGAGGCCA TGTTGAGAAACG[c]	+	18201–18227[j]
VTB-3	Norovirus GI	Human	96	VTB3-NoVGIf	GCYATGTTCCGYTGG ATGC	+	5282–5300[k]
				VTB3-NoVGIr	GTCCTTAGACGCCAT CATCATT	−	5357–5378[k]
				VTB3-NoVGIprobe	TCGGGCAGGAGATY GCGRTCYC[a]	−	5328–5349[k]
	Norovirus GII	Human, pig	88	VTB3-NoVGIIf	ATGTTYAGRTGGATG AGRTTYTC	+	5012–5034[l]
				SW GII rev[r]	TMGAYGCCATCWTC ATTCAC	−	5081–5100[l]
				SW GII probe[r]	CACRTGGGAGGGCG ATCGCAATC[b]	+	5044–5066[l]
	Norovirus GIII	Cattle, sheep	92	SW GIII forw[r]	CGCTCCATGTTYGC BTGG	+	4972–4989[m]
				VTB-3NoVGIIIr	TCASTCATCTTCATTT ACAAAATCG	−	5040–5064[m]
				SW GIII probe[r]	TGTGGGAAGGTAGT CGCGACRYC[c]	+	5012–5034[m]
VTB-4	F+ RNA bacteriophage GI	Mainly animal waste associated	91	VTB4-FphGIf[s]	GTCCTGCTCRACTTC CTGT	+	20–38[n]
				VTB4-FphGIr	ATGGAATTSCGGCTA CCTACA	−	91–111[n]

Continued on following page

TABLE 1—*Continued*

Assay	Target virus	Host(s)	Product length (bp)	Primer or probe	Sequence	Sense	Position
				VTB4-FphGIprobe	CGAGACGCTACCWTGGCTATCGC[a]	+	68–90[n]
	F+ RNA bacteriophage GII	Mainly human waste associated	145	VTB4-FphGIIf	ACCTATGTTCCGATTCASAGAG	+	1745–1766[o]
				VTB4-FphGIIr	GGTAGGCAAGTCCATCAAAGT	−	1870–1890[o]
				VTB4-FphGIIprobe	CACTCGCGATTGTGCTGTCCGATT[d]	+	1793–1816[o]
	F+ RNA bacteriophage GIII	Mainly human waste associated	112–118	VTB4-FphGIII(MX1)f	TTTGAGGCTRTGTTGCGACA	+	2744–2763[p]
				VTB4-FphGIII(Qβ)f	CCGTCCGTTGAGGGTATGTT	+	2738–2757[p]
				VTB4-FphGIII(MX1)r	CCGTGGSGTACACTCTTG	−	2840–2856[p]
				VTB4-FphGIII(Qβ)r	CGAGGSGTACACGCTTG	−	2840–2857[p]
				VTB4-FphGIIIprobe	CGGYCATCCGTCCTTCAAGTTTGC[b]	+	2809–2832[p]
	F+ RNA bacteriophage GIV	Mainly animal waste associated	86	VTB4-FphGIVf	AAGACWGGTCGGTACAAAGT	+	4076–4095[q]
				VTB4-FphGIVr[s]	ARCTTCACCTCGGGAAKTC	−	4144–4162[q]
				VTB4-FphGIVprobe[s]	CCGGATGAAGGCACTGTCCTGAATC[c]	−	4100–4124[q]

[a] Probe was labeled with 6-carboxyfluorescein (FAM)-BHQ1.
[b] Probe was labeled with Cal fluor red 610-BHQ2.
[c] Probe was labeled with Quasar 705-BHQ2.
[d] Probe was labeled with Cal fluor gold 540-BHQ1.
[e] Genome location of primers and probe based on GenBank accession number AB026117.
[f] Genome location of primers and probe based on GenBank accession number AF252854.
[g] Genome location of primers and probe based on GenBank accession number X51783.
[h] Genome location of primers and probe based on GenBank accession number DQ149613.
[i] Genome location of primers and probe based on GenBank accession number AF036092.
[j] Genome location of primers and probe based on GenBank accession number AF289262.
[k] Genome location of primers and probe based on GenBank accession number M87661.
[l] Genome location of primers and probe based on GenBank accession number X86557.
[m] Genome location of primers and probe based on GenBank accession number AJ011099.
[n] Genome location of primers and probe based on GenBank accession number NC_001417.
[o] Genome location of primers and probe based on GenBank accession number NC001426.
[p] Genome location of primers and probe based on GenBank accession number AY099114.
[q] Genome location of primers and probe based on GenBank accession number X07489.
[r] Primers sequences were reported in reference 5.
[s] Primers sequences were reported in reference 6.

PAdV-5 DNA plasmids at a concentration of 100 copies per reaction could be reliably detected in the presence of 10 to 10^5 copies of HAdV-5 (HAdV species C [HAdV-C]) plasmids. With increasing concentrations of HAdV-5, qPCR amplification curves for PAdV-5 appeared flatter, with lower fluorescence signal maxima at the end of the qPCR reaction (data not shown). However, this appeared to have only a limited effect on the quantitation of PAdV-5. Although the presence of a 1,000-fold excess of HAdV-5 plas-

TABLE 2. C_T values and quantified copy numbers of PAdV-5 DNA plasmids in qPCR reaction mixtures containing 100 copies of PAdV-5 DNA plasmids and 0 to 10^5 copies of HAdV-C DNA plasmids using VTB-2[a]

Copy no. of HAdV-C DNA plasmid per reaction	Obtained C_T values (SD) for PAdV-5	Estimated copy no. per reaction (SD) of PAdV-5
0	32.3 (1.6)	100 (20.6)
10^2	32.8 (0.6)	72 (24.0)
10^3	31.7 (0.3)	143 (33.0)
10^4	32.0 (0.6)	118 (44.1)
10^5	34.1 (1.4)	32 (24.2)

[a] $n = 4$.

TABLE 3. Detection of viruses targeted in the viral tool box in animal fecal specimens

Fecal specimen source	No. of samples	Positive sample(s) (% positive)
Human	15	HAdV-C (7), NoV GI (40) and GII (67), F+ RNA bacteriophage GI (7)
Pig	5	PAdV-3 (60), PAdV-5 (60), F+ RNA bacteriophages GI (80) and GII (80)
Sheep	2	OAdV (100), AtAdV (50), NoV GIII (50)
Deer	1	F+ RNA bacteriophage GII (100)
Cattle	2	NoV GIII (100), F+ RNA bacteriophage GII (50)
Canada goose	14	None
Black swan	16	None
Duck	16	F+ RNA bacteriophage GI (6) and GII (6)

TABLE 4. Prevalence and concentration of human and animal NoV and AdV and F+ RNA bacteriophages in environmental samples as detected by the viral tool box[b]

Sample type	No. of samples	% Positive samples (mean $\log_{10}$ genome copy no. of positive samples ± SD [min–max])					
		VTB-1			VTB-2		
		Porcine AdV-5	Ovine AdV	Human AdV-F	Human AdV-C	AtAdV	Porcine AdV-5
Shellfish	15	13 (3.3 ± 0.1 [3.2–3.4])	13 (2.1 ± 0.4 [1.8–2.3])	20 2.8 ± 0.4 [2.4–3.2])	7 (2.4[a])	7 (2.4[a])	0
Sewage influent	11	0	55 (4.2 ± 0.7 [3.4–5.3])	100 (6.0 ± 1.0 [4.0–7.1])	36 (2.7 ± 0.4 [2.2–3.1])	0	0
Abattoir effluent	2	0	50 (5.4[a])	0	0	0	0
Biosolids	4	0	75 (3.2 ± 0.6 [2.8–3.9])	75 (4.9 ± 0.7 [4.3–5.7])	50 (2.8 ± 0.3 [2.6–3.0])	0	0
River water	6	50 (2.8 ± 0.3 [2.6–3.1])	50 (1.5 ± 0.9 [0.6–2.3])	50 (2.6 ± 0.4 [2.1–3.0])	0	0	0

[a] Only one sample was positive.
[b] Concentrations are per gram digestive tract for shellfish (fresh weight), per liter river water and sewage influent and effluent, and per gram biosolids (dry weight), respectively.

mids resulted in an average shift of +1.8 in cycle threshold (C_T) values, translating to a decrease to 32 copies of PAdV-5 detected (Table 2), this shift was not significant according to the Student *t* test ($P = 0.151$).

Specificity and test panel. Target viruses were amplified as expected, and none of the VTB assays detected nontarget viruses. Fecal specimens from humans, sheep, pigs, and cattle ($n = 25$) tested positive for host-specific viruses only, including HAdV species C, OAdV, PAdV types 3 and 5, atadenovirus (AtAdV), and NoV GI to GIII (Table 3). All wild-bird fecal specimens ($n = 48$) tested negative for human and animal NoV and AdV. One duck fecal specimen tested positive for GI and one tested positive for GII F+ RNA bacteriophage.

HAdV species C and F and NoV GI and GII were frequently detected in municipal sewage and biosolid samples, with average concentrations of positive samples ranging from $\log_{10}$ 2.7 to $\log_{10}$ 6.0 genome copies per liter and $\log_{10}$ 1.5 to $\log_{10}$ 4.9 genome copies per gram dry weight, respectively (Table 4). Interestingly, high percentages of municipal sewage (55%) and biosolid (75%) samples tested positive for OAdV, and one municipal sewage sample (9%) tested positive for NoV GIII (confirmed by nucleotide sequencing of the qPCR products). In terms of absolute concentrations, the contribution of animal AdV and NoV to municipal sewage and biosolids was not negligible, but concentrations were generally lower than those of human AdV and NoV. Compared to OAdV, HAdV species F was found at $\log_{10}$-1.5- to $\log_{10}$-1.8- and $\log_{10}$-1.0- to $\log_{10}$-6.7-higher concentrations in biosolids and municipal sewage samples, respectively (data not shown). In both municipal sewage and biosolid samples, F+ RNA bacteriophage GII and GIII dominated in both prevalence and average concentration, suggesting predominantly human sources of pollution. Sheep and cattle abattoir effluent samples tested positive for OAdV and NoV GIII, with average concentrations between $\log_{10}$ 5.1 and $\log_{10}$ 5.4 genome copies per liter. One abattoir effluent sample tested positive for F+ RNA bacteriophage GI. Nearly all target viruses of the VTB could be detected in samples from contaminated shellfish collected from various locations around New Zealand ($n = 15$) and in water from two New Zealand rivers ($n = 6$), indicating a high prevalence of VTB viruses in environmental samples associated with fecal contamination. Virus concentrations in positive shellfish and river water ranged from $\log_{10}$ 1.4 to $\log_{10}$ 3.7 genome copies per gram digestive tract and $\log_{10}$ 0.6 to $\log_{10}$ 4.2 genome copies per liter river water, respectively. AtAdV was detected in only one shellfish sample, and PAdV-5 was not detected in any environmental samples (Table 4).

DISCUSSION

The VTB for the specific detection of human and animal AdV and NoV specifically targets some of the most common human and animal enteric or respiratory viral pathogens (13, 16, 25, 38, 41) as well as F+ RNA bacteriophages, which are generally found as part of the normal gut flora of humans and animals (3). Once released into the environment, AdV, NoV, and F+ RNA bacteriophages are considered very stable in the environment and are found frequently in contaminated rivers, freshwater, marine water, groundwater, drinking water, and shellfish (4, 7, 10–12, 26, 27, 36, 39, 49).

Viruses targeted by the VTB were detected in high concentrations in municipal raw sewage, biosolids, abattoir wastes, fecally contaminated environmental samples, and human and animal fecal specimens. PAdV-5 and AtAdV were found least frequently in environmental samples, although both viruses were found to be present in fecal specimens of pigs and sheep, respectively. The confirmed presence of OAdV and NoV GIII in raw municipal sewage was unexpected but may reflect the fact that in New Zealand, many abattoirs discharge into municipal sewage treatment plants, and almost all treatment plants in New Zealand are in close proximity to farming activities. However, this apparent limitation, which potentially affects all MST strategies, may be offset by the broad range of specific quantitative assays included in the VTB. The VTB targets at least two different viruses per host species; e.g., NoV GIII, AtAdV, and OAdV are found in cattle, and NoV GI and GII and HAdV species C and F are found in humans. Thus, a high level of confidence on the pollution source can be obtained if more than one host-specific virus is detected. This strategy may be useful for areas that are contaminated by only a few individuals (e.g., leaking septic tanks) or livestock.

All VTB assays showed high analytical sensitivity and specificity. Although many studies suggested competitive effects in multiplex PCR assays (6, 43), we showed that the presence of a 1,000-fold excess of one target virus DNA plasmid did not result in a loss of sensitivity for a second low-copy-number virus DNA plasmid. This is in concordance with data reported previously by Molenkamp and colleagues (35), who found no difference in the quantitation of four different synthetic DNA constructs in a multiplex qPCR even when three of the four

TABLE 4—*Continued*

% Positive samples (mean $\log_{10}$ genome copy no. of positive samples ± SD [min–max])						
VTB-3			VTB-4			
NoV GI	NoV GII	NoV GIII	F+ RNA GI	F+ RNA GII	F+ RNA GIII	F+ RNA GIV
27 (3.6 ± 0.4 [3.1–4.1])	40 (3.2 ± 0.3 [2.9–3.7])	47 (2.4 ± 0.7 [1.4–3.1])	20 (2.5 ± 0.9 [1.5–3.1])	73 (3.2 ± 1.0 [1.9–5.1])	7 (4.8[a])	0
82 (3.1 ± 1.1 [1.3–4.6])	82 (4.4 ± 1.0 [2.5–5.7])	9 (4.4[a])	55 (5.4 ± 0.5 [4.9–6.0])	91 (6.2 ± 0.9 [4.2–7.1])	100 (6.1 ± 1.0 [4.0–7.4])	18 (4.3 ± 1.4 [3.4–5.3])
0	0	100 (5.2 ± 0.2 [5.1–5.3])	50 (5.7[a])	0	0	0
50 (1.5 ± 0.7 [1.0–2.0])	75 (2.8 ± 1.0 [2.0–3.9])	0	0	75 (2.5 ± 0.5 [2.1–3.0])	75 (3.6 ± 0.6 [2.9–3.9])	0
0	17 (2.3[a])	17 (2.9[a])	50 (2.4 ± 0.7 [1.8–3.9])	100 (3.5 ± 0.6 [2.7–4.2])	33 (3.1 ± 0.8 [2.6–3.7])	0

target DNAs were in a 1,000-fold excess. However, interpretations of quantitative data in MST studies are challenging because variable host-specific rates of virus shedding (47), seasonal or geographic variations in viral infections (37), and virus survival in the environment (1, 23, 39) can all influence the presence and concentration of enteric viruses in the environment. Virus detection by molecular methods does not show virus infectivity, and so it is not possible to estimate the impact of various virus types on human and animal health from the quantitative data generated. Therefore, the viral loads detected may not reflect the respective contributions to the total fecal pollution. In this study, the concentrations of HAdV and human NoV in municipal sewage were shown to be highly variable and ranged from 10^4 to 10^7 copies for HAdV-F and from "not detected" to 10^6 copies per liter for NoV GII, respectively, whereas the concentrations of bovine and ovine NoV GIII strains in the two abattoir waste samples were relatively constant at 10^5 copies per liter.

Inhibition can be a major problem with RT and PCRs for environmental samples and was described previously in several studies (4, 11, 15). In this study, inhibition may have generated biased quantitative data or false-negative results, especially for complex samples such as concentrated sewage and shellfish. The identification of inhibition problems in PCR assays can be overcome by the use of internal controls and diluted viral RNA. Therefore, the inclusion of an appropriate process control is required for future studies.

The application of quantitative virus detection methods for MST of contaminated shellfish may complicate the picture further, as virus uptake, bioaccumulation, and persistence in shellfish were previously shown to be virus specific and to vary between seasons and shellfish species (2, 28, 33). The specific binding of NoV to certain carbohydrates in the digestive tract of oysters, mussels, and clams was demonstrated recently (28, 46).

In conclusion, we believe that our viral toolbox of quantitative assays for the specific detection of human and animal enteric and respiratory viruses in the environment is a promising tool for future virus source tracking studies. The VTB assays were shown to be highly sensitive and specific. The multiplex format facilitates efficient time and cost analyses of large numbers of samples. Assays for less prevalent viruses (e.g., PAdV-5 and AtAdV) may be substituted or complemented with assays for viruses (including emerging viruses) that are more relevant for a given geographic region, sample type, environment, or time period. The application and specificity of this VTB-MST method require further evaluation with a larger number of samples from a range of environmental matrices. Further studies are planned to obtain information on the prevalence of viruses from animal- and human-derived fecal sources in different environmental matrices.

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