Water Research
November 2013, Volume 47, Issue 18, Pages 6929–6943
http://dx.doi.org/10.1016/j.watres.2013.04.064

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

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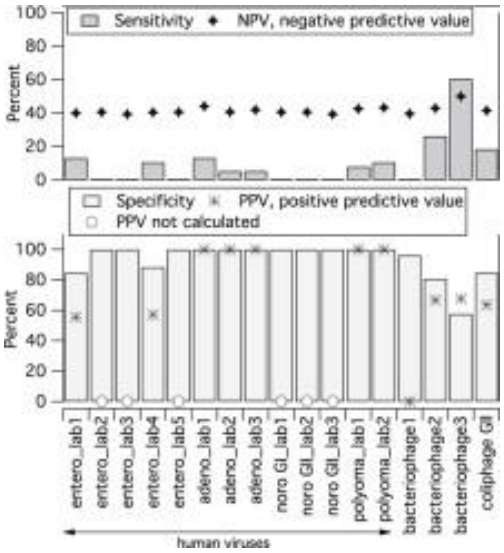
Abstract:

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 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 *Bacteroides* 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 ~40–70% 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.

Graphical abstract



Highlights

► A multi-lab comparison of virus and bacteriophage accuracy toward human feces and sewage. ► Human viruses were host-specific, but lacked sensitivity and negative predictive value. ► Bacteriophage methods either lacked sensitivity to human fecal material or were non-specific. ► Performance of insensitive methods should be improved by larger sample volumes. ► Use of larger samples requires better concentration methods that do not concentrate inhibitors.

Keywords: Virus ; Bacteriophage ; Water quality ; Fecal pollution ; Validation

1. 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 (Wade et al. 2006, Wade et al. 2003, Kay et al. 1994), other studies have found no relationship between FIB and the presence of human pathogens (Boehm et al. 2003, Noble and Fuhrman 2001, Jiang and Chu 2004, McQuaig et al. 2012, Jiang et al. 2001) 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 (McQuaig et al. 2012, Noble et al. 2003).

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 (McQuaig et al. 2012, Noble et al. 2003, Cox et al. 2005, McQuaig et al. 2006, McQuaig et al. 2009), which improves confidence in identification of pollution sources. Virus concentrations in waste can be similar to that of FIB, e.g. human polyomaviruses at ~$10^4 \cdot ml^{-1}$ in untreated sewage (McQuaig et al. 2009) and human adenoviruses at ~$10^5 \cdot ml^{-1}$ (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 (Staley et al. 2012, Harwood et al. 2009, 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, Wong et al. 2012, Wyn-Jones et al. 2011, Stewart et al. 2008).

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 (Soller et al. 2010, Sinclair et al. 2009, Atmar and Estes 2006, Svraka et al. 2007). Studies worldwide have also frequently detected enteroviruses (Noble and Fuhrman 2001, Reynolds et al. 1998, Moce-Llivina et al. 2005, Sassoubre et al. 2012) and adenoviruses in recreational waters (Jiang et al. 2001, Wyn-Jones et al. 2011, Hundesa et al. 2006). Enteroviruses can tolerate a range of temperatures and salinities (Wetz et al. 2004, Skraber 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 (Hundesa et al. 2006, Bofill-Mas et al. 2000, Markowitz et al. 1993, Polo et al. 2004, Vanchiere et al. 2009, Vanchiere et al. 2005). HPyVs have successfully been used as MST tools, and are highly human-specific (McQuaig et al. 2012, McQuaig et al. 2006, McQuaig et al. 2009, Bofill-Mas et al. 2006, 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 (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 (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 (Gourmelon et al. 2010, Hsu et al. 1995).

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.

2. Materials and Methods

2.1. *Participants*

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

157 **2.2. *Sample handling, concentration, and nucleic acid extraction***

158 All collection and preparation of fecal ("challenge") samples were carried out by the
159 Southern California Coastal Water Research Project (SCCWRP) in Costa Mesa, CA. Sample
160 collection, preparation, and shipping procedures are detailed in a companion paper that provides
161 an overview of the entire inter-laboratory study (Boehm et al. 2013). Briefly, artificial freshwater
162 (distilled water with 0.3 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 1.4 mM $NaHCO_3$ (Boehm et al. 2013))
163 was intentionally contaminated with fecal and/or sewage samples from various sources. All fecal
164 samples were composites from at least twelve individuals. Nineteen "singleton" samples were
165 inoculated with one fecal source (chicken, deer, dog, goose, gull, horse, pig, pigeon, cow, human
166 feces, septage or sewage), and 13 doubleton samples were inoculated with two fecal sources each
167 at volumetric ratios of 9:1. Six of the singleton samples contained human fecal material. Seven
168 of the singleton samples were created at both full strength and at 1:10 diluted strength. All of the
169 doubleton samples contained a human source. A full list of the samples can be found in Table 2
170 of Boehm et al. (2012). 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 s 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 min at maximum speed and centrifuged for 1 min at 12,000 x g. Supernatant were transferred to 1.7 mL microtubes and centrifuged again for 5 min 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 μm, 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 Section 2.3). FRNAPH genotyping was carried out on isolated plaques obtained using a previously published protocol (Mauffret et al. 2012).

2.3. *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) (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 (Walters et al. 2009, Gregory et al. 2006, De Leon et al. 1990). Cycling parameters included a 15 min RT step at 48 °C, followed by a 10 min denaturation step at 95 °C and then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. 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. 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. 2005). Each sample was run in triplicate. Thermocycling parameters included 95 °C for 10 min followed by 45 cycles of 95 °C

for 15 s and 55 °C for 1 min. 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 (Applied Biosystems, CA) using previously published protocols (Walters et al. 2009, Gregory et al. 2006, De Leon et al. 1990). Samples were run in triplicate. Cycling parameters included a 15 min RT step at 48 °C, followed by a 10 min denaturation step at 95 °C and then 45 cycles of 95 °C for 15 s and 60 °C for 60 s. 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 (Thermo Scientific, Wilmington, DE) 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-Ct™ 1-Step Kit (Applied Biosystems, CA), according to previously described methods (da Silva et al. 2007, Jothikumar et al. 2005b, Kageyama et al. 2003). Samples were run in triplicate. Cycling parameters included a 15 min RT step at 48 °C, followed by a 10 min denaturation step at 95 °C and then 45 cycles of 95 °C for 15 s and 60 °C for 60 s. 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 (Thermo Scientific, Wilmington, DE) and serially diluted to make standard curves. Standard curves were run in triplicate with every set of

284 samples, and then pooled.

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

300 **UNC-CH.** EV was detected by reverse transcription-PCR (RT-PCR) on a Cepheid
301 SmartCycler thermocycler using a Quantitect Probe RT-PCR kit (Qiagen) using previously
302 published primers and probes (Donaldson et al. 2002). The RT-PCR reaction mixture contained
303 2 µl of sample, each primer at a concentration of 500 nM, each probe mixture at a concentration
304 of 120 nM, 12.5 µl of 2X RT-PCR buffer, 0.3 µl of 25X RT-PCR enzyme mix, and nuclease-free
305 water for a total reaction mixture of 25 µL. The reaction mixture was subjected to a one-step
306 assay on using the following conditions: (i) RT for 30 min at 50°C, (ii) 15 min at 95°C, (iii) 45

cycles of 15 s at 94°C and 1 min 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: (i) RT for 30 min at 50°C, (ii) 15 min at 95°C, (iii) 45 cycles of 15 s at 94°C and 1 min 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 min, and (ii) 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min. PCR products were visualized on

330 a 1.0% agarose gel stained with ethidium bromide and visualized on a GelDoc imaging system
331 (BioRad, Hercules, CA).

332 **Bacteriophage Analysis (IFREMER. FOPH, UB, WSU).** Enumeration of somatic
333 coliphages was carried in accordance with standard methods (Anon 2000) using the host strain *E.*
334 *coli* WG-5, and was based on a double agar plaque count procedure similar to that described
335 below for *Bacteroides* phage detection (Anon 2001). Screw-topped glass tubes (Hach, UK)
336 containing BPRM broth were used to grow strain GB-124 (1 ml host in 12ml broth) to the
337 correct optical density (approx. 0.33 at 620 nm) for phage detection. Once the correct optical
338 density was reached (usually within 3 h), strain GB-124 was placed on melting ice and used
339 within 4h. All samples were filtered using 0.22 mm polyvinylidene difluoride (PVDF) membrane
340 syringe filters (Millipore, US) to remove any background bacterial contamination before phage
341 detection. On each occasion, 1ml of the filtrate (or dilution thereof) and 1ml of host GB-124
342 were added to a sterile 10 ml disposable test tube containing 2.5 ml of semi-solid BPRM agar
343 (Ebdon et al. 2007); log phase *Bacteroides* strains (GB-124, GA-17) and *Enterococcus faecium*
344 strains (HB-73, and MB-55) (1 mL) and 5mL of sample filtrate were added to 4 mL of 2 X
345 BPRM agar (Vijayavel et al. 2010) and mixed gently to avoid bubble formation. The contents
346 were then poured onto the surface of BPRM agar and left to solidify. The plates were inverted
347 and incubated at 36 ºC (± 2 ºC) for 18 (± 2) h in anaerobic jars containing anaerobic sachets
348 (Anaerogen, Oxoid, UK). The presence of phages resulted in the production of visible plaques
349 (zones of lysis) in a confluent lawn of the host bacterium. All samples were analyzed in at least
350 duplicate and expressed as the mean number of plaque forming units (PFU) $\cdot 100\ ml^{-1}$.

351 FRNAPH were counted according to the ISO 10705-1 method by analyzing 2 mL of each
352 sampleusing 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."

2.3. 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.

2.4. 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 either 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). Specificity, or the ability of a test to detect only the target contaminant source and no other, was calculated as specificity = TN/(FP+TN). Positive predictive value, or the frequency at which a positive test result is a true positive, was calculated as PPV = TP/(TP+FP). Negative predictive value, or the frequency at which a negative test result is a true negative, was calculated as NPV = TN/(TN+FN). Variables were treated as binary observations (+ or -) for all of these calculations.

399 **3. Results**

400 Relatively small volumes were used in the PCR and bacteriophage assays; therefore the
401 theoretical limits of detection (LOD_T) were relatively insensitive, ranging from 10 - 50 gene
402 copies·filter^{-1} or plaques·50 ml^{-1}. The sensitivity and NPV of the virus methods toward challenge
403 samples containing target fecal material tended to be rather low, while specificity and PPV was
404 generally much higher (Tables 2 and 3). Table 2 shows performance measures for the dataset that
405 includes all challenge samples, including singletons and doubletons (n=64), and Table 3 contains
406 the results for the dataset that includes only singleton challenge samples (n=38).
407 The human viruses were generally highly specific toward human fecal sources (Tables 2 and
408 3), ranging from 84.6% to 100% when considering the dataset containing all samples (Table 2).
409 Cross-reactivity of the assays was observed for two of the EV methods (USC and SCCWRP)
410 toward pig feces, and lowered both specificity and PPV compared to the other human virus
411 methods. In fact, USC and SCCWRP both detected EV in three samples contaminated with pig
412 feces and in all four sewage/pig doubleton samples, while the other two laboratories did not
413 detect EV in these samples. The incomplete specificity of the two EV assays was still evident
414 when considering results from only singleton challenge samples (Table 3); however, only pig
415 fecal sources produced false-positive results.
416 None of the human virus methods displayed good sensitivity toward human fecal sources
417 (Tables 2 and 3); however, each of the methods except NoVI and NoVII detected the target in at
418 least two samples containing human fecal sources. The method with the best combination of
419 sensitivity and specificity was HAdV (TetraTech), which detected human fecal contamination in
420 five samples (13.2% sensitivity) in the complete dataset and was 100% specific (Table 2).
421 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.

444 Although quantitative methods such as qPCR or plaque counting were used for most of the
445 methods (Table 1), the usefulness of the quantitative data is limited by the low frequency of
446 detection of the viruses. Gene copies·filter^{-1} (50 mL of sample was applied to each filter) and
447 fecal source are shown in Table 4 for samples in which the target was reported at concentrations
448 above the LOD_T. Most of the quantities detected were on the order of 10^2 gene copies·filter^{-1},
449 with the exception of EV_USC, which was on average $6.8*10^5$ gene copies·filter^{-1} when detected
450 in samples from human fecal sources (Table 4); however, this method lacked specificity, making
451 the quantitative data unreliable.
452 Agreement of results among laboratories for viruses that were measured by more than one
453 laboratory was assessed by correlation. Results indicating the presence/absence of target in the
454 64 challenge samples were positively associated in some instances, e.g. EV results from
455 SCCWRP and USC (phi coefficient = 0.87, $p<0.05$). HAdV run by UNC-CH-IMS was also
456 positively associated with EV run by USC and by SCCWRP (phi coefficient = 0.44 and 0.51,
457 respectively, $p<0.05$ for both). HAdV measured by Stanford and TetraTech were positively
458 associated (phi coefficient = 0.28, $p<0.05$), but there was no agreement with HAdV data from
459 UNC-CH-IMS. Results from the HB-73 bacteriophage were positively associated with HAdV
460 results from TetraTech (phi coefficient = 0.25, $p>0.05$), but the human-associated bacteriophage
461 results were not correlated with each other. Results from the two labs that ran HPyV were not
462 correlated, however, the results for HPyV from USF were positively correlated with the results
463 from the three labs running human adenovirus (phi coefficient = 0.41, 0.32, 0.32 for HAdV
464 measured by TetraTech, Stanford, and UNC-CH-IMS, respectively, $p<0.05$).
465 Levels of enterococci and somatic coliphages, both of which are general indicators of fecal
466 contamination, are graphed in Figure 1 along with instances of detection of human viruses and

467 human-associated bacteriophages in challenge samples. Enterococci concentrations tended to be
468 higher in the fecal samples compared to the sewage and septage samples, and were particularly
469 high in dog and pigeon fecal samples (~10^6 CFU·filter^{-1}). The relationship between enterococci
470 concentrations and virus detection was determined using ANOVA, in which virus presence or
471 absence was used as a treatment. Several of the methods showed a relationship with enterococci
472 concentrations, i.e. EV_USC detections were associated with lower enterococci levels ($\log_{10}$ 2.69
473 when EV detected vs. $\log_{10}$ 3.96 when EV not detected; P = 0.002). A similar relationship was
474 found for EV_SCCWRP ($\log_{10}$ 2.81 when EV detected vs. $\log_{10}$ 3.90 when EV not detected; P =
475 0.019). In contrast, when FRNAPH typing indicated the presence of a human fecal source,
476 somatic coliphage and enterococci concentrations were both significantly higher (P<0.001 and
477 P=0.004, respectively). FRNAPH detection was associated with significantly higher enterococci
478 levels ($\log_{10}$ 4.40 when FRNAPH detected vs. $\log_{10}$ 3.58 when FRHAPH not detected; P =
479 0.015).

480 **4. Discussion**

481 If MST methods are to be useful in a regulatory and/or management context, one must
482 have good confidence in the performance of the method(s), e.g. that a positive or a negative
483 result is a reliable predictor, at a minimum, of the presence/absence of the targeted source of
484 fecal contamination. The proliferation of MST methods over the last decade has produced a
485 potpourri of possible assays for fecal source identification whose performance is rarely directly
486 compared. This study provided the opportunity for direct comparison; however, because of the
487 many methods and laboratories involved, compromises in sample composition and processing
488 had to be made that were not optimal approaches for some methods. The virus methods, in
489 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 X 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. 2009, Vanchiere et al. 2005, 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 contamination a human source, 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 35-45%, 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

513 assumed to have no human source of pollution. These results agree with recent calculations of
514 detection limits of HPyVs in sewage diluted in environmental water samples. QPCR for HPyVs
515 was generally not sensitive enough to detect sewage contamination at levels corresponding to
516 elevated risk of norovirus infection, which was (calculated by quantitative microbial risk
517 assessment (Staley et al. 2012).

518 The performance of bacteriophage-based methods for detecting human contamination
519 varied from highly specific but insensitive (MB-55) to relatively sensitive but nonspecific
520 (GB124). FRNAPH genotype II had the best performance of the bacteriophages, but, like many
521 of the methods, was not sensitive toward singleton samples containing a human fecal source,
522 indicating that these phages are not ubiquitously distributed in the human population sampled.
523 An advantage of the bacteriophage methods as performed in this study is that the viruses are
524 known to be viable, as a culture step is utilized. The disadvantage of culture methods is that they
525 do not return rapid results, unlike the several-hour turnaround time of qPCR methods that are
526 directly applied to nucleic acid extracted from a water sample. FRNAPH genotyping as
527 performed here requires manipulations for reverse-trancriptase PCR that adds to the time and
528 labor of the culture method. Detection of specific FRNAPH genotypes directly by RT-PCR,
529 without the culture step, has been demonstrated in wastewater (Ogorzaly and Gantzer 2006) and
530 highly polluted river water (Ogorzaly et al. 2009); however, the direct RT-PCR methodology can
531 be less sensitive than the method which includes a culture step, and was therefore not employed
532 here.

533 Some of the viruses were measured by more than one method or laboratory, i.e. EV was
534 tested by four labs and four methods, HAdV by three labs and two methods, and NoV GII by two
535 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

559 from all three laboratories running HAdV. It is not surprising that agreement at the sample level
560 was not generally achieved due to level of target viruses in the samples, which was generally
561 near the LOD for the methods.

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

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

582 study, 500 ml rather than 50 ml of diluted sewage was processed; therefore, consistent detection
583 of HPyVs and, presumable, the other viruses requires more concentrated sewage samples than
584 those used in the current study.

585 While the specificity and PPV of many of the virus methods is encouraging, the low
586 sensitivity and NPV indicate that better concentration methods are needed if they are to be
587 reliable MST markers. Observed human virus concentrations in sewage estimated by qPCR span
588 a broad range (all concentrations expressed in gene copies•L^{-1}), e.g. enteroviruses from ~10^1 -
589 10^8 (Katayama et al. 2008, Wolf et al. 2010); noroviruses from ~$5 \times 10^3 - 10^9$ (da Silva et al.
590 2007, Katayama et al. 2008), adenoviruses from ~$10^1 - 5 \times 10^5$ (Katayama et al. 2008, Wolf et al.
591 2010, Fong et al. 2010), and HPyVs ~10^7 (McQuaig et al. 2009) (reviewed in Chapter 5: Viruses
592 as Tracers of Fecal Contamination (McQuaig and Noble 2011). Virus concentrations in surface
593 waters tend to be low except when sewage contamination is present (all concentrations expressed
594 in gene copies•L^{-1}), e.g. adenoviruses detectable to ~ 10^2 and enteroviruses at 10^1 -10^2 (Sassoubre
595 et al. 2012); HPyVs undetectable to ~10^6 (McQuaig et al. 2012, McQuaig et al. 2009); norovirus
596 Group I at 10^3 (Sauer et al. 2011). The practice of concentrating large volumes (40 L or more) of
597 surface water is commonly used for quantifying viruses in surface waters (e.g. (Jiang et al.
598 2001)), although the strategy of capturing viruses on negatively charged membrane filters has
599 allowed effective use of 500 to 1,000 ml volumes (McQuaig et al. 2012, Katayama et al. 2002,
600 Rigotto et al. 2009), which is less expensive and labor-intensive than using larger volumes. The
601 results of this study and others (Staley et al. 2012, Harwood et al. 2009), however, indicate that
602 greater concentration factors from surface water samples must be achieved for viral targets to be
603 effective MST markers. Alternative methods for concentration of viruses from surface waters
604 include hollow fiber ultrafiltration (Leskinen et al. 2010, Rajal et al. 2007, Liu et al. 2012,

Rhodes et al. 2011), 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).

5. Conclusions

- Host-specific viruses tended to have high specificity and PPV, but low sensitivity and NPV owing to their uncommon occurrence in their hosts. These findings suggest that when viral markers are detected they can be useful tools for identifying human sources of pollution, however, when viral markers are not detected, human sources of pollution cannot be ruled out.
- Host-specific bacteriophage and FRNAPH genotyping methods had moderate specificity and sensitivity; however, they tended to cross react with various non-target hosts. Larger sample sizes may improve sensitivity and negative predictive values.
- Some host-associated viruses were tested by more than one laboratory, and while their results did not agree completely, several were significantly associated in challenge fecal samples. Inter-laboratory variation is not surprising given the low levels of viruses presumably in the samples.
- Concentration methods that allow quantitative recovery of viruses while avoiding concentration of substances that inhibit the PCR will be necessary to allow researchers and regulatory agencies to take advantage of the specificity of viruses in MST efforts.

Acknowledgments

Funding for this project has been provided in part through an agreement with the California State

628 Water Resources Control Board. The contents of this document do not necessarily reflect the
629 views and policies of the California State Water Resources Control Board, nor does mention of
630 trade names or commercial products constitute endorsement or recommendation for use. UNC-
631 CH acknowledges the help of Neil Bhathela in analyses. Tetra Tech acknowledges Qiagen for
632 the sponsoring of Qiagen QIAamp MinElute Virus Spin Kit and Quantifast Pathogen PCR + IC
633 kits.

634 **6. References**

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891 **Figure Legend**

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

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

Human Virus Designation & 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	JJV2F: CAA GAG TCA ATG TTT AGG TGG ATG AG COG2R: TCG ACG CCA TCT TCA TTC ACA	(Jothikumar et al. 2005b, Kageyama et al. 2003)	15

Human Virus Designation & Lab	**Virus Type**	**Target**	**Method**	**Primer/Probe sequences**	**Reference**	**LOD_T**
			QPCR	RING2-TP: FAM-TGG GAG GGC GAT CGC AAT CT-BHQ		
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 GGG 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
Bacteriophage and Lab						
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	*Enterococcus* 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-	General	Double Layer Agar	NA	(ISO 1995)	25

Human Virus Designation & Lab	**Virus Type**	**Target**	**Method**	**Primer/Probe sequences**	**Reference**	**LOD_T**
	49					
FRNAPH II_ IFREMER	Genotype II FRNAPH	Human	Reverse Transcription QPCR on isolated plaques	GIIF : TGCAAACCTAACTCGGAATGG GIIR : AGGAGAGAACGCAGGCCTCTA GIIP : FAM-TCCCTCTATTTCCTC-MGBNFQ	(Ogorzaly and Gantzer 2006)	NA

[a]Samples were acidified prior to filtration

[b]Not applicable

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%	67.7%	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%	84.6%	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%	88.5%	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

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**	**Sample Type**	**Gene Copies**	**Sample Type**	**Gene Copies**	**Sample Type**	**Gene Copies**
pig 1:10	$2.1x10^5$	pig 1:10	$3.1 x10^1$	sewage	$1.1 x10^2$	septage	$7.5 x10^1$	human/cow 10/90	$1.4 x10^2$
pig	$8.3 x10^5$	pig	$3.3 x10^1$	septage	$7.8 x10^1$	septage	$3.8 x10^1$	sewage	$3.4 x10^2$
sewage/pig 90/10	$2.7 x10^5$	sewage/pig 90/10	$4.0 x10^1$	sewage/gull 10/90	$2.9 x10^2$			human/dog 10/90	$6.3 x10^2$
sewage	$1.0 x10^6$	sewage/pig 10/90	$1.3 x10^2$	sewage/pig 90/10	$6.7 x10^1$				
sewage/pig 10/90	$8.1 x10^5$	sewage/pig 10/90	$3.2 x10^2$	sewage/gull 90/10	$5.2 x10^1$				
sewage/pig 90/10	$7.5 x10^4$								
sewage/pig 10/90	$1.2 x10^6$								
pig	$2.4 x10^5$								
pig 1:10	$4.9x10^4$								

[a]Gene copies·filter^{-1}

Virus Types
HPyVs
HAdV
EV
FRNA type
phage
Enterococci (CFU/filter)
10^0
10^2
10^4
10^6
Somatic phage (PFU/50 mL)
chicken
chicken
deer
deer
dog
dog
dog 1:10
dog 1:10
goose
goose
gull
gull
gull
gull 1:10
gull 1:10
horse
horse
pig
pig
pig 1:10
pig 1:10
pigeon
pigeon
cow
cow
cow 1:10
cow 1:10
human
human
human 1:10
human 1:10
human/cow 10/90
human/cow 10/90
human/cow 90/10
human/cow 90/10
human/dog 10/90
human/dog 10/90
human/dog 90/10
human/dog 90/10
human/goose 10/90
human/goose 10/90
human/gull 10/90
human/gull 10/90
human/gull 90/10
human/gull 90/10
septage
septage
septage 1:10
septage 1:10
septage/horse 10/90
septage/horse 10/90
sewage
sewage
sewage 1:10
sewage 1:10
sewage/chicken 10/90
sewage/chicken 10/90
sewage/gull 10/90
sewage/gull 10/90
sewage/gull 90/10
sewage/gull 90/10
sewage/pig 10/90
sewage/pig 10/90
sewage/pig 90/10
sewage/pig 90/10