GLOBAL WATER PATHOGEN PROJECT

PART TWO. INDICATORS AND MICROBIAL SOURCE TRACKING MARKERS

HUMAN AND ANIMAL ENTERIC VIRAL MARKERS FOR TRACKING THE SOURCES OF FAECAL POLLUTION

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Summary

Viruses are a major cause of waterborne diseases in humans. Their effects range from self-limiting gastroenteritis (nausea, diarrhoea, vomiting) to severe diseases like hepatitis, meningitis, and polio. The presence of human viruses in water is a relatively definitive indication of human wastewater pollution. Tracking the source(s) of faecal pollution in environmental water resources used for recreation or aquaculture is imperative to minimize human health impacts and regulate water quality. Microbial source tracking (MST) is increasingly employed to determine the source(s) of faecal pollution in environmental waters. Among the MST techniques, application of enteric viruses has received significant attention due to their high abundance in the faeces and urine of humans and animals, low infectious doses, high persistency in environmental waters and, typically, strict host-specificity. Human adenoviruses (HAdVs), human polyomaviruses (HPyVs), and pepper mild mottle viruses (PMMoVs) are frequent targets for the polymerase chain reaction (PCR) and quantitative PCR (qPCR) based assays used in MST field studies to identify human wastewater pollution in environmental waters. Animal viruses have also been used to identify faecal pollution from specific animal hosts. This chapter outlines the concentrations of human and animal (where available) wastewater-associated viruses in point source materials (untreated human wastewater, secondary effluent, treated wastewater and sludge) and receiving waters (recreational water, storm water and drinking water reservoirs). The chapter also sheds light on the correlation between faecal indicator bacteria (FIB) and viral MST markers, and the relevance of these markers to public health risks. Finally, expanding the application of enteric viruses for MST field application through new technologies and efficient virus recovery methods are discussed.

1.0 Performance Characteristics of the Viral MST Markers

1.1 Overview

The human enteric viruses, adenoviruses, polyomaviruses, and a plant virus, the pepper mild mottle viruses (PMMoVs) have received significant attention as library-independent microbial source tracking (MST) markers of human wastewater due to their high abundance in the faeces and urine of humans, high persistency in environmental waters, and strict host-specificity (Wong et al., 2012). To identify the sources of human wastewater pollution, PCR/qPCR detection of human adenoviruses (HAdVs), human polyomaviruses (HPyVs), and pepper mild mottle viruses (PMMoVs) have been the most commonly used tools in MST studies (Fong et al., 2005; McQuaig et al., 2009; Rosario et al., 2009), whereas, animal viruses such as porcine adenoviruses (PAdVs), bovine adenoviruses (BAdVs), bovine polyomaviruses (BPyVs), chicken/turkey parvoviruses (ChPVs/TuPVs) have also been used at some extent to identify animal wastewater pollution in environmental waters (Hundesa et al., 2006; Carratalá et al., 2012). For the detection of human and animal viral MST markers, PCR and qPCR based assays are used over cell culture assays. Cell culture assays provide information on the infectivity of the viral markers, which may not be considered important from the MST point of view.

The host-sensitivity, host-specificity are important aspects of the characteristics of these viruses as well as the possible correlation between faecal indicator bacteria and viral MST markers, and their relevance to public health risks. Their concentrations of human and animal wastewater-associated viral markers in point sources (raw human wastewater, secondary effluent, treated wastewater and sludge samples), and receiving waters (recreational water, storm water and drinking water reservoirs) are summarized. The application of enteric viruses have been included using relevant MST studies Extending the use of enteric viruses for MST field application through new technologies such as metagenomics, and the importance of efficient virus recovery methods are included.

1.2 Host-sensitivity and -specificity of human viral markers

Host-sensitivity and -specificity are considered as the two most important performance characteristics of MST markers. Non-specific (found in non-target hosts) and non-prevalent (rare) markers tend to yield false positive or negative results in field studies (Stoeckel and Harwood, 2007; Ahmed et al., 2013). It should be pointed out that in PCR-based assays, the precise sequence targeted and the primers/probes used can play a major role in sensitivity and specificity of a given marker/assay as similar sequences may be amplified by poorly designed oligonucleotides. Thus, all assays for a given target may not be equally effective.

Table 1 summarizes the host-sensitivity and -specificity values for human wastewater associated-viral markers reported in the literature. Several studies have investigated the host-sensitivity and -specificity of HAdVs and HPyVs. It is apparent that broad range of HAdVs comprising all six species (A-F) are highly prevalent in raw wastewater samples (Hundesa et al., 2006; Ahmed et al., 2010a; Rusiñol et al., 2014) rather than a particular species of HAdVs (Wolf et al., 2010). Therefore, assays targeting a broad range of HAdVs would be more suitable for detecting human wastewater pollution in environmental studies. However, the broad range of HAdVs would not provide information on the health risks, which can be obtained by analysing HAdVs species F, which contains two fastidious serotypes Ad40 and 41 responsible for gastroenteritis in children and adults.

Several studies have reported 100% host-specificity of HAdVs by analysing non-human faecal samples (Table 1). For example, Ahmed et al., (2010) investigated the host-specificity of HAdVs by analysing 106 non-human faecal samples from bird, cattle, chicken, dog, duck, goat, horse, kangaroo, pig and sheep. All the non-human faecal samples were PCR negative for the HAdVs. Similarly, Wolf et al., (2010) also screened 56 faecal samples from black swan, cattle, Canada goose, deer, duck, pig, sheep for the presence of HAdVs, however, none of the samples was PCR

positive. studies also determined the host-specificity values of HAdVs by analysing only a handful of non-human faecal samples from a small number of hosts (Noble et al., 2003; Hundesa et al., 2006; Li et al., 2015).

Table 1. Host-sensitivity and -specificity values reported in the literature for human-associated viral MST markers

MST Viral Markers	Types of Assay	Host-sensitivity		Host-specificity		Reference
		Human Host	Percent Sensitivity (# of Samples)	Non-human Hosts	Percent Specificity (# of Samples)	
HAdVs[a] (A-F)	Nested PCR	Raw wastewater	100% (9/9)	Slaughterhouse wastewater	100% (22/22)	Hundesa et al., 2006
HAdVs (A-F)	Microarray detection	Raw and diluted wastewater	100% (5/5)	Cattle faeces, poultry litter, swine faeces, wild avian faeces	0% (0/7)	Li et al., 2015
HAdVs (A-F)	qPCR	Raw wastewater	100% (9/9)	-	NR	Hundesa et al., 2009
HAdVs	Nested PCR	Raw wastewater	92% (11/12)	-	-	De Motes et al.,2004
HAdVs (A-F)	Nested PCR	Raw wastewater	100% (30/30)	Bird faeces, cattle faeces, cattle wastewater, chicken faeces, dog faeces, duck faeces, goat faeces, horse faeces, kangaroo faeces, pig faeces, sheep faeces	100% (106/106)	Ahmed et al., 2010a
		Primary effluent	100% (18/18)			
		Secondary effluent	13% (2/16)			
		Septic wastewater	80% (8/10)			
HAdVs (A-F)	qPCR	Raw wastewater	100% (33/33)	-	-	Rusiñol et al., 2014
		Secondary wastewater	100% (32/32)			
HAdVs-F	qPCR	Raw wastewater	100% (11/11)	Black swan faeces, cattle faeces, Canada goose faeces, deer faeces, duck faeces, pig faeces, sheep faeces, sheep and calf processing abattoirs	100% (56/56)	Wolf et al., 2010
		Biosolids	75% (3/4)			
		Human faeces	0% (0/15)			
HAdVs-C	qPCR	Raw wastewater	36% (4/11)	Black swan faeces, cattle faeces, Canada goose faeces, deer faeces, duck faeces, pig faeces, sheep faeces, sheep and calf processing abattoirs	100% (56/56)	Wolf et al., 2010
		Biosolids	50% (2/4)			
		Human faeces	47% (7/15)			
HAdVs	Nested PCR	Raw wastewater	50% (4/8)	Cow faeces, dog faeces, gull faeces	100% (3/3)	Noble et al., 2003
HPyVs[b] (JCVs)	Real-time PCR	Raw wastewater	100% (9/9)	-	-	Hundesa et al., 2006
HPyVs (JCVs and BKVs)	Microarray detection	Raw and diluted wastewater	100% (5/5)	Cattle faeces, poultry litter, swine faeces, wild avian faeces	100% (7/7)	Li et al., 2015
HPyVs (JCVs and BKVs)	PCR	Raw wastewater	100% (40/40)	Chicken faeces, dog faeces,duck faeces, kangaroo faeces, wild bird faeces, cattle wastewater, pig wastewater, sheep wastewater	99% (80/81)	Ahmed et al., 2010b
		Secondary effluent	100% (23/23)			

MST Viral Markers	Types of Assay	Host-sensitivity		Host-specificity		Reference
		Human Host	**Percent Sensitivity (# of Samples)**	**Non-human Hosts**	**Percent Specificity (# of Samples)**	
HPyVs (JCVs and BKVs)	qPCR	Raw wastewater	100% (41/41)	Cat faeces, chicken faeces, cow faeces, sand hill crane faeces, deer faeces, dog faeces, duck faeces, fox faeces, horse faeces, racoon faeces, seagull faeces, sparrow faeces, wild pig faeces, cow wastewater, pig wastewater, dog urine, cat urine	100% (127/127)	McQuaig et al., 2009
		Tertiary effluent	22% (2/9)			
		Septic tank pump truck	100% (9/9)			
		Septic tank	60% (3/5)			
		Human urine	23% (6/26)			
HPyVs (JCVs and BKVs)	Nested PCR	Raw wastewater	100% (36/36)	Cow wastewater, pig wastewater,	100% (25/25)	McQuaig et al., 2006
		Septic tank pump truck	100% (14/14)			
HPyVs (JCVs and BKVs)	PCR	Raw wastewater	100% (17/17)	Cat faeces, chicken faeces, chicken effluents, cow faeces, cow lagoon wastewater, dog faeces, duck faeces, seagull faeces, bird faeces	100% (332/332)	Harwood et al., 2009
		Lift stations	100% (21/21)			
		Septic systems	100% (3/3)			
HPyVs (JCVs and BKVs)	PCR	Raw wastewater	100% (6/6)	Bird faeces (Black swan, chicken, duck, pukeko, seagull, unidentified birds), dairy farm wastewater, horse faeces, marsupial faeces (kangaroo, possum, rabbit, wallabies), pet faeces (cat and dog), rodent faeces (guinea pig, mice, rat), ruminant faeces (Alpaca, cow, deer, goat, sheep)	100% (71/71)	Kirs et al., 2011
		Human faeces	18% (2/11)			
		Oxidation pond	100% (5/5)			
HPyVs (JCVs and BKVs)	qPCR	-		Bird faeces, cattle faeces, dog faeces	100% (32/32)	Staley et al., 2012a
HPyVs (JCVs)	qPCR	Hospital raw wastewater	100% (10/10)	-	-	Rusiñol et al., 2013
		Raw wastewater	100% (4/4)			
HPyVs (JCVs)	qPCR	Raw wastewater	100% (33/33)	-	-	Rusiñol et al., 2014
		Secondary wastewater	81% (26/32)			

MST Viral Markers	Types of Assay	Host-sensitivity		Host-specificity		Reference
		Human Host	Percent Sensitivity (# of Samples)	Non-human Hosts	Percent Specificity (# of Samples)	
HPyVs (JCVs and BKVs)	PCR	Raw wastewater	100% (15/15)	Cat faeces, chicken faeces, cow faeces, dog faeces, duck faeces, geece faeces, pig faeces, seagull faeces	100% (173/173)	Hellein et al., 2011
PMMoVs[c]	RT-qPCR	Raw wastewater	100% (12/12)	Cow faeces, chicken faeces, coyote faeces, dog faeces, gull faeces, horse faeces, hog faeces, pig faeces, sheep faeces, racoon faeces, intestinal chicken homogenates, intestinal turkey homogenates	90% (71/79)	Rosario et al., 2009
		Treated wastewater	100% (12/12)			
PMMoVs	Microarray	Raw and diluted wastewater	0% (0/4)	Cattle faeces, swine faeces, poultry litter, wild avian faeces	67% (2/3)	Li et al., 2015
HEV[d]	PCR	Raw wastewater	38% (3/8)	Cow faeces, dog faeces, gull faeces	100% (3/3)	Noble et al., 2003
HNoVs[e]-GI	RT-qPCR	Raw wastewater	82% (9/11)	Black swan faeces, cattle faeces, Canada goose faeces, deer faeces, duck faeces, pig faeces, sheep faeces, sheep and calf processing abattoirs	100% (56/56)	Wolf et al., 2010
		Biosolids	50% (2/4)			
		Human feces	40% (6/15)			
HNoVs-GII	RT-qPCR	Raw wastewater	82% (9/11)	Black swan faeces, cattle faeces, Canada goose faeces, deer faeces, duck faeces, pig faeces, sheep faeces, sheep and calf processing abattoirs	100% (58/58)	Wolf et al., 2010
		Biosolids	75% (3/4)			
		Human feces	67% (10/15)			

NR: Not Reported; RT: Reverse-transcriptase; -: Not tested; [a]HAdVs: Human Adenoviruses; [b]HPyVs: Human Polyomaviruses; [c]MMoVs:Pepper mild mottle viruses [d]HEV hepatitis E Virus; [e]HNoVs Human norovirus

The performance characteristics of HPyVs have been evaluated more rigorously than HAdVs. HPyVs are reported to be highly prevalent (100% host-sensitivity) in raw and septic wastewater samples in Australia, New Zealand, Spain and the USA (McQuaig et al., 2006; Ahmed et al., 2010b; Kirs et al., 2011; Rusiñol et al., 2014). McQuaig et al. (2006) developed a PCR assay for the detection of HPyVs in environmental waters. This assay was later upgraded to a TaqMan-based qPCR for the quantification of HPyVs in raw wastewater, human urine and environmental samples (McQuaig et al., 2009). These two PCR/qPCR assays have been most commonly used to determine the host-sensitivity and -specificity of HPyVs in human and non-human wastewater and faecal samples. Merging faecal and wastewater samples from studies (McQuaig et al., 2006; Harwood et al., 2009; McQuaig et al., 2009; Ahmed et al., 2010b; Kirs et al., 2011; Staley et al., 2012a; Hellein et al., 2011), the presence of HPyVs was tested by analysing 841 non-human faecal and wastewater samples. HPyVs were 100% specific to human wastewater and urine samples. Less is known regarding the host-sensitivity and -specificity of other human wastewater associated viruses such as PMMoVs, human enteroviruses (HEVs), human noroviruses group I and II (HNoVs-GI/GII). Some of these viruses although appear to be host-specific, but they have low host-sensitivity. For example, HEVs were detected in 38% of eight wastewater samples (Noble et al., 2003), whereas, HNoVs-GI and HNoVs-GII were detected in 82% of 11 raw wastewater samples (Wolf et al., 2010).

1.3 Host-sensitivity and -specificity of animal viral markers

Table 2 summarizes the host-sensitivity and -specificity values for animal faecal-associated viral markers in the literature. PAdVs, BAdVs and BPyVs have been used as targets to identify porcine and bovine faecal pollution in field studies. The prevalence of these viruses can be high in slaughterhouse wastewater (a mixture of faeces and urine from thousand animals) compared to a faecal sample from an individual animal. In reality, the chance of slaughterhouse or farm wastewater reaching environmental waters is higher than waste from an individual animal. To date, only three studies have investigated the host-specificity of PAdVs (De Motes et al., 2004; Hundesa et al., 2009; Wolf et al., 2010). Merging faecal samples from these studies, the presence of PAdVs was determined by analysing 87 non-porcine faecal samples. PAdVs were 100% specific to faeces tested. BAdVs also appear to be highly host-specific (100%) when merging 329 non-bovine faecal samples from studies by De Motes et al., 2004; Ahmed et al., 2010a; Wolf et al., 2010; Wong and Xagoraraki, 2010; Ahmed et al., 2013). Little has been published regarding the host-sensitivity and -specificity of ovine polyomaviruses (OPyVs), bovine noroviruses (BNoVs), bovine enteroviruses (BEVs) and porcine teschoviruses (PTVs) (Ley et al., 2002; Wolf et al., 2010; Rusiñol et al., 2013). Nonetheless, these viral markers have shown potential to indicate bovine and porcine faecal pollution in environmental waters. Their application along with commonly used BAdVs, PAdVs and BPyVs in a “toolbox” format may provide additional information on the presence of animal wastewater in environmental water samples.

Table 2. Host-sensitivity and -specificity values reported in the literature for animal-associated viral MST markers

MST Viral Markers	Types of Assay	Host-sensitivity		Host-specificity		Reference
		Animal Host	Percent Sensitivity (# of Samples)	Non-target Hosts	# of Samples Tested (% Specificity)	
PAdVs[a]	Nested PCR	Slaughterhouse wastewater	100% (100/100)	-	-	Hundesa et al., 2006
PAdVs	qPCR	Pig faeces	87% (33/38)	Raw human wastewater	100% (9/9)	Hundesa et al., 2009
		Slaughterhouse wastewater	100% (8/8)			
PAdVs	Nested PCR	Pig faeces	84% (32/38)	-	-	Hundesa et al., 2009
		Slaughterhouse wastewater	100% (8/8)			
PAdVs	qPCR	Slaughterhouse wastewater	100% (11/11)	-	-	Hundesa et al., 2010
PAdVs	Nested PCR	Pooled swine faeces	71% (16/23)	Raw human wastewater	100% (12/12)	De Motes et al., 2004
PAdVs-5	qPCR	Pig faeces	60% (3/5)	Black swan faeces, Canada goose faeces, cattle faeces, duck faeces, deer faeces, human faeces, sheep faeces	100% (66/66)	Wolf et al., 2010
BAdVs[b]	Nested PCR	Slaughterhouse wastewater	5% (1/22)	-	-	Hundesa et al., 2006
BAdVs/OAdVs[c]	Nested PCR	Cattle faeces	75% (6/8)	Raw human wastewater	100% (12/12)	De Motes et al., 2004
BAdVs/OAdVs	qPCR	Slaughterhouse effluent wastewater	50% (1/2)	Black swan faeces, Canada goose faeces, duck faeces, deer faeces, human faeces, pig faeces	100% (67/67)	Wolf et al., 2010
BAdVs	qPCR	Dairy manure	85% (22/26)	-	-	Wong and Xagoraraki, 2011
		Cow faeces	22% (4/18)			
BAdVs	Nested PCR	Cattle faeces	30% (6/20)	Bird faeces, cattle faeces, cattle wastewater, chicken faeces, dog faeces, horse faeces, human wastewater, duck faeces, kangaroo faeces, pig faeces, possum faeces	100% (90/90)	Ahmed et al., 2013
		Cattle wastewater	90% (18/20)			
BAdVs	Nested PCR	Cattle faeces	30% (3/10)	Bird faeces, chicken faeces, dog faeces, duck faeces, goat faeces, horse faeces, kangaroo faeces, pig faeces, raw human wastewater, septic wastewater, sheep faeces	100% (154/154)	Ahmed et al., 2010a
		Cattle wastewater	100% (16/16)			

MST Viral Markers	Types of Assay	Host-sensitivity		Host-specificity		Reference
		Animal Host	Percent Sensitivity (# of Samples)	Non-target Hosts	# of Samples Tested (% Specificity)	
BAdVs (serotypes 4-8)	qPCR	Dairy manure	19% (3/16)	Swine faeces	100% (6/6)	Wong and Xagoraraki, 2010
		Cattle faeces	0% (0/20)			
BAdVs (BAdV 1)	Duplex FRET PCR	Dairy manure	100% (16/16)	Swine faeces	100% (6/6)	Wong and Xagoraraki, 2010
		Cattle faeces	20% (4/20)			
BAdVs (BAdV 2)	Duplex FRET PCR	Dairy manure	100% (16/16)	Swine faeces	100% (6/6)	Wong and Xagoraraki, 2010
		Cattle faeces	20% (4/20)			
BPyVs[d]	Nested PCR	Slaughterhouse wastewater	94% (17/18)	-	-	Hundesa et al., 2006
BPyVs	qPCR	Bovine urine	31% (8/26)	Raw human wastewater, porcine faeces, porcine urine	100% (23/23)	Hundesa et al., 2010
		Bovine faeces	0% (0/10)			
		Slaughterhouse wastewater	91% (10/11)			
BPyVs	qPCR	Dairy manure	100% (26/26)	Raw human wastewater, swine faeces	100% (26/26)	Wong and Xagoraraki, 2011
		Cow faeces	6% (1/18)			
BPyVs	qPCR	Bovine urine	100% (5/5)			Rusiñol et al., 2013
		Bovine slaughterhouse wastewater	100% (5/5)			
OPyVs[e]	qPCR	Sheep urine	69% (9/13)	Bovine urine, bovine slaughterhouse wastewater, chicken faeces, chicken slaughterhouse wastewater, goat faeces, goat wet straw beds, human hospital wastewater, human raw wastewater, porcine faeces, porcine slaughterhouse wastewater	100% (61/61)	Rusiñol et al., 2013
		Sheep wet straw beds	50% (21/42)			
		Sheep faeces	54% (7/13)			
		Slaughterhouse wastewater	75% (3/4)			
		Slaughterhouse treated wastewater	50% (1/2)			
BNoVs/ONoVs	RT-qPCR	Sheep faeces	50% (1/2)	Black swan faeces, Canada goose faeces, duck faeces, deer faeces, human faeces, pig faeces	100% (67/67)	Wolf et al., 2010
		Cattle faeces	100% (2/2)			
		Abattoir effluent	100% (2/2)			
		Chicken faeces	73% (22/30)			
		Hen faeces	72% (5/7)			

MST Viral Markers	Types of Assay	Host-sensitivity		Host-specificity		Reference
		Animal Host	Percent Sensitivity (# of Samples)	Non-target Hosts	# of Samples Tested (% Specificity)	
ChPVs/TuPVs[f]	Nested PCR	Turkey faeces	100% (3/3)	Biosolids, duck faeces, partridge faeces, raw human wastewater, seagull faeces, treated human wastewater	100% (48/48)	Carratalá et al., 2012
		Chicken wastewater	100% (5/5)			
		Chicken treated wastewater	80% (4/5)			
		Raw wastewater with poultry influents	44% (4/9)			
AtAdVs[g] (OAdVs, BAdVs, OdAdVs, CaAdVs)	qPCR	Sheep faeces	50% (1/2)	Black swan faeces, Canada goose faeces, duck faeces, human faeces, pig faeces	100% (66/66)	Wolf et al., 2010
		Deer faeces	0% (0/1)			
		Cattle faeces	0% (0/2)			
		Abattoir effluent	0% (0/2)			
BEVs	RT-qPCR	Cattle faeces	78% (78/100)	Donkey faeces, goat faeces, horse faeces, sheep faeces	42% (21/50)	Jimenez-Clavaro et al., 2005
BEVs[h]	RT-PCR	Cattle faeces	76% (106/139)	Deer faeces, Geese faeces	37% (20/54)	Ley et al., 2002
PTVs[i]	RT-qPCR	Pig wastewater	100% (5/5)	Cattle faeces, sheep faeces, goat faeces	100% (3/3)	Jimenez-Clavaro et al., 2003

RT: Reverse-transcriptase; -: Not tested; [a]PAdVs: Porcine Adenoviruses; [b]BAdVs: Bovine Adenoviruses; [c]BAdVs/OAdVs: Bovine Adenoviruses/Ovine Adenoviruses; [d]BPyVs:Bovine Polyomaviruses; [e]OPyVs: ovine polyomaviruses; [f]ChPVs/TuPVs: chicken/turkey parvoviruses; [g]Atadenoviruses; [h]BEVs:bovine enteroviruses; [i]PTVs:porcine teschoviruses

Human and animal enteric viruses are highly host-specific, with the exception of two studies that reported the occasional presence of host-specific viruses in non-target hosts. For example, BEVs (cattle) have been detected in goose, deer, sheep, goat and horse faecal samples (Ley et al., 2002; Jimenez-Clavero et al., 2005). Similarly, PMMoVs have been detected in chicken and seagull faecal samples (Rosario et al., 2009). The non-specific markers are unreliable for field studies due to the possibility of yielding false positive detection, which may result in the wasted capital investment for mitigation activities. In such scenario, obtaining additional information on the concentrations of non-specific markers in faecal samples may be important. Considering the high concentration (10^7/10^8 gene copies per L) of a marker in its host, it is unlikely that if it is detected at a low concentration (10^1/10^2 gene copies per L) in the non-target host(s) would be a limitation to MST results interpretation (Weidhaas et al., 2010).

Notably, most of the host-sensitivity and -specificity assays have been undertaken in Australia, New Zealand, Spain and the USA. A little is known regarding the host-sensitivity and-specificity of viral markers in other continents such as Asia, Africa and South America. Further host-sensitivity and -specificity testing on the currently used markers should be undertaken prior to their application in new geographical locations. In addition, new assays need to be developed specifically for MST studies, since many currently used qPCR assays used for source tracking purposes have been originally developed to monitor enteric viruses in clinical studies and wastewater treatment efficacy monitoring (Heim et al., 2003; He and Jiang, 2005; Wong et al., 2012). Priority should also be given to develop new assays to detect faecal pollution from a wide range of wild, pet and domesticated animals.

1.4 Correlation among FIB, pathogens and viral MST markers and the relevance to public health risks

An ideal MST marker should correlate with FIB that is used as regulatory standards. Several studies have reported negative correlations between FIB concentrations and viral markers in environmental waters (Fong et al., 2005; Korajkic et al., 2010; Staley et al., 2012a; Ahmed et al., 2013; Sidhu et al., 2013). Several factors such as types of water, dilution effect, turbidity, differences in analytical methods (culture-based vs. qPCR), sources of faecal inputs, differential decay and numbers of samples may account for the lack of correlations observed. Harwood et al., (2009) compared *Enterococcus* spp. concentrations in undiluted human wastewater and the dilution corresponding to the limit of detection of HPyVs by PCR, with the rationale that the observations should be correlated if the FIB and marker co-vary in the undiluted human wastewater (Harwood et al., 2009). The study found no significant correlation between HPyVs and *Enterococcus* spp. concentrations in human wastewater and the dilution necessary to achieve the limit of detection. McQuaig et al., (2009) determined the correlations among FIB (faecal coliforms, *E. coli* and *Enterococcus* spp.) and HPyVs for human, disinfected and septic wastewater samples. HPyVs were poorly or negatively correlated with all three FIB tested. Poor correlations between FIB and viral MST markers may not necessarily hinder their application as MST tools if the objective of the study is to determine the sources of faecal pollution for mitigation purpose.

From public health point of view, the relationship between viral markers and pathogens is more critical than that of the markers to FIB. One of the important aspects of using viral markers for MST field studies is that their presence indicates potential health risks because some of the viral targets such as HAdVs, HEVs and HNoVs are capable of causing illnesses in humans. Further studies would be required to obtain information on the correlations of viral markers to pathogens to translate the health outcomes of human wastewater pollution in environmental waters.

2.0 Overview of Methods for Recovery of Viral MST Markers from Environmental Matrices

Enteric viruses are relatively difficult to concentrate from environmental waters due to typically low concentrations and their small size (Maier et al., 2008). Although rapid enumeration of viruses by quantitative PCR (qPCR) has the potential to greatly improve water quality analysis and risk assessment, the upstream steps of capturing and recovering viruses from environmental water sources along with removing PCR inhibitors from extracted nucleic acids remain formidable barriers to routine use. A wide range of methods has been developed and used to recover viruses from various types of environmental waters (Casas and Sunen, 2002; Katayama et al., 2002; Lambertini et al., 2008; Nordgren et al., 2009; Rodriguez-Diaz et al., 2009; Bennett et al., 2010; Leskinen et al., 2010). Virus adsorption and elution (VIRADEL) methods such as negatively charged HA membranes (Katayama et al., 2002; Haramoto et al., 2005), positively or negatively charged 1MDS cartridge filters (Lukasik et al., 2000), filterite filters (Wetz et al., 2004), electropositive nanoCeram filters (Lee et al., 2011) and glass wool (Lambertini et al., 2008) have been most commonly used to recover viruses from environmental waters. The mechanisms of these methods have been described in a review paper (Wong et al., 2012).

Besides the VIRADEL methods, virus recovery techniques such as hollow-fiber ultrafiltration (HFUF) has been used widely to recover viruses from environmental waters. Research studies by several groups (Morales-Morales et al., 2003; Hill et al., 2005; Hill et al., 2007; Polaczyk et al., 2008;) reported that HFUF can be effective for higher recovery (50-90%) of viruses, bacteria and parasites from various water matrices. In addition, HFUF is rapid, and it does not require the use of extensive chemicals. The method also simultaneously retains bacteria, protozoa and viruses in a single step, which is an added advantage when analysis of multiple MST markers is required (Kfir et al., 1995; Morales-Morales et al., 2003; Wong et al., 2012).

It has been suggested that capturing viruses on membranes followed by direct nucleic acid extraction may result in higher recoveries compared to protocols that require viral elution from membranes (Wong et al., 2012). Ahmed et al., (2015) compared the efficiency of virus recovery for three rapid methods of concentrating HAdVs and HPyVs from river water samples on HA membranes. Samples were spiked with raw wastewater, and viral adsorption to membranes was promoted by acidification or addition of $MgCl_2$. Viral nucleic acid was extracted directly from membranes, or viruses were eluted with NaOH and concentrated by centrifugal ultrafiltration. Recovery efficiencies of HAdVs and HPyVs were approximately ten-fold greater for a method that involved direct DNA extraction compared to the frequently-used strategy of viral absorption with added cations (Mg^{2+}) and elution with acid, with mean recovery efficiency ranging from 31-78%.

Recovery of viruses from environmental water requires filtration on the scale of 1-100 L of the sample. Processing larger volume of samples can be difficult to accomplish in the field, and many methods require expensive, expendable filters that cannot be reused. When large volumes are processed, it is always necessary to use a secondary concentration method. Reconcentration methods such as organic flocculation (Katzenelson et al., 1976), and polyethylene glycol (PEG) precipitation (Lewis and Metcalf, 1988) have some disadvantages, *e.g.* these methods do not produce consistent recovery efficiency for different viruses and the sample processing time can be lengthy (Lewis and Metcalf, 1988). Alternatively, specifically designed ultrafilters, which retain viruses based on molecular weight cut-off can be used as a secondary concentration step. In a previous study, Centriprep Filter Concentrators provided high and stable recovery yields (74%) of seeded polioviruses (Haramoto et al., 2004). Another study reported the 35% recovery of HAdV 41 through Centricon filters (Wu et al., 2011). Ultracentrifugation is another alternative method for virus concentration from environmental water samples. This method requires minimal sample manipulation and samples can be processed under natural pH and an elution step is not needed. By ultracentrifugation it is possible to concentrate all viruses in a sample, by using a sufficient g-force. 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 downstream analysis. Alternatively, a number of recent studies have taken the optional approach of concentrating 1-2 L volumes of surface water by membrane filtration to test for enteric viruses (Katayama et al., 2002; Fong et al., 2005; Haramoto et al., 2005; De Paula et al., 2007; Haramoto et al., 2008; Miagostovich et al., 2008; Victoria et al., 2009; Ahmed et al., 2010a; Ahmed et al., 2010b). Application of these methods may be suitable for MST field studies, which warrant delivering the results rapidly for remediation. If the concentration of a chosen viral marker is high in its host, then using a small volume of water sample may not be problematic to its detection in the environment.

One method that accomplishes highly efficient concentration of all viruses has not yet been found. Variations in several factors such as adsorption of viruses to membranes, membrane type, elution buffer, seeding materials (strains vs. wastewater), seeded concentrations, sample type, sample volume and sensitivity of qPCR assays can influence recovery efficiency (Bofill-Mas et al., 2006; Albinana-Gimenez et al., 2009; Li et al., 2010). A good recovery method should fulfil several criteria: it should be simple, rapid, provide high recovery, consistently recover a wide range of viruses, provide a small volume of concentrate, be cost effective and not alter viral community structure (Bosch, 1998; Angly et al., 2006). More studies are needed for the development of new recovery methods and comparing with the existing methods for the effective recovery of viruses from environmental waters.

3.0 Environmental Occurrence and Persistence

3.1 Concentrations of viral MST markers in human wastewater, faeces, and urine

The concentration of a viral marker in its host is an important factor because it is likely that a marker whose concentration is high will be consistently and more easily detected in polluted water samples. Markers whose concentrations are highly variable or low can be difficult to detect in the environment due to factors such as dilution, turbidity and different decay rates. Tables 3 and 4 summarize the concentrations of human and animal markers in target host groups and point sources in the published studies. Several studies have provided quantitative data on HAdVs and HPyVs in raw, treated and septic tank wastewater. The mean concentrations of HAdVs A-F in raw wastewater samples tested in Australia, New Zealand and Spain ranged from 1.0×10^6 to 8.7×10^6 gene copies per L. However, the mean concentration of HAdV species C in raw wastewater samples was four orders of magnitude lower than the HAdVs group (species A-F) (Wolf et al., 2010). Such data clearly indicate that application of assays that are targeting one or two species of HAdVs may not be sensitive enough to detect human wastewater pollution in environmental samples. In general, the concentration of HAdVs in secondary wastewater was one to two orders of magnitude lower than raw wastewater, which is expected in effective wastewater treatment processes.

Table 3. Concentrations of human viral MST markers in wastewater, faeces and urine determined using qPCR assays

Area	Viral Markers	Sample Type	Percent Positive (# of Samples)	Average Concentration GC (Gene Copy) per L, (± SD[a] or Range)	Reference
Australia	HAdVs[b] (A-F)	Raw wastewater	100% (3/3)	3.9E+06 ± 7.9E+05	Ahmed et al., 2015
Australia	HPyVs[c] (JCVs and BKVs)	Raw wastewater	100% (3/3)	2.5E+06 ± 3.9E+05	Ahmed et al., 2015
New Zealand	HAdVs-F	Raw wastewater	100% (11/11)	1.0E+06 ± 10	Wolf et al., 2010
New Zealand	HAdVs-F	Biosolids	75% (3/4)	7.9E+04 ± 5.0	Wolf et al., 2010
New Zealand	HAdVs-C	Raw wastewater	36% (4/11)	5.0E+02 ± 2.5	Wolf et al., 2010
New Zealand	HAdVs-C	Biosolids	50% (2/4)	6.3E+02 ± 2.0	Wolf et al., 2010
Spain	HAdVs (A-F)	Raw wastewater	100% (9/9)	2.1E+06	Hundesa et al., 2009
Spain	HAdVs (A-F)	Raw wastewater	100% (8/8)	2.1E+06	Hundesa et al., 2010
Spain	HAdVs (A-F)	Raw wastewater	100% (33/33)	8.7E+06	Rusiñol et al., 2014
Spain	HAdVs (A-F)	Secondary wastewater	81% (26/32)	1.3 E+05	Rusiñol et al., 2014
Spain	HPyVs (JCVs)	Raw wastewater	100% (33/33)	9.2E+06	Rusiñol et al., 2014
Spain	HPyVs (JCVs)	Secondary wastewater	81% (26/32)	1.2E+05	Rusiñol et al., 2014
Spain	HPyVs	Raw wastewater	100% (8/8)	2.4E+06	Hundesa et al., 2010
Spain	HPyVs (JCVs)	Hospital raw wastewater	100% (10/10)	1.6E+05	Rusiñol et al., 2013
Spain	HPyVs (JCVs)	Raw wastewater	100% (4/4)	3.3E+06	Rusiñol et al., 2013
USA	HPyVs (JCVs and BKVs)	Raw wastewater	100% (41/41)	3.0E+07 ± 1.7E+07	McQuaig et al., 2009
USA	HPyVs (JCVs and BKVs)	Tertiary effluent	22% (2/9)	1.2E+03 ± 2.5E+03	McQuaig et al., 2009
USA	HPyVs (JCVs and BKVs)	Septic tank pump truck	100% (9/9)	1.1E+07 ± 1.0E+07	McQuaig et al., 2009
USA	HPyVs (JCVs and BKVs)	Septic tank	60% (3/5)	1.4E+07 ± 1.6E+07	McQuaig et al., 2009
USA	HPyVs (JCVs and BKVs)	Human urine	23% (6/26)	2.7E+09 ± 4.7E+09	McQuaig et al., 2009
USA	HPyVs	Raw wastewater	100% (3/3)	4.7E+06 to 2.7E+07	Staley et al., 2012a
USA	PMMoVs[d]	Raw human wastewater	100% (12/12)	1.5E+09 to 2.2E+10	Rosario et al., 2009
USA	PMMoVs	Treated wastewater	100% (12/12)	1.1E+07 to 7.0E+09	Rosario et al., 2009

[a]SD:Standard Deviations; [b]HAdVs human adenoviruses; [c]HPyVs: Human Polyomaviruses; [d]PMMoVs:Pepper mild mottle viruses

Table 4. Concentrations of animal viral MST markers in wastewater, faeces and urine determined using qPCR assays

Area	Viral Markers	Sample Type	Percent Positive (# of Samples)	Average Concentration GC (Gene Copies) per L or g, (± SD[a] or Range)	Reference
New Zealand	BAdVs/OAdVs[b]	Slaughterhouse effluent	50% (1/2)	2.5E+05 per L	Wolf et al., 2010
New Zealand	BNoVs/ONoVs[c]	Slaughterhouse effluent	100% (2/2)	1.6E+05 ± 1.6 per L	Wolf et al., 2010
Spain	PAdVs[d]	Slaughterhouse wastewater	100% (10/10)	1.0E+04 to 1.0E+06 per L	Hundesa et al., 2006
Spain	PAdVs	Slaughterhouse wastewater	11	1.6E+06 per L	Hundesa et al., 2010
Spain	PAdVs	Pig faeces	87% (33/38)	5.6E+05 to 7.3E+05 per g	Hundesa et al., 2009
Spain	PAdVs	Slaughterhouse wastewater	100% (8/8)	1.6E+06 per L	Hundesa et al., 2009
Spain	PAdVs	Swine faeces	17% (4/23)	10 to 1E+03 per g	De Motes et al., 2004
Spain	BAdVs/OAdVs	Cattle faeces	75% (6/8)	10 to 1.0E+04 per g	De Motes et al., 2004
Spain	BPyVs[e]	Slaughterhouse wastewater	94% (17/18)	1.0E+04 to 1.0E+05 per L	Hundesa et al., 2006
Spain	BPyVs	Bovine urine	31% (8/26)	2.2E+04 per L	Hundesa et al., 2010
Spain	BPyVs	Slaughterhouse wastewater	91% (10/11)	3.0E+03 per L	Hundesa et al., 2010
Spain	BPyVs	Bovine urine	100% (5/5)	8.3E+04 per L	Rusinol et al., 2013
Spain	BPyVs	Bovine slaughterhouse wastewater	100% (5/5)	7.8E+05 per L	Rusinol et al., 2013
Spain	OPyVs[f]	Sheep urine	69% (9/13)	1.6E+05 per L	Rusiñol et al., 2013
Spain	OPyVs	Sheep wet straw beds	50% (21/42)	1.3E+05 per L	Rusiñol et al., 2013
Spain	OPyVs	Sheep faeces	54% (7/13)	7.6E+04 per g	Rusiñol et al., 2013
Spain	OPyVs	Slaughterhouse wastewater	75% (3/4)	9.8E+05 per L	Rusiñol et al., 2013
Spain	OPyVs	Slaughterhouse treated wastewater	50% (1/2)	4.9E+04 per L	Rusiñol et al., 2013
Spain	ChPVs/TuPVs[g]	Chicken faeces	81% (17/21)	9.1E+08 per g	Carratalá et al., 2012
Spain	ChPVs/TuPVs	Slaughterhouse raw wastewater	100% (3/3)	4.6E+08 per L	Carratalá et al., 2012
USA	BAdVs	Dairy manure	85% (22/26)	1.1E+07 to 1.4E+07 per L	Wong and Xagoraraki, 2011
USA	BAdVs	Cow faeces	22% (4/18)	7.8E+02 per g	Wong and Xagoraraki, 2011
USA	BAdVs (serotypes 4-8)	Dairy manure	100% (16/16)	1.0E+05 to 1.0E+07 per L	Wong and Xagoraraki, 2010

Area	Viral Markers	Sample Type	Percent Positive (# of Samples)	Average Concentration GC (Gene Copies) per L or g, (± SD[a] or Range)	Reference
USA	BAdVs (serotypes 4-8)	Cattle faeces	10% (2/20)	1.0E+03 to 1.0E+04 per g	Wong and Xagoraraki, 2010
USA	BAdVs (BAdV 2)	Dairy manure	56% (9/16)	1.0E+05 per L	Wong and Xagoraraki, 2010
USA	BPyVs	Dairy manure	100% (26/26)	1.2E+08 to 2.8E+09 per L	Wong and Xagoraraki, 2011
USA	BPyVs	Cattle faeces	6% (1/18)	5.5E+02 per g	Wong and Xagoraraki, 2011

[a]SD: Standard deviations; [b]BAdVs/OAdVs: Bovine Adenoviruses/Ovine Adenoviruses; [c]BNoVs/ONoVs: Bovine Noroviruses/Ovine Norovirues; [d]PAdVs: Porcine Adenoviruses; [e]BPyVs: Bovine Polyomaviruses; [f]OPyVs: ovine polyomaviruses; [g]ChPVs/TuPVs: chicken/turkey parvoviruses

The concentration of HPyVs in human wastewater was similar to HAdVs, although one order of magnitude higher concentration has been reported in wastewater samples from Florida, USA (McQuaig et al., 2009). HPyVs in human urine samples were approximately two orders of magnitude higher than raw wastewater. This is because HPyVs are shed mainly through human urine. Among the human wastewater-associated viral markers, the concentrations of PMMoVs have been reported to be two to three orders of magnitude higher than HAdVs and HPyVs (Rosario et al., 2009). Such high concentrations may results in the overestimation of the magnitude of wastewater pollution in environmental waters; however, they may also be useful for tracing more dilute pollution. More studies would be required to investigate the usefulness of PMMoVs for MST field studies and associated health risks.

3.2 Concentrations of viral MST markers in animal wastewater, faeces, and urine

Compared to human wastewater viral markers, information on the concentration of animal markers is scant. PAdVs and BAdVs in composite slaughterhouse wastewater and dairy manure samples ranged from 1.0×10^5 to 1.4×10^7 gene copies per L (Hundesa et al., 2006; Wong and Xagoraraki, 2011). However, in the faeces of individual animals, the concentrations were two to three orders of magnitude lower than in wastewater (De Motes et al., 2004; Wong and Xagoraraki, 2010). Hundesa et al., (2006) developed a BPyV assay for the determination of bovine faecal pollution in the environment. The concentration of BPyVs in slaughterhouse wastewater estimated to be 1.0×10^4 to 1.0×10^5 per L. Similar concentrations of BPyVs in bovine urine and slaughterhouse wastewater have been reported in subsequent studies by the same group. In contrast, Wong and Xagoraraki (2011) reported much higher concentrations (1.2×10^8 to 2.8×10^9 per L) of BPyVs in dairy manure samples. It is recommended that a marker with high concentrations in its host should be used to determine the sources of faecal pollution in environmental waters (Ahmed et al., 2016). More studies are warranted to determine the concentrations of the viral markers in new geographical locations in order to identify their potential for global utility.

3.3 Concentrations of viral MST markers in the water environment

Tables 5 and 6 summarize the prevalence and concentrations of human and animal viral markers in various environmental matrices. HAdVs are reported to be highly prevalent in environmental water samples collected from Australia, Brazil, Greece, Hungary, Spain, Sweden, New Zealand and the USA. Only a handful of MST studies has provided the concentrations of HAdVs in environmental waters (Table 5). Among these studies, Rusiñol et al., (2014) investigated the concentrations of HAdVs in 792 water samples from diverse watersheds in Greece, Spain, Sweden, Hungary and Brazil. The concentrations of HAdVs were as high as 5.1×10^7 per L which is similar to that found in wastewater. The prevalence and concentrations of HPyVs are reported in the published articles are similar to HAdVs (Chase et al., 2012; Rusiñol et al., 2014). The high prevalence of these two viruses in the environments could be attributed to the fact that both are double-stranded DNA viruses, which can persist lengthy periods in the environment (Love et al., 2010). Therefore, their presence may not indicate the recent pollution or any adverse health risks. The information on the recent pollution can be obtained by testing human RNA viruses such as PMMoVs or HEVs, which do not persist longer in the environment (Lipp et al., 2007).

Table 5. Prevalence and concentrations of human viral markers in environmental waters

Area	Viral Markers	Types of Assay	Percent Positive (# of Samples)	Average Concentrations GC (Gene Copies) per L, (± SD[a] or Range)	Reference
Australia	HAdVs[b] (A-F)	Nested PCR	10% (4/40)	NR	Ahmed et al., 2010a
Australia	HPyVs[c] (JCVs and BKVs)	PCR	25% (5/20)	NR	Ahmed et al., 2010b
Australia	HAdVs (A-F)	PCR	50% (8/16)	NR	Sidhu et al., 2012
Australia	HAdVs (A-F)	PCR	91% (21/23)	NR	Sidhu et al., 2013
Australia	HPyVs (JCVs and BKVs)	PCR	52% (12/23)	NR	Sidhu et al., 2013
Brazil	HAdVs	qPCR	92% (250/272)	1.1E+02 to 2.8E+06	Rusiñol et al., 2014
Brazil	HPyVs (JCVs)	qPCR	68% (185/272)	2.3E+02 to 6.3E+05	Rusiñol et al., 2014
Greece	HAdVs	qPCR	28% (39/140)	8.8 E+01 to 1.3E+04	Rusiñol et al., 2014
Greece	HPyVs (JCVs)	qPCR	16% (22/140)	1.4E+02 to 3.4E+04	Rusiñol et al., 2014
Hungary	HAdVs	qPCR	99% (128/129)	3.1E+02 to 5.1E+07	Rusiñol et al., 2014
Hungary	HPyVs (JCVs)	qPCR	70% (90/129)	3.4E+02 to 1.5E+08	Rusiñol et al., 2014
New Zealand	HAdVs-F	qPCR	50% (3/6)	1.3E+02 ± 3.0E+03	Wolf et al., 2010
New Zealand	HNoVs[d]-GII	RT-qPCR	17% (1/6)	2.0E+02	Wolf et al., 2010
New Zealand	HPyVs (JCVs and BKVs)	PCR	67% (10/15)	NR	Kirs et al., 2011
New Zealand	HPyVs (JCVs and BKVs)	PCR	0% (0/3)	NR	Kirs et al., 2011
Spain	HAdVs (A-F)	Nested PCR	100% (5/5)	NR	Hundesa et al., 2006
Spain	HAdVs (A-F)	qPCR	100% (6/6)	4.5E+02	Hundesa et al., 2009
Spain	HAdVs (A-F)	qPCR	100% (6/6)	1.2E+04	Hundesa et al., 2010
Spain	HPyVs	qPCR	33% (2/6)	2.6E+02	Hundesa et al., 2010
Spain	HPyVs (JCVs)	qPCR	52% (46/89)	6.3 to 3.0E+05	Rusiñol et al., 2014
Spain	HAdVs	qPCR	78% (69/89)	1.7E+01 to 3.4E+05	Rusiñol et al., 2014
Sweden	HAdVs	qPCR	14% (23/162)	5.5 E+01 to 6.5E+02	Rusiñol et al., 2014
Sweden	HPyVs (JCVs)	qPCR	10% (16/162)	6.0E+01 to 1.4E+03	Rusiñol et al., 2014
USA	HAdVs (A-F)	Nested PCR	37% (11/30)	NR	Fong et al., 2005
USA	HAdVs (A-F)	Nested PCR	20% (1/5)	NR	Fong et al., 2005
USA	HAdVs (A-F)	Nested PCR	4% (2/57)	NR	Jiang et al., 2007

Area	Viral Markers	Types of Assay	Percent Positive (# of Samples)	Average Concentrations GC (Gene Copies) per L, (± SD[a] or Range)	Reference
USA	HAdVs (A-F)	Nested PCR	4% (10/250)	NR	McQuaig et al., 2012
USA	HPyVs (JCVs and BKVs)	qPCR	12% (30/250)	5.0E+02 ± 3.5E+05	McQuaig et al., 2012
USA	HAdVs	qPCR	12% (9/73)	NR	Bambic et al., 2015
USA	HPyVs (JCVs and BKVs)	qPCR	100% (5/5)	9.2E+01 ± 1.4E+06	McQuaig et al., 2009
USA	HPyVs (JCVs and BKVs)	qPCR	4% (2/56)	NR	Staley et al., 2013
USA	HPyVs (JCVs and BKVs)	Nested PCR	25% (3/12)	NR	Abdelzaher et al., 2010
USA	HPyVs (JCVs and BKVs)	PCR	15% (12/80)	NR	Korajkic et al., 2010
USA	HPyVs (JCVs and BKVs)	Nested PCR	40% (62/156)	NR	McQuaig et al., 2006
USA	HPyVs	qPCR	6% (2/35)	1.4E+03 to 1.7E+03	Chase et al., 2012
USA	HPyVs (JCVs and BKVs)	PCR	0% (0/14)	NR	Hellein et al., 2011
USA	HEVs[e]	RT-nested PCR	57% (17/30)	NR	Fong et al., 2005
USA	HEVs	RT-nested PCR	40% (2/5)	NR	Fong et al., 2005
USA	PMMoVs[f]	RT-qPCR	57% (4/7)	4.1E+05 to 6.0E+07	Rosario et al., 2009

NR: Not Reported; RT: Reverse-transcriptase; -: Not tested; [a]SD: Standard deviations; [b]HAdVs human adenoviruses; [c]HPyVs: Human Polyomaviruses; [d]HNoVs: Human Noroviruses; [e]HEV: Human Enteroviruses; [f]PMMoVs: Pepper mild mottle viruses

Table 6. Prevalence and concentrations of animal viral markers in environmental waters

Area	Viral Markers	Types of Assay	Sample Type	Percent Positive (# of Samples)	Average Concentration GC (Gene Copies) per L, (± SD[a] or Range)	Reference
Australia	BAdVs[b]	Nested PCR	River water	10% (4/40)	-	Ahmed et al., 2010a
Australia	BAdVs	Nested PCR	Reservoir water	6% (2/36)	-	Ahmed et al., 2013
Brazil	PAdVs[c]	qPCR	River water	1% (3/272)	1.5E+02 to 2.3E+02	Rusiñol et al., 2014
Brazil	BPyVs[d]	qPCR	River water	2% (5/272)	35 to 70	Rusiñol et al., 2014
Greece	PAdVs	qPCR	River and sea water	19% (27/140)	1.5E+03 to 4.8E+03	Rusiñol et al., 2014
Greece	BPyVs	qPCR	River and sea water	22% (31/140)	1.4E+02 to 6.6E+03	Rusiñol et al., 2014
Greece	OPyVs[e]	qPCR	River water	75% (3/4)	11	Rusiñol et al., 2013
Hungary	PAdVs	qPCR	River water	91% (117/129)	7.6E+02 to 4.2E+08	Rusiñol et al., 2014
Hungary	BPyVs	qPCR	River water	96% (124/129)	2.0E+02 to 3.9E+07	Rusiñol et al., 2014
New Zealand	PAdVs-5	qPCR	River water	50% (3/6)	4.0E+02 to 1.3E+03	Wolf et al., 2010
New Zealand	BAdVs/OAdVs[f]	qPCR	River water	50% (3/6)	4.0 to 2.0E+02	Wolf et al., 2010
New Zealand	BNoVs/ONoVs[g]	RT-qPCR	River water	17% (1/6)	8.0E+02	Wolf et al., 2010
Spain	PAdVs	qPCR	River and sea water	9% (8/89)	1.1E+03 to 9.5E+03	Rusiñol et al., 2014
Spain	PAdVs	qPCR	River water	100% (6/6)	8.3	Hundesa et al., 2010
Spain	PAdVs	Nested PCR	River water	80% (4/5)	-	Hundesa et al., 2006
Spain	BPyVs	Nested PCR	River water	56% (5/9)	-	Hundesa et al., 2006
Spain	BPyVs	qPCR	River water	50% (3/6)	3.1E+02	Hundesa et al., 2010
Spain	BPyVs	qPCR	River and sea water	2% (2/89)	3.0E+02 to 6.0E+02	Rusiñol et al., 2014
Spain	OPyVs	qPCR	River water	13% (1/8)	1.1E+02	Rusiñol et al., 2013
Spain	BEVs[h]	RT-qPCR	Watering tank	66% (2/3)	-	Jimenez-Clavaro et al., 2005
Spain	BEVs	RT-qPCR	Creek water	66% (4/6)	-	Jimenez-Clavaro et al., 2005
Spain	BEVs	RT-qPCR	Swamp water	100% (1/1)	-	Jimenez-Clavaro et al., 2005
Spain	BEVs	RT-qPCR	Pool water	0% (0/4)	-	Jimenez-Clavaro et al., 2005

Area	Viral Markers	Types of Assay	Sample Type	Percent Positive (# of Samples)	Average Concentration GC (Gene Copies) per L, (± SD[a] or Range)	Reference
Spain	BEVs	RT-qPCR	River water	100% (1/1)	-	Jimenez-Clavaro et al., 2005
Spain	BEVs	RT-qPCR	Reservoir water	0% (0/1)	-	Jimenez-Clavaro et al., 2005
Spain	PTVs[i]	RT-qPCR	Duct water	100% (6/6)	2.6E+07 to 1.3E+08	Jimenez-Clavaro et al., 2003
Spain	PTVs	RT-qPCR	Stream water	40% (6/15)	1.3E+05 to 1.6E+06	Jimenez-Clavaro et al., 2003
Spain	PTVs	RT-qPCR	River water	0% (0/2)	-	Jimenez-Clavaro et al., 2003
Spain	PTVs	RT-qPCR	Spring water	0% (0/1)	-	Jimenez-Clavaro et al., 2003
Sweden	PAdVs	qPCR	River and sea water	NR	6.0E+03 to 3.7E+03	Rusiñol et al., 2014
Sweden	PAdVs	qPCR	River and sea water	4% (7/162)	6.0E+03 to 3.7E+03	Rusiñol et al., 2014
Sweden	BPyVs	qPCR	River and sea water	60% (97/162)	1.0 to 2.5E+04	Rusiñol et al., 2014
USA	BEVs	RT-PCR	Pastures pool	100% (3/3)	-	Ley et al., 2002
USA	BEVs	RT-PCR	Animal watering tank	50% (1/2)	-	Ley et al., 2002
USA	BEVs	RT-PCR	Stream water	100% (2/2)	-	Ley et al., 2002
USA	BEVs	RT-PCR	River water	50% (2/4)	-	Ley et al., 2002
USA	BEVs	RT-PCR	Surface water	37% (11/30)	-	Fong et al., 2005
USA	BEVs	RT-PCR	Bottom water	0% (0/5)	-	Fong et al., 2005

NR: Not Reported; RT: Reverse-transcriptase; -: Not tested; [a]SD: Standard deviations; [b]BAdVs: Bovine Adenoviruses; [c]PAdVs: Porcine Adenoviruses; [d]BPyVs:Bovine Polyomaviruses; [e]OPyVs: ovine polyomaviruses; [f]BAdVs/OAdVs: Bovine Adenoviruses/Ovine Adenoviruses; [g]BNoVs/OnoVs: Bovine Noroviruses/Ovine Norovirues; [h]BEVs: bovine enteroviruses; [i]PTVs: porcine teschoviruses

Compared to human viral markers, less information is available on the prevalence and concentrations of animal wastewater markers (Table 6). PAdVs, BAdVs and BPyVs have been most commonly used in MST field studies in the South Pacific, Europe and North America. Rusiñol et al., (2014) reported the wide prevalence of PAdVs and BPyVs in a river in Hungary with concentrations as high as 4.2×10^8 and 3.9×10^7 per L of water, respectively. Low to moderate prevalence of PAdVs and BPyVs have also been detected in river waters in Spain, Greece, Sweden and Brazil. Other animal viral markers such as BEVs, PTVs, OPyVs have also been detected in various types of surface water samples with low to high prevalence rates.

The presence/absence results of any given marker in a sample should be interpreted with care. Lack of detection does not necessarily indicate the sample is free from pollutants and safe for human exposure. As shown in Tables 4 and 5, viral concentrations vary widely in environmental samples, and Tables 3 and 4 demonstrate the variability in host faecal and wastewater. Therefore, detection of these markers in environmental waters can be difficult due to factors such as dilution, sorption to particulate matters, environmental persistence, loss due to recovery and DNA extraction, and the fact that only a small volume of DNA sample is used for PCR/qPCR analysis (Horswell et al., 2010; Wong et al., 2012). It is therefore, recommended that a “toolbox” approach should be used for the accurate identification of polluting source(s) (Noble et al., 2006; Ahmed et al., 2012; Mauffret et al., 2012). None of the field studies reported the presence of viral markers in soils and sediments. It is well known that these environmental

matrices may harbour enteric viruses and other faecal microorganisms (Wheeler et al., 2003; Staggemeier et al., 2015). Viruses associated with particular matter in suspension or solid matrices tend to remain viable for a longer time than if they were dispersed in water (Schenewski and Julich, 2001). These results have implications for the accuracy of MST tools as regulatory standards for the protection of water quality.

4.0 Future Applications and Directions

The currently used MST viral tools rely on detecting or quantifying a single viral marker in a water sample. This is a significant limitation for better management of water quality. Detecting a single marker does not rule out the presence of potential faecal pollution from other sources. Viral community analysis may ultimately allow for a more comprehensive assessment of source contributions by identifying multiple sources of faecal pollution in a single sample. This could be advantageous for scenarios where the pollution sources are not known or mixed sources of pollution impacting waterways. Microarray technology where hundreds of probes targeting multiple markers and pathogens can be tested simultaneously by hybridization makes it a powerful tool for MST studies. Li et al. (2015) developed a custom microarray targeting pathogens, MST markers and antibiotic resistance genes. The array was able to detect HAdVs, bacteriophage, bocavirus, influenza C, HNoVs, HPyVs, PMMoVs and torqueteno viruses (TTVs) in human wastewater and animal faeces. The authors also noted that the host-sensitivity values of human markers were somewhat low, ranging from 21 to 33%. However, the host-specificity values were much higher ranging from 83 to 90%. Therefore, further studies are warranted in order to improve the detection sensitivity of this technology.

The recent advances in metagenomics using next generation sequencing allow specific identification of sequences of enteric viruses in human faeces and environmental waters (Breitbart et al., 2002; Breitbart et al., 2003). Viral metagenomics provides information on the viral community of a sample including culturable, non-culturable, known and unknown (Mokili et al., 2012; Wong et al., 2012). A metagenomic approach can be a powerful tool to identify novel host-associated viruses in faecal and wastewater samples based on their relative abundance (Wong et al., 2012). For example, HAdVs were thought to be the most abundant enteric viruses in human wastewater, however, a viral metagenomic sequencing analysis identified parechovirus is being more abundant than other enteric viruses (Bibby et al., 2011). One of the most important aspects of metagenomic analysis is that it has the potential to detect multiple human and animal viruses. However, because the abundance of most enteric viruses in the environment can be low, an increase in sequence depth and further improvements in bioinformatics will be required to detect enteric viruses in the environment (Wong et al., 2012).

4.1 Final Conclusions

Application of enteric viruses for MST field studies is promising due to their high host-specificity. Among the human wastewater-associated viral markers, HAdVs and HPyVs have been shown to be useful tools for tracking human wastewater in environmental waters. High persistence of HAdVs in the environment, and the fact that some serotypes can be pathogenic, make it a very useful viral marker. However, based on the performance characteristics, we recommend the use of both HAdVs and HPyVs in a tandem fashion for the accurate and sensitive detection of human wastewater pollution in environmental waters.

Little research has been undertaken on the host-specificity and -sensitivity of most animal viruses, particularly OPyVs, BNoVs, BEVs and PTVs. Some animal markers were also found to cross-react with faecal samples from non-target hosts. Further evaluation of performance characteristics should be undertaken to determine the broader applicability of these markers. Non-specific markers may still be useful for source tracking if information is available on the contributing sources and if possible testing should be accompanied with additional markers in a toolbox format.

Limited numbers of PCR/qPCR-based assays have been developed, and field tested. Several currently used qPCR assays have been originally developed to monitor enteric viruses in clinical samples and wastewater monitoring. Focus should be given on the development of new assays specifically for MST field studies. Priority should also be given to develop new assays to detect faecal pollution from wild animals, pets, birds, and domesticated animals.

Quantitative data on the occurrence of human and animal viruses in their hosts is limited. Such data is important to determine the suitability of a marker for detecting faecal pollution in environmental waters. Baseline concentrations which are appropriate to detect faecal pollution and also indicate health risks to some extent need to be established.

The high prevalence and concentrations of human wastewater associated markers in environmental waters in Australia, New Zealand, Spain, USA and several other European countries indicate chronic human wastewater pollution in environmental waters. The application of these tools is encouraged in continents such as Asia and Africa where wastewater pollution due to improper sanitation and gastrointestinal diseases is a major concern.

Regulatory and public health concerns mandate that more studies should be undertaken to gain an understanding of how viral markers correlate with FIB and pathogens. The absence of correlations does not necessarily impede the utility of these markers to identify faecal pollution and potential for mitigation of faecal pollution.

The most significant challenge associated with the field application of viral markers is effective, quantitative recovery of these viruses from environmental samples. Recent developments in virus recovery methods indicate that small volume (1 to 2 L) of an environmental water sample can be analysed for their potential presence making the analysis more rapid and cost-effective. Also recent

developments in qPCR technology such as digital droplet PCR will enable sensitive detection of these viruses from environmental waters. Incorporation of quantitative microbial risk assessment (QMRA) with MST science will improve our understanding of the relative public health risks associated with human vs. animal faecal pollution.

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