Use of viral pathogens and indicators to differentiate between human and non-human fecal contamination in a microbial source tracking comparison study

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ABSTRACT

Assays for the detection and typing of adenoviruses, enteroviruses and F+ specific coliphages were performed on samples created as part of a national microbial source tracking methods comparison study. The samples were created blind to the researchers, and were inoculated with a variety of types of fecal contamination source (human, sewage, dog, seagull and cow) and mixtures of sources. Viral tracer and pathogen assays demonstrated a general ability to discriminate human from non-human fecal contamination. For example, samples inoculated with sewage were correctly identified as containing human fecal contamination because they contained human adenovirus or human enterovirus. In samples containing fecal material from individual humans, human pathogen analysis yielded negative results probably because the stool samples were taken from healthy individuals. False positive rates for the virus-based methods (0–8%) were among the lowest observed during the methods comparison study. It is suggested that virus-based source tracking methods are useful for identification of sewage contamination, and that these methods may also be useful as an indication of the public health risk associated with viral pathogens. Overall, virus-based source tracking methods are an important approach to include in the microbial source tracking 'toolbox'.

Key words | bacterial indicators, coliphage, fecal, polymerase chain reaction, viral indicators

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INTRODUCTION

Microbial source tracking (MST) techniques have been developed in order to determine the sources of fecal pollution and pathogens that affect a particular water body or watershed. Microbial contamination of water has been determined for decades by measuring bacterial indicators in drinking water sources, recreational waters and shellfish harvesting waters. However, measurements of bacterial indicators such as total and fecal coliforms, and enterococci (a subset of the fecal streptococci) alone do not provide information relating to the source of the fecal contamination. In addition, these bacteria are not necessarily adequate predictors of human pathogenic viruses (Wyer *et al.* 1995; Noble & Fuhrman 2001), which are important aetiological agents of waterborne disease.

There are several types of microbiological techniques and approaches that can be used for MST. Many of the MST methods, such as multiple antibiotic resistance (MAR) testing, ribotyping and pulse field gel

electrophoresis (PFGE), are based upon the bacterial indicators previously mentioned. The aforementioned MST methods largely depend upon development of a library of fingerprint profiles of fecal bacteria, and characterization of unknown sources based upon the existing library.

Given that the host range of most viruses is narrow (generally limited to a single species), and that they are prevalent in sewage and fecal material, other MST techniques have been developed utilizing both pathogenic and indicator viruses as tracers of specific types of fecal contamination. An advantage of the virus-based MST methods is that they are library-independent. A disadvantage of the virus-based methods is that the relationship between the viruses and fecal indicator bacteria, which are often the targets of total maximum daily load (TMDL) implementation, is not well understood.

Human-specific viruses can potentially be used as a tracer of human fecal contamination. For example, human adenoviruses and enteroviruses have been frequently found in urban rivers associated with human fecal contamination (Tani *et al.* 1995; Castingnolles *et al.* 1998; Chapron *et al.* 2000) as well as in polluted coastal waters (Puig *et al.* 1994; Pina *et al.* 1998; Jiang *et al.* 2001). Studies conducted in Europe have suggested using adenovirus as an index of human viral pollution since this virus has often been detected in samples contaminated with human fecal material (Pina *et al.* 1998). Similarly, the human enterovirus family includes poliovirus, echovirus, and Coxsackie A and B viruses, which have been found in activated sludge, sewage outfalls, and fresh and marine waters associated with human fecal contamination (Kopecka *et al.* 1993; Reynolds *et al.* 1998; Griffin *et al.* 1999; Noble & Fuhrman 2001; Jiang *et al.* 2001). Animal-specific viruses, such as bovine enteroviruses, have also been used to indicate the origin of animal fecal contamination (Ley *et al.* 2002).

Similarly, coliphages, viruses that infect *Escherichia coli*, have been suggested as candidate tracers of specific types of fecal contamination (Havelaar *et al.* 1986). Four genetically distinct subtypes of F^+RNA coliphages have been identified and appear to be somewhat host-specific. Types II and III are generally associated with human sources of fecal contamination, type IV is generally associated with animal sources of fecal contamination, and type I has been associated with both human and animal wastes (Furuse 1987; Hsu *et al.* 1995). These viruses have been consistently isolated from domestic, hospital and slaughterhouse wastewaters (Funderburg & Sorber 1985) and from treated wastewaters (Gantzer *et al.* 1998). F + RNA coliphages appear to be present in fecally polluted waters (Borrego *et al.* 1987) and did not appear to be present in non-fecally polluted waters (Toranzos *et al.* 1988).

Polymerase chain reaction (PCR) and other molecular methods for genotyping viruses have become more common over the past few years. For viral pathogens, PCR methods are much more rapid than traditional cell culture methods, which are also not typically sensitive enough to be used for the detection of many types of enteric viruses. For coliphages, genotyping is replacing serotyping, which occasionally gives ambiguous results. Direct sequence analysis, as demonstrated by this study, can also be used for identification of particular viral subtypes.

The purpose of this study was to apply some of the newly developed real-time PCR and genotyping methods to identify human-specific adenoviruses, enteroviruses and coliphages from blind samples seeded with fecal contamination from different sources. The sources included sewage, humans, dogs, seagulls and cows.

MATERIALS AND METHODS

Sample preparation

Twenty-two researchers performing 12 methods participated in the overall study (Griffith *et al.* 2003). Each laboratory processed samples and conducted data analysis using its own operating procedures. There was no attempt made to standardize protocols within or across methods. Samples were provided to four laboratories for coliphage and viral analysis. Twelve freshwater samples were sent to the Sobsey laboratory at the University of North Carolina at Chapel Hill (UNC/Sobsey) for coliphage analysis. Twelve freshwater and 12 mixed matrix (0.22 μm filtered seawater or freshwater amended with humic acids) samples were sent to the National Oceanographic and Atmospheric Administration Center for Coastal

Environmental Health and Biomolecular Research (NOAACCEHBR) laboratory for coliphage analysis. Twelve large volume freshwater samples were transported to the Jiang laboratory at the University of California at Irvine (UCI) for analysis of human adenovirus and enterovirus by both real-time PCR and conventional PCR. Twelve freshwater and 12 mixed matrix samples were shipped to the Noble laboratory at UNC (UNC/Noble) for analysis of enteroviruses by real-time PCR.

For sample preparation, human fecal material for the study was obtained from 12 healthy adult volunteers residing in various locations throughout southern California. Canine fecal material was obtained from three dogs and cattle fecal material was obtained from three cows. Composite guano samples were obtained from separate flocks of western gulls at Seal Beach, Bolsa Chica State Beach, Huntington State Beach and Newport Beach, all located in Orange County, California. Samples from all sources were collected on 8 October 2002, stored on ice out of direct sunlight and transported to the laboratory in ice chests. The blind test samples were created from the fecal samples by first creating source-specific stock solutions prepared by dissolving equal portions (by mass) of each scat into sterile water. Source-specific fecal stocks were then diluted with sterile water, 0.22 μm filtered seawater or sterile water amended with 0.01% w/v humic acids to produce source/matrix-specific stock solutions, which were then combined to form the blind samples. The blind samples were stored overnight at 4°C prior to packing and shipping on the morning of 9 October 2002. For further details on the preparation of samples see Griffith *et al.* (2003).

Coliphage isolation

Two methods were used for the detection of coliphages. The single agar layer (SAL) method (US EPA 2001*b*; Method 1602) was enumerative and the two-step enrichment method (US EPA 2001*a*; Method 1601) provided presence/absence results. *Escherichia coli* CN13 was used as a host for isolating somatic coliphages and *E. coli* F_{amp} was used as a host for F+ specific coliphages.

At the NOAA laboratory all samples were analysed by the SAL method using sample volumes of 100 ml. Samples negative for F+ specific coliphage by the SAL method were further tested by enrichment using 100 ml sample volumes. Standard methods were followed with a few exceptions. $CaCl_2$ was used to provide a divalent cation instead of $MgCl_2$. For the SAL method, the 2× base medium was prepared by combining tryptic soy agar with tryptic soy broth, retaining the recommended double strength nutrient concentrations but allowing a lower agar concentration (1.5% instead of 3%). For the enrichment procedure, a 100 ml sample volume was used instead of 1 l, with a proportional decrease in other enrichment components. This decrease was necessary because of the limited sample volumes provided during the study.

All 12 samples were analysed by both the SAL and the enrichment methods at the UNC/Sobsey laboratory. Sample aliquots were altered for these two methods because of available sample volumes. For Method 1601, a total of 9.99 ml of sample was used, split as a 3 × 3 dilution array for the purpose of Most Probable Number (MPN) calculation (3 replicates each of 3, 0.3 and 0.03 ml). Each sterile filtered enrichment filtrate was spotted onto a spot plate containing the corresponding host, and incubated overnight. Lysis zones were counted, and MPN was computed from the combination of positives of the 3 × 3 dilution matrix. For Method 1602, serial dilutions using 10.0, 1.0 and 0.1 ml volumes of the samples were analysed.

Plaques were picked from lysis zones on *E. coli* F_{amp} host to make virus suspensions for further characterization. Tryptic soy broth (TSB) with 15% glycerol (by volume) was used as the suspension medium at NOAA and 20% glycerol used at UNC/Sobsey. Sterile wooden applicator sticks were used to transfer phage particles directly from the plates to the suspension tubes, and the tubes were vortexed. A negative control suspension was made in the same manner, but by touching the host lawn outside of any lysis zones. The tubes were stored at 4°C during characterization (2 days to 2 months) and at −70°C for long-term storage.

Coliphage characterization

At NOAA, F^+ RNA coliphage isolates were distinguished from F^+ DNA isolates by testing for inhibition of plaque formation in the presence of RNase A. Confirmed F^+ RNA

coliphages were then genotyped into groups I, II, III or IV following the method of Hsu *et al.* (1995) with a few exceptions. Post-hybridization washes included two 5 min washes in 2 × Saline Sodium Citrate (SSC) with 0.1% Sodium Dodecyl Sulfate (SDS) at room temperature, followed by two 15 min washes in 0.5% SSC and 0.1% SDS at hybridization temperature (45°C). Also, sequences for probes II and III were adopted from Beekwilder *et al.* (1996) while the recommended sequences for probes I and IV were retained from Hsu *et al.* (1995). Identification of group II or III from water samples was scored as indicative of human source contamination. Identification of group I or IV from water samples was considered generally indicative of animal source contamination (Hsu *et al.* 1995).

At the UNC/Sobsey laboratory, coliphages were typed by sequence analysis. Reverse transcriptase (RT) PCR was performed on viral suspensions after a number of them failed to propagate for further characterization. One μl aliquots of enrichment cultures were added to 25 μl reaction mixtures using reagents from the OneStep RT-PCR kit (Qiagen, Inc.), supplemented with 20 units of ribonuclease inhibitor (Promega, Inc.). RT-PCR was carried out on all enrichment culture material using each of two primer pairs. Primers JV80 and JV81 (Oudejans *et al.* 2003) are specific for the *Leviviridae* genus of F^+RNA coliphages, including members of serogroups I and II. The levivirus RT-PCR cycling profile consisted of an initial 30 min reverse transcription step at 42°C, a 15 min reverse transcriptase inactivation step at 95°C, followed by 40 amplification cycles as follows: denaturation (1 min at 94°C), annealing (1 min at 45°C) and extension (1 min at 72°C). A 10 min elongation step at 72°C completed the reaction. The resulting 336 base pair amplicons were visualized in a 2% agarose gel stained with ethidium bromide. Primer pair JV40 and JV41 (Oudejans *et al.* 2003) is specific for the *Allolevivirida*e genus consisting of serogroups III and IV. The cycling profile for the JV40/JV41 primer set was identical to the levivirus primer profile except that primer annealing occurred at 40°C. The JV40/JV41 primer pair yielded a 256 base pair amplicon.

Amplicon DNA was purified from either the RT-PCR reaction mixture or from agarose gel slices using the Qiagen QIAquick PCR Purification kit. Primers JV80 and JV40 were used in the sequencing reactions. DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility on a 3100 Genetic Analyzer (Applied Biosystems, Inc.). Amplicon sequences were compared with an extensive database of F^+RNA coliphage isolate sequences using a local BLAST search in order to separate levivirus amplicons into groups I and II, and allolevirus amplicons into groups III or IV.

Human pathogenic viruses

Concentration of viruses

Twelve freshwater samples were processed at the UCI laboratory. Samples A, C, E, F, G, I, K, N were concentrated from 10 l to 100 ml using a Centramate Tangential Flow recirculation ultrafiltration system (Pall Life Science) with a 30 kDa molecular weight cut-off Omega filtration cassette following the manufacturer's instruction. The efficiency of viral recovery with this system averaged 55.5% as determined by a bacteriophage seeding study (Jiang, unpublished results). The filtration systems were disinfected between each sample with chlorine bleach, followed by flushing with 2 to 4 l of diH_2O. This was followed by recirculation of 1 l 0.1 N NaOH for 30 min, and a repeat flush with 4–6 l of diH_2O. The pH and the membrane cassette flow rates were measured before use with each sample processing (membrane recovery >90% and pH neutral). However, samples J and L were only concentrated from 10 l to 8 l and 4.35 l, respectively, because of the presence of a heavy load of particulates in the samples. Samples P and U were concentrated using Centricon Plus-80 ultracentrifugation units (with 100 kDa molecular weight membrane, Millipore, Inc.) from a volume of 300 ml to 22 ml. The viral recovery with this system is comparable to the Centramate system with an average phage recovery of 60.4% (Jiang, unpublished results). The concentrates were frozen immediately in aliquots. Viral nucleic acid was purified from concentrates using a QIAamp viral nucleic acid purification kit (Qiagen, Inc.) before PCR analysis.

At the UNC/Noble laboratory the starting volume was tenfold lower than that transported to UCI because of the proximity of the laboratories to the original sample set-up. All 24–100 ml samples were filtered with 47 mm

Whatman A/E glass fibre filters at low vacuum (<5 mm Hg), and the filters were immediately frozen at −80°C. During previous recovery experiments, these filters have been found to recover 5–95% of the virus in environmental samples (Noble, unpublished data). In order to capture all viruses in the sample, the filtrate was subsequently concentrated using either Macrosep (Pall Gelman) or Centriprep (Millipore) 30 kDa molecular weight cut-off ultraconcentration units to final volumes of 1–3.5 ml. The final concentrate was frozen immediately at −80°C.

PCR detection of human viruses

At UCI, nested-PCR for human adenovirus was performed following the protocol of Pina *et al.* (1998) with minor modifications as in Jiang *et al.* (2001). Human adenovirus specific primers were 5′-GCCGCAGTGGTCTTACATGC ACATC-3′ and 5′-CAGCACGCCGCGGATGTCAAAGT-3′, yielding an amplicon of 300 bp in size. The nested primers were 5′-GCCACCGAGACGTACTTCAGCCTG-3′ and 5′-TTGTACGAGTACGCGGTATCCTCGCGGTC-3′; the resulting amplicon was 143 bp. PCR products were resolved on 2% agarose.

RT-PCR for enteroviruses was as described by Tsai *et al.* (1993) with a minor modification. The primers used were 5′-CCTCCGGCCCCTGAATG-3′ and 5′-ACCGGA TGGCCAATCCAA-3′, yielding a 197 bp amplicon. An internal probe 5′-TACTTTGGGTGTCCGTGTTTC-3′ was used for confirmation of the amplicon and to improve the sensitivity of detection. Four μl of sample were used in a 10 μl reverse transcription reaction followed by PCR using the conditions described by Tsai *et al.* (1993). Amplicons were resolved on 2% agarose and transferred to nylon membrane for probe hybridization. Hybridization was carried out overnight at 44°C with triple membrane washing at room temperature in a solution containing 6 × SSC, 1% SDS and 0.05% sodium pyrophosphate, followed with two washes at 55°C for 1 h each.

Real-time quantitative PCR detection of human adenovirus 40 and enterovirus

Real-time quantitative PCR detection of adenovirus 40 (AD40) was performed on the 12 freshwater samples at UCI. The PCR primers and Taqman® probe were those presented by Dezfulian *et al.* (2003). This set of primers and probe is specific to AD40 based on a similarity search algorithm (BLAST).

PCR amplification for adenovirus was performed in a 25-μl reaction mixture with a PCR core reagent (Applied Biosystems). The reaction mixture contained 9 μl of viral extract; 1 × Taqman core buffer; 5 mM $MgCl_2$; 200 μM each of dATP, dCTP and dGTP; 400 μM dUTP; 300/900 nM (reverse/forward) of each primer; 250 nM probe; 0.25 U of AmpErase uracil *N*-glycosylase; and 1 U of *Taq* Gold polymerase. Following activation of the uracil *N*-glycosylase (2 min, 50°C) and activation of the AmpliTaq Gold for 10 min at 95°C, 40 PCR cycles of 15 sec at 95°C and 1 min at 60°C were performed with an ABI 7000 sequence detection system (Applied Biosystems). A five-point standard curve was constructed for each run using serial dilutions of titrated plasmid containing a single AD40 hexon gene insert. Samples were run in triplicate and each point of the standard curve was run in quadruplicate. Each run also contained at least three no-template controls to establish the baseline emission intensity of the quenched reporter dye.

Real-time RT-PCR detection of enterovirus was performed at the UNC/Noble lab using the primers: forward 5′-CCCTGAATGCGGCTAAT-3′ and reverse 5′-TGTCAC CATAAGCAGCCA-3′, with Taqman® probe 5′-6FAM-ACGGACACCCAAAGTAGTCGGTTC-TAMRA-3′ (G. Shay Fout, USEPA). The test was originally performed on the blind samples as a one-step RT-PCR reaction which took place in a 25 μl reaction mixture containing 5 μl of extracted RNA; Access RT-PCR system (Promega), with 1 × RT buffer; 4 mM Mg, 500 μM dNTPs, 500 nM reverse primer, 400 nM forward primer, 160 nM probe, 2.5 U TFL polymerase, 2.5 U AMV reverse transcriptase, 10 U RNase inhibitor. The real-time RT-PCR reactions were run with a 10 min room temperature hold, 45 min at 50°C, activation of the Taq polymerase for 4 min at 95°C, 45 PCR cycles of 15 sec at 94°C and 1 min at 60°C and 72°C for 30s, on a Cepheid Smart Cycler® Real-time PCR machine. Four-point standard curves were constructed for each run using serial dilutions of titred Sabin vaccine strain 1 poliovirus. Samples and standards were run in duplicate.

Subsequent reanalysis of samples involved use of a 2-step RT-PCR method, utilizing Omniscript™ (Qiagen, Inc.) for the reverse transcription step, on a Genius® Thermal Cycler using 1 × Buffer RT, 0.5 mM each dNTP, 1 μm EV1R reverse primer, 8 U/reaction RNase inhibitor, and 4 U/reaction Omniscript™ Reverse Transcriptase. Real-time PCR was performed using Cepheid's Smart Cycler™. Five μl of each cDNA was added to a 25 μl reaction with final concentrations of 1 × *Taq* DNA Polymerase Buffer, 4 mM $MgCl_2$, 500 μm each dNTP, 400 nM EVIF forward primer, 400 nM EV1R reverse primer, 250 nM EV1Probe and 2.5 U/reaction *Taq* DNA Polymerase (Takara, Inc.). Real-time PCR was run with an initial 2 min denaturing hold at 95°C, followed by 45 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec.

RESULTS

Coliphage analysis

Coliphage enumeration

All 12 (100%) of the freshwater samples were positive for somatic coliphage, at concentrations ranging from 10^2 to 10^5 per 100 ml (Table 1a). F+ specific coliphage concentrations ranged from <1 to 2.5×10^3 per 100 ml in the tested freshwater samples. Five of 12 (42%) freshwater samples were positive for F+ specific coliphage using the SAL method (Table 1a). The enrichment technique identified an additional two samples that were positive for F+ coliphage. F+ specific RNA coliphage were identified from six of the eight (75%) samples positive for F+ specific coliphage (sample C was positive for F+ specific coliphage by UNC/Sobsey, Table 1a). All 12 of the mixed matrix samples were also positive for somatic coliphage (Table 1b). Six of the 12 mixed matrix samples were positive as determined using the enrichment technique (Table 1b).

Coliphage characterization by genotyping

Genotyping was performed for all samples positive for F^+ RNA coliphages, including 5 of 12 (42%) freshwater samples and 4 of 12 (33%) matrix samples (Table 2a and b). Among the freshwater samples, A, J, N and P contained group II and/or III coliphages, suggesting the samples had been seeded with a human source of fecal contamination. Only one coliphage was isolated from sample K. This isolate typed as group I suggesting that the sample had been seeded with animal source fecal contamination. Comparison of blind results with the contaminants key showed that F^+ RNA coliphages had been isolated from four of four (100%) samples seeded with sewage (A, J, K and N), and that a human source designation had been accurately made for three (75%) of them. The fourth sample for which a human source designation was made (P) had been seeded with cow and gull fecal material. Type III coliphages were isolated from the gull feces provided during this study (Table 3) so the gull material probably contributed the group III coliphage isolated from this sample.

Among matrix samples, genotyping was performed for all four samples positive for F^+ RNA coliphages. Comparison with the contaminants key showed that coliphages were again isolated from all samples (Q, R and W) seeded with sewage. Two of these samples (R and W) contained type II and/or III coliphages, and a third sample (Q) contained one type I coliphage. One group III isolate was also identified from a sample (B) seeded with human and gull feces.

Coliphage characterization by RT-PCR and sequencing

F-specific RNA coliphages were detected in coliphage enrichment cultures from 5 of 12 freshwater samples (data not shown). Samples C and N contained coliphages from only Group III, indicating that the fecal contamination in those samples was predominantly human in origin. Comparison of these results with the contaminants key showed that sample C had been seeded with human and gull fecal material, and sample N had been seeded with sewage and dog materials. Samples A, J and K contained F^+ RNA coliphages from coliphage groups associated with human (Groups II and III) and non-human (Groups I and IV) sources, indicating that these samples had been seeded by fecal contamination from non-human as well as human sources. The contaminants key showed that all three of

Table 1 | Coliphage concentrations (per 100 ml) for aqueous samples

Sample ID	SAL[a] somatic (F^-)	SAL[a] F+ specific	Enrichment F+ specific
(a) freshwater samples			
A	1.8E + 03	2.5E + 03	ND[b]
C	1.6E + 04	< 1	Negative[c]/positive[d]
E	3.3E + 02	< 1	Positive
F	2.4E + 04	< 1	Negative
G	7.1E + 04	< 1	Negative
I	1.3E + 02	< 1	Negative
J	1.0E + 03	2.5 + E02	ND
K	4.6E + 03	2.1 + E03	ND
L	2.7E + 02	< 1	Negative
N	1.6E + 03	2.6 + E02	ND
P	4.9E + 04	2	Positive
U	1.5E + 04	< 1	Positive
(b) matrix samples			
B	9.6E + 03	1	Negative
D	1.4E + 03	< 1	Positive
H	2.1E + 05	< 1	Positive
M	2.8E + 02	< 1	Negative
O	2.9E + 02	< 1	Positive
Q	3.2E + 02	20	Positive
R	4.0E + 03	1.2 + E03	ND
S	5.0E + 05	< 1	Positive
T	9.6E + 04	1	Positive
V	3.6E + 05	< 1	Negative
W	2.6E + 03	9.5 + E02	ND
X	4.2E + 04	< 1	Negative

[a]Concentrations determined at NOAA using single agar layer (SAL) method.
[b]Not determined because samples had tested positive by SAL method.
[c]Result from NOAA.
[d]Result from UNC/Sobsey.

these samples had been seeded with sewage. Overall, sequencing of F^+ RNA coliphages correctly identified a human signal in four of four (100%) samples seeded with sewage and one of four (25%) samples seeded with fecal material from individual humans.

Pathogen analysis

Adenoviruses

Four of the samples of the 12 analysed were positive for adenoviruses by nested-PCR (Table 2a). Two of the samples (A and K) contained sewage as the sole source of fecal contamination. The third sample (N) contained sewage and dog feces. The fourth positive sample (F) contained individual human feces and sea gull feces. Of the four positive results for adenovirus by nested-PCR, two of them (A and N) were also positive by real-time PCR. Using a standard curve for quantification of the adenovirus load, we detected 320 and 120 adenovirus per l for samples A and N, respectively. Adenoviruses were not detected in any samples that did not contain human fecal contamination, i.e. there were no false positives. However, there were four samples, one (J) that contained sewage, and three (K, C and I) that contained human feces that did not yield a positive result (Table 2a). This is probably because the number of adenoviruses in this sample was below the detection limit, or because the individual humans that yielded the fecal material were not infected with adenoviruses.

Enteroviruses

At UCI, three of four (75%) samples that contained sewage were positive for enteroviruses using conventional RT-PCR, which corresponded to samples that had been inoculated solely with human sewage (A and K) and a sample containing sewage, dog and cattle feces (J, Table 2a). None of the samples that contained individual human fecal contamination was positive for enteroviruses. However, there were several samples (L, N, P) that yielded inconclusive results, one (L) that contained human feces, and the other two which did not contain any human feces or sewage. At the UNC/Noble lab, no enteroviruses were

Table 2 | F+RNA coliphage typing, adenovirus and enterovirus results

	F+RNA coliphage type (no. of isolates)		Viral pathogen results		Contaminants key
Sample	**Genotyping (NOAA)**	**Sequence analysis (UNC/Sobsey)**	**Adenovirus (l^{-1})**	**Enterovirus**	
(a) freshwater samples					
A	I(1), III(1)	I(8), III(5)	320[a]	+	Sewage
C	—	III(1)	—	—	Human and gull
E	—	—	—	—	Dog and cow
F	—	—	+	—	Human and gull
G	—	—	—	—	Gull
I	—	—	—	—	Human
J	I(1), II(11), III(9)	I(1), II(1), III(6)	—	+	Sewage, dog and cow
K	I(1)	I(3), III(9)	+	+	Sewage
L	—	—	—	—	Human, dog and cow
N	III(3)	III(7)	120[a]	—	Sewage and dog
P	III(1)	—	—	—	Cow and gull
U	—	—	ND	—	Cow

Sample	**F+RNA coliphage type (no. of isolates) Genotyping (NOAA)**	**Matrix**	**Contaminants key**
(b) matrix samples			
B	III(1)	Humic acid	Human and gull
D	—	Seawater	Cow
H	—	Seawater	Cow and gull
M	—	Seawater	Human
O	—	Humic acid	Cow
Q	I(1)	Seawater	Sewage, dog, and cow
R	II(9), III(9)	Humic acid	Sewage
S	—	Seawater	Gull
T	—	Seawater	Human and gull
V	—	Seawater	Human and gull
W	II(2)	Seawater	Sewage
X	—	Humic acid	Human and gull

[a]Samples with adenovirus load determined using quantitative PCR.

Table 3 | F^+RNA coliphage typing results for known-source samples

Sample type (number of samples)	Number of plaques picked	Number of F^+RNA isolates	F^+RNA coliphage type (no. of isolates)			
			I	II	III	IV
Raw sewage (1)	60	21	5	7	9	
Humans (9)	Negative	0				
Dogs (10)	Negative	0				
Cows (10)	17	0				
Gulls (4 flocks)	60	31	5		23	3

detected during the original blinded sample analysis by real-time one-step RT-PCR. Conventional RT-PCR was not applied to these samples. Reanalysis of samples using a 2-step real time RT-PCR method for enteroviruses demonstrated that the original sewage influent sample was positive for enteroviruses, and by real-time PCR was determined to contain 1.2 enterovirus infectious units per ml.

DISCUSSION

Coliphage analysis

The inability to culture F^+ RNA coliphages from 6 of 12 freshwater samples is probably explained by an apparent absence of these viruses in most of the known source samples used to seed study waters (Table 1a, Table 3). It is known that coliphages, as with most enteric viruses, are only harboured by a proportion of individuals within a population. F^+RNA coliphages are reportedly isolated in less than 10% of human fecal samples and at variable rates in non-human animal feces (Havelaar *et al.* 1986; Cornax *et al.* 1994; Calci *et al.* 1998). The limited number of individual humans included in the 'human' source category (9) appears to have been insufficient to contribute viral loads to every water sample. The source categories representing population composites, the sewage and seagull flock samples, were positive for F^+RNA coliphages. All samples seeded with sewage were positively identified at NOAA and UNC/Sobsey. Typing results successfully identified a human source of fecal contamination in five of seven (71%) of these samples at NOAA and four of four (100%) sewage samples at UNC/Sobsey.

It has previously been argued that coliphages may be a better indicator of sewage than fecal contamination (IAWPRC 1991). The results of this study demonstrate that these viruses are a better tracer of wastes from human populations than individuals. However, water quality problems are more likely to stem from population-based contamination. The authors reason that coliphage and viral pathogen analyses are better suited for most real world scenarios than they were for tracing contamination from individuals during this study.

Exceptions to the associations of coliphage types with particular host sources, groups II and III with humans and groups I and IV with animals, have been reported (Schaper *et al.* 2002; Stewart 2002). The identification of group III coliphages associated with gull feces during this study provided further evidence of these exceptions. The general associations appear to be statistically significant (Schaper *et al.* 2002), and use of this method has proved useful to resource managers (Stewart 2002). It must be understood, however, that designations by phage typing are not absolute, and empirical studies to verify

categorization of coliphage groups should be conducted in each study.

Pathogen analysis

Adenovirus results indicated the presence of human fecal contamination in 75% of the samples inoculated with sewage. These results are consistent with our expectation that a population of humans needs to be sampled to yield positive results for human pathogens (whereas the individual human scat samples were taken from known healthy individuals who would not be expected to harbour viral pathogens). The results of this study are the first demonstration of real-time quantitative PCR detection of adenoviruses in environmental samples (diluted sewage). AD40 was only detected by real-time PCR in one of two samples contaminated with the same volume of sewage (both A and K contained sewage from the same origin). These results may be due to the heterogeneous nature of sewage. Even though the detection by real-time PCR was specifically for AD40, we would expect that the amount of AD40 in our samples (as compared with all adenovirus) would be high, because adenovirus 40/41 are the major cause of childhood gastroenteritis (50% of the adenovirus that are shed in feces belong to serotype 40 and 41; Knipe & Howley 2001).

Enterovirus results accurately detected the presence of sewage in three of four (75%) freshwater samples, and two of two (100%) samples when human sewage was the sole source of contamination. The enterovirus test yielded a positive in one mixed sample (J) that had a very low percentage of sewage influent added to it (1% based upon *Enterococcus* enumeration, Griffith *et al.* 2003), but did not pick up the sewage signal in another mixed sample (N, contained 58% sewage influent as determined by *Enterococcus* enumeration, Griffith *et al.* 2003). Conversely, adenoviruses were detected in mixed sample N, but not in the mixed sample J. It is possible that the heterogeneous nature of sewage, and the small volume of material used for the PCR reactions could cause these inconsistent results. The primers and probe used at UCI for enterovirus detection were designed over 13 years ago by DeLeon *et al.* (1990). A loop structure at the end of one of the primers was found recently, which may contribute to the low PCR amplification efficiency (M. Sobsey, pers. comm.). Enterovirus results that were reported as inconclusive at the time of data submission indicated that one of the four tests yielded weak positive results from a sample that contained only cow and seagull feces when all quality assurance and quality control measures were satisfactory.

In samples analysed by UNC/Noble, newly designed primers and a Taqman® probe were used for real-time PCR detection of enteroviruses. However, no viruses were detected in any of the blind samples. Enteroviruses (1.2 infectious units per ml) were detected in the sewage influent sample. The inability to detect enteroviruses in the blind samples was probably due to three issues: (1) transport of materials across the country with improper storage conditions culminating in arrival of the samples at room temperature; (2) receipt of only 100 ml of each sample for concentration; and (3) use of only a one-step real-time RT-PCR reaction for analysis.

In this study, we demonstrate that the real-time PCR assay was less sensitive than the nested PCR assay used for adenovirus detection. Viruses in sample F were not detected by real-time PCR assays while the sample was positive using the nested PCR assay. Enteroviruses were detected in the blind samples using a conventional RT-PCR assay followed by hybridization with an internal probe by the UCI lab, but not detected in the blind samples by real-time RT-PCR at UNC/Noble. Subsequent reanalysis of the original sewage influent samples by a two-step real-time RT-PCR assay demonstrated positive detection of enteroviruses in the sample at a concentration of 1.2 infectious units per ml (determined using freshly titred poliovirus stock as the standard). The two-step real-time PCR assay appears to be more sensitive, especially with analysis of complex samples, a finding which has been supported by analysis of other complex samples (Noble, unpublished data).

PCR is a rapid method for the detection of virus genomes. PCR assays can easily be completed within 24 h of sampling, although sample concentrates can be held at −80°C for long periods of time for batch processing if necessary. Traditional cell culture methods, on the other hand, require 1–2 weeks, and are not nearly as sensitive for the detection of all types of enteric viruses. However,

PCR-based methods provide information on the presence of a specific type of viral nucleic acid only, in this case enteroviral RNA and adenoviral DNA. It does not provide any estimate of the level of infective viruses in the water sample. Other studies have examined the relationship between the presence of viral genomes and respective infectivity of those viruses in environmental samples, and have found that viruses detected by PCR-based methods are typically infective (Dubois *et al.* 1997). Pichard & Paul (1991) have shown that free RNA degrades relatively rapidly in seawater, so it is unlikely that the RT-PCR products resulted from the amplification of free viral RNA. Because of their presence in high numbers in feces of infected humans, their pathogenicity and their potential use as models of pathogen degradation, viral pathogens and indicators alike are good candidates for creating indices of human fecal contamination. Real-time PCR is a promising method for determination of viral load in complex environmental samples. In order for real-time PCR methods to be considered fully quantitative, however, sample collection, extraction and amplification efficiency must be clearly tested for a variety of environments, and internal and external standards and careful quality control procedures must be used.

CONCLUSIONS

Viral pathogens and indicators show promise for identification of human fecal contamination, and for discrimination between human and non-human sources in environmental waters. Furthermore, use of these methods may more accurately predict potential public health risk as viral pathogens, not indicator bacteria, are a major causative agent of waterborne disease. Virus-based methods were generally successful in this study in indicating the presence of human sewage in samples, and in differentiating between human and non-human sources of fecal contamination. False positives were not a problem with the methods utilized here, although virus-based methods were generally not as effective when applied to individual healthy human fecal contamination versus sewage (whole population-based source). Use of virus-based methods paired with novel molecular approaches can be advantageous for source tracking because databases are not necessary and results can be obtained within a relatively short period (hours to days). The persistence of viruses relative to bacterial indicators also means that viral analysis could be advantageous for situations requiring tracking of fecal contamination post-treatment, or at a distance downstream from inputs. Additionally, these approaches evaluate a stable part of the virus genomes. That is, the genotype to which a virus belongs does not change appreciably with time or varying environmental conditions. These details make viral analysis an appropriate and valuable addition to the MST toolbox.

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