Performance of viruses and bacteriophages for fecal source determination in a multi-laboratory, comparative study

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Abstract

An inter-laboratory study of the accuracy of microbial source tracking (MST) methods was conducted using challenge fecal and sewage samples that were spiked into artificial freshwater and provided as unknowns (blind test samples) to the laboratories. The results of the Source Identification Protocol Project (SIPP) are presented in a series of papers that cover 41 MST methods. This contribution details the results of the virus and bacteriophage methods targeting human fecal or sewage contamination. Human viruses used as source identifiers included adenoviruses (HAdV), enteroviruses (EV), norovirus Groups I and II (NoVI and NoVII), and polyomaviruses (HPyVs). Bacteriophages were also employed, including somatic coliphages and F-specific RNA bacteriophages (FRNAPH) as general indicators

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of fecal contamination. Bacteriophage methods targeting human fecal sources included genotyping of FRNAPH isolates and plaque formation on bacterial hosts *Enterococcus faecium* MB-55, *Bacteroides* HB-73 and GB-124. The use of small sample volumes (≤50 ml) resulted in relatively insensitive theoretical limits of detection (10 - 50 gene copies or plaques·50 ml^{-1}) which, coupled with low virus concentrations in samples, resulted in high false-negative rates, low sensitivity, and low negative predictive values. On the other hand, the specificity of the human virus methods was generally close to 100% and positive predictive values were ~40 to 70% with the exception of NoVs, which were not detected. The bacteriophage methods were generally much less specific toward human sewage than virus methods, although FRNAPH II genotyping was relatively successful, with 18% sensitivity and 85% specificity. While the specificity of the human virus methods engenders great confidence in a positive result, better concentration methods and larger sample volumes must be utilized for greater accuracy of negative results, i.e., the prediction that a human contamination source is absent.

Introduction

Coastal waters impacted by fecal contamination pose a health risk to recreational users and shellfish consumers. To identify contaminated waters, fecal indicator bacteria (FIB) such as enterococci are typically monitored. However, analysis for conventional FIB cannot distinguish sources of contamination. The Source Identification Protocol Project (SIPP) was conducted to evaluate methods that may be useful to identify sources of fecal contamination in water. The strategy of the study was to share samples that were intentionally contaminated with fecal material and blinded with respect to source with multiple laboratories to determine if methods under development could correctly identify sources of fecal pollution. This study constitutes the largest multi-laboratory study on microbial source tracking (MST) conducted to date, and provides a rare opportunity to compare the performance of a variety of viral markers for specific contamination sources on a head-to-head basis.

While some studies have shown a positive relationship between FIB levels and gastrointestinal (GI) illness (Kay *et al.* 1994; Wade *et al.* 2003, 2006), other studies have found no relationship between FIB and the presence of human pathogens (Jiang *et al.* 2001, Noble and Fuhrman 2001, Boehm *et al.* 2003, Jiang and Chu 2004, McQuaig *et al.* 2012,) or with GI illness (Colford *et al.* 2007). Viruses are implicated as important, or even dominant etiological agents of waterborne and shellfish-borne disease (WHO 2003, Westrell *et al.* 2010), and their fate and transport in aquatic environments may well be very different than that of bacteria. Viruses are therefore increasingly used as MST tools (Noble *et al.* 2003, McQuaig *et al.* 2012).

The use of viruses for MST has a number of advantages over using bacterial markers. First, measuring pathogenic viruses directly may provide a more accurate measure of GI risk, eliminating errors introduced by weak correlations between bacteria and GI illness or viral pathogens. Second, the morphology of many non-pathogenic viruses is similar to that of viral pathogens, and some studies have found that they exhibit similar fate and transport in the environment (Savichtcheva and Okabe 2006). Thus, their decay rate through wastewater treatment and/or in polluted waters may be more similar to viral pathogens than other indicators (Walters *et al.* 2009), although some studies on drinking water have found that adhesion characteristics (Pelleieux *et al.* 2012) and removal rates (Boudaud *et al.* 2012) for bacteriophages MS2, Qβ and GA differ among these phages. Third, many pathogenic and non-pathogenic viruses are highly host-specific (Noble *et al.* 2003, Cox *et al.* 2005, McQuaig *et al.* 2006, McQuaig *et al.* 2009, McQuaig *et al.* 2012), which improves confidence in identification of pollution sources. Virus concentrations in waste can be similar to that of FIB, e.g., human polyomaviruses at ~10^4·ml^{-1} in untreated sewage (McQuaig *et al.* 2009) and human adenoviruses at ~10^5·ml^{-1} (Bofill-Mas *et al.* 2006).

A disadvantage of the use of viruses for MST is the relatively low concentrations of some viruses in polluted waters, which can lead to low sensitivity in analysis of environmental samples (Harwood *et al.* 2009, Staley *et al.* 2012, Wong *et al.* 2012). Certain viruses are shed in high numbers in the feces of infected individuals (Melnick and Rennick 1980). However, the number of infected individuals within a population varies depending on the season and etiological agent, as well as the general health of the population. Dilution after waste enters the environment can also lead to low viral concentrations in aquatic environments. This issue is compounded by the methodological challenges encountered in concentrating and enumerating viruses. The basic

steps for virological analysis of water include sample concentration, nucleic acid extraction, and molecular detection. These procedures can be expensive, time-consuming, and they often have poor to mediocre recovery rates, particularly when viral concentrations are low (McQuaig *et al.* 2009, Stewart *et al.* 2008, Wyn-Jones *et al.* 2011, Wong *et al.* 2012).

The pathogenic viruses used in the SIPP study, including adenoviruses, enteroviruses, and noroviruses, were chosen for their association with water-related GI illness, their ability to persist in sewage, sometimes through water treatment processes, and their widespread distribution in human populations. Norovirus is thought to be the dominant etiological agent for GI illness from exposure to recreational waters in developed countries (Atmar and Estes 2006, Svraka *et al.* 2007, Sinclair *et al.* 2009, Soller *et al.* 2010). Studies worldwide have also frequently detected enteroviruses (Reynolds *et al.* 1998, Noble and Fuhrman 2001, Moce-Llivina *et al.* 2005, Sassoubre *et al.* 2012) and adenoviruses in recreational waters (Jiang *et al.* 2001, Hundesa *et al.* 2006, Wyn-Jones *et al.* 2011). Enteroviruses can tolerate a range of temperatures and salinities (Skraber *et al.* 2004, Wetz *et al.* 2004) as well as residual chlorine (Keswick *et al.* 1984). Adenoviruses have been found to be more resistant to UV disinfection than other viruses (Thurston-Enriquez *et al.* 2003).

Nonpathogenic human polyomaviruses (BK and JC) and bacteriophages were also used as source-specific or general markers of contamination in the SIPP study. HPyVs are rarely pathogenic and they are prevalent in sewage influent and onsite wastewater disposal system (septic) tanks due to their wide distribution in human populations and excretion in urine and feces (Markowitz *et al.* 1993, Bofill-Mas *et al.* 2000, Polo *et al.* 2004, Vanchiere *et al.* 2005, Hundesa *et al.* 2006, Vanchiere *et al.* 2009). HPyVs have successfully been used as MST tools, and are highly human-specific (Bofill-Mas *et al.* 2006; McQuaig *et al.* 2006, 2009, 2012; Harwood *et al.* 2009, Gourmelon *et al.* 2010). Bacteriophages are viruses that infect bacteria, and they have been used for decades as indicators of enteric viruses in sewage (reviewed in Chapter 6: Phage Methods in Jofre *et al.* 2011). Relatively simple and inexpensive culture-based assays for bacteriophage enumeration have been developed as standard methods in the European Union (Anon 2000, 2001). Some bacteriophages, including FRNAPH and *Bacteroides* phages, can be useful for microbial source tracking of human fecal contamination (reviewed in Chapter 6: Phage Methods in Jofre *et al.* 2011). FRNAPH, for example are classified into four main genotypes, two of which (II, and III) predominate in wastewater effluents and human fecal samples and two of which (I and IV) are mainly associated with animal feces and effluents from animal-rearing facilities or slaughterhouses (Hsu *et al.* 1995, Gourmelon *et al.* 2010).

This paper focuses on the performance of the viral markers measured during the SIPP study. Water samples contaminated with single-source or mixed-source fecal material were evaluated by laboratories which volunteered for the study, and which were already using the methods. No effort was made to harmonize virus enumeration methodologies across participating laboratories, as a major goal of the study was to incorporate variation at the laboratory scale into viral marker performance. Due to logistical limitations of the very large study, sample sizes were standardized across bacterial and viral methods (Boehm *et al.* 2013). Crucial performance characteristics including specificity, sensitivity and detection limits (Stoeckel and Harwood 2007) of viral markers were compared to help identify methods with the most promise for identifying sources of fecal waste in water. These results are intended to help provide the best tools to water resource managers and policy makers who work to protect public health in coastal areas.

Methods

Participants

The methods are organized by participating laboratories. The institutions, locations, and abbreviations used are given below, and the method(s) performed by each laboratory are provided in Table 1. The laboratories involved in this study were: Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), France; Federal Office of Public Health (FOPH), Switzerland; Southern California Coastal Water Research Project (SCCWRP), USA; Stanford University (Stanford), USA; TetraTech, USA; University of Brighton (UB), United Kingdom; University of North Carolina Chapel Hill (UNC-CH), USA; University of North Carolina Chapel Hill Institute of Maine Science (UNC-CH-IMS), USA; University of South Florida (USF), USA; University of Southern California (USC), USA; Wayne State University (WSU), USA.

Table 1. Summary of virus, targeted host, and method type. The theoretical limit of detection (LOD_T) is expressed as gene copies per filter (50 ml samples were filtered) or plaques per 50 ml.

Human Virus Designation and Lab	Virus Type	Target	Method	Primer/Probe Sequences	Reference	LOD_T
EV_Stanford EV_SCCWRP EV_SCCWRP[a]	Enterovirus	Human	Reverse Transcription QPCR	EVupstream: CCT CCG GCC CCT GAA TG EVdownstream: ACC GGA TGG CCA ATC CAA Pan-enterovirus Probe: FAM- ACG GAC ACC CAA AGT AGT CGG TTC–BHQ	(Walters et al. 2009, Gregory et al. 2006, De Leon et al. 1990)	20
EV_USC	Enterovirus	Human	Reverse Transcription QPCR	EV1 (reverse): GAT TGT CAC CAT AAG CAG C EV 2 (forward): CCC CTG AAT GCG GCT AAT C EV Probe: FAM-CGG AAC CGA CTA CTT TGG GTG TCC GT-BHQ	(Fuhrman et al. 2005, Monpoeho et al. 2001)	10
EV_UNC-CH	Enterovirus	Human	Reverse Transcription QPCR	Upstream: GGC CCC TGA ATG CGG CTA AT Downstream: CAC CGG ATG GCC AAT CCA A Probe: FAM-CGG ACA CCC AAA GTA GTC GGT TCC G-TAMRA	(Donaldson et al. 2002)	15
HAdV_Stanford HAdV_TetraTech[a]	Adenovirus	Human	QPCR	JTVXF: GGA CGC CTC GGA GTA CCT GAG JTVXR: ACI GTG GGG TTT CTG AAC TTG TT JTVXP: FAM-CTG GTG CAG TTC GCC CGT GCCA-BHQ	(Jothikumar et al. 2005)	20
HAdV_UNC-CH_IMS	Adenovirus	Human	Conventional PCR	Ad1: TTCCCCATGGCICAYAACAC Ad2: CCCTGGTAKCCRATRTTGTA	(Xu et al. 2001)	20
NoV GI_UNC-CH	Norovirus (Group I)	Human	Reverse Transcription QPCR	JJV1F: GCC ATG TTC CGI TGG ATG JJV1R: TCC TTA GAC G CC ATC ATC AT JJV1P: FAM-TGT GGA CAG GAG ATC GCA ATC TC-BHQ	(Jothikumar et al. 2005b)	15
NoV GII_UNC-CH	Norovirus (Group II)	Human	Reverse Transcription QPCR	JJV2F: CAA GAG TCA ATG TTT AGG TGG ATG AG COG2R: TCG ACG CCA TCT TCA TTC ACA RING2-TP: FAM-TGG GAG GGC GAT CGC AAT CT-BHQ	(Jothikumar et al. 2005b, Kageyama et al. 2003)	15
NoV GII_ Stanford	Norovirus (Group II)	Human	Reverse Transcription QPCR	QNIF2d: ATG TTC AGR TGG ATG AGR TTC TCW GA COG2R: TCG ACG CCA TCT TCA TTC ACA QNIFS - FAM- AGC ACG TGG GAG GGC ATC G–TAMRA	(da Silva et al. 2007, Jothikumar et al. 2005b, Kageyama et al. 2003)	20
HPyV_USF HPyV_TetraTech	Polyomaviruses BK and JC	Human	QPCR	SM2:AGT CTT TAG GGT CTT CTA CCT TT KGJ3:TCA TCA CTG GCA AAC AT P6:GGT GCC AAC CTA TGG AAC AG	(McQuaig et al. 2009)	20

[a] Samples were acidified prior to filtration.

Table 1. Continued

Bacteriophage and Lab	Phage Type	Target	Method	Primer/Probe Sequences	Reference	LOD_t
GB-124_UB & FOPH	*Bacteroides* GB-124 Phage	Human	Double Layer Agar	NA	(Anon 2000, Ebdon et al. 2007)	50
WG-5_UB	Somatic Coliphage on host WG-5	Human	Double Layer Agar	NA	(Anon 2001)	50
MB-55_WSU	*Enterococcus* Phage on host MB-55	Human	Double Layer Agar	NA	(Vijayavel et al. 2010)	10
HB-73_WSU	*Bacteroides* Phage on host HB-73	Human	Double Layer Agar	NA	(Vijayavel et al. 2010)	10
FRNAPH_IFREMER	F-specific RNA Bacteriophage on host *S. enterica* WG-49	General	Double Layer Agar	NA	(ISO 1995)	25
FRNAPH_IFREMER	Genotype II FRNAPH	Human	Reverse Transcription QPCR on isolated plaques	GIIF : TGCAAACCTAACTCGGAATGG GIIR : AGGAGAGAACGCAGGCCTCTA GIIP : FAM-TCCCTCTATTTCCTC-MGBNFQ	(Ogorzaly and Gantzer 2006)	NA

Sample Handling, Concentration, and Nucleic Acid Extraction

All collection and preparation of fecal ("challenge") samples were carried out by SCCWRP, in Costa Mesa, CA. Sample collection, preparation, and shipping procedures are detailed in a companion paper that provides an overview of the entire inter-laboratory study (Boehm *et al.* 2013). Briefly, artificial freshwater (distilled water with 0.3 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 1.4 mM $NaHCO_3$; Boehm *et al.* 2013) was intentionally contaminated with fecal and/or sewage samples from various sources. All fecal samples were composites from at least twelve individuals. Nineteen "singleton" samples were inoculated with one fecal source (chicken, deer, dog, goose, gull, horse, pig, pigeon, cow, human feces, septage or sewage), and 13 doubleton samples were inoculated with two fecal sources each at volumetric ratios of 9:1. Six of the singleton samples contained human fecal material. Seven of the singleton samples were created at both full strength and at 1:10 diluted strength. All of the doubleton samples contained a human source. A full list of the samples can be found in Table 2 of Boehm *et al.* (2013). Duplicates of each sample were processed as described below so that each participating lab received 64 filters or liquid samples for processing with their method. All filters (see below for method details) were frozen in liquid nitrogen and shipped on dry ice, while liquid samples for bacteriophages were shipped on blue ice. Procedures are organized below by participating laboratory. Viral targets used in the study, laboratories, primers, probes and citations for methods are presented in Table 1. Enterococci (ENT) concentrations were also measured using membrane filtration in each sample by USEPA Method 1600 (USEPA 2002), with method details reported elsewhere (Boehm *et al.* 2013).

SCCWRP

Human enteroviruses (EV) were enumerated in 50 ml challenge samples filtered through 0.45 µm mixed cellulose filters (Millipore, MA). Replicate volumes of each sample were acidified with 10% HCl until a pH of 3.5 was reached and then filtered as before. Filters were stored at -80°C until extraction. Viral nucleic acids were extracted using the QIAamp MinElute Virus Spin Kit (Qiagen, Valencia, CA). The extraction was performed according to manufacturer instructions and 40 µl was eluted. One unamended filter and one acidified

filter were extracted for each sample. Nucleic acid extracts were stored at -80°C until analysis.

Stanford

Human enteroviruses (EV), adenoviruses (HAdV), and norovirus II (NoV GII) were enumerated in 50 ml challenge samples filtered through 0.45 μm mixed cellulose filters (Millipore, MA). Magnesium chloride ($MgCl_2$) was added to samples before membrane filtration to increase viral recover by facilitating virus attachment to the filters (Mendez *et al.* 2004). Briefly, 1 ml of 5 M $MgCl_2$ was added to 50 ml of sample for a final concentration of 0.1 M $MgCl_2$ before membrane filtration. Filters were stored at -80°C until extraction. Viral nucleic acids were extracted using the QIAamp MinElute Virus Spin Kit (Qiagen, Valencia, CA) according to manufacturer instructions and 40 μl was eluted. For each sample, nucleic acids were extracted from two filters and the eluants combined to provide enough volume for all the viral assays being run. Nucleic acid extracts were stored at -80°C until analysis.

Tetra Tech

Human polyomaviruses (HPvYs) and HAdV were concentrated according to a previously published protocol (Katayama *et al.* 2002). Samples were acidified to pH 3.5 with HCl and were then filtered through type HA, negatively charged membranes (Millipore, Billerica, Mass.) with a 47 mm diameter and a 0.45 μm pore size. Filters were stored in 1.5 ml microcentrifuge tubes and shipped on ice to analytical laboratory. At the analytical laboratory, filters were stored at -80°C prior to further processing. Viral nucleic acid was extracted and purified using Qiagen QIAamp MinElute Virus Spin Kit (Qiagen, Valencia, CA) following the manufacturer's protocol with minor modifications. Upon removal from the freezer, 400 μl of RNAse free water was added into each tube with filter and pulse-vortex for 30 seconds to release viral particles from the filters. Purification steps were then performed according to manufacturer's protocol. Purified viral RNA/DNA was eluted in 100 μl of RNase-free water and stored at -20°C.

UNC-CH

Human enteroviruses (EV), human norovirus I (NoV GI) and norovirus II (NoV GII) were enumerated in 50 ml challenge samples filtered through 0.45 μm mixed cellulose filters (Millipore, MA). Filters were stored at -80°C until extraction. Viral nucleic acids were extracted using a modified version of the RNeasy Mini Kit (Qiagen, Valencia, CA) as described previously (Gregory *et al.* 2006). Nucleic acid extracts were eluted into 30 μl of DNase- and RNase-free water and stored at -80°C until analysis.

UNC-CH-IMS

Human adenoviruses (HAdV) were enumerated in 50 ml challenge samples filtered through 47 mm HA filters with a pore size of 0.45 μm. Filters were stored at -80°C until further processing. Frozen filters were transferred to 2 ml semi-conical screw-cap tubes loaded with 0.3 g of 0.1 mm glass beads (BioSpec, Bartlesville, OK) and 990 μl of AE Buffer (Qiagen, Valencia, CA). Tubes were bead beaten for 2 minutes at maximum speed and centrifuged for 1 minute at 12,000 x g. Supernatant were transferred to 1.7 ml microtubes and centrifuged again for 5 minutes at 12,000 x g. Supernatant was transferred carefully to new 1.7 ml microtubes, and DNA was extracted using DNA-EZ RW01 kits (GeneRite, New Brunswick, NJ) following manufacturer instructions.

USC

Human enteroviruses (EV) were enumerated in 50 ml challenge samples filtered through 47-mm nitrocellulose filters with a pore size of 0.45 μm (Millipore, MA). RNA was extracted from filters using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer instructions with modifications as specified in (Fuhrman *et al.* 2005).

USF

Human polyomaviruses BK and JC (HPyV) were enumerated in 50 ml challenge samples filtered through 47 mm nitrocellulose filters, pore size 0.45 μμm, after sample pH was adjusted to 3.5 using 2.0 N HCl (McQuaig *et al.* 2009). Filters were immediately frozen at -80°C until they were analyzed (within 30 days of receipt). DNA was extracted from filters by mechanical disruption (bead beating) using GeneRite bead tubes (North Brunswick, NJ).

Bacteriophage Analysis (IFREMER, FOPH, UB, WSU)

Laboratories received 50 ml of each raw (unfiltered) challenge sample. Samples were shipped on blue ice. One to 5 ml of sample was added to a

suspension of the appropriate host for enumeration of *Bacteroides* phages, somatic coliphages, FRNAPH and *Enterococcus* phages (see the Analytical Methods section). FRNAPH genotyping was carried out on isolated plaques obtained using a previously published protocol (Mauffret *et al.* 2012).

Analytical Methods

Challenge samples were tested for inhibition of qPCR reactions prior to shipment from SCCWRP to the individual laboratories (see Boehm *et al.* 2013 for details). Individual laboratories also tested for inhibition using qualitative methods such as running conventional PCR for 16S rRNA or general *Bacteroidales* on the sample, semi-quantitative methods such as diluting samples 1:5 or 1:10 and comparing C_T values to those obtained for undiluted samples (Cao *et al.* 2012), or quantitative methods using a commercially-supplied internal control (QuantiFast Pathogen +IC Kit; Qiagen, Valencia, CA; data not shown). Few instances of inhibition were noted by any of the laboratories and when they were, samples were diluted 1:5 or 1:10 and re-analyzed.

SCCWRP

EV were enumerated by reverse transcription-QPCR (RT-QPCR) on a BioRad CFX 96 thermocycler using TaqMan® RNA-to-Ct™ 1-Step Kit (Applied Biosystems, CA) using the protocols cited (De Leon *et al.* 1990, Gregory *et al.* 2006, Walters *et al.* 2009). Cycling parameters included a 15-minute RT step at 48°C, followed by a 10-minute denaturation step at 95°C, then 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Fluorescence data were analyzed using BioRad CFX96 software v2.0 with a threshold fluorescence value of 100. RNA standards were made by *in vitro* transcription of plasmids extracted from an *E. coli* clone. Standards were quantified using a Nanodrop-1000 (Thermo Scientific, Wilmington, DE) and serially diluted to make standard curves. The highest concentration of enterovirus standard was $1.5*10^6$ PFU ml^{-1}. Standard curves were run in triplicate on every qPCR plate containing samples. All enterovirus standard curves were 'pooled' and the 'pooled' standard curves were then used to relate quantification cycles (C_q) to copy numbers and quantify samples (Sivaganesan *et al.* 2010).

Stanford

HAdV were enumerated by QPCR on an Applied Biosystems StepOnePlus real-time PCR system using TaqMan chemistry (Jothikumar *et al.* 2005a). Each sample was run in triplicate. Thermocycling parameters included 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 55°C for 1 minute. Fluorescence data were analyzed using Applied Biosystems StepOnePlus software v2.0 with a threshold of 0.03. Standard curves were generated from *E. coli* plasmid DNA and run in triplicate with every set of samples, and then pooled by the same method as the SCCWRP protocol described above.

EV were enumerated by reverse transcription-QPCR (RT-QPCR) on an Applied Biosystems StepOnePlus thermocycler using TaqMan RNA-to-Ct 1-Step Kit using previously published protocols (De Leon *et al.* 1990, Gregory *et al.* 2006, Walters *et al.* 2009). Samples were run in triplicate. Cycling parameters included a 15-minute RT step at 48°C, followed by a 10-minute denaturation step at 95°C, then 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Fluorescence data were analyzed using Applied Biosystems StepOnePlus software v2.0 with a threshold of 0.03. RNA standards were made by *in vitro* transcription of plasmids extracted from an *E. coli* clone. Standards were quantified using a Nanodrop-1000 and serially diluted to make standard curves. Standard curves were run in triplicate with every set of samples and then pooled.

NoV GII were enumerated by reverse transcription-QPCR (RT-QPCR) on an Applied Biosystems StepOnePlus thermocycler using TaqMan RNA-to-C 1-Step Kit according to previously described methods (Kageyama *et al.* 2003, Jothikumar *et al.* 2005b, da Silva *et al.* 2007). Samples were run in triplicate. Cycling parameters included a 15-minute RT step at 48°C, followed by a 10-minute denaturation step at 95 °C, then 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Fluorescence data were analyzed with a threshold of 0.005. RNA standards were made by *in vitro* transcription of plasmids extracted from an *E. coli* clone. Standards were quantified using a Nanodrop-1000 and serially diluted to make standard curves. Standard curves were run in triplicate with every set of samples, and then pooled.

Tetra Tech

HPyV and HAdV analyses were performed by QPCR using the Quantifast Pathogen PCR + IC kits

following the manufacturer's suggestion for PCR cycling conditions. Primers and probes sequences for each target organisms were adapted from the literature (Table 1). The Quantifast Pathogen PCR kit was supplemented with an Internal Control DNA and a standardized Internal Control assay. The presence of PCR inhibitor in the samples was determined by the deviation of ±3 threshold cycles of mean Ct value of the internal control. Samples that showed PCR inhibition were diluted and reanalyzed. Non-linearized plasmids with target DNA inserts (DNA2.0, Menlo Park, CA) were used as DNA standards for all target organisms. Concentration of each DNA standard was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). DNA standards were serially diluted to obtain standard curves. All real-time PCR reactions were performed on RotorGene Q (Qiagen, Valencia, CA). Sensitivities of these assays range between 10^1 to10^2 plasmid copies per PCR reaction. PCR analyses of all samples were performed in duplicate. All qPCR runs included at least one negative control reaction (PCR-grade H_2O without template) and a positive control reaction.

UNC-CH

EV was detected by reverse transcription-PCR (RT-PCR) on a Cepheid SmartCycler thermocycler using a Quantitect Probe RT-PCR kit (Qiagen) using previously published primers and probes (Donaldson *et al.* 2002). The RT-PCR reaction mixture contained 2 µl of sample, each primer at a concentration of 500 nM, each probe mixture at a concentration of 120 nM, 12.5 µl of 2X RT-PCR buffer, 0.3 µl of 25X RT-PCR enzyme mix, and nuclease-free water for a total reaction mixture of 25 µl. The reaction mixture was subjected to a one-step assay on using the following conditions: RT for 30 minutes at 50°C and 15 minutes at 95°C, followed by 45 cycles of 15 seconds at 94°C and 1 minute at 60°C. All amplification reactions were carried out in duplicate. Fluorescence data was analyzed using Cepheid SmartCycler software with a threshold of 30. All amplification reactions were carried out in duplicate. Samples that gave a positive result in either or both of the duplicate reactions were amplified by RT-PCR again. Only after a sample gave a second positive result was it counted as an overall positive.

NoV GI and GII were enumerated by reverse transcription-QPCR (RT-QPCR) on a Cepheid SmartCyler using a Quantitect Probe RT-PCR kit using previously published primers and probes (Jothikumar *et al.* 2005b). The RT-PCR reaction mixture was the same as for EV run by this lab. The reaction mixture was subjected to a one-step assay using the following conditions: RT for 30 minutes at 50°C and 15 minutes at 95°C, followed by 45 cycles of 15 seconds at 94°C and 1 minute at 60°C. All amplification reactions were carried out in duplicate. Fluorescence data was analyzed using Cepheid SmartCycler software with a threshold of 30. RNA standards were a NoV GI.4 RNA transcript and a NoV GII.1 RNA transcript (courtesy J. Vinjé, CDC) that were serially diluted to make standard curves.

USC

EV were enumerated by reverse transcription-QPCR (RT-QPCR) on a Stratagene MX3000, by a modification of the 2-step protocol from Monpoeho *et al.* (2001) as described by Fuhrman *et al.* (2005).

UNC-CH-IMS

HAdV were detected using a conventional PCR assay targeting the hexon gene (Xu *et al.* 2001). Each sample was run in duplicate. Each 50 µl reaction contained 5 µl of sample DNA extract, each primer at a concentration of 0.2 µM, 50 mM $MgSO_4$, 0.2mM (each) dNTP, and one unit of Platinum Taq HiFi (Invitrogen, Grand Island, NY). Reactions were thermal cycled on a MyCycler (BioRad, Hercules, CA) in two stages: i) 94°C for 2 minutes, and ii) 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 1 minute. PCR products were visualized on a 1.0% agarose gel stained with ethidium bromide and visualized on a GelDoc imaging system (BioRad, Hercules, CA).

Bacteriophage Analysis (IFREMER, FOPH, UB, WSU)

Enumeration of somatic coliphages was carried in accordance with standard methods (Anon 2000) using the host strain *E. coli* WG-5, and was based on a double agar plaque count procedure similar to that described below for *Bacteroides* phage detection (Anon 2001). Screw-topped glass tubes (Hach, UK) containing BPRM broth were used to grow host strains GB-124, HB-73, & MB-55 (1 ml host in 12 ml broth) to the correct optical density (approx. 0.33 at 620 nm) for phage detection. Once the correct optical density was reached (usually within 3 hour), host strains were placed on melting ice and used within 4 hours. All samples were

filtered using 0.22 mm polyvinylidene difluoride (PVDF) membrane syringe filters (Millipore, US) to remove any background bacterial contamination before phage detection. On each occasion, 1ml of the filtrate (or dilution thereof) and 1ml of log phase *Bacteroides* host strain GB-124 was added to a sterile 10 ml disposable test tube containing 2.5 ml of semi-solid BPRM agar (Ebdon *et al.* 2007); log phase *Bacteroides* strain (HB-73) and *Enterococcus faecium* strain (MB-55; 1 ml) and 5 ml of sample filtrate were added to 4 ml of 2 X BPRM agar (Vijayavel *et al.* 2010) and mixed gently to avoid bubble formation. The contents were then poured onto the surface of BPRM agar and left to solidify. The plates were inverted and incubated at 36ºC (±2 ºC) for 18 (±2) hours in anaerobic jars containing anaerobic sachets (Anaerogen, Oxoid, UK). The presence of phages resulted in the production of visible plaques (zones of lysis) in a confluent lawn of the host bacterium. All samples were analyzed in at least duplicate and expressed as the mean number of plaque forming units (PFU)·100 ml^{-1}.

FRNAPH were counted according to the ISO 10705-1 method by analyzing 2 ml of each sample using the host strain *Salmonella enterica* Typhimurium WG-49. In addition, for samples with low virus levels, a concentration step was performed on the remaining 48 ml of sample by flocculation with $MgCl_2$ (0.05 M) followed by filtration through 0.22 µm filters (GSWP047S0, Millipore, St Quentin en Yvelines, France). The viruses on the filter were then recovered in 5 ml eluent (Gourmelon *et al.* 2007) and analyzed as described above, however, bacteriophage isolated following the secondary concentration step were not used in the estimation of concentration, but were used for typing. Plaques were individually picked and stored in 15% PBS-glycerol at -20°C until genotyping. Isolates were cultivated on Petri dishes with or without RNase. Isolates that were not sensitive to this treatment corresponded to DNA bacteriophages and were removed from the analysis. FRNAPH were genotyped using a QuantiTech probe RT-PCR kit (Qiagen, France) and previously published primers (Ogorzaly and Gantzer 2006). When less than five plaques could be isolated for typing, the result was reported as "non-conclusive".

Data Reporting

All data were reported by the participating laboratories on a common spreadsheet. The units for QPCR methods were gene copies·$filter^{-1}$ and those for bacteriophage methods were plaque forming units (PFU)·50 ml^{-1}. The theoretical limit of detection (LOD_T) was calculated by assuming that 1 gene copy or PFU could be detected in a given test (PCR reaction or plate), and subsequently calculating the minimum quantity that must be present on a filter (or in 50 ml) of sample to be detected given the concentration factor through processing and the volume used in each method. The LOD_T calculation also assumes 100% recovery through processing, and is therefore an optimistic estimate of the LOD. Results for samples in which target was detected, but reported by the participating laboratories at levels below the LOD_T were considered positive, but were not quantified. Conventional (binary) PCR methods were reported as +/- results.

Statistical Analysis

All virus and bacteriophage data sets were translated into binary data indicating presence or absence. The FRNAPH typing data was translated into a binary data set indicating the presence or absence of human feces (i.e., genotype II): if the typing indicated human was present (either alone or with animals), then it was considered positive for human feces; if the typing was inconclusive (less than five plaques available for typing), or if no plaques were present, it was considered negative for human feces. Statistical analyses were carried out using SPSS Statistics version 20.0.0 (IBM, Foster City, CA, USA). The agreement between the binary data sets was determined using the phi coefficient. A one-way ANOVA was used to assess whether somatic coliphage and enterococci concentrations were significantly higher when individual human viruses, human-associated bacteriophages, or FRNAPH were present. Somatic coliphage and enterococci concentrations were log_{10}- transformed for statistical analysis. Statistical significance was determined at $\alpha = 0.05$.

The sensitivity, specificity, positive predictive value, and negative predictive value were calculated for each assay using Matlab version 2009b (Natick, MA). The formulas for these metrics are provided below, where true positive is abbreviated TP, false positive is FP, true negative in TN, and false negative is FN, and all are expressed as percentages. Sensitivity, or the ability of the test to detect a contamination source when it is present, was calculated as sensitivity = TP $(TP+FN)^{-1}$. Specificity, or the ability of a test to detect only the target contaminant

source and no other, was calculated as specificity = TN $(FP+TN)^{-1}$. Positive predictive value, or the frequency at which a positive test result is a true positive, was calculated as PPV = TP $(TP+FP)^{-1}$. Negative predictive value, or the frequency at which a negative test result is a true negative, was calculated as NPV = TN $(TN+FN)^{-1}$. Variables were treated as binary observations (+ or -) for all of these calculations.

Results

Relatively small volumes were used in the PCR and bacteriophage assays; therefore the theoretical limits of detection (LOD_T) were relatively insensitive, ranging from 10 to 50 gene copies·filter^{-1} or plaques·50 ml^{-1}. The sensitivity and NPV of the virus methods toward challenge samples containing target fecal material tended to be rather low, while specificity and PPV was generally much higher (Tables 2 and 3). Table 2 shows performance measures for the dataset that includes all challenge samples, including singletons and doubletons (n = 64), and Table 3 contains the results for the dataset that includes only singleton challenge samples (n = 38).

The human viruses were generally highly specific toward human fecal sources (Tables 2 and 3), ranging from 84.6 to 100% when considering the dataset containing all samples (Table 2). Cross-reactivity of the assays was observed for two of the EV methods (USC and SCCWRP) toward pig feces, and lowered both specificity and PPV compared to the other human virus methods. In fact, USC and SCCWRP both detected EV in three samples contaminated with pig feces and in all four sewage/pig doubleton samples, while the other two laboratories did not detect EV in these samples. The incomplete specificity of the two EV assays was still evident when considering results from only singleton challenge samples (Table

Table 2. All samples (38 singletons and 26 doubletons) used to calculate performance measures including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Data for general (non host-specific) fecal indicators are in shaded rows.

Human Viruses	Sensitivity %	Specificity %	PPV %	NPV %
EV_USC (qPCR)	13.2	84.6	55.6	40.0
EV_UNC-CH (qPCR)	0.0	100.0	NC[a]	40.6
EV_Stanford (qPCR)	0.0	100.0	NC	39.3
EV_SCCWRP (qPCR)	10.5	88.5	57.1	40.4
EV_SCCWRP_acid (qPCR)	0.0	100.0	NC	40.6
AdV_TetraTech (qPCR)	13.2	100.0	100.0	44.1
AdV_Stanford (qPCR)	5.4	100.0	100.0	40.7
AdV_UNC-CH-IMS (PCR)	5.3	100.0	100.0	41.9
NoV GI_UNC-CH (qPCR)	0.0	100.0[c]	NC	40.6
NoV GII _UNC-CH (qPCR)	0.0	100.0[c]	NC	40.6
NoV GII _Stanford (qPCR)	0.0	100.0[c]	NC	39.3
HPyVs_TetraTech (qPCR)	7.9	100.0	100.0	42.6
HPyVs_USF (qPCR)	10.5	100.0	100.0	43.3
Bacteriophage				
MB-55	0.0	96.2	0.0	39.7
HB-73	26.3	80.8	66.7	42.9
GB-124	60.5	57.7	66.7	50.0
FRNAPH Group II	18.4	84.6	63.6	41.5
FRNAPH[b]	21.1	69.2	50.0	37.5
Somatic coliphage[b]	71.1	38.5	62.8	47.6

[a]NC designates no calculation, as the formula would require division by 0.

[b]These methods are intended to be general indicators of fecal contamination, therefore sensitivity and specificity are provided only for comparison

Table 3. Singleton samples only (n = 38) used to calculate performance measures including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Data for general (non host-specific) fecal indicators are in shaded rows.

Human Viruses	Sensitivity %	Specificity %	PPV %	NPV %
EV_USC (qPCR)	8.3	84.60%	20.0	66.7
EV_UNC-CH (qPCR)	0.0	100.0[c]	NC[a]	68.4
EV_Stanford (qPCR)	0.0	100.0[c]	NC	66.7
EV_SCCWRP (qPCR)	0.0	88.50%	0.0	65.7
EV_SCCWRP_acid (qPCR)	0.0	100.0	NC	68.4
AdV_TetraTech (qPCR)	16.7	100.0	100.0	72.2
AdV_Stanford (qPCR)	16.7	100.0	100.0	70.6
AdV_UNC-CH-IMS (PCR)	0.0	100.0	NC	68.4
NoV GI_UNC-CH (qPCR)	0.0	100.0[c]	NC	40.6
NoV GII _UNC-CH (qPCR)	0.0	100.0[c]	NC	68.4
NoV GII _Stanford (qPCR)	0.0	100.0[c]	NC	66.7
HPyVs_TetraTech (qPCR)	8.3	100.0	100.0	70.3
HPyVs_USF (qPCR)	8.3	100.0	100.0	70.3
Bacteriophage				
MB-55	0.0	96.2	0.0	67.6
HB-73	25.0	80.8	37.5	70.0
GB-124	66.7	57.7	42.1	78.9
FRNAPH Group II	0.0	84.6	0.0	64.7
FRNAPH[b]	8.3	69.2	11.1	62.1
Somatic coliphage[b]	58.3	38.5	30.4	66.7

[a]NC designates no calculation, as the formula would require division by 0.

[b]These methods are intended to be general indicators of fecal contamination.

[c]Specificity was 100% because no false-positive results occurred, however, no true-positive results were observed

3); however, only pig fecal sources produced false-positive results.

None of the human virus methods displayed good sensitivity toward human fecal sources (Tables 2 and 3); however, each of the methods except NoVI and NoVII detected the target in at least two samples containing human fecal sources. The method with the best combination of sensitivity and specificity was HAdV (TetraTech), which detected human fecal contamination in five samples (13.2% sensitivity) in the complete dataset and was 100% specific (Table 2). Results for the singleton dataset (Table 3) were characterized by higher NPVs (mean 68.6% for all human viruses) compared to the complete dataset (mean 41.1%) due to the lower frequency of false-negative results in the singleton samples compared to the doubletons. The majority of human virus detections were in sewage samples (76%), followed by septage (16%), and lastly human feces (8%).

The somatic coliphage and FRNAPH methods are general indicators of fecal pollution, rather than source-specific markers. The performance measures shown in Tables 2 and 3 for these coliphages were calculated with respect to human fecal source. Somatic coliphages were present in most of the samples containing human fecal material (sensitivity = 71%), while FRNAPH were present in only 21% (Table 2). Note that the sensitivity of somatic coliphages and FRNAPH toward human fecal material decreased greatly in singleton samples (8.3%; Table 3). The human-associated bacteriophage method with the greatest sensitivity toward samples containing human waste in the complete dataset was the GB-124 bacteriophage assay (60.5%); however, this method also had low specificity and NPV, indicating a high proportion (>50%) of false-positive results (Table 2). GB-124 cross-reacted with all non-human fecal sources except deer and goose. GB-124 levels

in singleton samples from both human-derived and animal fecal samples ranged from undetectable to 700 PFU·100 ml^{-1}.

The human-associated bacteriophage methods HB-73 and FRNAPH II genotyping were more specific than GB-124 (80.8 and 84.6%, respectively for the complete dataset), but were not very sensitive toward human fecal sources (~25%), and were not as specific as most of the human virus methods. HB-73 cross-reacted with dog, goose, gull, horse, and pigeon feces, while the FRNAPH typing method identified human-associated genotype II phages in gull and pigeon feces. MB-55 was detected in only one sample, which contained cow feces.

Although quantitative methods such as qPCR or plaque counting were used for most of the methods (Table 1), the usefulness of the quantitative data is limited by the low frequency of detection of the viruses. Gene copies·filter^{-1} (50 ml of sample was applied to each filter) and fecal source are shown in Table 4 for samples in which the target was reported at concentrations above the LOD_T. Most of the quantities detected were on the order of 10^2 gene copies·filter^{-1}, with the exception of EV_USC, which was on average 6.8×10^5 gene copies·filter^{-1} when detected in samples from human fecal sources (Table 4); however, this method lacked specificity, making the quantitative data unreliable.

Agreement of results among laboratories for viruses that were measured by more than one laboratory was assessed by correlation. Results indicating the presence/absence of target in the 64 challenge samples were positively associated in some instances, e.g., EV results from SCCWRP and USC (phi coefficient = 0.87; $p < 0.05$). HAdV run by UNC-CH-IMS was also positively associated with EV run by USC and by SCCWRP (phi coefficient = 0.44 and 0.51, respectively; $p < 0.05$ for both). HAdV measured by Stanford and TetraTech were positively associated (phi coefficient = 0.28; $p < 0.05$), but there was no agreement with HAdV data from UNC-CH-IMS. Results from the HB-73 bacteriophage were positively associated with HAdV results from TetraTech (phi coefficient = 0.25; $p > 0.05$), but the human-associated bacteriophage results were not correlated with each other. Results from the two labs that ran HPyV were not correlated, however, the results for HPyV from USF were positively correlated with the results from the three labs running human adenovirus (phi coefficient = 0.41, 0.32, 0.32

Table 4. Results by laboratory for host-associated viruses from methods and samples with results above the LOD_T.

EV_USC		EV_SCCWRP		HAdV_TT		HAdV_Stanford		HPyV_TT	
Sample Type	Gene Copies[a]	Sample Type	Gene Copies[a]	Sample Type	Gene Copies	Sample Type	Gene Copies	Sample Type	Gene Copies
pig 1:10	2.1×10^5	pig 1:10	3.1×10^1	sewage	1.1×10^2	septage	7.5×10^1	human/cow 10/90	1.4×10^2
pig	8.3×10^5	pig	3.3×10^1	septage	7.8×10^1	septage	3.8×10^1	sewage	3.4×10^2
sewage/pig 90/10	2.7×10^5	sewage/pig 90/10	4.0×10^1	sewage/gull 10/90	2.9×10^2			human/dog 10/90	6.3×10^2
sewage	1.0×10^6	sewage/pig 10/90	1.3×10^2	sewage/pig 90/10	6.7×10^1				
sewage/pig 10/90	8.1×10^5	sewage/pig 10/90	3.2×10^2	sewage/gull 90/10	5.2×10^1				
sewage/pig 90/10	7.5×10^4								
sewage/pig 10/90	1.2×10^6								
pig	2.4×10^5								
pig 1:10	4.9×10^4								

[a]Gene copies·filter^{-1}

for HAdV measured by TetraTech, Stanford, and UNC-CH-IMS, respectively; p <0.05).

Levels of enterococci and somatic coliphages, both of which are general indicators of fecal contamination, are graphed in Figure 1 along with instances of detection of human viruses and human-associated bacteriophages in challenge samples. Enterococci concentrations tended to be higher in the fecal samples compared to the sewage and septage samples, and were particularly high in dog and pigeon fecal samples (~10^6 CFU·filter^{-1}). The relationship between enterococci concentrations and virus detection was determined using ANOVA, in which virus presence or absence was used as a treatment. Several of the methods showed a relationship with enterococci concentrations, i.e., EV_USC detections were associated with lower enterococci levels ($\log_{10}$ 2.69 when EV detected vs. $\log_{10}$ 3.96 when EV not detected; $P = 0.002$). A similar relationship was found for EV_SCCWRP ($\log_{10}$ 2.81 when EV detected vs. $\log_{10}$ 3.90 when EV not detected; $P = 0.019$). In contrast, when FRNAPH typing indicated the presence of a human fecal source, somatic coliphage and enterococci concentrations were both significantly higher (P <0.001 and $P = 0.004$, respectively). FRNAPH detection was associated with significantly higher enterococci levels ($\log_{10}$ 4.40 when FRNAPH detected vs. $\log_{10}$ 3.58 when FRHAPH not detected; $P = 0.015$).

Discussion

If MST methods are to be useful in a regulatory and/or management context, one must have good confidence in the performance of the method(s), e.g., that a positive or a negative result is a reliable predictor, at a minimum, of the presence/absence of the targeted source of fecal contamination. The proliferation of MST methods over the last decade has produced a potpourri of possible assays for fecal source identification whose performance is rarely directly compared. This study provided the opportunity for direct comparison; however, because of the many methods and laboratories involved, compromises in

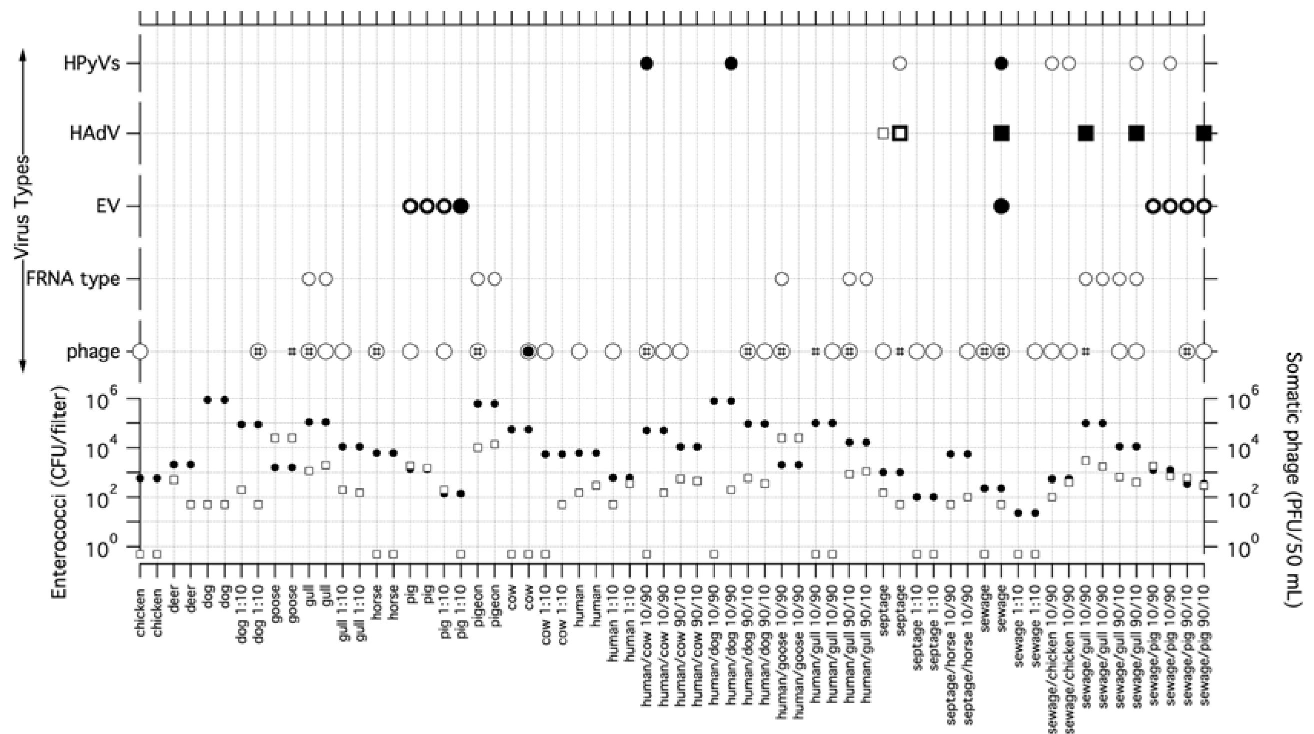

Figure 1. Concentrations of enterococci (●) and somatic coliphage (□) per filter or 50 ml challenge samples. Note that all samples containing human feces are on the right of the bottom axis. Presence of phage/virus in the challenge samples is indicated above the enterococci and phage data with different symbols for the different laboratories measuring the virus. Detection of the putative human-specific phage HB73 (#), MB55 (●), and GB124 (○) is indicated. Detection of human-associated Group II F+ RNA coliphages (○) is indicated. Detection of EV in the USC (●) and SCCWRP non-acid method (○) laboratories is indicated; no EV detected by other laboratories. The presence of HAdv in the TetraTech (■) and Stanford (□) laboratories is indicated; no HAdV detected by UNC. The presence of HPyVs in the TetraTech (●) and USF (○) laboratories is indicated.

sample composition and processing had to be made that were not optimal approaches for some methods. The virus methods, in particular, suffered in terms of sensitivity from the relatively small sample size utilized, although these manufactured samples carried quite a high level of contamination as measured by conventional FIB (median enterococci level of 6 × 10^3 CFU·filter^{-1}; Boehm *et al.* 2013). Membrane filtration of water samples does not always result in good recovery of viruses; however, McQuaig *et al.* (2012) demonstrated an average efficiency of 90.9% recovery for HPyVs with the methods utilized here. Furthermore, some viral targets such as HPyVs are shed in urine (McQuaig *et al.* 2009, Bialasiewicz *et al.* 2009) and in feces (Vanchiere *et al.* 2005, 2009; Bialasiewicz *et al.* 2009), therefore sewage rather than feces is probably a more appropriate source of reference material for such viruses. Larger sample sizes and more efficient concentration methods, such as a recently-published organic flocculation method (Calgua *et al.* 2013) would very likely improve the effectiveness of the viral markers for detecting sewage contamination.

The performance measures calculated in this study show that many of the methods designed to detect a human source of contamination, i.e., human viruses and FRNAPH genotype II, had good (>80%) to excellent specificity, meaning that they did not detect their target in waste from other host species (false-positives were infrequent). On the other hand, NPV provided a much more critical assessment of the markers in the face of a high percentage of non-detect results from the viruses. NPV measures the proportion of true-negative results among all negative results, and is therefore lowered by false-negative results (failure to detect the target when it should be present). NPVs for the human viruses in all samples were generally in the range of 35 to 45%, even though specificity was generally >85 to 100%. This result suggests that when viruses are detected in water samples they can be useful tools for identifying the source of pollution; however, when using the sample volumes and concentration methods employed in this study, water samples with undetectable or non-quantifiable viral concentrations cannot be assumed to have no human source of pollution. These results agree with recent calculations of detection limits of HPyVs in sewage diluted in environmental water samples. QPCR for HPyVs was generally not sensitive enough to detect sewage contamination at levels corresponding to elevated risk of norovirus infection, which was (calculated by quantitative microbial risk assessment (Staley *et al.* 2012).

The performance of bacteriophage-based methods for detecting human contamination varied from highly specific but insensitive (MB-55) to relatively sensitive but nonspecific (GB-124). FRNAPH genotype II had the best performance of the bacteriophages; however, like many of the methods, was not sensitive toward singleton samples containing a human fecal source, indicating that these phages are not ubiquitously distributed in the human population sampled. An advantage of the bacteriophage methods as performed in this study is that the viruses are known to be viable, as a culture step is utilized. The disadvantage of culture methods is that they do not return rapid results, unlike the several-hour turnaround time of qPCR methods that are directly applied to nucleic acid extracted from a water sample. FRNAPH genotyping as performed here requires manipulations for reverse-trancriptase PCR that adds to the time and labor of the culture method. Detection of specific FRNAPH genotypes directly by RT-PCR, without the culture step, has been demonstrated in wastewater (Ogorzaly and Gantzer 2006) and highly polluted river water (Ogorzaly *et al.* 2009); however, the direct RT-PCR methodology can be less sensitive than the method which includes a culture step, and was therefore not employed here.

Some of the viruses were measured by more than one method or laboratory, i.e., EV was tested by four labs and four methods, HAdV by three labs and two methods, and NoV GII by two labs and two methods. EV was the most subject to performance variability, as the EV_USC and EV_SCCWRP (unacidified) results were more sensitive than those of the other labs when all samples were considered; however, their specificity notably PPVs were significantly lower than those of other laboratories. The false-positive results for EV_USC and EV_SCCWRP were exclusively against challenge samples containing pig feces, and these two methods used a common set of primers and probe. Sensitivity and PPV were notably lower for the EV_USC and EV-SCCWRP methods in singleton samples than for the dataset containing all samples, which included four samples of combined human and pig waste that were not part of the singleton dataset. HAdV was more sensitive to human contamination in singleton samples than any of the EV methods, and results were consistent for

the two labs that measured HAdV by qPCR. Note that one laboratory (TetraTech) acidified samples prior to filtration while the other did not; however, the performance characteristics were not different between the two methods, which used the same primer and probe combination. HPyV was detected at about the same frequency by the two laboratories that tested them, both of which employed the same methods, and showed the typical virus pattern of low sensitivity and NPV, but high specificity and PPV. NoV GII was not detected by either laboratory that tested it (Stanford or UNC-CH).

The agreement at the sample level among the tests and laboratories reveals some interesting results. Only the norovirus methods, which failed to identify any positive samples, were in complete agreement for each sample, and this included NoV GI and NoVGII. Results for the two EV methods with high false-positive rates were significantly associated; however, much of the agreement was due to false-positive results from pig samples. Results of HAdV testing from the two laboratories that ran qPCR assays (Stanford and TetraTech) were correlated, but not results from UNC-CH-IMS, which ran conventional PCR. HPyV results were not correlated at the sample level, but results from one laboratory running HPyV were correlated with results from all three laboratories running HAdV. It is not surprising that agreement at the sample level was not generally achieved due to level of target viruses in the samples, which was generally near the LOD for the methods.

Another interesting aspect of this study was differences among the laboratories in data generation and handling. Some laboratories ran analyses in duplicate, while others ran triplicates. Although most laboratories required either two positive duplicates or two of three triplicates to call a positive result, two participants called samples with signal in one of two duplicates positive. This discrepancy highlights the need for method standardization across laboratories that carry out MST.

In this study, water was spiked with a level of fecal waste intended to mimic a plausible level of surface water contamination, i.e., an amount that resulted in ~2,000 CFU 100 ml^{-1} enterococci in the challenge samples (Boehm *et al.* 2013). This goal was not always achieved because of the inherent variability of enterococci in the waste, and enterococci concentrations in challenge samples spiked only with sewage or septage ranged from 23 (1:10 dilution of sewage) to 1015 (septage) CFU 100 ml^{-1}. Although the singleton challenge samples containing human feces had higher levels of enterococci than sewage and septage samples (~6000 CFU 100 ml^{-1}), viruses were more often detected in septage and sewage than in human feces. Septage and sewage are composite samples from many individuals; therefore, they are more likely to contain a target that is sporadically distributed in the population than a fecal sample from an individual, although it should be noted that the human fecal sample used here was a composite from twelve individuals. The inconsistent detection of human viruses in this study is supported by a previous study in which the LOD for HPyVs corresponded to 1,000 to 10,000-fold dilution of sewage containing between ~800 and 5,000 CFU 100 ml^{-1} enterococci (Harwood *et al.* 2009). In the 2009 study, 500 ml rather than 50 ml of diluted sewage was processed; therefore, consistent detection of HPyVs and, presumably, the other viruses requires more concentrated sewage samples than those used in the current study.

While the specificity and PPV of many of the virus methods is encouraging, the low sensitivity and NPV indicate that better concentration methods are needed if they are to be reliable MST markers. Observed human virus concentrations in sewage estimated by qPCR span a broad range (all concentrations expressed in gene copies L^{-1}), e.g., enteroviruses from ~10^1 to 10^8 (Katayama *et al.* 2008, Wolf *et al.* 2010); noroviruses from ~5×10^3 to 10^9 (da Silva *et al.* 2007, Katayama *et al.* 2008), adenoviruses from ~10^1 to 5×10^5 (Katayama *et al.* 2008, Fong *et al.* 2010, Wolf *et al.* 2010), and HPyVs ~10^7 (McQuaig *et al.* 2009; reviewed in Chapter 5: Viruses as Tracers of Fecal Contamination in McQuaig and Noble 2011). Virus concentrations in surface waters tend to be low except when sewage contamination is present (all concentrations expressed in gene copies L^{-1}), e.g., adenoviruses detectable to ~10^2 and enteroviruses at 10^1 to 10^2 (Sassoubre *et al.* 2012); HPyVs undetectable to ~10^6 (McQuaig *et al.* 2009, 2012); norovirus Group I at 10^3 (Sauer *et al.* 2011). The practice of concentrating large volumes (40 L or more) of surface water is commonly used for quantifying viruses in surface waters (e.g., Jiang *et al.* 2001), although the strategy of capturing viruses on negatively charged membrane filters has allowed effective use of 500 to 1,000 ml volumes (Katayama *et al.* 2002, Rigotto *et al.* 2009, McQuaig *et al.* 2012), which is less expensive and labor-intensive than using larger volumes. The results of this study and others (Harwood *et al.* 2009,

Staley *et al.* 2012), however, indicate that greater concentration factors from surface water samples must be achieved for viral targets to be effective MST markers. Alternative methods for concentration of viruses from surface waters include hollow fiber ultrafiltration (Rajal *et al.* 2007, Rhodes *et al.* 2011, Leskinen *et al.* 2010, Liu *et al.* 2012), electropositive filters such as the NanoCeram® cartridge specified in USEPA Method 1615 (USEPA 2010) and skim milk flocculation (Calgua *et al.* 2008). The necessity for secondary and/or tertiary steps to concentrate nucleic acids and/or to remove inhibitors must also be taken into consideration (Jiang *et al.* 2001, McQuaig and Noble 2011, Rhodes *et al.* 2011).

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Acknowledgements

Funding for this project has been provided in part through an agreement with the California State Water Resources Control Board. The contents of this document do not necessarily reflect the views and policies of the California State Water Resources Control Board, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. UNC-CH acknowledges the help of Neil Bhathela in analyses. Tetra Tech acknowledges Qiagen for the sponsoring of Qiagen QIAamp MinElute Virus Spin Kit and Quantifast Pathogen PCR + IC kits. WSU acknowledges funding from the Department of Biological Sciences.