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Review

Application of enteric viruses for fecal pollution source tracking in environmental waters

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ABSTRACT

Microbial source tracking (MST) tools are used to identify sources of fecal pollution for accurately assessing public health risk and implementing best management practices (BMPs). This review focuses on the potential of enteric viruses for MST applications. Following host infection, enteric viruses replicate and are excreted in high numbers in the hosts' feces and urine. Due to the specificity in host infection, enteric viruses have been considered one of the most accurate library-independent culture-independent MST tools. In an assessment of molecular viral assays based on sensitivity, specificity and the density of the target virus in fecal-impacted samples, human adenovirus and human polyomavirus were found to be the most promising human-specific viral markers. However, more research is needed to identify promising viral markers for livestock because of cross-reactions that were observed among livestock species or the limited number of samples tested for specificity. Other viral indicators of fecal origin, F+ RNA coliphage and pepper mild mottle virus, have also been proposed as potential targets for developing MST markers. Enhancing the utility of enteric viruses for MST applications through next generation sequencing (NGS) and virus concentration technology is discussed in the latter part of this review. The massive sequence databases generated by shotgun and gene-targeted metagenomics enable more efficient and reliable design of MST assays. Finally, recent studies revealed that alternative virus concentration methodologies may be more cost-effective than standard technologies such as 1MDS; however, improvements in the recovery efficiency and consistency are still needed. Overall, developments in metagenomic information combined with efficient concentration methodologies, as well as high host-specificity, make enteric viruses a promising tool in MST applications.

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1. Introduction

Fecal pollution of environmental waters is a major concern for the general public and can lead to severe impacts on health, as well as economic and societal burdens. Identifying dominant sources of fecal pollution is critical for accurate assessment of public health risks and implementation of best management practices (BMPs). Recently, microbial source tracking (MST) tools have been developed to track the source of fecal pollution in aquatic environments by identifying a microbial and/or chemical indicator associated with fecal materials from a specific host (or host group) (Hagedorn et al., 2011). Various indicators have been introduced and their analytical approaches fall into four categories: library-dependent culture-dependent (Field et al., 2003; Griffith et al., 2003), library-dependent culture-independent (Griffith et al., 2003; Stoeckel et al., 2004) library-independent culture-dependent (Blanch et al., 2006; Noble et al., 2003) and library-independent culture-independent (Bernhard and Field, 2000; Fong et al., 2005; McQuaig et al., 2009; Scott et al., 2005).

Library-dependent methods rely upon a database of fingerprints from bacterial isolates obtained from known fecal environmental sources or specific hosts to determine the source of fecal contamination in environmental waters (Harwood et al., 2003; Stoeckel and Harwood, 2007; USEPA, 2005). Bacterial isolate fingerprints are developed using phenotypic or genotypic approaches. Examples of library-dependent phenotypic approaches include the use of antibiotic resistance analysis (ARA), multiple antibiotic resistance (MAR) or Kirby–Bauer antibiotic resistance analysis (KB-ARA), and carbon source utilization. Examples of library-dependent genotypic approaches include methods such as ribotyping or pulse-field gel electrophoresis, among others (Casarez et al., 2007; Parveen et al., 1999, 2001; Wiggins et al., 2003). While library-based methods may provide high rates of correct classification, they generally require collection of hundreds to thousands of isolates from multiple known sources in a watershed, may be sensitive to temporal, spatial and geographic variability of sources, and are resource- and time-consuming (Casarez et al., 2007; Choi et al., 2003). Additionally, some library-based methods rely on expensive equipment or complicated software for library construction and data analysis (Casarez et al., 2007).

A library-independent culture-independent method does not require either cultivation of a target microorganism or development of a fingerprint database. It is a genotypic-based approach that identifies sources through amplification of host specific marker genes by PCR, also called “host-specific PCR” (Field and Samadpour, 2007). The advantage of a library-independent method is that it is less laborious and results are obtained more quickly. Culture-dependent methods require cultivation of microbial indicators that are often difficult to grow or that may be viable but non-culturable; culture-independent methods avoid the limitations of cultivation procedures, saving time and resources.

Enteric viruses, as well as *Bacteroides*/*Prevotella, Bifidobacteria*, *Enterococcus*, mitochondrial DNA, and F+ coliphages, have all been proposed as promising library-independent culture-independent MST tools (Blanch et al., 2006; Caldwell et al., 2007; Fong et al., 2005; Long et al., 2005; Scott et al., 2005). The biggest advantage of utilizing enteric virus genes as MST markers is that enteric viruses of different host species are easily identified and differentiated based on sequence differences in genus-common genes. For example, hexon and fiber genes of adenoviruses have been widely used for detection and speciation of adenoviruses (La Rosa et al., 2011; Rux et al., 2003). Phylogenetic analyses of five main genes (VP1, VP2, VP3, large T-antigen and small T-antigen) in the polyomavirus genome showed that polyomaviruses are highly host-specific and co-evolved with their avian and mammalian hosts (Perez-Losada et al., 2006). In addition, enteric virus markers may be used to differentiate between ongoing and recent fecal contamination by selecting either DNA (i.e. adenovirus) or RNA (i.e. enterovirus) based enteric viruses which have different environmental persistent rates due to their nucleic acid composition and structure (Lipp et al., 2007; Love et al., 2010; Mena and Gerba, 2009; Wetz et al., 2004). Challenges associated with commonly used MST markers, including enteric viruses are listed in Table 1. Therefore, a “tool box” approach, which targets multiple makers, has been suggested to improve the reliability for identifying the source of fecal pollution (Plummer and Long, 2009; Roslev and Bukh, 2011).

Enteric viruses are frequently detected in the environment. Of more than 150 enteric viruses, the most commonly reported enteric viruses in fecal-polluted water are adenoviruses (AdV), enteroviruses (EV), noroviruses (NoV), rotaviruses (RV), hepatitis viruses (HepV) and polyomavirus (PyV). Their genomes and physical sizes are summarized in Table 2. AdV and PyV are double-stranded DNA viruses, while EV, NoV, HepV and RV are RNA viruses. Enteric viruses are excreted in the feces and urine of infected hosts and have been found in different water environments such as marine, river, ground, drinking, recreational and wastewater (Borchardt et al., 2003; Fong et al., 2007; Haramoto et al., 2005; Katayama et al., 2002; Kuo et al., 2010; Xagoraraki et al., 2007). Lipp et al. (2007) and Futch et al. (2010) used human adenovirus (HAdV) and human enteroviruses (HEV) to track human fecal contaminant movement to a coral reef environment in the Florida Keys. High concentrations of viruses in groundwater and coral mucus, especially during the wet summer season, suggested that the migration of fecal contamination from on-site septic systems into groundwater is a plausible source of microbes found in offshore reef environments (Lipp and Griffin, 2004).

In this review, we offer a brief overview of enteric virus detection techniques, followed by a review of the specificity and sensitivity of currently available host-specific enteric virus assays, validation protocols, and density/prevalence of enteric viruses in environmental media. A discussion of alternative viral indicators is included. In the second half of this review, we discuss the enhancement of the utility of enteric viruses for MST by recent and future developments in metagenomics and virus concentration technology.

2. Analytical methods for detection and quantification of enteric viruses

Traditionally, cell culture has been recognized as the gold standard for infectious enteric virus detection and quantification. However, cell culture is labor intensive and lacks the ability to differentiate specific types of enteric viruses in environmental samples. For example, the Buffalo Green Monkey (BGM) cell line is currently recommended by the USEPA to propagate total culturable viruses from environmental samples. Viruses that can be propagated on BGM cells include HAdV, HEV, human rotaviruses and other enteric viruses (Chapron et al., 2000; Dahling and Wright, 1986; Lee et al., 2004). BGM cells are known to favor HEV, and other slow-growing enteric viruses may be out-competed by HEV in environmental assays (Dahling and Wright, 1986; Lee and Kim, 2002). The invention of new molecular pathogen detection techniques, such as polymerase chain reaction (PCR), makes it possible to detect and quantify specific enteric viruses directly from the environment. At the beginning of the 1990s,

Table 1
Challenges associated with selected library independent markers.

Type of marker	Challenges	Reference
Enteric virus	Low target number in environmental matrices Tedious sampling procedure	USEPA (2005)
F+ RNA coliphage	Low target number in environmental matrices Specificity issue between human and animal feces Cannot identify absolute animal host	USEPA (2005); Wolf et al. (2010)
Bacteroidetes 16S rRNA gene	Cross amplification among closely related animal species (i.e., human, cat, dog) Cross amplification among animals with close digestive physiologies (i.e., human and swine)	Kildare et al. (2007) Layton et al. (2006); Okabe et al. (2007)
ESP gene	Low target number in environmental matrices Gene could be present in animal feces Seems to work better for wastewater contamination sources than septic systems	Whitman et al. (2007)
Mitochondrial DNA	False positive by non-fecal source. (i.e., skin cells) False positive for animal signal in human feces due to human consumption of meat	Caldwell et al. (2007)

researchers began to use end point PCR to detect enteric viruses in water and wastewater samples (Abbaszadegan et al., 1993; Puig et al., 1994). In the last ten years, many environmental virology studies have shifted from using end point PCR to quantitative PCR (qPCR) because it provides both qualitative and quantitative results. Moreover, qPCR assays are more sensitive than conventional PCR (usually requiring less than 100 copies per reaction) and no post-PCR handling step is required to view the results (Fontaine and Guillot, 2002; Heid et al., 1996). In addition to cell culture and PCR, flow cytometry, fluorescence-activated cell sorting assay, microarray, PCR coupled with propidium (or ethidium) monoazide and next generation sequencing have been developed in recent years to detect virus genome and/or infectious viruses from environmental samples (Bibby et al., 2011; Cantera et al., 2010; Gardner et al., 2010; Li et al., 2010a; Parshionikar et al., 2010).

3. Application of enteric viruses for microbial source tracking

Enteric viruses are promising MST tools due to their prevalence in host feces and host specificity, making validation of these markers simpler in concept than bacterial and library-dependent markers (Scott et al., 2002; Stoeckel and Harwood, 2007). The high prevalence of HAdV in contaminated waters in Europe has made it the recommended index virus for human contamination (Albinana-Gimenez et al., 2009; Bofill-Mas et al., 2006; Pina et al., 1998; Wyn-Jones et al., 2011). Although interspecies transmission of some enteric viruses, i.e. RV, NoV, and hepatitis E, has been documented, such phenomena are rare and, in most instances, zoonotic transmission is limited to certain genotypes of a virus species (Bank-Wolf et al., 2010; Martella et al., 2010; Pavio et al., 2010). In 2001, a large-scale, multiple-lab MST method comparison study was performed, in which blind samples were inoculated with mixtures of fecal materials including human, sewage, dog, seagull and cow (Griffith et al., 2003; Noble et al., 2003). Among the MST methods evaluated in this study, virus-based library-independent methods had the lowest false positive rates (0–8%), confirming their ability to differentiate between human and non-human fecal sources. The fecal sources differentiated in recent MST enteric virus studies included human, bovine, porcine and ovine (Table 3). To the best of our knowledge, no bird- or fowl-specific enteric virus assay has been used for any MST study. Current studies have mostly focused on virus groups that have been extensively studied, such as AdV, PyV and EV, with the exception of a few that included NoV and teschoviruses (Jimenez-Clavero et al., 2003; Wolf et al., 2010).

3.1. Host specificity of viral markers

Sensitivity and specificity are two parameters that commonly describe the performance of an MST assay. In MST, specificity is defined as "the ability to detect a source when fecal material is not present and is calculated by dividing the number of true-negative results by the number of samples that should not contain the target" (Stoeckel and Harwood, 2007). Table 3 summarizes the sensitivity and specificity testing of enteric virus assays in previous MST studies. It shows that most of enteric virus MST assays have high specificity, but the number of samples used for specificity testing varied. Among the assays, the human polyomavirus (HPyV) end point PCR assay described in McQuaig et al. (2006), which was originally developed in Askamit (1993), has been tested most extensively (Table 3). Combining studies by McQuaig et al. (2006), Harwood et al. (2009), Ahmed et al. (2010b) and Kirs et al. (2011), this assay was tested with 505 non-human fecal samples and no false positive was observed (100% specificity), showing higher specificity than *Bacteroidetes* and *M. smithii* assays (Harwood et al., 2009). Besides the end point PCR assay, the HPyV qPCR assay by McQuaig et al. (2009) also showed promising results after the marker was found to be PCR negative with 127 waste samples (feces and urine) from 14 different species of animals.

Selection of a target gene or genome region is critical to ensure host specificity of a MST assay. Though it is generally believed that enteric viruses are host-specific, some studies have detected highly similar strains of enteric viruses in different animal species (Jimenez-Clavero et al., 2005; Ley et al., 2002). One example is the bovine enterovirus (BEV) assays described in Ley et al. (2002) and Jimenez-Clavero et al. (2005); these assays were designed to target the NTR region of BEV and results showed BEV-like sequences were present in the feces of other animals such as goose, deer, sheep, goat and horse. Therefore, it is unlikely that BEV can be used as a reliable MST marker unless future studies identify another marker in

Table 2
Characteristics of waterborne enteric viruses.

Enteric virus	Family	Nature of genome	Genome size (kbp or kb)	Dimension (nm)	Reference
Adenovirus	Adenoviridae	dsDNA	28–45	60–90	Wagner et al. (2008); Thomas et al. (2004)
Astrovirus	Astroviridae	ssRNA	7–8	28	Wagner et al. (2008); Maier et al. (2008)
Enterovirus	Picornaviridae	ssRNA	7–8	27–32	Wagner et al. (2008); Maier et al. (2008)
Hepatitis A	Picornaviridae	ssRNA	7–8	27–32	Wagner et al. (2008); Maier et al. (2008)
Norovirus	Caliciviridae	ssRNA	7–8	27–38	Wagner et al. (2008); Maier et al. (2008)
Polyomavirus	Polyomaviridae	dsDNA	5	35–40	Wagner et al. (2008); de Ligny et al. (2000)
Rotavirus	Reoviridae	dsRNA	19	70	Wagner et al. (2008); Maier et al. (2008)

Table 3
Sensitivity and specificity testing of enteric virus assays from previous MST studies.

Enteric virus	Assay type	Target gene	Samples tested for sensitivity	Sensitivity (n)	Samples tested for specificity	Specificity (n)	Reference
HAdV	Nested	Hexon	Sewage	1.00 (30)	Chicken (10), dog (10), duck (10), kangaroo (10), horse (10), bird (5),	1.00 (106)	Ahmed et al. (2010a)
			Septic wastewater	0.80 (10)	cattle (10), pig (10), sheep (10), goat (5) feces; cattle wastewater (16)		
HAdV-F	Real time	Hexon	Feces	0.00 (15)	Pig (5), sheep (2), deer (1), cattle (2), Canada goose (14), black swan (16),	1.00 (56)	Wolf et al. (2010)
			Sewage	1.00 (11)	duck (16) feces		
HAdV-C			Feces	0.47 (15)		1.00 (56)	
			Sewage	0.36 (11)			
HAdV	Real time	Hexon	Sewage	1.00 (9)	Pig feces (38); slaughterhouse wastewater (8)	1.00 (46)	Hundesa et al. (2009)
	Nested	Hexon	NA	NA	Swine feces (23) , bovine feces (8)	1.00 (31)	Hundesa et al. (2006)
	Nested	Hexon	Sewage	0.50 (8)	Dog (1), cow (1) and gull (1) feces	1.00 (3)	Noble et al. (2003)
BAdV	Real time[a]	Hexon	Feces	0.22 (18)	NA	NA	Wong and Xagoraraki (2011)
	Real time[a]	Hexon	Feces	0.20 (20)	HAdV culture (7)	1.00 (7)	Wong and Xagoraraki (2010)
			Manure	1.00 (16)			
	Nested[b]	Hexon	Feces	0.30 (10)	Influent (30), primary (18) and secondary effluent (16), septic wastewater (10),	1.00 (154)	Ahmed et al. (2010a)
			Wastewater	1.00 (16)	Feces from Chicken (10), dog (10), duck (10), kangaroo (10), horse (10), bird (5), pig (10), sheep (10), goat (5) feces		
BAdV/OAdV	Real time	Hexon	Feces	0.50 (4)	Human (15), pig (5), deer (1), Canada goose (14), black swan (16),	1.00 (67)	Wolf et al. (2010)
			Wastewater	0.50 (2)	duck (16) feces		
BAdV	Nested[b]	Hexon	Wastewater	1.00 (10)	Swine feces (23) , sewage (12) and HAdV culture (4)	1.00 (39)	Hundesa et al. (2006)
	Nested[b]	Hexon and protease	Pooled feces	0.75 (8)	HAdV culture (4) and sewage (12)	1.00 (16)	de Motes et al. (2004)
AtAdV	Real time	Hexon	Feces	0.20 (5)	Human (15), pig (5), Canada goose (14), black swan (16), duck (16) feces	1.00 (66)	Wolf et al. (2010)
			Wastewater	0.00 (2)			
PAdV-5	Real time	Hexon	Feces	0.60 (5)	Human (15), sheep (2), deer (1), cattle (2), Canada goose (14), black swan (16),	1.00 (66)	Wolf et al. (2010)
PAdV-3			Feces	0.60 (5)	duck (16) feces	1.00 (66)	
PAdV	Real time	Hexon	Pooled feces	0.87 (38)	HAdV culture (6), bovine feces tested positive with BAdV-2/4/7 (≥3), and urban sewage (9)	1.00 (≥18*)	Hundesa et al. (2009)
	Nested[c]	Hexon	Wastewater	0.05 (22)	Cattle feces (8), sewage (12) and HAdV culture (4)	1.00 (24)	Hundesa et al. (2006)
	Nested[c]	Hexon and protease	Pooled feces	0.71 (24)	HAdV culture (4) and urban-sewage (12)	1.00 (16)	de Motes et al. (2004)
HPyV	Nested[d]	Conserved T antigen	Feces	0.18 (11)	Birds (22) ruminants (27), horse (3), cat (1), dog (1), rodents (4),	1.00 (67)	Kirs et al. (2011)
			Sewage	1.00 (11)	marsupials (9) feces		
	Real time	Conserved T antigen	Sewage	1.00 (8)	NA	NA	Hundesa et al. (2010)
	Real time	Conserved T antigen	Sewage	1.00 (39)	Fecal associated samples from cat (5), chicken (1), cow (25), dog (55), sandhill crane (2), deer (3), duck (4), fox (1), horse (8), raccoon (1), seagull (6), sparrow (3), pig (3); urine from dog (9) and cat (1); HAdV (1)	1.00 (128)	McQuaig et al. (2009)
	Conventional[d]	Conserved T antigen	Sewage	1.00 (55)	Chicken (10), dog (10), duck (10), kangaroo (10), wild bird (5) feces; wastewater from cattle (10), pig (10), sheep (16)	1.00 (81)	Ahmed et al. (2010b)
	Conventional[d]	Conserved T antigen	Sewage	1.00 (55)	Fecal associated samples from cat (25), chicken (37), cow (78), dog (76), duck (35), seagull (58), wild (7) and birds (16).	1.00 (332)	Harwood et al. (2009)
	Nested[d]	Conserved T antigen	NA	NA	Dairy cow and composite wastes (25)	1.00 (25)	McQuaig et al. (2006)
BPyV	Real time	VP1	Feces	0.06 (18)	JCPyV (1) and BKPyV (1) culture, urban sewage (16), pig feces (12)	1.00 (30)	Wong and Xagoraraki (2011)
			Manure	1.00 (16)			
	Real time	VP1	Feces	0 (10)	Urban sewage (8)	1.00 (8)	Hundesa et al. (2010)
			Wastewater	1.00 (11)			
	Nested	VP1	Wastewater	0.94 (18)	JCPyV (1) and BKPyV (1) culture	1.00 (2)	Hundesa et al. (2006)
HEV	Conventional	NTR	Sewage	0.38 (8)	Dog (1), cow (1) and gull (1) feces	1.00 (3)	Noble et al. (2003)
BEV	Real time	NTR	Feces	0.78 (100)	Sheep (23), goats (10), horses (10), donkeys (7) feces	0.42 (50)	Jimenez-Clavero et al. (2005)
	Conventional	NTR	Feces	0.76 (139)	Deer (50) and geese (4) feces	0.37 (54)	Ley et al. (2002)
NoVGI (HNoV)	Real time	Capsid protein	Feces	0.40 (15)	Pig (5), sheep (2), deer (1), cattle (2), Canada goose (14), black swan (16),	1.00 (56)	Wolf et al. (2010)
			Sewage	0.82 (11)	duck (16) feces		
NoVGII (HNoV, PNoV)			Feces	0.50 (20)	Sheep (2), deer (1), cattle (2), Canada goose (14), black swan (16),	1.00 (51)	
			Sewage	0.82 (11)	duck (16) feces		
NoVGIII (BNoV, ONoV)		RNA polymerase	Feces	0.75 (4)	Human (15), pig (5), deer (1), Canada goose (14), black swan (16),	1.00 (67)	
			Sewage	1.00 (2)	duck (16) feces		
PTV	Real time	Polyprotein	Wastewater	1.00 (6)	Cattle (1), sheep (1), and goats (1) feces tested positive for BEV	1.00 (3)	Jimenez-Clavero et al. (2003)

(*Notes to Table 3 are on next page*)

BEV that is only bovine-specific. Also, the nucleotide sequence of the qPCR assay by Wong and Xagoraraki (2010) and the nested assay by de Motes et al. (2004), which were designed to target conserved region of bovine adenovirus (BAdV), may potentially react with some serotypes of ovine and deer adenovirus based on sequence homology. Despite these potential limitations, these assays may be used for "livestock" source tracking instead of specifically targeting cattle. After conducting an ecological study of AdV from different livestock, Sibley et al. (2011) concluded that animal AdV infecting a given livestock may not be monophyletic and suggested that the design of BAdV assay specifically targeting cattle should focus on hypervariable regions instead of conserved regions. In addition, the norovirus presented in swine and bovine feces may have similar sequence to human norovirus GII.4 strain (Mattison et al., 2007).

Further specificity testing on previously published human enteric virus PCR assays is warranted. Many qPCR assays have been used for environmental monitoring of HAdV (Formiga-Cruz et al., 2002; He and Jiang, 2005; Heim et al., 2003; Hernroth et al., 2002; Jiang et al., 2005; Jothikumar et al., 2005; Ko et al., 2005). Even though most researchers were able to show high specificity and sensitivity detecting HAdV, not enough testing has been performed to demonstrate no/low cross-reaction with other animal adenoviruses or fecal associated materials [except for the assay by Hernroth et al., 2002, tested by Hundesa et al., 2009 and assay by Jiang et al., 2005]. When these assays were developed, specificity testing with animal fecal associated samples was not deemed necessary because some of them were designed for clinical studies and wastewater treatment monitoring (He and Jiang, 2005; Heim et al., 2003). When using the assays for MST applications in open watersheds, however, specificity testing becomes an extremely important aspect to include in the study. The same recommendation extends to published assays for other human enteric viruses such as HEV and HNoV.

Strains of microorganisms may vary by geographic region; therefore, multiple lab evaluations and validations including mixtures of fecal samples from different geographical regions are recommended when testing a new MST marker assay. It is also recommended to validate the assay with other markers to establish a parameter for comparison. Some animal enteric viruses (e.g. BAdV and bovine polyomavirus (BPyV)) are shed mainly through urine (see discussion in sensitivity section); thus, enteric virus MST assay tests should consider including urine or materials associated with both feces and urine (such as livestock manure or wastewater) when testing for specificity.

3.2. Sensitivity of Viral MST markers

Sensitivity is defined as "the ability to detect a source when the fecal materials are present, and is calculated by dividing the number of true-positive results by the number of samples that should contain the target" (Stoeckel and Harwood, 2007). In addition to those included in Table 3, numerous additional assays have been developed to detect HAdV, HEV, HNoV and HPyV in feces and/or wastewater influent samples (Albinana-Gimenez et al., 2006; Haramoto et al., 2007; Heim et al., 2003; Katayama et al., 2008; Kuo et al., 2010; Simmons et al., 2011). The occurrence of HAdV in wastewater influent and sludge was found to be significantly higher than HEV, HNoV and HPyV (Katayama et al., 2008; Simmons et al., 2011; Wong et al., 2010). The sensitivity results of animal enteric virus assays such as BEV, porcine adenovirus (PAdV), teschoviruses (PTV) and NoVGIII indicate that these viruses are present in high numbers in fecal materials (Table 3), however, the number of host fecal materials tested with PTV and NoVGIII was limited ($n<10$).

Adenovirus and polyomavirus can also be both fecal- and urine-associated. Two types of human polyomaviruses, JCPyV and BKPyV, have been found in urine samples (Arthur et al., 1989; Bofill-Mas et al., 2001; Polo et al., 2004) and studies have reported that HAdV can be excreted in urine, as well (Echavarria et al., 1998; Henderson et al., 1998). Other studies have demonstrated that BAdV and BPyV are mainly shed in cattle by urination, which resulted in their assays having much higher sensitivity with urine-associated fecal samples (cattle manure and wastewater) than with individual cattle feces (Hundesa et al., 2010; Sibley et al., 2011; Wong and Xagoraraki, 2010, 2011). In addition, Hundesa et al. (2010) and Sibley et al. (2011) determined that the occurrence of these viruses was higher in urine than in feces; BPyV was detected in 31% of the urine samples, but not in any fecal samples (Hundesa et al., 2010). Sibley et al. (2011) found that only 13% of individual bovine fecal samples tested positive for BAdV, but BAdV was found in 90% and 100% of urine and manure samples, respectively. In Wong and Xagoraraki (2011), all 26 manure samples tested positive for BPyV and BAdV, although BPyV and BAdV were positive only in ~6% and ~22% of fecal samples ($n=18$), respectively. The authors speculated that the high prevalence of BPyV in manure was likely due to the contribution from urine. However, this should not affect the potential of BAdV and BPyV as viral MST tools because composited manure, not individual feces, is commonly used for land application, so fecal contaminants that end up in aquatic environments are most likely mixtures of feces and urine.

3.3. Density and prevalence of enteric virus in fecal associated environmental samples

The density and prevalence of MST markers in fecal-associated environmental samples are important because the recovery of virus concentration procedures is usually low (see Section 5.2) and the sensitivity of MST assays is highly affected by the density of the target. In addition to its high assay sensitivity, HAdV was reported to have higher density than other enteric viruses in wastewater associated samples (Katayama et al., 2008; Simmons et al., 2011; Wong et al., 2010) and was detected more often than other enteric viruses in the natural water samples (Futch et al., 2010; Lipp et al., 2007). The greater prevalence of HAdV in the environment may be related to prolonged persistence of double-stranded DNA relative to RNA viruses (Love et al., 2010; Mena and Gerba, 2009); recent studies reported that HAdV can persist for a long time under natural environmental conditions (Ogorzaly et al., 2010; Rigotto et al., 2011). On the other hand, observations of loss of poliovirus (an RNA virus) genetic material and infectivity occur at similar rates, which suggest that enteroviruses may be better at indicating recent contamination (Lipp et al., 2007; Wetz et al., 2004). Enterovirus detection in the environment was observed to be consistent with clinical infection rates and patterns of excretion (Khetsuriani et al., 2006; Sedmak et al., 2003).

Recently, several studies compared the density and prevalence of HPyV and HAdV in different environmental matrices because they are both double-stranded DNA enteric viruses with high persistence

Notes to Table 3

* Number of bovine fecal samples was not specified in the study but there should be at least three samples based on the fecal samples that tested positive for three different serotypes of bovine adenovirus.

(n) = number of observations.

Sensitivity was calculated by "dividing the number of true-positive results by the number of samples that should contain the target" (Stoeckel and Harwood, 2007).

Specificity was calculated by "dividing the number of true-negative results by the number of samples that should not contain the target" (Stoeckel and Harwood, 2007).

($^{a, b, c \text{ or } d}$) Same letter indicates same assay under assay type.

NA = not available.

HAdV = human adenovirus; BAdV = bovine adenovirus; OAdV = ovine adenovirus; AtAdV = atadenovirus; PAdV = porcine adenovirus; HPyV = human polyomavirus; BPyV = bovine polyomavirus; HEV = human enterovirus; BEV = bovine enterovirus; HNoV = human norovirus; PNoV = porcine norovirus; ONoV = ovine norovirus; PTV = porcine teschovirus.

Table 4
Comparison between the density/occurrence of human adenovirus and polyomavirus in environmental samples.

Sample type	Human adenovirus		Human polyomavirus				Reference
			JCPyV		BKPyV		
	Density[a]	Occurrence	Density[a]	Occurrence	Density	Occurrence	
Biosolids (GC logs/g)	5.9	13/15	4.9	10/15	–	–	Wong et al. (2010)[b]
River (GC logs/L)	4.6	11/18	3.3	2/18	ND	0/18	Haramoto et al. (2010)
River (GC logs/L)	0.9–4.1	23/27	ND–3.1	13/27	NA	NA	Albinana-Gimenez et al. (2009)
Sewage (GC logs/L)	7.1	5/5	6.4	5/5	6.2	5/5	Albinana-Gimenez et al. (2006)
River (GC logs/L)	2.5–2.6	13/14	1.41–1.43	14/14	NA	4/6	
Sludge (GC logs/L)	5.3	5/5	4.1	1/5	ND	0/5	
GAC-filtered (GC logs/L)	0.7	9/9	0.1	5/9	ND	0/6	
Sewage (GC logs/L)	5.7–8.1	6/6	6.1–7.0	6/6	NA	NA	Bofill-Mas et al. (2006)
Sludge/biosolids (GC logs/g)	2.3–6.3	15/15	2.1–5.3	14/14	NA	NA	
Effluent (GC logs/L)	2.8–4.0	3/3	2.4–3.0	3/3	NA	NA	

ND = non-detected; NA = not available.
GC = genomic copies.
[a] Density value is either mean or range.
[b] HPyV assay used in this study is able to detect both JCPyV and BKPyV.

in the environment (Table 4). Based on reported values, HAdVs occur more frequently and in higher densities than HPyVs, but not to a great extent. Among HPyVs, the JCPyV may have higher density and occurrence than BKPyV (Albinana-Gimenez et al., 2006; Haramoto et al., 2010).

Seasonal distribution of various human enteric viruses has been suggested by previous studies (Fong et al., 2005; Jiang et al., 2007; Katayama et al., 2008; Tani et al., 1995). Katayama et al. (2008) observed that the highest density of HNoV in raw sewage occurred in winter months (November to March). Tani et al. (1995) suggested that the peak density of HEV in urban river water occurred in summer months (May to September). Katayama et al. (2008), however, did not observe seasonal distribution of either HEV or HAdV in sewage. Jiang et al. (2007) found higher detection of HAdV in the summer season (dry weather) than during the winter season (wet weather), but Fong et al. (2005) detected HAdV, as well as HEV and BEV, more frequently in the winter months. The different observations from these studies could be due to variations in water temperature, host excretion and/or transmission rate, suggesting that seasonal distribution of enteric viruses is geography- or climate-dependent.

Compared to human enteric viruses, less information is available on the density and prevalence of animal enteric viruses in excreta and environmental samples (Table 5). Wolf et al. (2010) developed viral tool boxes that consist of multiplex qPCR assays targeting human, porcine and ruminant hosts to characterize primary sources of fecal contaminants in environmental samples; they observed that

Table 5
Density of enteric viruses in animal waste associated samples.

Enteric virus	Type of samples	Collection site	Occurrence	Density[a] (mean or range)	Reference
BAdV	Manure	Michigan, US	22/26	7.1	Wong and Xagoraraki (2011)
BAdV	Manure	Michigan, US	16/16	5–7	Wong and Xagoraraki (2010)
	Drainage		2/2	5	
	Feces		4/20	3–4	
OAdV-2/3/4/5, BAdV-2	Shellfish	New Zealand	2/15	2.1	Wolf et al. (2010)
	Sewage influent		6/11	4.2	
	Abattoir effluent		1/2	5.4	
	Biosolids		3/4	3.2	
	River water		3/6	1.5	
BPyV	Manure	Michigan, US	26/26	8.6	Wong and Xagoraraki (2011)
	Feces		1/18	5.0	
BPyV	Urine	Spain	8/26	4.5	Hundesa et al. (2010)
	Feces		0/10	NA	
	Wastewater		11/11	3.5	
	River water		5/6	2.5	
BNoV and/or ONoV	Shellfish	New Zealand	7/15	2.4	Wolf et al. (2010)
	Sewage influent		1/11	4.4	
	Abattoir effluent		2/2	5.2	
	River water		1/6	2.6	
AtAdV	Shellfish	New Zealand	1/15	2.4	Wolf et al. (2010)
PAdV	Pooled Feces	Spain	33/38	5.7–5.9	Hundesa et al. (2009)
	Wastewater		8/8	6.2	
	River water		6/6	0.9	
PAdV-5	Shellfish	New Zealand	2/15	3.3	Wolf et al. (2010)
	River water		3/6	2.8	
PNoV, HNoV	Shellfish	New Zealand	4/15	3.2	Wolf et al. (2010)
	Sewage influent		9/11	4.4	
	Biosolids		2/4	4.4	
	River water		1/6	2.3	
	River water		3/6	2.8	
PTV	Open duct	Spain	6/6	7.6–8.1	Jimenez-Clavero et al. (2003)
	Stream		6/16	2.1–3.1	

Solid samples (shellfish, feces and biosolids) were expressed in genomic copies (GC) logs per gram; water samples were expressed in GC logs per L.
NA = not available.
[a] Positive samples only.

densities of viruses from different families (i.e. AdVs versus NoVs) were comparable across various environmental samples. For example, both ovine-specific markers, OAdV and NoV GIII, were detected in similar concentrations (~5 logs (Genome Copies) per L) in abattoir effluent samples. However, the prevalence of virus subgroups for a specific host group may vary. While porcine AdV-5 was detected in 13% (2/15) and 50% (3/6) of shellfish and river water samples, respectively, PAdV-3 was not detected in any of these samples (Wolf et al., 2010) (Table 5). Wong and Xagoraraki (2011) surveyed 26 manure samples collected from three different locations in Michigan; results indicated that concentrations of bovine polyomavirus (BPyV) were significantly higher (1.4 logs) than concentrations of bovine adenovirus (BAdV). Although certain enteric viruses are excreted in high numbers in the feces and urine of their hosts, their environmental densities may not be high enough for detection due to factors such as dilution, sorption to particulate matters/soil particles and environmental persistence (Horswell et al., 2010; Love et al., 2010; Sobsey et al., 1980; Wong and Xagoraraki, 2010).

Overall, studies show that HPyV assays have the best specificity and HAdV assays have the highest prevalence in human fecal wastes; nevertheless, more specificity testing on published human viral (q) PCR assays is warranted. PAdV and BPyV have shown promising results; however, these two markers should be tested with more urine-associated samples from other animals to confirm their specificity. Knowledge gaps, such as the development of avian and porcine viral marker assays, should also be addressed to advance the application of enteric viruses for MST.

4. Alternative viral indicators

4.1. F+ RNA coliphages

F+ RNA coliphages can be classified genetically into four different subtypes (GI to GIV). GI and GIV are mostly animal fecal-associated and GII and III are mostly human fecal-associated (Furuse, 1987; Hsu et al., 1995). Multiple studies have used F+ RNA coliphages for MST (Cole et al., 2003; Kirs and Smith, 2007; Lee et al., 2009, 2011b; Long et al., 2005; Noble et al., 2003; Rahman et al., 2009; Stewart et al., 2006; Stewart-Pullaro et al., 2006; Wolf et al., 2010). One advantage of using F+ RNA coliphages is that a simple bacteriophage assay can enumerate the numbers of coliphage in samples with low concentrations. However, each subtype is not exclusively associated with either human or animal hosts, therefore, specificity has been an issue. For example, human feces tested positive with the GI assay, and the GII assay cross-reacts with pig, sheep deer and duck feces (Wolf et al., 2010), and GIII genotype was identified in cow and gull feces (Noble et al., 2003). Despite specificity issues, Lee et al. (2009 and 2011b) demonstrated that genotyping followed by principle coordinate analysis (a statistical analysis) was able to differentiate the fecal origins from human and animals. This approach describes how to utilize statistical analysis to differentiate between genotype clusters of F+ RNA coliphage originated from human and animal. Future studies can investigate the possibility of using genotyping of F+ RNA coliphage followed by statistical analysis to differentiate among animal fecal source.

4.2. Pepper mild mottle virus

High prevalence of pepper mild mottle virus (PMMoV), a single-stranded RNA plant virus, in human feces, sewage and sewage polluted water was recently reported (Hamza et al., 2011; Rosario et al., 2009b; Zhang et al., 2006). After pyrosequencing the RNA viral community of human feces, Zhang et al. (2006) found that PMMoV was the most abundant virus. In a study by Rosario et al. (2009b), PMMoV was detected in all wastewater samples and its concentration in raw sewage averaged more than 10^6 copies/ml. PMMoV also had a higher concentration in wastewater than HAdV, HPyV, human picobirnaviruses and torque teno virus (TTV) (Hamza et al., 2011). PMMoV is present in pepper products, so it can be found with greater frequency in healthy human feces than viruses that cause human disease (Rosario et al., 2009b). The prevalence of PMMoV should not have seasonal variations because its presence in human fecal materials is mainly of dietary origin. Hamza et al. (2011) showed that PMMoV also had greater stability in sewage than enteric viruses like HAdV, which may be due to its capsid structure. However, PMMoV was also found in chicken and seagull feces (Hamza et al., 2011; Rosario et al., 2009b) but the concentrations in those animals' feces were 3 to 4 logs lower than in human feces. Future studies should investigate the specificity of PMMoV to determine the potential of utilizing PMMoV for human fecal source tracking.

5. Recent/future developments in sequencing and virus concentration technology

5.1. Metagenomic analysis by next generation sequencing — applications for viral MST

The invention and growth of next-generation DNA sequencing (NGS) (Margulies et al., 2005) have revolutionized the molecular biological sciences. With cost-effective production of large amounts of sequence data, researchers are now able to dig deeper into genomic information than ever before, expanding into areas that were previously unavailable. The sequencing revolution extends to environmental microbiology, including the potential for MST applications (Lee et al., 2010; Shanks et al., 2011), where the production of a large number of total sequences allows specific identification of enteric virus sequences in environmental samples (Bibby et al., 2011; Rosario et al., 2009a). Two NGS approaches applicable to viral MST are shotgun (random sequencing of nucleic acid fragments) and gene-targeted (sequencing of PCR amplicon; also known as tagged sequencing) metagenomics (Peccia et al., 2011; Scholz et al., 2012). Both enhance the development of enteric virus MST tools by deepening the understanding of viral ecology, expanding the database of viral genomic information, detecting abundant enteric viral types in environmental samples, and enabling more efficient design of MST assays.

The experimental procedure of shotgun and gene-based metagenomics for environmental viruses is illustrated in Fig. 1. In shotgun metagenomic approaches, virus-like particles (VLPs) are first isolated, typically by size or density exclusion, via methods such as filtration or CsCl centrifugation; this is followed by the removal of naked nucleic acid by nuclease digestion. Viral nucleic acids are extracted, viral RNA is reverse-transcribed to cDNA, DNA and cDNA are randomly fragmented to technology-appropriate lengths (i.e. 125 nt for Illumina hi-seq or 500 nt for 454 titanium technology), and ligated with sequencing adaptor and sequenced. The short sequences of this approach are typically assembled into larger contiguous sequences (contigs) and identified through bioinformatic comparison to a reference database. Bioinformatic approaches are being optimized to ensure correct identification of viral sequences. Shotgun metagenomics allows for estimation of the relative abundance of a virus since the likelihood that a viral fragment is sequenced depends on that virus's concentration and genome size (Angly et al., 2006). To fully capture viral diversity, shotgun metagenomic approaches are necessary because viruses do not have a ubiquitously conserved gene (Rohwer and Edwards, 2002) that can be used for phylogenetic studies, such as the 16S rRNA gene in bacteria and archaea.

Gene-targeted metagenomics involves targeted sequencing of a gene or genome region amplified by PCR. The PCR primers may also include barcodes or tags to allow simultaneous sequencing and differentiation of multiple samples of interest. The barcodes or tags are unique sequence identifiers upstream from the primer region; following sequencing, the sequences may be parsed and separated

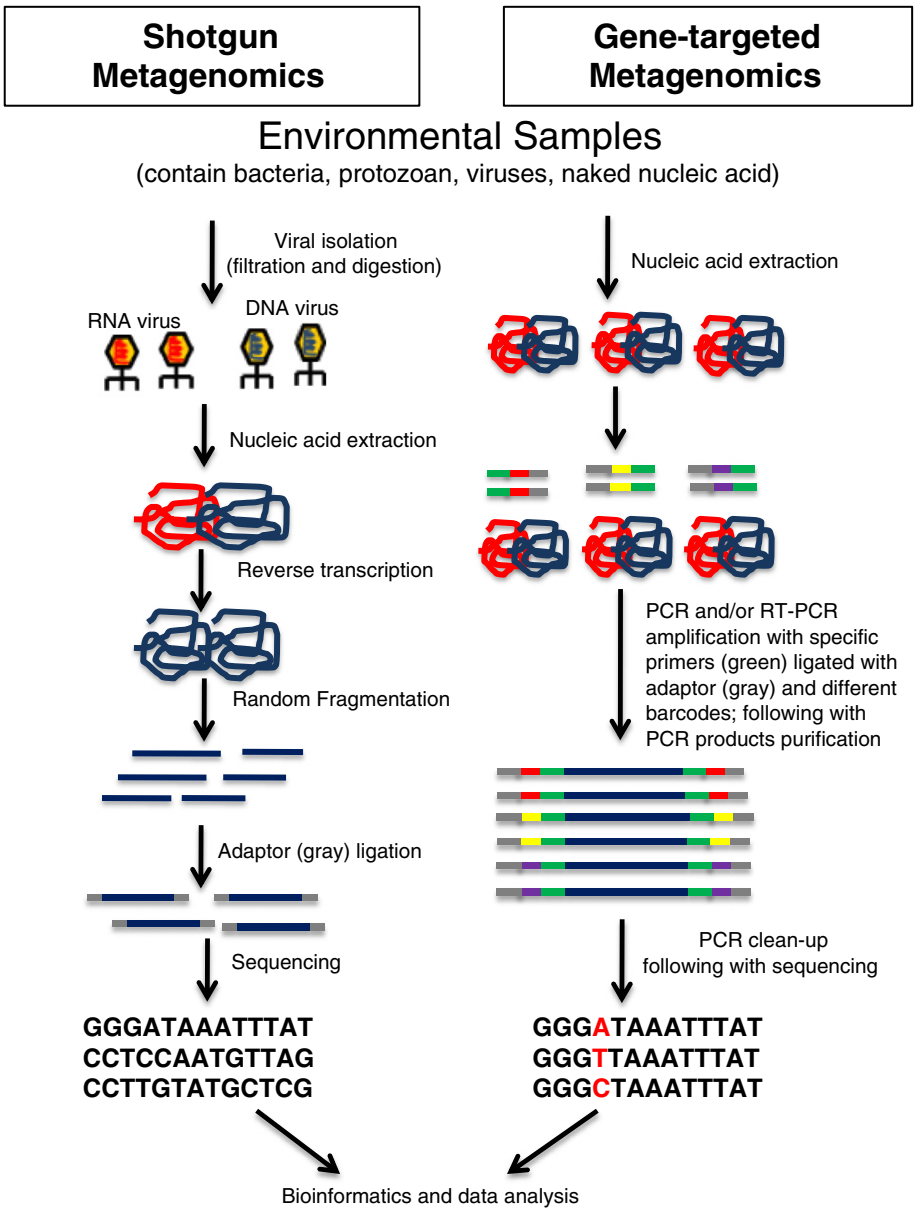

Fig. 1. The experimental procedure of shotgun and gene-targeted metagenomics for viruses.

based on the barcodes. Due to the PCR amplification step, gene-targeted metagenomics is specific to the selected target. One caveat is that PCR amplification may introduce biases from amplification and primer specificity. This approach is highly analogous to the 16S rRNA gene pyrosequencing method for describing bacterial diversity (Bibby et al., 2010).

Both shotgun and gene-targeted approaches present advantages and challenges for studying viral ecology and thus have different potential applications in the development of enteric virus MST tools or MST in general. The shotgun approaches have the advantages of allowing a less biased view of complete viral diversity and estimating relative viral abundance. A challenge of shotgun metagenomics is the bioinformatic classification of short sequences, due mainly to the large diversity of viruses in the environment and their limited representation in available sequence databases. Another is the limited environmental concentration of certain viruses of interest. A recent study found that viral pathogens comprise <0.1% of sequences in a viral metagenome derived from an environmental sample (Bibby et al., 2011). Thus, while enteric viruses may exist at significant concentrations in the environment, the abundance of certain enteric viruses may not be high enough (when contrasted with bacteriophages and other viruses) to be detected by a metagenomic approach.

The shotgun approaches advance the development of enteric virus MST tools in three ways. First, it may suggest the viruses that should be targeted in the field, based on the relative abundance of enteric viruses within a metagenome. For example, a recent viral metagenomic sequencing study by Bibby et al. (2011) suggested that parechovirus was actually more abundant than other enteric viruses – contrary to previous findings that adenovirus was the most abundant (Viau and Peccia, 2009; Wong et al., 2010) – indicating the potential of parechovirus for enteric virus monitoring and as an MST tool. An important gap in current library-independent studies, including enteric virus MST, is the inability to estimate the proportion of sources; determining relative abundance by shotgun metagenomics can possibly overcome this limitation.

Second, the whole genome sequence of certain enteric viruses (i.e., bovine polyomavirus) is still very limited. Designing a reliable PCR assay capable of efficiently target viruses of interest from different sources requires a comprehensive genome sequence database; NGS promises to expand this database. Compared to previous

sequencing methods, shotgun metagenomics can sequence the whole genome of isolated or highly enriched viruses in less time and at less cost. Since viral genome sizes are typically much smaller and simpler than bacterial and eukaryotic genomes, and the bioinformatic methods for assembly and annotation are reasonably well developed at this stage, expanding viral genome sequence databases should be an attainable goal. Many viruses cannot be cultured, making isolation extremely difficult; thus, shotgun approach may be the most accessible method for genomic information. Near-complete viral genomes have been successfully assembled from viral metagenomes (Skennerton et al., 2011).

Lastly, the shotgun approach has potential to simultaneously detect multiple targets, which is ideal for the MST toolbox approach. PCR-based methods require individual design, verification, and performance evaluations; in contrast, a single viral metagenome may be able to detect all highly enriched enteric viruses of concern in the environment. However, because the abundance of most enteric viruses in the environment is usually low, increases in sequence depth (number of sequences) and improvement of bioinformatic methods will still be required to robustly utilize whole viral metagenomics to detect environmental enteric viruses.

Gene-targeted approaches overcome the limitation of low enrichment of pathogens by only targeting individual pathogen sequences of interest, potentially giving a much deeper view of enteric virus diversity within the sample. Due to limited scope of gene-targeted approach, bioinformatic classification is more straightforward although it can introduce PCR bias and is limited by target selection (i.e., only selected viruses will be identified). Therefore, the gene or genome region amplified by PCR should be carefully chosen for sufficient variability to visualize the desired differences. At the same time, the primer regions must be sufficiently conserved to avoid PCR bias. The HAdV hexon region amplified by the PCR assay developed by Lu and Erdman (2006) can be a good candidate because the primer region sequence is conserved and the amplicon contains six hypervariable regions, enabling differentiation of various HAdV serotypes.

Gene-targeted metagenomics has several applications for the development of enteric virus MST tools. The first one is to determine the relative abundance of various viral serotypes. This information allows efficient selection of serotype-specific MST assays for detecting dominant serotypes of enteric virus associated with fecal materials. Like shotgun approach, gene-targeted metagenomics can also enhance the design of a reliable MST assay by increasing the database upon which the design of PCR assays is based. A new coliphage-based MST approach, genotyping F+ RNA coliphage following with statistical analysis, was described in Section 4.1. Since genotyping with gene-targeted metagenomics is very robust and the data provides an in-depth view of coliphage diversity, this approach can be used during the genotyping step in the coliphage-based MST described by Lee et al. (2009 and 2011b).

Even though metagenomic approaches may not yet be feasible for routine MST, owing to high cost and complicated data analysis, the cost associated with NGS has decreased substantially (e.g., from early costs of >$15,000 to current costs of ~$1000 per Illumina HiSeq lane), and more powerful bioinformatics software development in the future should decrease efforts to analyze and interpret NGS data. These improvements will make NGS more widely available and encourage development of varied NGS-based metagenomics for MST. This will also assemble genome data to design more effective PCR assays for MST, suggesting that NGS will be a powerful part of developing MST tools.

5.2. Virus concentration technology

Recovery of enteric viruses from water has always been challenging due to their low concentration in the environment and small size (mostly between 0.01 and 0.3 μm) (Maier et al., 2008). Different filters have been developed for concentrating viruses and an overview of the characteristics of these filters is provided in Table 6. The most common virus recovery method is the virus adsorption and elution (VIRADEL) technique where virus capturing is based on electrostatic interaction between viruses and filters. Isoelectric point (pI) is the pH at which the net charge of a virus surface is zero (Maier et al., 2008). Virus surface will be negatively charged when the solution's pH > pI and will be positively charged when the solution pH < pI. Since pI of most viruses is below natural water's pH (~7), the surface charge of most viruses under normal environmental conditions is usually negative (Michen and Graule, 2010).

In a VIRADEL method, the net surface charge of either the filters or viruses is manipulated to promote adsorption and subsequent elution of viruses from filters. Positively or negatively charged cartridge filters, such as the Zeta Plus™ 1MDS (1MDS) cartridge filters (CUNO, Inc.) and Filterite filters that allow concentration of large volumes (up to 1000 L) have been the top choice in drinking water and environmental virus monitoring since the 1980s (Farrah et al., 1976; Gerba et al., 1978; Sobsey and Glass, 1980, 1984; Sobsey and Jones, 1979). pH manipulation is usually not required when using an electropositive filter, such as the 1MDS filter. However, 1MDS filters are very costly (Table 6), and low recovery of viruses from marine water has been reported (Lukasik et al., 2000). Sobsey and Glass (1980) also reported that the 1MDS filter could not be used when water pH was above 8.0–8.5, and acidification of a water sample is needed when pH is above 8.0 (Fout et al., 1996). The use of an electronegative filter for fresh water sampling requires reversing the surface charge of the virus from negative to positive by either lowering the pH of the water sample to below the pI of most enteric viruses (~3.5) or by adding cationic salt (e.g. $MgCl_2$). Adjusting the pH and salt concentration of a large volume of sample can be time-consuming, tedious and requires additional testing in the field (i.e., measuring water pH).

Table 6
Characteristics of different filters.

	Mechanism	Ability to handle high turbidity water[a]	Technical difficulty	Recovery sensitive to pH	Recovery sensitive to salinity	Cost per filter	Commercial availability	Field application references
HA (membrane)	VIRADEL	Low	Low	Yes	Yes	~$3	Yes	Fong et al. (2005); Kirs et al. (2011)
1MDS	VIRADEL	High	Medium	Yes	Yes	>$200	Yes	Xagoraraki et al. (2007); Verheyen et al. (2009)
NanoCeram	VIRADEL	High	Medium	Yes	Yes	~$50	Yes	Deboosere et al. (2011); Rodríguez et al. (2012)
Glass wool	VIRADEL	High	Medium	Yes	Yes	~$5	No	Kiulia et al. (2010); Hunt et al. (2010)
Filterite	VIRADEL	High	Medium	Yes	Yes	~$50	Yes	Rao et al. (1984); Wetz et al. (2004)
Tangential ultrafiltration	Entrapment	Medium	High	No	No	~$20	Yes	Grassi et al. (2010); Gibson and Schwab (2011)
Dead end ultrafiltration	Entrapment	Medium	Medium	No	No	~$20	Yes	Leskinen et al. (2010)

[a] Evaluations were based on cartridge filters except for HA membrane.

5.2.1. NanoCeram

There has been an increased attention on the NanoCeram (NC) filter, a new electropositive filter, for concentrating viruses (Gibbons et al., 2010; Ikner et al., 2011; Karim et al., 2009; Lau et al., 2004; Lee et al., 2011a; Li et al., 2010b; Sibley, 2011; Tepper and Kaledin, 2006). The active component of a NC filter consists of an electropositive nanoalumina fibrille that is 2 nm in diameter, and end-bonded to a microglass fiber (Tepper and Kaledin, 2006). The pI of the nanoalumina fibrille is ~9.7 (Sibley, 2011). The average pore size of this filter is 2–3 μm. Recently, EPA suggested using the NC cartridge filter to concentrate waterborne enterovirus and norovirus (USEPA, 2010). The NC filter showed a comparable virus recovery to 1MDS filter at substantial cost savings to the user (Karim et al., 2009; Lee et al., 2011a). Lau et al. (2004) indicated that using nanoporous aluminium oxide can increase surface area by a factor of 100, compared to other coating materials. Table 7 illustrates the recovery of different viruses using NC cartridge and micro filters. Studies show that the NC cartridge filter can be used for monitoring HEV, HNoV and bacteriophage. Both sodium polyphosphate/phosphate buffer/glycine and beef extract/glycine can be used for eluting these viruses from NC filter. The recoveries were not significantly different for waters with pH between 6 and 9.5 and flow rates between 5.5 L/min to 20 L/min (Karim et al., 2009). To compare performance of NC and 1MDS cartridge filters, poliovirus and Norwalk virus were seeded into tap and Ohio River water. Results showed that the recoveries by NC were equal to or higher than 1MDS (Karim et al., 2009).

Low recoveries of adenovirus (AdV) using the NC cartridge filter were reported in several studies (Gibbons et al., 2010; Ikner et al., 2011; Sibley, 2011) and the strong attachment between AdV and the NC filter was speculated (Gibbons et al., 2010; Sibley, 2011). After filtering water seeded with AdV, both Gibbons et al. (2010) and Sibley (2011) found that only minimal amounts of AdV were left in the filtrate, which indicated that the NC cartridge filter was able to capture most of the AdV seeded in the sample. However, the eluents, which had good results in recovering other viruses from the filter, did not effectively elute AdV from the NC cartridge filter. Interestingly, Li et al. (2010b) compared the recovery of AdV from the NC microfilter using an eluent with different pHs and found that eluents with pH 5 and 6 had much higher recovery of AdV than the eluent at pH 9 (Table 7), the pH used in studies by Ikner et al. (2011), Gibbons et al. (2010), and Sibley (2011). This observation was unexpected because high pH eluent is usually more effective eluting viruses from electropositive filters, due to both the virus and filter becoming negatively charged when the pH of the eluent is above their pIs. More studies are warranted on the performance of filters and methods with a focus on: (1) interaction between AdV and NC filter surface, (2) the isoelectric point of AdV, and (3) whether eluents with lower pH can achieve higher recovery of AdV from NC cartridge filters. In conclusion, there are promising results using NC filters for concentrating enteric viruses. Future studies may focus on the performance of this type of filter with other promising enteric viruses for MST applications, such as polyomavirus.

Table 7
Recovery efficiency of NanoCeram filter.

Virus	Type of water	Recovery %	Eluate; secondary concentration	Reference
Murine norovirus	Distilled water	23	BE/glycine/Tween 80 pH 9.5; none	Lee et al. (2011a)[a]
GII — 4 NoV		86		
MS2	Tap water	45–56	Polyphosphate/ phosphate buffer/ glycine; centricon	Ikner et al. (2011)[b]
Poliovirus 1		66		
Echovirus 1		83		
Coxsackievirus B5		77		
Adenovirus 2		14		
Adenovirus 41	Seawater, source water, finished water	1.4–2.5	BE/glycine; none	Gibbons et al. (2010)[b]
Q_B coliphage		34–96		
Norovirus	Seawater	111	BE	
Adenovirus 5	RO water	~10[c]	BE pH 9; Amicon	Li et al. (2010b)[a]
		~80[c]	BE pH 5; Amicon	
		64–91	BE pH 6; Amicon	
	Seawater	37–82		
	Treated sewage	44–86		
Poliovirus 1	Tap water[d]	54	BE/glycine; celite elution	Karim et al. (2009)[b]
Coxsackievirus B5		27		
Echovirus 7		32		
Poliovirus 1	Tap water[e]	51–277		
	River water[e]	38–65		

RO = reverse osmosis.
BE = beef extract.
[a] Microfilters were used.
[b] Cartridge filters were used.
[c] Estimated from graph.
[d] Number of seeded virus = 2×10^5 to 9×10^5 PFU.
[e] Number of seeded virus = 94 to 318 PFU.

5.2.2. Glass wool

Another VIRADEL technique reported in the literature is a novel glass wool filter validated by researchers from the Marshfield Clinic and the University of California-Davis during a large groundwater epidemiological study (Lambertini et al., 2008). This filter consists of packed glass wool held together by a binding agent and coated with mineral oil, providing both hydrophobic and electropositive sites on its surface. Glass wool has been used in several virus monitoring studies of wastewater, drinking water, ground water and river water (Deboosere et al., 2011; Gantzer et al., 1997; Lambertini et al., 2008; Powell et al., 2003; van Heerden et al., 2004). Recovery efficiencies for enteric viruses (i.e. poliovirus, coxsackievirus, echovirus, adenovirus and norovirus) averaged between 14% and 70% (Lambertini et al., 2008). Efficiency of these filters is largely affected by virus type, water matrix and high pH. The cost of a glass wool filter is low (Table 6), substantially cheaper than other VIRADEL-based cartridge filters. These filters are not manufactured on a large scale and technical skills are required to assemble them; however, a recent visual-based article illustrated the technique to pack glass wool column (Millen et al., 2012).

5.2.3. HA membrane

While filtering a large volume of water allows viruses to be concentrated and isolated from the environment, the techniques often suffer from clogging and low recovery. With the widespread use of sensitive detection assays that can detect as few as 10 copies of a viral genome, large volumes of water samples may not be necessary. Alternatively, positively or negatively charged membrane filters have been evaluated and used successfully in concentrating viruses from 0.5 to 10 L of water (Fong et al., 2005; Haramoto et al., 2005; Katayama et al., 2002; Lipp et al., 2007; Lukasik et al., 2000). Katayama et al. (2002) validated a virus concentration and elution method with a high virus recovery and minimal PCR inhibitory effects. In this method, freshwater with 25 mM $MgCl_2$ added or marine water is filtered through a negatively charged HA membrane (Millipore, Billerica, MA), followed by weak acid rinsing to remove cation and other inhibitors. An inorganic eluting solution (NaOH) that has fewer inhibitory effects in PCR/qPCR assays than beef extract is used to elute viruses from the membrane. Katayama et al. (2002) observed poliovirus recoveries between 33 and 90% from purified water and between 38 and 89% from natural seawater. Victoria et al. (2009) evaluated recoveries of HNoV and human astrovirus (HAstV) from tap water, seawater, river water and mineral water with varying $MgCl_2$ concentrations. While there was no consistent trend between $MgCl_2$ concentrations and virus recovery, the best recovery for both HNoV and HAstV was observed in mineral water. Recovery of HNoV ranged between 0.8% in seawater and 22.8% in mineral water,

whereas recovery of HAstV ranged between 0.5% in tap water and 63.5% in mineral water.

In 2005, the same research group developed a positively charged membrane concentration method by coating the HA filter with $AlCl_3$ (Haramoto et al., 2005). Haramoto et al. (2009) compared recovery of HNoV and HEV from five water matrices (MilliQ water, bottled water, pond water, river and tap water samples), using three virus concentration methods: HA membrane with $MgCl_2$ amendment; Al^{3+}-coated HA membrane; and 1MDS membrane. With $MgCl_2$ amendment, norovirus recovery ranged from 15% in river water to 186% in MilliQ water, and a similar trend was observed for poliovirus recovery. Al^{3+}-coated HA membrane showed moderate recovery, with recovery ranging from 32 to 138% for both viruses. Recovery from 1MDS membrane was the lowest among the three methods, ranging from 5 to 92%.

5.2.4. Ultrafiltration and ultracentrifugation

Besides the VIRADEL method, virus recovery techniques based on entrapment, such as hollow-fiber ultrafiltration and ultracentrifugation, have also been used. One advantage of using ultrafiltration and ultracentrifugation for sampling in MST studies is that they allow simultaneous recovery of various types of microorganisms which, in turn, allow the user to apply a "tool box" approach in MST targeting both bacterial and viral markers. Studies have used both tangential and dead-end flow ultrafiltration to sample pathogens and indicators from environmental water (Gibson and Schwab, 2011; Hill et al., 2005, 2007; Leskinen et al., 2010; Smith and Hill, 2009). The advantage of tangential-flow (also known as cross-flow) over dead-end-flow is reduced filter fouling since solids accumulating on the filter are substantially washed away by the cross-flow. However, tangential-flow requires more training to operate (Smith and Hill, 2009). Recent studies showed that both tangential and dead-end ultrafiltration are able to concentrate viruses, bacterial pathogen, fecal indicators, and protozoan parasites simultaneously with decent recovery rates (Gibson and Schwab, 2011; Polaczyk et al., 2008; Smith and Hill, 2009). The recovery range for bacteria and virus were 57–94% and 57–73%, respectively, by Smith and Hill (2009), 30–183% and 16–84%, respectively, by Gibson and Schwab (2011). Mean recoveries of seeded MS2 bacteriophage, echovirus 1, *Salmonella enterica* subsp. enterica serovar Typhimurium, *Bacillus atrophaeus* subsp. *globigii* endospores, and *Cryptosporidium parvum* oocysts ranged between 51 and 94% (Polaczyk et al., 2008). These studies were able to concentrate up to 100 L of tap and surface water. A drawback of this method is that fine organic matter present in certain environmental matrices is also concentrated during the ultrafiltration procedure, and can cause PCR inhibition issues during sample analysis. More studies are needed to investigate the extent of PCR inhibition in environmental water samples concentrated by ultrafiltration and potential solution scenarios.

5.2.5. Direct nucleic acid extraction from filter membrane

Finally, future research should investigate on the possibility of performing nucleic acid (NA) extraction directly from the filter membrane. As mentioned earlier, the main applications and advantages of using enteric viruses for MST have been their use as a culture-independent approach. If the objective of the study is only to determine the fecal source and the viability of the viruses is not an issue, there is really no need to elute the "intact" virus before the NA extraction. Direct nucleic acid extraction from the filter may result in higher overall recoveries, but attention should be given to other issues, such as larger degree of inhibition and more rapid degradation of filter-attached viruses (Haramoto et al., 2008), if direct extraction of NA from filters is considered. Future studies will require investigation of whether direct NA extraction will indeed produce higher overall recoveries and identify issues of inhibition with different types of environmental matrices.

6. Conclusion

Enteric viruses show great potential as MST markers, especially when used in "tool box" approaches where viruses of different hosts are tested in the same sample. In addition, the higher persistence of these viruses relative to bacterial indicators (Fujioka and Yoneyama, 2002) suggests that viral analysis could be advantageous in situations that require tracking of fecal contamination at a distance downstream from the source. Viruses are generally host-specific, but the selection of a target gene is important in deciding inclusivity and exclusivity of a detection assay. Human polyomavirus has been shown to be a good tool for tracking human fecal contamination and for discriminating between human and non-human contamination sources in aquatic environments. However, high occurrence, density and environmental persistence give human adenovirus multiple advantages over other enteric viruses for MST. Additional specificity testing of human enteric virus assays originally designed for medical or treatment purposes is warranted. More investigation of the potential of applying avian enteric viruses for MST, as well as the prevalence and specificity of ruminant and porcine enteric viruses, is also warranted.

Next generation sequencing (NGS) provides a platform for a better understanding of virus ecology and viral genomes in specific environments. The information on relative abundance produced by shotgun and gene-targeted metagenomic approaches can serve as a screening tool to determine virus types/serotypes that should be targeted in the field. An important gap in current library-independent studies, including enteric virus MST, is the inability to estimate the proportion of the sources contributing fecal contamination. Shotgun metagenomic approaches potentially can overcome this issue by providing a broad overview of the whole viral community, including relative abundances of the different host groups present in a sample. The massive sequence database generated by NGS-based metagenomics can definitely enable more efficient and reliable design of MST assays.

In the past decade, advancements have been made not only in virus detection techniques, but also in sample concentration methods. Large volume concentration methods such as NanoCeram, glass wool, and ultra-filters can achieve equal – or better – results than 1MDS filters and at a much lower cost. Electronegative (HA) membrane filters offer a simple, but efficient, method to concentrate waterborne viruses. Sampling that allows co-concentration of several pathogen groups (i.e., ultrafiltration) makes comparisons of multiple MST markers feasible; however, inconsistent recoveries associated with these methods greatly affect viral quantification. Therefore, one area for future development in environmental virology is to improve virus concentration technology by focusing on surface interactions between viruses and filter materials. Specifically, there is a need to develop efficient elution methodologies that can disrupt the attachment between filter materials and viruses to increase recovery.

Overall, high specificity and sensitivity, along with higher environmental stability make enteric virus MST assays a promising tool as part of regular monitoring of impaired surface and recreational waters. Consideration should be given to using enteric virus assays for Total Maximum Daily Load (TMDL) applications where allocation of contamination sources is extremely important in implementing best management practices. As technology advances in the field of molecular biology, we expect enteric virus MST assays to become more robust and reliable for identifying fecal contamination sources.

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