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Human and bovine adenoviruses for the detection of source-specific fecal pollution in coastal waters in Australia

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ABSTRACT

In this study, the host-specificity and -sensitivity of human- and bovine-specific adenoviruses (HS-AVs and BS-AVs) were evaluated by testing wastewater/fecal samples from various animal species in Southeast, Queensland, Australia. The overall specificity and sensitivity of the HS-AVs marker were 1.0 and 0.78, respectively. These figures for the BS-AVs were 1.0 and 0.73, respectively. Twenty environmental water samples were collected during wet conditions and 20 samples were colleted during dry conditions from the Maroochy Coastal River and tested for the presence of fecal indicator bacteria (FIB), host-specific viral markers, zoonotic bacterial and protozoan pathogens using PCR/qPCR. The concentrations of FIB in water samples collected after wet conditions were generally higher compared to dry conditions. HS-AVs was detected in 20% water samples collected during wet conditions and whereas BS-AVs was detected in both wet (i.e., 10%) and dry (i.e., 10%) conditions. Both *Campylobacter jejuni mapA* and *Salmonella invA* genes detected in 10% samples collected during dry conditions. The concentrations of *Salmonella invA* ranged between 3.5×10^2 and 4.3×10^2 genomic copies per 500 ml of water *Giardia lamblia* β-giardin gene was detected only in one sample (5%) collected during the dry conditions. Weak or significant correlations were observed between FIB with viral markers and zoonotic pathogens. However, during dry conditions, no significant correlations were observed between FIB concentrations with viral markers and zoonotic pathogens. The prevalence of HS-AVs in samples collected from the study river suggests that the quality of water is affected by human fecal pollution and as well as bovine fecal pollution. The results suggest that HS-AVs and BS-AVs detection using PCR could be a useful tool for the identification of human sourced fecal pollution in coastal waters.

1. Introduction

Tracking sources of fecal pollution in water resources used for recreational or aquaculture is imperative to minimize human health impacts. Human enteric pathogens such as *Salmonella* spp., *Shigella* spp. (Savichtcheva et al., 2007), *Escherichia coli* O157:H7 (Ibekwe and Grieve, 2003), *Cryptosporidium* spp. (Hörman et al., 2004) and enteric viruses (Haramoto et al., 2005) have been found in environmental waters as a result of fecal pollution. Non-point sources such as defective septic systems, stormwater drainage systems, runoff from animal feedlots and/or point sources such as industrial effluent and

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municipal waste are known to be sources of fecal pollution (Ahmed et al., 2005; Aslan-Yilmaz et al., 2004; O'Shea and Field, 1992). Fecal indicator bacteria (FIB) such as fecal coliforms, *E. coli* and/or enterococci have been widely used as indicators of the microbiological quality of surface and ground waters. These are commonly found in the gastrointestinal tracts of all warm-blooded animals including humans (Harwood et al., 1999). One major limitation of FIB is their inability to predict the presence of pathogenic microorganisms, especially protozoans and enteric viruses (Hörman et al., 2004; McQuaig et al., 2006). Another shortcoming of FIB is that they cannot be used to distinguish among the sources of fecal pollution.

In recent years, microbial source tracking (MST) methods have been developed to identify, and in some cases, quantify the sources of fecal pollution in environmental waters. Most commonly used MST methods are primarily PCR-based, and include host-specific *Bacteroides* markers (Bernhard and Field, 2000; Gourmelon et al., 2007), toxin/virulence gene markers (Khatib et al., 2003; Scott et al., 2005), and host-specific (i.e., humans and animals) viruses (Fong et al., 2005; McQuaig et al., 2006; Love and Sobsey, 2007).

More than 100 types of enteric viruses could be present in environmental waters due to fecal pollution (Haramoto et al., 2005; Lee and Kim, 2002; Sobsey et al., 1986). Such viruses are generally transmitted via the fecal-oral route, and they infect and replicate in the intestine of the hosts. Infected humans and animals may excrete high concentrations (i.e., 10^5–10^{11} viral particles per gram of feces) of enteric viruses through defecation. One notable feature of these viruses is that they are more resistant to extreme environmental conditions and treatment processes, such as chlorination, UV radiation, and filtration compared to FIB and other pathogens (Thurston-Enriquez et al., 2003). It has been reported that these viruses can remain infective for lengthy periods (i.e., 100–130 days) in environmental waters (Wetz et al., 2004).

Among human enteric viruses, adenovirus is the only DNA virus, and 51 adenovirus serotypes have been identified which can be classified into six species (i.e., species A–F) (Jothikumar et al., 2005). Species F contains two fastidious enteric serotypes, 40 and 41, which are among the leading causes of childhood diarrhea. It has been suggested that adenoviruses could be used as an index of human viral pollution in environmental waters (Pina et al., 1998). Recently human specific adenoviruses (HS-AVs) and bovine-specific adenoviruses (BS-AVs) have been identified, and used to track the sources of fecal pollution derived from sewage and cattle farms in the USA and Spain (Fong et al., 2005; He and Jiang, 2005; Xogararaki et al., 2007; Maluquer de Motes et al., 2004).

The primary objective of the study discussed in the paper was to evaluate the prevalence and specificity of host-specific HS-AVs and BS-AVs in fecal samples collected from a sewage treatment plant (STP), an abattoir and from common animal species in Australia including farm and domesticated animals. Samples were also collected from a coastal river potentially affected by fecal pollution and tested for the presence of HS-AVs and BS-AVs to identify their likely sources. In addition, samples were also tested for FIB (*E. coli* and enterococci) using culture based methods and zoonotic pathogens (i.e., *Salmonella* spp., *Campylobacter jejuni* and *Giardia lamblia*) using quantitative PCR (qPCR) in order to investigate the microbial quality of water and to establish a correlation among these host-specific viruses, FIB and zoonotic pathogens.

2. Materials and methods

2.1. Oligonucleotides

For the PCR detection of host-specific HS-AVs, BS-AVs and qPCR detection of *Salmonella* spp., *C. jejuni* and *G. lamblia*, previously published primers were used. To detect HS-AVs, a nested primer set was used to identify 47 serotypes including types 2, 40, and 41 (Fong et al., 2005). To detect BS-AVs, a degenerate primer set designed by Maluquer de Motes et al., (2004) was used. The primer sets were designed based on alignments of all available sequences of the hexon gene

Table 1 – Primers used in this study.

Target	Gene	Primers	Amplicon size (bp)	Primer source
Human-specific adenovirus[a]	Hexon	GCC GCA GTG GTC TTA CAT GCA CATC CAC GCC GCG GAT GTC AAA GT	300	Fong et al., 2005
		GCC ACC GAG ACG TAC TTC AGC CTG	143	
		TTG TAC GAG TAC GCG GTA TTC TCG CGG TC		
Bovine specific adenovirus	Hexon	GRT GGT CIY TRG ATR TRA TGGA AAG YCT RTC ATC YCC DGG CCA	641	Maluquer de Motes et al., 2004
C. jejuni	*mapA*	GCT AGA GGA ATA GTT GTG CTT AA TTA CTC ACA TAA GGT GAA TTT TGA	72	Price et al., 2006
Salmonella spp.	*invA*	ACA GTG CTC GTT TAC GAC CTG AAT AGA CGA CTG GTA CTG ATC GAT AAT	244	Chiu and Ou, 1996
G. lamblia	β-giardin gene	CCT CAA GAG CCT GAA CGA TCTC AGC TGG TCG TAC ATC TTC TTC CTT	74	Guy et al., 2003

Y = C + T; R = A + G; D = G + A + T

a Serotypes 1–5, 9, 16, 17, 19, 21, 28, 37, 40, 41, and simian adenovirus 25.

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present in the Genbank and EMBL databases. The primer sequence and annealing temperature for all host-specific viruses, bacterial and protozoan targets are shown in Table 1.

2.2. PCR positive controls

For HS-AVs and BS-AVs PCR assays, DNA positive controls were isolated from raw sewage and cattle fecal slurries. In summary, the PCR amplified product was purified using the QIAquick PCR purification kit (Qiagen), and cloned into the pGEM®-T Easy Vector System (Promega, Madison, WI, USA), transferred into *E. coli* JM109 competent cells, and plated on LB agar plates containing ampicillin, IPTG (isopropyl-β-D-thio-galactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as recommended by the manufacturer. Plasmid was purified using plasmid mini kit (Qiagen). DNA sequencing was carried out at the Australian Genome Research Facility (St. Lucia, Queensland, Australia). For *Salmonella* and *C. jejuni* qPCR assays, genomic DNA was isolated from *Salmonella enterica* serovar Typhimurium ATCC 14028 and *C. jejuni* NCTC 11168 respectively. For *G. lamblia* PCR assay, genomic DNA 30888D was purchased from ATCC.

2.3. Host groups sampling

To determine the host-specificity and sensitivity of HS-AVs and BS-AVs, 182 wastewater/fecal samples were collected from 11 host groups. Wastewater samples (approximately 100 ml) were collected from the influent, primary effluent and secondary effluent of a sewage treatment plant (STP), and septic tanks. Horse fecal samples were collected from a horse racecourse. Cattle, sheep, and pig fecal samples, and cattle-wastewater samples (approximately 100 ml) were collected from an abattoir. Goat and chicken fecal samples were collected from various farms within the region. Dog fecal samples were collected from a dog park. Kangaroo fecal samples were collected from University of the Sunshine Coast (USC) where a larger number of kangaroos roam. Duck and wild bird fecal samples were collected from the City botanical garden adjacent to Queensland University of Technology (QUT). A fresh fecal sample (approximately 500 mg) was collected from the defecation of each individual animal with sterile swabs and inserted into a sterile container, transported on ice to the laboratory, stored at 4 °C and processed within 6 h.

2.4. Concentration and viral DNA extraction from fecal samples

Both fecal (approximately 400 mg) and wastewater samples (approximately 50 ml) were concentrated using a previously published method (Katayama et al., 2002). Each fecal sample was transferred into 15-ml tubes containing 10 ml of phosphate buffer saline (PBS). Briefly each sample was supplemented with 2.5 mM $MgCl_2$ and then passed through a HA electronegative filter (0·45 μm pore size, 90 mm diameter; Millipore, Tokyo, Japan) attached to a glass filter holder (Advantec, Tokyo, Japan). Subsequently, 100 ml of 0.5 mM H_2SO_4 solution (pH 3) was passed through the filter to remove magnesium ions and other electropositive substances, followed by filtration with of 10 ml of 1 mM NaOH solution (pH 11) for elution of viruses from the filter. The filtrate was recovered in a tube containing 100 μl of 100 mM H_2SO_4 solution (pH 1) and 100 μl of 100 × Tris-EDTA buffer (pH 8) for neutralization. All 10 ml elutes were stored at −20 °C until further processing. The concentrated samples were further purified, concentrated, and desalted with Centriprep YM-50 concentrator columns (Millipore). Samples were added to the Centriprep YM-50 and centrifuged at 1000 g for 10 min, followed by removal of the sample that passed through the ultrafiltration membrane (8 ml) and further centrifugation at 1000 g for 10 min to obtain a final volume of 400 μl. Concentrates were split in half (i.e., 2 × 200 μl) and stored at −80 °C. DNA was extracted from each concentrated sample (200 μl) using DNeasy blood and tissue kit (Qiagen, Valencia, CA). Extracted viral DNA was resuspended in 200 μl buffer AE, and stored at −80 °C until processed.

2.5. Water sampling sites

Environmental water samples were collected from the Maroochy River, Sunshine Coast Region located approximately 100 km from Brisbane, Queensland (Fig. 1). A total number of 40 samples were collected from five sites (i.e., MR1–MR5). Among these, 20 were collected after wet conditions (i.e., 25–100 mm rainfall), and the remaining 20 samples were collected in dry conditions from the same sites. The sampling sites are characterised by intensive recreational activities such as fishing, swimming and water sports. Salinity was measured during sampling and ranged between 28 ± 2 and 32 ± 2 PPT for all sites. Sampling site MR1 was located close to the mouth of the Maroochy River. Sampling sites MR2 and MR3 were located near stormwater outlets discharging into the Maroochy River and receiving runoff from urban areas. Sampling sites MR2 and MR3 were approximately 300 m apart and were potentially affected by human sourced wastewater pollution as determined by a sanitary survey. Sampling site MR4 was located downstream of the Maroochy STP and sampling site MR5 was located upstream of the STP. Site MR5 was potentially affected by bovine fecal pollution as determined by a sanitary survey. Samples were generally collected during the low tide except on sampling occasion two when samples were collected during high tide. Samples were collected in 20 L plastic containers and transported to the laboratory on ice for microbiological analysis.

2.6. Enumeration of fecal indicator bacteria

The membrane filtration method was used to process the water samples for FIB enumeration. Serial dilutions were made, and filtered through 0.45-μm pore size (47 mm diameter) nitrocellulose membranes (Advantec, Tokyo, Japan), and placed on modified mTEC agar (Difco, Detroit, MI, USA) and mEI agar (Difco, Detroit, MI, USA) for the isolation of *E. coli* and enterococci, respectively. Modified mTEC agar plates were incubated at 35 °C for 2 h to recover stressed cells, followed by incubation at 44 °C for 22 h (US EPA, 2002). mEI agar plates were incubated at 41 °C for 48 h (US EPA, 1997). After incubation the colonies were enumerated. For FIB enumeration, all the samples were tested in triplicate.

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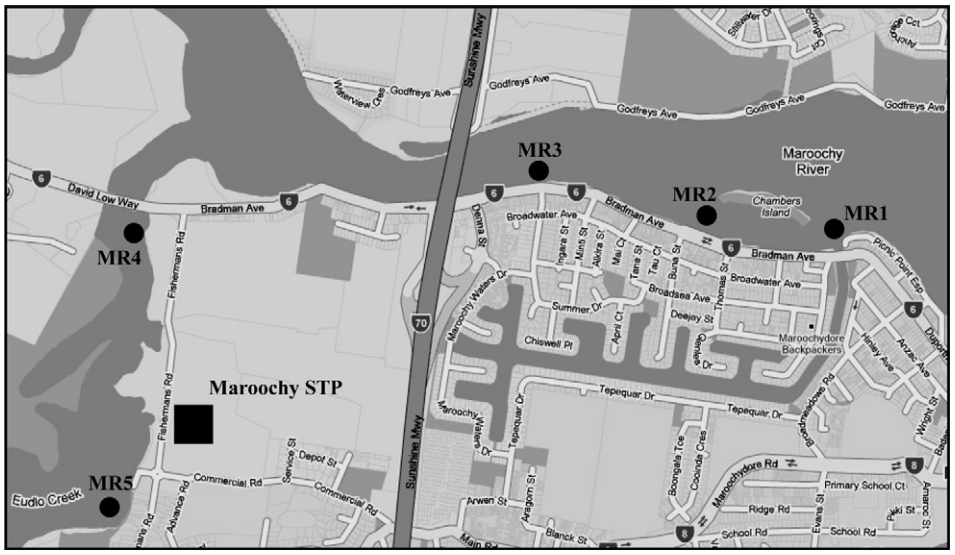

Fig. 1 – Map of the Maroochy River showing sampling sites MR1–MR5 (●), and the STP (■).

2.7. *Host-specific viral, bacterial and protozoan DNA extraction from environmental water samples*

Viruses were concentrated from water samples according to the same method described above except the volumes of water filtered ranged between 4 and 5 L depending upon the turbidity of the water. In case of filter paper clogging, a second filter paper was used. Concentrates were stored at −80 °C, and DNA was extracted from each concentrated samples by using DNA blood and tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Extracted DNA was eluted and resuspended in 200 μl of AE buffer, serially diluted, and stored at −20 °C until use.

For qPCR of *Salmonella invA* gene and *C. jejuni mapA* gene, 500 ml of water sample was filtered through 0.45-μm pore size membrane (Advantec). In case of membrane clogging during filtration, multiple membranes were used. The membranes were immediately transferred into 15 ml screw cap tubes containing 10 ml of sterile STE buffer (0.1 M NaCl, 10 mM Tris, and 1 mM EDTA [pH 7.6]). The tubes were vortexed vigorously for 8–10 min to detach the bacteria from the membranes followed by centrifugation at 15,000 rpm for 30 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 2 ml of sterile distilled water. DNA was extracted using DNeasy blood and tissue kit (Qiagen), eluted and resuspended in 200 μl of AE buffer, serially diluted, and stored at −20 °C until use.

For qPCR of *G. lamblia* β-giardin gene, 5 L of water sample was filtered through a 3-μm-pore-size membrane (47-mm diameter; Advantec). In case of membrane clogging during filtration, multiple membranes were used. After filtration, the membrane was transferred to a 2 ml sterile microcentrifuge tube. DNA was extracted directly onto the filter, using DNeasy blood and tissue kit (Qiagen). In summary, 360 μl of buffer ATL was added to each sample and subjected to three cycles of freezing (−80 °C) and thawing (56 °C) in a water bath. After the freezing–thawing, 40 μl of proteinase K was added to each tube which was then incubated overnight at 56 °C. After incubation, the DNA was extracted according to the manufacturer's instructions. Each DNA sample was eluted and resuspended in 200 μl of AE buffer, serially diluted, and stored at −20 °C until use.

2.8. *Preparation of standard curves*

Standards for qPCR of *C. jejuni mapA*, *Salmonella invA*, and *G. lamblia* β-giardin genes were prepared from the genomic DNA of the selected pathogens. The concentration of genomic DNA was determined by measuring the absorbance at A_{260} using Beckman Coulter DU® 730 spectrophotometer. The genomic copies were calculated, and a tenfold dilution was prepared from the genomic DNA, ranging from 10^6 to 10^0 copies per μl of DNA extract using CAS-1200™ precision liquid handling system (Corbett Life Sciences, Brisbane, Australia), and stored at −20 °C until use. For each standard, the concentration was plotted against the cycle number at which the fluorescence signal increased above the threshold value (C_T value). The amplification efficiency (E) was determined by running the standards, and was estimated from the slope of the standard curve by the formula $E = (10^{-1/\text{slope}}) - 1$. A reaction with 100% efficiency generates a slope of −3.32.

2.9. *PCR detection and quantification*

PCR analyses were performed using a Rotor-Gene 6000 real-time cycler (Corbett Research, Mortlake, Australia). Amplification was performed in either 50-μl reaction mixtures (for HS-AVs and BS-AVs detection) containing 45 μl of platinum blue SuperMix (Invitrogen), 200–400 nM of each primer, and 3 μl of template DNA or 25-μl reaction mixtures (for *Salmonella* spp., *C. jejuni*, and *G. lamblia*) containing 12.5 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA), 200–400 nM of each primer, and 2 μl of template DNA. For the detection of HS-AVs (Fong et al. 2005) and BS-AVs (Maluquer de Motes et al., 2004), a nested PCR protocol (i.e., two rounds) was used. Both rounds of BS-AVs PCR consisted of 4

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min at 94 °C followed by 30 cycles of 60 s at 92 °C, 30 s at 52 °C and 75 s at 72 °C, and a final extension of 7 min at 72 °C. *Salmonella* PCR consisted of 5 min at 94 °C followed by 45 cycles of 30 s at 94 °C, 35 s at 59 °C and 2 min at 72 °C, and a final extension of 5 min at 72 °C. *C. jejuni* PCR consisted of 10 min at 95 °C followed by 50 cycles of 15 s at 95 °C, 30 s at 59 °C. *G. lamblia* PCR consisted of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 60 s at 59 °C.

2.10. Testing for PCR inhibitors in environmental samples

An experiment was conducted to determine the effects of PCR inhibitory substances on the PCR detection/quantification of host-specific viral, bacterial and protozoan targets in environmental samples ($n = 5$) collected from the Maroochy River. Three sets of DNA were extracted from each sample according to the methods described above, and tested with the PCR. DNA was also extracted from ultra pure DNAse and RNase free sterile distilled water (Invitrogen) in the same manner. All three sets of DNA samples were spiked with 10^3 gene copies of the sewage-associated HF183 *Bacteroides* markers (Bernhard and Field, 2000; Seurinck et al., 2005). Before spiking, all DNA samples were tested to determine the background level of the HF183 markers. None of the samples were positive for the marker.

The PCR was performed using a Rotor-Gene 6000 real-time cycler (Corbett Research, Mortlake, Australia). Amplification was performed in 25 μl reaction mixtures using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA). The PCR mixture contained 12.5 μl of SuperMix, 300 nM of each primer (for primers sequence see Bernhard and Field, 2000; Seurinck et al., 2005), 1 μl of corresponding environmental DNA, and 1 μl of the HF183 DNA. The HF183 PCR consisted of 2 min at 50 °C, 10 min at 95 °C followed by 45 cycles of 30 s at 95 °C, 1 min at 53 °C, and then extension of 1 min at 60 °C. The threshold cycle (C_T) values of these spiked environmental DNA samples were compared to those of the DNA sample of distilled water spiked with the same concentration of the HF183 marker. The C_T value reflects the PCR cycle number at which the fluorescence generated crosses the threshold. It is inversely correlated to the logarithm of the initial copy number.

2.11. PCR detection limits

To determine the lower limits of the PCR detection, plasmid DNA (i.e., HS-AVs and BS-AVs) and genomic DNA (*Salmonella* serovar Typhimurium, *C. jejuni* and *G. lamblia*) were quantified using a spectrophotometer. Ten-fold serial dilutions were made and tested with the PCR.

2.12. Recovery efficiency

The recovery efficiencies were determined only for *Salmonella* and *G. lamblia* qPCR assays. The recovery efficiency of *C. jejuni* was assumed to be similar to that of *Salmonella* qPCR assay. Deionised water ($n = 3$) and coastal environmental water samples ($n = 3$) were spiked with known concentrations of *Salmonella Typhimurium* cells and *G. lamblia* cysts (obtained from Biotechnology Frontiers, New South Wales, Australia). Initially, samples ($n = 5$) were tested for the presence of *Salmonella* spp. and *G. lamblia* using PCR detection. Water samples which showed the absence of *Salmonella* spp. and *G. lamblia* were selected for this experiment. The samples were autoclaved to destroy background microbial flora and kept under UV light to minimize any background DNA that could be present. The *S.* Typhimurium strain was grown overnight in LB broth, and cell concentrations were determined using microscopic counts. Ten-fold serial dilutions were made and spiked into 500 ml of deionised and rainwater samples. Similarly, known concentrations of *G. lamblia* cysts were serially diluted and spiked into 5 L of deionised and environmental samples. The samples were filtered through membranes and DNA extraction was performed according to the method described above. Samples were tested in triplicate for each concentration, and the recovery efficiency (%) was calculated using the following equation: Recovery (%) = No. of cells after filtration/No. of cells before filtration $\times 100$. All results were corrected according to their relevant recovery ratios.

2.13. Quality control

To minimize PCR contamination, DNA extraction, PCR set up, and gel electrophoresis were performed in separate laboratories. To prevent false positive results for environmental samples, a method blank was included for each batch of environmental samples. For each PCR experiment, corresponding positive (i.e., target plasmid DNA) and negative controls (i.e., DNase and RNase free water) were included. To separate the specific product from non-specific products, DNA melting curve analysis was performed for each PCR experiment. During melting curve analysis, the temperature was increased from 57 °C to 95 °C at approximately 2 °C/min. Amplified products (for HS-AVs and BS-AVs) were also visualized by electrophoresis through 2% E-gel® (Invitrogen), and exposure to UV light. Samples were considered to be positive when the visible band was the same as that of the positive control strain, and had the same melting temperature as the positive control.

2.14. Statistical analysis

The Pearson's correlation was used to test the relationship between *E. coli* and enterococci concentrations in environmental water samples colleted during wet and dry conditions. A binary logistic regression (BLR) (SPSS version 12.0) analysis was also performed to obtain correlations between the presence/absence of the host-specific markers and zoonotic pathogens with the concentrations of FIB. BLR is the technique most commonly used to model such a binary (i.e., presence/absence) response. The presence/absence of pathogens was treated as the dependent variable (i.e., a binary variable). When a target organism was present, it was assigned the value 1, and when a target organism was absent, it was assigned the value 0. Nagelkerke's *R* square, which can range from 0.0 to 1.0, denotes the effect size (the strength of the relationship) where stronger associations have values closer to 1.0. Relationships were considered significant when the *p* value for the model chi square was <0.05 and the confidence

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interval for the odds ratio did not include 1.0. Greater odds ratios indicate a higher probability of change in the dependent variable with a change in the independent variable.

3. Results

3.1. Host-specificity and sensitivity of host-specific viral markers

Of the 74 human sourced wastewater samples tested, 58 (78%) were positive for the HS-AVs (Table 2). However, 14 (87%) out of 16 secondary effluent samples were negative for this marker. Most (i.e., 80%) of the septic samples were also positive for the marker. All human-sourced wastewater DNA samples which gave PCR negative results were serially diluted, and tested with the PCR to rule out the possibility of the presence of PCR inhibitors and their effects on the PCR detection. No discrepancies were observed between undiluted and diluted DNA samples. Of the 106 animal fecal DNA samples tested, none (i.e., undiluted DNA, 10-fold and 100-fold dilutions) were positive for the HS-AVs. All cattle-wastewater DNA samples were positive for the BS-AVs. However, only 3 out of 10 individual cattle fecal DNA samples colleted were positive for this marker. The BS-AVs marker could not be detected in DNA samples from the feces of other animals and human wastewater.

Host-specificity is the probability of detection when a source is not present and sensitivity is the probability of detection when it is present. The overall specificity of the HS-AVs marker to differentiate between humans and animals was 1.0, and the overall sensitivity of this marker in human wastewater was 0.78. Similarly, the overall specificity and sensitivity of the BS-AVs maker were 1.0 and 0.73, respectively.

3.2. PCR inhibitors

Host-specific viral, bacterial and protozoan DNA was spiked with 10^3 gene copies of the sewage-associated HF183 *Bacteroides* marker. The C_T values were compared to those obtained from the same concentration (i.e., 10^3) of DNA that was used to spike distilled water. For the spiked distilled water, the mean C_T value for the HF183 marker was 25.0 ± 0.6. For surface water samples, the mean C_T values for viral, bacterial and protozoan undiluted, 10-fold, and 100-fold diluted DNA are shown in Table 3. One-way ANOVA was performed to determine the differences between the C_T values obtained for distilled water and those obtained for viral, bacterial and protozoan DNA isolated from surface water samples. No significant differences ($p > 0.05$) were observed between the C_T values for spiked distilled water and undiluted viral DNA. However, significant ($p < 0.001$) differences were observed between the C_T values for spiked distilled water and undiluted bacterial and protozoan DNA from surface water samples, indicating that the undiluted bacterial and protozoan DNA extracted from surface water samples contained PCR inhibitory substances. However, no significant differences ($p > 0.05$) were observed between the C_T values for spiked distilled water and serially diluted (i.e., 10-fold) bacterial DNA and protozoan DNA (100-fold) indicating that 10- and 100-fold dilution of DNA is required to remove the effects of PCR inhibitory substances.

Table 2 – Host-specificity and sensitivity of human and bovine specific adenoviruses in various host groups.

Host groups	Number of samples	PCR positive results for human and bovine specific adenoviruses at various dilutions					
		Human-specific adenoviruses			Bovine-specific adenoviruses		
		Undiluted DNA	Diluted DNA		Undiluted DNA	Diluted DNA	
			10^{-1}	10^{-2}		10^{-1}	10^{-2}
Humans							
Influent	30	30/30	–	–	0/30	0/30	0/30
Primary effluent	18	18/18	–	–	0/18	0/18	0/18
Secondary effluent	16	2/16	2/16	2/16	0/16	0/16	0/16
Septic wastewater	10	8/10	8/10	8/10	0/10	0/10	0/10
Animals							
Kangaroos	10	0/10	0/10	0/10	0/10	0/10	0/10
Dogs	10	0/10	0/10	0/10	0/10	0/10	0/10
Ducks	10	0/10	0/10	0/10	0/10	0/10	0/10
Horses	10	0/10	0/10	0/10	0/10	0/10	0/10
Birds	5	0/5	0/5	0/5	0/5	0/5	0/5
Chickens	10	0/10	0/10	0/10	0/10	0/10	0/10
Cattle	10	0/10	0/10	0/10	3/10	3/10	3/10
Sheep	10	0/10	0/10	0/10	0/10	0/10	0/10
Pigs	10	0/10	0/10	0/10	0/10	0/10	0/10
Pooled cattle wastewater[a]	16	0/16	0/16	0/16	16/16	–	–
Goat	5	0/5	0/5	0/5	0/5	0/5	0/5

– Not tested.
a Cattle wastewater.

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Table 3 – Effects of PCR inhibitors on the PCR detection of spiked sewage-associated HF183 *Bacteroides* markers in viral, bacterial and protozoan DNA isolated from surface water samples as opposed to distilled water samples.

Samples	Threshold cycle (C_T) value for the real-time PCR								
	Undiluted DNA			10-fold dilution			100-fold dilution		
	Viral DNA	Bacterial DNA	Protozoan DNA	Viral DNA	Bacterial DNA	Protozoan DNA	Viral DNA	Bacterial DNA	Protozoan DNA
Distilled water	25.0 ± 0.6	–	–	–	–	–	–	–	–
MR1	24.6 ± 0.6	28.8 ± 0.3	32.7 ± 0.3	24.8 ± 0.3	25.1 ± 0.4	27.8 ± 0.6	24.3 ± 0.9	25.3 ± 0.6	24.2 ± 0.6
MR2	25.4 ± 0.3	28.0 ± 0.4	33.0 ± 0.4	25.0 ± 0.5	26.0 ± 0.6	28.3 ± 0.6	25.8 ± 0.6	25.9 ± 0.8	24.8 ± 0.4
MR3	25.6 ± 0.2	29.2 ± 0.7	32.1 ± 0.6	25.2 ± 0.7	25.9 ± 0.6	28.2 ± 0.4	25.9 ± 0.7	25.9 ± 0.6	23.9 ± 0.7
MR4	23.5 ± 1.2	27.9 ± 0.7	31.6 ± 0.8	23.9 ± 0.9	24.0 ± 0.9	27.9 ± 0.3	23.8 ± 0.8	26.9 ± 0.7	23.6 ± 0.8
MR5	24.9 ± 0.6	28.7 ± 0.6	32.7 ± 0.6	24.7 ± 0.4	25.0 ± 0.6	27.0 ± 0.4	24.3 ± 0.6	25.3 ± 0.6	24.5 ± 0.5

3.3. PCR limit of detection and recovery efficiency

The PCR limit of detection assays were performed by analysing purified plasmid and genomic DNA isolated from the viral, bacterial and protozoan targets. To determine the reproducibility of the assay, several replicates ($n = 10$) of diluted DNA ranging from 10^3 to 10^0 were tested. The PCR detection limits were as low as five (for *C. jejuni mapA* and *Salmonella invA* genes) to seven (for *G. lamblia* β-giardin gene) gene copies per reaction. For both HS-AVs and BS-AVs, the limits of detection were 10 gene copies per reaction. Lower levels (i.e., one copy) were detected, but the results were not reproducible for all replicates.

The estimated recovery efficiency in autoclaved distilled water samples ranged between 93% and 48% (for *Salmonella*) and 43% and 23% (for *G. lamblia*) with the greatest variability occurring at lower cell and cyst counts. The mean recovery efficiencies were 69% ± 13% (for *Salmonella*) and 31% ± 10% (for *G. lamblia*). The estimated recovery efficiency in autoclaved coastal water samples ranged between 81% and 55% (for *Salmonella*) and 39% and 16% (for *G. lamblia*) with the greatest variability occurring at lower cell and cyst counts. The mean recovery efficiencies were 68% ± 14% (for *Salmonella*) and 31% ± 8% (for *G. lamblia*).

3.4. Concentrations of fecal indicators

The concentrations of FIB in water samples collected after wet conditions ranged from 48 ± 11 to 2906 ± 300 (for *E. coli*) and from 60 ± 20 to 1586 ± 180 (for enterococci) (Table 4). Upstream sites had higher concentrations of FIB than downstream sites. Site MR5 had higher *E. coli* and enterococci counts than other sites on all occasions. The concentrations of FIB in water samples collected during dry conditions ranged from <1 to 103 ± 11 (for *E. coli*) and from <1 to 220 ± 60 (for enterococci) (Table 5). The concentrations of both FIB were generally higher in samples collected after wet conditions compared to dry conditions. Of the 20 samples tested during wet conditions, 16 (80%) *E. coli* and 20 (100%) enterococci exceeded the Australian and New Zealand Environment and Conservation Council (ANZECC) water quality guidelines of 150 fecal coliforms and 35 enterococci per 100 ml of water for primary contact. During dry conditions, the *E. coli* value did not exceeded the ANZECC guidelines value. However, 13 samples (65%) exceeded the enterococci guidelines value for primary contact. Pearson's correlation was used to test the relationship between *E. coli* and enterococci concentrations. The concentrations of *E. coli* and enterococci correlated significantly during wet conditions ($p < 0.0005$). However, during dry conditions, there was no significant ($p > 0.323$) correlation observed between the concentrations *E. coli* and enterococci.

3.5. Prevalence of host-specific viruses

Of the 20 samples tested during wet conditions, four (25%) were positive for the HS-AVs marker (Table 4). HS-AVs marker was detected in MR2 and MR3 sites which are located near the stormwater outlets. Similarly, two samples (10%) were positive for the BS-AVs marker. However, this marker was detected in samples collected from upstream MR5 site. Six samples (30%) were positive for at least one marker tested. Among the 20 samples collected during dry conditions, none was positive for the HS-AVs. However, two samples (10%) at upstream sites MR4 and MR5 were positive for the BS-AVs marker. Overall (i.e., pooled data of wet and dry conditions) both HS-AVs and BS-AVs were detected in four samples (10%), and eight samples (20%) were positive for at least one marker.

3.6. Prevalence and concentrations of enteric pathogens

During wet conditions, *C. jejuni mapA* gene was detected in two samples (10%) collected from upstream site MR5 (i.e., occasions two and three) by PCR but was non-quantifiable. *Salmonella invA* gene was also detected in two samples (10%) collected from the same site (occasions one and three). The concentrations of *Salmonella invA* in these PCR positive samples ranged from 3.5×10^2 to 4.3×10^2 genomic copies per 500 ml of water. None of the samples colleted during the wet conditions were positive for *G. lamblia* β-giardin gene. In contrast, during dry conditions, none of the samples were positive for *C. jejuni mapA* and *Salmonella invA* genes. Only one sample was positive for *G. lamblia* β-giradin gene but was non-quantifiable.

3.7. Correlation between fecal indicators and presence absence of viral markers and zoonotic pathogens

BLR analysis was used to identify whether any correlation existed between the concentrations of FIB and the presence/

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Table 4 – Fecal indicators concentrations, PCR positive/negative results of host-specific viruses and qPCR results of zoonotic pathogens in water samples collected from the Maroochy River during wet conditions.

Sampling sites (occasion)	Concentrations (CFU/100 ml) of fecal indicators		Presence (+)/absence (–) of host-specific viruses		Concentrations of zoonotic pathogens		
	E. coli	Enterococci	HS-AVs	HS-BVs	*C. jejuni mapA*	*Salmonella invA*	*G. lamblia* β-giardin
MR1 (1)[a]	48 ± 11	73 ± 12	–	–	–	–	–
MR2 (1)[a]	52 ± 30	120 ± 20	+	–	–	–	–
MR3 (1)[a]	152 ± 55	229 ± 30	–	–	–	–	–
MR4 (1)[a]	360 ± 80	403 ± 105	–	–	–	–	–
MR5 (1)[a]	2906 ± 300	1586 ± 180	–	+	–	350	–
MR1 (2)[b]	248 ± 31	380 ± 57	–	–	–	–	–
MR2 (2)[b]	152 ± 38	230 ± 68	+	–	–	–	–
MR3 (2)[b]	56 ± 15	176 ± 70	+	–	–	–	–
MR4 (2)[b]	260 ± 35	430 ± 80	–	–	–	–	–
MR5 (2)[b]	1100 ± 230	1480 ± 186	–	–	+	–	–
MR1 (3)[c]	156 ± 31	386 ± 70	+	–	–	–	–
MR2 (3)[c]	120 ± 25	224 ± 35	–	–	–	–	–
MR3 (3)[c]	152 ± 35	110 ± 15	–	–	–	–	–
MR4 (3)[c]	156 ± 38	90 ± 24	–	–	–	–	–
MR5 (3)[c]	760 ± 134	1130 ± 220	–	+	+	430	–
MR1 (4)[d]	78 ± 11	60 ± 20	–	–	–	–	–
MR2 (4)[d]	152 ± 30	170 ± 55	–	–	–	–	–
MR3 (4)[d]	182 ± 55	80 ± 38	–	–	–	–	–
MR4 (4)[d]	450 ± 80	235 ± 67	–	–	–	–	–
MR5 (4)[d]	820 ± 300	480 ± 135	–	–	–	–	–

a Study area received >100 mm rainfall 2 days prior sampling.
b Study area received >25 mm rainfall 2 days prior sampling.
c Study area received >32 mm rainfall 2 days prior sampling.
d Study area received >15 mm rainfall 2 days prior sampling.

absence results of host-specific viral markers and zoonotic pathogens. During wet conditions, weak correlation was found between *E. coli* with HS-AVs ($p = 0.02$). However, significant correlations were observed between *E. coli* with BS-AVs ($p = 0.007$) and *Salmonella invA* gene ($p = 0.007$) (Table 6). The concentrations of enterococci also significantly correlated with BS-AVs ($p = 0.006$), *C. jejuni mapA* ($p = 0.01$) and *Salmonella invA* ($p = 0.006$) genes. BLR could not be performed for *G. lamblia* β-giardin gene as none of the samples gave a positive signal during wet conditions. During dry conditions, no significant correlations were observed between FIB concentrations with BS-AVs and *G. lamblia* β-giardin gene. BLR could not be performed for HS-AVs, *C. jejuni mapA* and *Salmonella invA* genes because none of the samples were positive. BLR was also performed on the pooled data of both wet and dry conditions. The concentrations of FIB did not correlate with HS-AVs. However, both *E. coli* ($p = 0.021$) and enterococci ($p = 0.025$) weakly correlated with the BS-AVs. The concentrations of enterococci significantly ($p = 0.002$) correlated with *C. jejuni mapA* gene. However, *E. coli* did not correlate with *C. jejuni mapA* gene. Significant correlations ($p = 0.002$) were also observed for both FIB with *Salmonella invA* gene. No correlations were observed between *E. coli* and enterococci with *G. lamblia* β-giardin gene.

4. Discussion

Specificity and sensitivity are two commonly adopted parameters used to evaluate the performance of host-specific markers. It is desirable that a marker should be highly host-specific and, any marker showing a value >0.95 could be considered as suitable for source tracking. It has been recommended that the specificity and sensitivity of MST markers need to be tested prior to their application for field studies especially for geographical locations where the specificity has never been tested (Field and Samadpour, 2007). This is important to prevent false positive and negative results. Recently, human specific bacterial markers such as human-specific *Bacteroides* and enterococci surface protein (*esp*) markers have been reported to have been found in fecal samples from animals, especially dogs (Ahmed et al., 2008; Whitman et al., 2007). In this study, both HS-AVs and BS-AVs showed the specificity value of 1.0 which is consistent with previous research findings (Maluquer de Motes et al., 2004). However, the sensitivities of the HS-AVs and BS-AVs were relatively low compared to other host-specific markers such as sewage-associated *Bacteroides* (Ahmed et al., 2008). All samples tested from the influent and primary effluents were positive for the HS-AVs marker. However, this marker could not be detected in all samples from the secondary effluent and some septic systems. The absence of the HS-AVs in secondary effluent indicates removal of viruses after treatment. Therefore, to detect these viruses in secondary effluent, a large volume of water needs to be analysed. The absence of HS-AVs in certain septic tanks is not surprising because these markers are generally present in infected humans. Therefore, septic tanks collecting wastewater from healthy humans may not have this marker.

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Table 5 – Fecal indicators concentrations, PCR positive and negative results of host-specific viruses and qPCR results of zoonotic pathogens in water samples collected from the Maroochy River during dry conditions.

Sampling sites (occasion)	Concentrations (CFU/100 ml) of fecal indicators		Presence (+)/absence (–) of host-specific viruses		Concentrations of zoonotic pathogens		
	E. coli	Enterococci	HS-AVs	HS-BVs	*C. jejuni mapA*	*Salmonella invA*	*G. lamblia* β-giardin
MR1 (1)	38 ± 14	60 ± 28	–	–	–	–	–
MR2 (1)	23 ± 8	34 ± 18	–	–	–	–	–
MR3 (1)	35 ± 10	90 ± 23	–	–	–	–	–
MR4 (1)	23 ± 10	110 ± 34	–	–	–	–	–
MR5 (1)	30 ± 8	76 ± 18	–	+	–	–	–
MR1 (2)	17 ± 10	156 ± 38	–	–	–	–	–
MR2 (2)	13 ± 5	220 ± 60	–	–	–	–	–
MR3 (2)	21 ± 7	33 ± 8	–	–	–	–	–
MR4 (2)	33 ± 7	62 ± 13	–	+	–	–	–
MR5 (2)	21 ± 6	133 ± 57	–	–	–	–	+
MR1 (3)	<1	60 ± 20	–	–	–	–	–
MR2 (3)	44 ± 5	20 ± 5	–	–	–	–	–
MR3 (3)	15 ± 5	30 ± 10	–	–	–	–	–
MR4 (3)	70 ± 15	65 ± 41	–	–	–	–	–
MR5 (3)	103 ± 11	<1	–	–	–	–	–
MR1 (4)	13 ± 5	40 ± 10	–	–	–	–	–
MR2 (4)	6 ± 4	15 ± 6	–	–	–	–	–
MR3 (4)	<1	18 ± 11	–	–	–	–	–
MR4 (4)	67 ± 15	40 ± 14	–	–	–	–	–
MR5 (4)	6 ± 2	42 ± 7	–	–	–	–	–

Similarly, BS-AVs marker was detected in all samples from cattle-wastewater, but could not be detected in samples from all individual cattle feces. These results are also consistent with a previous study which was undertaken in Spain (Maluquer de Motes et al., 2004).

For the HS-AVs and BS-AVs specificity assay, care was taken to prevent PCR false positive/negative results. DNA extracted from wastewater and fecal samples were diluted, and subsequently tested with the PCR to confirm that inhibitors did not mask the PCR amplification. Similarly, DNA isolated from Maroochy River water samples were also assessed for the presence of PCR inhibitors prior to testing for viral markers and zoonotic pathogens. The viral DNA extracted from water samples did not contain any PCR inhibitors, which is due to the fact that viral DNA extraction method used in this study involved membrane filtration followed by virus concentration and purification using a Centriprep column. Therefore DNA extracted from environmental water samples should not contain any PCR inhibitory substances (Haramoto et al., 2005). This method was previously used to extract DNA from raw sewage and none of the samples contained PCR inhibitors (Ahmed et al., 2009). However, bacterial and protozoan DNA extracted from the same water samples contained PCR inhibitors as the methods did not involve DNA purification. Ten-fold (for bacterial) and 100-fold (for protozoan) dilutions of DNA were required to remove PCR inhibitors. A large volume of water samples was processed to extract protozoan DNA compared to bacterial DNA, and therefore, protozoan DNA contained more PCR inhibitory substances.

In this study, environmental water samples were collected from a coastal river. Several sites were located in the vicinity of stormwater outlets and an STP which may create the potential for fecal pollution. The concentrations of FIB in most of the sites except site MR5 in wet conditions were generally low. This could be explained by the fact that the decay rates of FIB are higher in saltwater compared to freshwater, and as a result these indicators may not persist in water for a prolonged period (Anderson et al., 2005). It is also possible that more water flow may have diluted the concentrations of FIB. The concentrations of FIB in dry conditions were low compared to wet conditions. This is not unexpected because after rainfall events, FIB indicators are generally transported to the waterways via stormwater runoff from various point and non-point sources of fecal pollution.

During wet conditions, HS-AVs were detected in sites MR1, MR2 and MR3. These sites were located near the vicinity (i.e., MR2 and MR3) or downstream (i.e., MR1) of stormwater outlets discharging into the river. It is highly likely that these stormwater outlets which were identified as potential sources of human fecal pollution may have contributed HS-AVs in the Maroochy River. The HS-AVs could not be detected in samples from upstream sites such as MR4 and MR5 suggesting that STP discharges may not be the source of this marker. The HS-AVs could not be detected in none of the water samples colleted during dry conditions suggesting that human fecal pollution was not occurring during the sampling period. However, BS-AVs was detected in both wet and dry conditions suggesting that cattle fecal pollution is occurring. BS-AVs was only detected in samples from the upstream site, MR5, which was located downstream of the agricultural and cattle grazing land. *C. jejuni mapA* and *Salmonella invA* genes were detected on two occasions during wet conditions, but were not detected during dry conditions. In contrast, *G. lamblia* β-giardin could not be detected in wet conditions, but was detected on one occasion during dry conditions. *C. jejuni mapA* and *G. lamblia* β-

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Table 6 – Relationships between fecal indicator concentrations and PCR positive and negative results of host-specific viruses and zoonotic pathogens in water samples using binary logistic regression analysis.

Fecal indicators	Host-specific viral markers				Zoonotic pathogens					
	HS-AVs		BS-AVs		*C. jejuni mapA*		*Salmonella invA*		*G. lamblia* β-giradin gene	
	R square	Odd ratio	R square	Odd ratio	R square	Odd ratio	R square	Odd ratio	R square	Odd ratio
Wet conditions										
E. coli	0.364[b]	0.985	0.634[b]	1.004	0.100	1.001	0.630[b]	1.004	NA	NA
Enterococci	0.082	0.998	0.657[b]	1.004	0.592[b]	1.004	0.657[b]	1.004	NA	NA
Dry conditions										
E. coli	NA	NA	0.002	1.004	NA	NA	NA	NA	0.019	0.983
Enterococci	NA	NA	0.001	1.001	NA	NA	NA	NA	0.188	1.018
Wet and dry conditions[a]										
E. coli	0.024	0.999	0.260[b]	1.002	0.153	1.001	0.662[b]	1.005	0.157	0.970
Enterococci	0.005	1.000	0.247[b]	1.002	0.634[b]	1.004	0.692[b]	1.005	0.014	0.998

NA: Analysis was not undertaken because of the absence of at least one positive value.
a Pooled data of dry and wet conditions.
b Significant correlation for ($p < 0.05$ for chi-square, confidence interval for odds ratio does not include 1.0).

giardin genes were present in samples, but were not quantifiable. None of the zoonotic pathogens were detected in samples which were PCR positive for the HS-AVs. However, *Salmonella invA*, *C. jejuni mapA* and *Giardia* β-giardin was detected in samples that were also PCR positive for the BS-AVs. Overall, viral markers and zoonotic pathogens were more frequently detected in wet conditions than dry conditions.

It has been suggested that fecal coliform levels do not provide reliable information regarding the occurrence of human viruses (Griffin et al., 1999; Pina et al., 1998). BLR was used to identify the correlations between the concentrations of FIB (i.e., *E. coli* and enterococci) with viral markers and zoonotic pathogens. Concentrations of FIB did not correlate with the HS-AVs. However, both FIB concentrations significantly correlated with BS-AVs during wet conditions. When the datasets of both dry and wet conditions were pooled, a weak correlation was found between FIB concentrations and BS-AVs. The data obtained in this study also suggest that FIB may not be reliable as indicators of the presence of enteric viruses in coastal waters of Southeast Queensland, Australia.

The advantage of using HS-AVs and BS-AVs as an MST tool appears to be high-host specificity and geographical stability as reported in this study and others (Fong et al., 2005; Maluquer de Motes et al., 2004). These are double-stranded DNA viruses and more stable to environmental stresses and treatments compared to FIB commonly used to predict the presence of viral and protozoan pathogens. Therefore, these viruses are better suited as surrogates for human pathogens especially viruses and protozoan groups (Dorner et al., 2007; Harwood et al., 2005). In addition, PCR detection of viruses has advantages over cell culture assays as PCR offers high specificity and detection sensitivity compared to traditional cell culture (Chung et al., 1996; Jiang et al., 2001). Furthermore, PCR could be used to detect viruses that are difficult to culture such as noroviruses (Fong et al., 2005). The other advantages of using host-specific viral markers include cost-effectiveness (i.e., analysis is cheaper compared to library based and certain chemical methods) and the results can be obtained within two days.

A limitation of using viral markers is that their concentrations appear to be low in sewage compared to bacterial markers such as *Bacteroides*. Therefore, to detect these markers, a large volume of water samples needs to be analysed. In addition, their absence in a water sample does not rule out the presence of human fecal pollution. Nonetheless, their presence indicates potential health risks since only a small number of infective plaques is required to cause illness. For the identification of human fecal pollution accurately, it is recommended that multiple markers (viral and bacterial) should be used where necessary (McQuaig et al., 2006) to obtain confirmatory results. The current study is not quantitative and therefore, does not provide information regarding the magnitude of fecal pollution in the river under investigation. Currently, qPCR HS-AVs method is being developed in our laboratory for the quantitative detection of this marker in environmental waters. Furthermore, a little is known regarding the persistence of HS-AVs and BS-AVs in relation to FIB and pathogens. Further research needs to be undertaken in order to obtain information regarding their persistency in marine and freshwater.

5. Conclusions

- The HS-AVS and BS-AVs tested in this study were specific to human and bovine wastewater. The HS-AVs and BS-AVs detection using PCR appears to be a useful tool for the identification of human and cattle fecal pollution in coastal waters.
- The prevalence of HS-AVs in samples collected from the study river suggests that the quality of water is affected by human fecal pollution which could originate from defective septic systems and urban stormwater run off. BS-AVs was also detected in upstream sites suggesting that cattle also contribute to the fecal load in the river and the presence of this marker also indicates the presence of potential zoonotic pathogens. This is further supported by the presence of zoonotic pathogens such as *C. jejuni*, *Salmonella* spp., and *G. lamblia* in the river water samples.
- The concentrations of FIB and the occurrence of viral markers and pathogens were higher in wet conditions than

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dry conditions. None or little correlations were observed between the concentration of FIB and viral markers, thus indicating that FIB could not be reliably used to predict the presence of viruses in coastal waters.

- Further research is required to understand the persistency of these markers in environmental water samples in relation to traditional fecal indicators and pathogenic microorganisms. Additionally, quantitative PCR data would be required to assess the magnitude of fecal pollution and associated public health risks. Our future research will focus on evaluating the survival of these markers in various environmental waters along with the traditional fecal indicators and pathogens.

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