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Comparison of molecular markers to detect fresh sewage in environmental waters

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ABSTRACT

Human-specific *Bacteroides* HF183 (HS-HF183), human-specific *Enterococci faecium esp* (HS-*esp*), human-specific adenoviruses (HS-AVs) and human-specific polyomaviruses (HS-PVs) assays were evaluated in freshwater, seawater and distilled water to detect fresh sewage. The sewage spiked water samples were also tested for the concentrations of traditional fecal indicators (i.e., *Escherichia coli*, enterococci and *Clostridium perfringens*) and enteric viruses such as enteroviruses (EVs), sapoviruses (SVs), and torquetenoviruses (TVs). The overall host-specificity of the HS-HF183 marker to differentiate between humans and other animals was 98%. However, the HS-esp, HS-AVs and HS-PVs showed 100% hostspecificity. All the human-specific markers showed >97% sensitivity to detect human fecal pollution. *E. coli*, enterococci and, *C. perfringens* were detected up to dilutions of sewage 10_5, 10_4 and 10_3 respectively.HS-esp, HS-AVs, HS-PVs, SVs and TVs were detected up to dilution of sewage 10_4 whilst EVs were detected up to dilution 10_5. The ability of the HS-HF183 marker to detect freshsewagewas3–4 orders ofmagnitude higher than that of the HS-esp and viral markers. The ability to detect fresh sewage in freshwater, seawater and distilled water matrices was similar for human-specific bacterial and viral marker. Based on our data, it appears that human-specific molecular markers are sensitive measures of fresh sewage pollution, and the HS-HF183 marker appears to be the most sensitive among these markers in terms of detecting fresh sewage. However, the presence of the HS-HF183 marker in environmental waters may not necessarily indicate the presence of enteric viruses due to their

high abundance in sewage compared to enteric viruses. More research is required on the persistency of these markers in environmental water samples in relation to traditional fecal indicators and enteric pathogens.

Keywords: Microbial source tracking; Sewage pollution; Molecular markers; Enteric viruses; Water quality

1. Introduction

Fecal pollution is one of the major concerns in relation to water bodies used for drinking water supply, recreational activities and harvesting seafood due to likely exposure to a wide array of pathogenic bacteria, protozoa and viruses (Hörman et al., 2004; Fong et al., 2005). Various sources such as agricultural run-off, wild animals, combined sewer overflows (CSOs), sewage treatment plants (STPs), defective on-site wastewater treatment systems and industrial wastewater outlets are known to be potential sources of such pollution. The microbiological quality of water is generally assessed by enumerating fecal indicator bacteria such as *E. coli* and enterococci which are commonly found in the feces of warm-blooded animals including humans (USEPA 2000a). The presence of these indicators in water bodies generally points to fecal pollution and potential public health risks. The identification of indicator bacteria from major polluting source(s) is vitally important in order to implement appropriate mitigation strategies to minimise fecal pollution and associated public health risks (Scott et al., 2002). However, the assignment of indicator bacteria to human and animal sources in environmental waters is difficult due to their cosmopolitan nature (Field and Samadpour, 2007). In addition, environmental waters can be impacted by multiple sources of fecal pollution making it extremely difficult to implement a robust management plan without understanding the potential sources of pollution.

Over the last decade, microbial source tracking (MST) techniques have been developed to distinguish human from animal fecal pollution. The underlying assumption of MST is that the host specificity of microorganisms is influenced by selective pressure (Wiggins, 1996). The majority of the early MST methods are library-dependent which require the development of a collection of *E. coli* or enterococci isolates from suspected sources using various phenotypic and genotypic methods. Phenotypic or genotypic patterns of target strains are then compared to the library to identify their likely sources (Scott et al., 2002). There are several significant

limitations in library-dependent methods which have been widely reported in the research literature such as: (1) a large representative library is required for successful field application. The development of such a library is laborious, and usually costly when using phenotypic and genotypic methods (i.e., PFGE and carbon source utilization) (Field and Samadpour, 2007); (2) commonly used fecal indicator bacteria (*E. coli* and/or enterococci) lack host-specificity and clonal in nature (Gordon et al., 2002); (3) a library comprising of a small number of isolates cannot be readily used in multiple catchments, and therefore development of a separate library may be required for each catchment of interest (Ahmed et al., 2006; Hartel et al., 2002; Scott et al., 2003), and (4) library-dependent methods may yield both high false positive and negative results (Harwood et al., 2003; Moore et al., 2005).

Certain limitations of library-dependent methods could be overcome by using library-independent methods. These methods rely on detecting host-specific molecular markers in a given environmental sample using PCR assays. These methods are rapid and have shown to have higher accuracies in a method comparison study (Griffith et al., 2003). The most commonly used markers for MST can be categorised into three groups: (1) anaerobic bacterial markers (i.e., host-specific *Bacteroides* PCR) (Bernhard and Field 2000), (2) bacterial toxin markers (i.e., *Enterococci faecium esp* and *E. coli* toxin gene markers) (Scott et al., 2005; Khatib et al., 2002), and (3) viral markers (i.e., host-specific adenoviruses and polyomaviruses) (Fong et al., 2005; McQuaig et al., 2006). Several studies have reported high host specificities of these markers which makes them suitable to distinguish between sources of fecal pollution (Ahmed et al., 2008a; Seurinck et al., 2005; Bernhard and Field, 2000, Reischer et al., 2006; Scott et al., 2005; Khatib et al., 2002).

However, certain markers have also been detected in small number of non-target samples (Gourmelon et al., 2007; Carson et al., 2005; Gawler et al., 2007; Whitman et al., 2007). One major limitation of these markers is that they are not present in the feces of all individuals, and the concentrations may vary in targets (Field and Samadpour, 2007). For example, the concentration of human-specific *Bacteroides* markers in sewage samples could be 4 to 5 orders of magnitude higher than human-specific viral or toxin gene markers. Moreover, little is known regarding the persistency of these markers in environmental waters. In addition, the correlation between some of these markers with traditional fecal indicators and pathogens is not well documented. The absence of a particular marker in environmental waters does not completely rule out the presence of fecal pollution from that particular source. A general

consensus is that multiple markers should be used (where possible) to obtain accurate and confirmatory results. To-date, only a few studies have used multiple host-specific markers to identify the sources of fecal pollution in environmental waters (Ahmed et al., 2007; Gourmelon et al., 2007; McQuaig et al., 2006). These markers appear to be promising in identifying the sources of fecal pollution. However, more research is required prior to their application for routine monitoring of water quality. A recent review paper highlighted the various research gaps that need to be addressed for library-independent methods (Santo Domingo et al., 2007).

The aim of this study was to evaluate the real-time PCR minimum detection limit of the human-specific *Bacteroides* HF183 (HS-HF183), human-specific *E. faecium esp* (HS-*esp*), human-specific adenoviruses (HS-AVs), and human-specific polyomaviruses (HS-PVs) assays to detect fresh sewage pollution in sewage spiked freshwater, seawater and distilled water samples. Furthermore, the sewage spiked water samples were also tested for the concentrations of fecal indicators such as *E coli*, enterococci and *Clostridium perfringens*. In addition, real-time PCR minimum detection limit of enteric viruses such as emerging enteroviruses (EVs), sapoviruses (SVs), and torquetenoviruses (TVs) were also determined for sewage spiked water samples. These enteric viruses are excreted in extremely high numbers in the feces of infected individuals and can cause mild to severe gastroenteritis in humans. Humans could be exposed to enteric viruses via consumption of shellfish harvested in contaminated waters, sewage contaminated recreational and drinking waters. Faecal indicators concentrations and minimum detection limit of human-specific markers and enteric viruses were used to obtain a better understanding of which faecal indicators and human-specific marker(s) could potentially indicate the presence of enteric viruses in environmental waters polluted with fresh sewage.

2. Materials and Methods

2.1 Host-specificity and sensitivity of human-specific markers

To determine the host specificity and sensitivity of the human-specific markers, 82 fecal samples were collected from six host groups. Approximately 10 ml of sample from each human fecal source (n = 32) was collected from influent entering sewage treatment plants (STPs). Cattle (n = 10), pigs, (n =10), and sheep (n = 10) samples (i.e., individual and composite animal wastewater samples) were collected from an abattoir in Killarney, Brisbane. Dog fecal samples (n = 10) were collected from two dog parks. Duck fecal samples (n = 10)

were collected from Brisbane City Botanical Gardens where a large number of ducks roam. A fresh fecal sample (approximately 0.5 – 1.0 g) was collected from each individual animal (*n*= 50) with sterile swabs and inserted into a sterile container, transported on ice to the laboratory, stored at 4°C, and processed within 6 h. For DNA extraction three different methods were used according to the previously published methods (Ahmed et al., 2008a; Ahmed et al., 2008b; Haramoto et al., 2005). Approximately 180-200 mg of fresh animal feces was used for DNA extraction using each method separately.

2.2 Testing for real-time PCR inhibitors

Fecal and STP samples contain numerous organic and inorganic substances with the potential to inhibit PCR analyses (Wilson, 1997). An experiment was conducted to determine the potential presence of inhibitory substances in DNA extracted from animal fecal and STP samples for the HS-HF183, HS-*esp* and HS-AVs and HS-PVs assays. DNA was extracted from 1 L of ultrapure DNase- and RNase-free sterile distilled water (Invitrogen) after concentrating the sample. A representative number of pooled animal fecal samples (n = 5) and STP samples (n = 5) were spiked with 10^3 gene copies of the HS-HF183 and cattle-specific CF128 (CS-CF128) markers (Bernhard and Field 2000). The threshold cycle (C_T) values of these spiked DNA samples were compared to those of the DNA samples from distilled water spiked with the same concentration of the HS-HF183 and CS-CF128 markers.

2.3 Sample preparation

To determine the real-time PCR minimum detection limit of the human-specific markers, fresh sewage samples were collected from the primary influent of a STP. Sewage samples were suspended in freshwater (n=3), seawater (n=3), and distilled water (n=3) at a ratio 1:1 (62.5 ml water:62.5 ml fresh sewage), and a serial dilution (10^{-1} to 10^{-10}) was made for each sample type. Environmental water samples were autoclaved before spiking, and exposed under UV light for 1 h to minimise background target DNA that could be present due to fecal pollution. The numbers of *E. coli*, enterococci, and *C. perfringens* were enumerated using membrane filtration method for each dilution. DNA and RNA extraction was performed for each dilution to determine the real-time PCR minimum detection limit of the human-specific markers and enteric viruses.

2.4 Isolation and enumeration of fecal indicators

The membrane filtration method was used to process the sewage spiked water samples for *E. coli*, enterococci, and *C. perfringens* enumeration. Sample serial dilutions were made and filtered through 0.45-µm pore size (47-mm-diameter) nitrocellulose membranes (Advantec, Tokyo, Japan), and placed on modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC agar) (Difco, Detroit, MI, USA), membrane-Enterococcus indoxyl-D-glucoside (mEI) agar (Difco), and oleandomycin-polymyxin-sulfadiazine perfringens (OPSP) agar with supplement for the isolation of *E. coli*, enterococci, and *C. perfringens*, respectively. For the isolation of *C. perfringens* spores, water samples were heated at 60°C for 30 min before filtration. 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 (USEPA 2002), and mEI agar plates were incubated at 41°C for 48 h (US EPA 1997). The OPSP agar plates were overlaid with 15 ml of molten OPSP agar before incubation. OPSP agar plates (for *C. perfringens*) were incubated anaerobically at 44°C for 24 h. A confirmatory test for *C. perfringens* was performed according to the method described previously (Wohlsen et al., 2006).

2.5 Specificity of the real-time PCR primers

Previously published primers were used in this study. The primer sequence and annealing temperature for corresponding targets are shown in Table 1. Primer specificity was checked by searching for similar sequences in microbial genomes using the Basic Local Alignment Search Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/BLAST/).

2.6 DNA and RNA extraction

Anaerobic bacterial DNA (i.e., HS-HF183) was extracted for each dilution according to the previously published method (Bernhard and Field, 2000). In brief, 125 ml of each sewage spiked water sample was filtered through 0.45 µm pore size membranes (Advantec, Tokyo, Japan). The filters were transferred in sterile 2-ml tubes containing 500 µl of guanidine isothiocyanate (GITC) buffer [5 mol l^{-1}GITC, 100 mmol l^{-1} EDTA (pH 8.0), 0.5% Sarkosyl] and stored overnight at -80°C. DNA was extracted using DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA). Extracted DNA were resuspended in 200 µl AE buffer, and stored at -80°C until processed.

For the HS-*esp* marker, 125 ml of each sewage spiked water sample was filtered through 0.45-µm pore size membranes (Advantec). The membranes were then transferred to mEI agar (Difco, Detroit, MI) and incubated at 41°C for 48 h. After incubation, the filter papers were

suspended in tryptic soy broth (TSB) (Oxoid, London, UK) and incubated at 41°C for 3 h for enrichment (Scott et al., 2005). DNA was extracted from 2 ml of enriched culture by using QIAamp stool DNA kit (Qiagen) according to the manufacturer's instructions. Extracted DNA were resuspended in 200 µl AE buffer, and stored at -80°C until processed.

For viral markers (HS-AVs and HS-PVs) and enteric viruses (i.e., EVs, SVs and TVs), each dilution was concentrated by using the previously published method (Haramoto et al., 2005). Briefly 125 ml of each sewage spiked water sample was supplemented with 2.5 mM $MgCl_2$ and then passed through an 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 (pH 3) was passed through the filter to remove magnesium ions and other electropositive substances, followed by filtration of 10-ml of 1 mM NaOH (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 (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 (10 ml) and further centrifugation at 1000 g for 10 min to obtain a final volume of 700-µl. Concentrates were split in half (2 X 350 µl), and stored at –80°C. Viral DNA and RNA were extracted from each concentrated samples using DNeasy blood and tissue kit and QIAamp viral RNA mini kit (Qiagen) according to the manufacture's instructions. For viral DNA (HS-AVs, HS-PVs, TVs) and RNA (EVs and SVs) extraction respectively, 200 µl and 140 µl of concentrated samples were used. Extracted viral DNA were resuspended in 200 µl AE buffer, and extracted viral RNA were resuspended in 60 µl AVE buffer, respectively, and stored at -80°C until processed.

2.7 Real-time PCR positive controls

The HS-HF183, HS-AVs, HS-PVs, EVs, SVs and TVs positive controls were isolated from sewage. In brief, the real-time PCR-amplified product was purified using a QIAquick PCR purification kit (Qiagen) and cloned into a pGEM-T Easy vector system (Promega, Madison, WI), transferred into *E. coli* JM109-competent cells, and plated on LB agar plates containing ampicillin, isopropyl-β-D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, as recommended by the manufacturer. Recombinant plasmids with

corresponding inserts were purified using a plasmid mini kit (Qiagen). DNA sequencing was carried out at the Australian Genome Research Facility (St. Lucia, Queensland, Australia). For the HS-esp marker, genomic DNA was extracted from a positive control (*E. faecium* C68 strain, provided by Dr. Louis B. Rice of the Louis Stokes Cleveland veterans Affairs Medical Centre in Cleveland, OH, USA).

2.8 Real-time PCR analysis for bacteria, DNA and RNA viruses

The real-time PCRs were 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 HS-HF183 and the HS-*esp* real-time PCR mixture contained 12.5 µl of SuperMix, 300 nM of each primer, and 5 µl of DNA extract. For both HS-HF183 and the HS-*esp* markers, the real-time PCR consisted of 2 min at 50°C, 15 min at 95°C followed by 45 cycles of 30 s at 94°C, 60 s at 59°C and 60 s at 72°C, and a final extension of 5 min at 72°C.

For the HS-AVs detection a nested real-time PCR protocol was used (Fong et al., 2005). The first round of real-time PCR contained 12.5 µl of SuperMix, 300 nM of each primer, and 5 µl of DNA extract. The first round of real-time PCR consisted of 2 min at 50°C, 4 min at 94°C followed by 45 cycles of 30 s at 92°C, 30 s at 60°C and 60 s at 72°C, and a final extension of 5 min at 72°C. The second round of real-time PCR was run under the same reaction conditions and cycling parameters described above, with 3 µl of product from the first round as the template.

A nested real-time PCR protocol was also used for HS-PVs detection. The first round of real-time PCR contained 12.5 µl of SuperMix, 200 nM of each primer, and 5 µl of DNA extract. The first round of real-time PCR consisted of 2 min at 50°C, 2 min at 94°C followed by 50 cycles of 20 s at 94°C, 20 s at 55°C and 20 s at 72°C, and a final extension of 2 min at 72°C. The second round of real-time PCR was run under the same reaction conditions and cycling parameters described above, with 3 µl of product from the first round as the template. TVs real-time PCR contained 12.5 µl of SuperMix, 400 nM of each primer, and 5 µl of template DNA. The real-time PCR consisted of 2 min at 50°C, 10 min at 95°C followed by 45 cycles of 10 s at 95°C, 30 s at 62°C.

For the detection of EVs and SVs, eight microliters of extracted RNA was added to a reaction mixture containing 1 µl of annealing buffer and 1 µl of 50 ng/µl random hexamers and was incubated at 65°C for 5 min, then quenched at 4°C for 60 s. Subsequently, 10 µl of 2 X reaction mix, 2µl SuperScript III enzyme mix (Invitrogen), then were heated at 25°C for 10 min then 50°C for 50 min. The reaction was terminated with a 85°C incubation for 5 minutes. EVs real-time PCR contained 12.5 µl of SuperMix, 400 nM of each primer, and 2 µl of cDNA. The real-time PCR consisted of 2 min at 50°C, 10 min at 95°C followed by 50 cycles of for 15 s at 95°C, 60 s at 60°C. SVs real-time PCR contained 12.5 µl of SuperMix, 400 nM of each primer, and 5 µl of cDNA. The real-time PCR consisted of 2 min at 50°C, 10 min at 95°C followed by 50 cycles of 15 s at 95°C, 60 s at 56°C.

2.9 Real-time PCR quality control

For each real-time PCR experiment, corresponding positive (i.e., target DNA) and negative (sterile water) controls were included. Each DNA sample was tested in triplicate to obtain positive/negative results. To separate the specific product from non-specific products (if any), DNA melting curve analysis was performed for each real-time PCR experiment. During melting curve analysis, the temperature was increased from 53 to 95°C at approximately 2°C/min. Samples were considered 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. To minimise real-time PCR contamination, DNA extraction, the real-time PCR set up, and gel electrophoresis were performed in separate laboratories. To prevent false positive results for water samples, a method blank was included.

2.10 Real-time PCR detection limit

To determine the real-time PCR detection limits, genomic DNA and plasmid DNA were quantified using a spectrophotometer. Tenfold serial dilutions (i.e., 10^3 -10^0) were made using buffer AE (Qiagen) for each target and tested with the real-time PCR. The lowest concentration of gene copies detected consistently in replicate assays was considered as the real-time PCR detection limit.

3. Results

3.1 Specificity of the Real-time PCR primers

Primer specificity was determined by searching for similar sequences in microbial genomes using the Basic Local Alignment Search Tool (BLAST) program

(http://www.ncbi.nlm.nih.gov/BLAST/). No homology was observed with known gene sequences of other pathogenic microorganisms commonly found in environmental waters.

3.2 Real-time PCR inhibitors

To detect the presence of inhibitors, DNA from animal fecal and STP samples were spiked with 10^3 gene copies of the HS-HF183 and CS-CF128 markers. The real-time PCR C_T values were compared to those obtained from the same concentrations of DNA that was used to spike 1 L of distilled water. For the spiked distilled water, the mean C_T values for the HS-HF183, HS-esp, HS-AVs and HS-PVs are shown in Table 2.

For STP DNA samples (extracted for the HS-HF183 specificity and sensitivity assay), the mean C_T values were 28 ± 0.5 when undiluted DNA was spiked with cattle-specific CF128 markers. For ten-fold and 100-fold dilutions of DNA, these values were 23 ± 0.7, and 23 ± 0.5, respectively. Similarly, for animal fecal DNA samples (extracted for the HS-HF183 specificity and sensitivity assay), the mean C_T values were 26 ±1.1 when undiluted DNA was spiked with the HS-HF183 markers. For ten-fold and 100-fold dilutions of DNA, these values were 22 ± 0.5, and 22 ± 0.8, respectively. One-way ANOVA was performed to determine the differences between the C_T values obtained for distilled water and those obtained for animal fecal DNA samples extracted for the HS-HF183 assay. Significant (P <0.001) differences were observed between the C_T values for spiked distilled water and undiluted DNA from animal fecal samples, indicating that the undiluted DNA extracted from animal fecal 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 DNA (i.e., ten-fold and 100-fold) indicating that ten fold dilution of DNA is required to remove the effects of real-time PCR inhibitory substances from animal fecal samples. For STP and animal fecal DNA samples (extracted for the HS-*esp*, HS-AVs and HS-PVs specificity and sensitivity assays), the mean C_T values for undiluted DNA were similar to the values obtained for distilled water indicating the samples were inhibitors free.

3.3 Host-specificity and sensitivity of human specific molecular markers

Host-specificity is the probability to detect when a source is not present and sensitivity is the probability to detect a source when it is present (Stoeckel and Harwood, 2007). The HS-HF183 marker was detected in all sewage samples and was not detected in 50 animal fecal samples (Table 3). Only one fecal sample from a dog was found to be positive. However, the

band was very faint on the gel analysis. The overall host-specificity of the HS-HF183 marker to differentiate between humans and animals was 98%, and the overall sensitivity of these markers in sewage samples was 100%. Similarly, the HS-*esp* marker was detected in all STP samples, and was not detected in any animal fecal sample. The overall host-specificity and sensitivity of the HS-*esp* markers was 100%. The HS-AVs were detected in 31 out of 32 STP samples, and HS-PVs were detected in all STP samples. However, these markers could not be detected in any of the 50 animal fecal samples tested. The overall specificity of both HS-AVs and PVs was 100%. The overall sensitivity of the HS-AVs was 97%. This figure for HS-PVs was 100%.

3.4 Real-time PCR limit of detection

The real-time PCR limits of detection were performed by analysing purified plasmid/genomic DNA isolated from the bacterial and viral targets. To determine the reproducibility of the assay, several replicates (n = 10) were tested. The results of these assays are summarised in Table 4. The real-time PCR detection limits were as low as one gene copy per reaction for the HS-HF183 and HS-*esp* markers. For HS-AVs, HS-PVs, EVs, SVs and TVs, the detection limits were ten gene copies per reaction. Lower levels (i.e., one copy) were detected, but the results were not reproducible for all replicates.

3.5 Concentrations of fecal indicators in sewage spiked water samples

The concentration of *E. coli* at sewage spiked freshwater dilution of 10^{-1} (i.e., contains 6.25 ml of sewage) was $4.3 \pm 0.3 \times 10^5$ colony forming units (CFUs) (Table 5). For enterococci and *C. perfringens* spores these figures were $9.6 \pm 0.2 \times 10^4$ and $5.0 \pm 1.0 \times 10^2$, respectively. Culturable *E. coli* were detected at dilution up to 10^{-6} and the concentration of *E. coli* at this dilution was $3.0 \pm 2.0 \times 10^0$ CFUs. Enterococci and *C. perfringens* spores were detected at dilutions of up to 10^{-5} and 10^{-3} respectively, and the concentrations at these dilutions were $7.1 \pm 3.0 \times 10^0$ and $5.1 \pm 3.0 \times 10^0$. The concentration of *E. coli* was higher than enterococci and *C. perfringens* spores.

3.6 The real-time PCR minimum detection limit of molecular markers in sewage spiked water samples

For freshwater, the real-time PCR minimum detection limit assay resulted in the detection of the HS-HF183 marker up to dilution 10^{-8}. At this dilution, no culturable *E. coli*, enterococci and *C. perfringens* spores were found. The HS-*esp* marker was detected up to dilution 10^{-4}

and the concentration of *E. coli* and enterococci at this dilution were $3.9 \pm 0.8 \times 10^2$ and $8.1 \pm 6.0 \times 10^1$ CFUs. However, no *C. perfringens* spores were found at this dilution. The HS-AVs and HS-PVs were detected up to dilution 10^{-4} by nested real-time PCR protocol. Real-time PCR minimum detection limit of enteric viruses was similar to HS-*esp*, HS-AVs and HS-PVs. However, EVs were detected up to dilution 10^{-5} and the concentrations of *E. coli* and enterococci at this dilution was only $4.0 \pm 1.0 \times 10^1$ and $7.1 \pm 6.0 \times 10^1$ CFUs. The real-time PCR minimum detection limit of human-specific markers and enteric viruses for seawater and distilled water were similar to that of freshwater (see Table 5).

4. Discussion

The host-specificity and sensitivity of the human-specific markers were assessed by testing fecal DNA samples from target (i.e., sewage samples) and non-target (i.e., animal fecal samples) sources. In our previous studies, we evaluated the host-specificity and sensitivity of the HS-HF183 and HS-*esp* markers by testing more than 150 animal fecal samples. Both the markers showed 100% specificity to sewage samples (Ahmed et al., 2008a; Ahmed et al., 2008b). Despite this fact, the host-specificity and sensitivity of the HS-HF183 and HS-*esp* markers were further evaluated along with the viral markers (i.e., HS-AVs and HS-PVs) to confirm the high host-specificity of these markers . The HS-HF183 marker was detected in one animal fecal sample out of 50 samples tested. Previous studies also reported the presence of the host-specific *Bacteroides* markers in a small number of non target samples (Ahmed et al., 2008a; Carson et al., 2005; Gawler et al., 2007; Gourmelon et al., 2007). It has been reported that horizontal transfer of fecal bacteria may occur among species in close contact such as humans and their pets (Dick et al., 2005). A recent review recommended testing the specificity of the host-specific *Bacteroides* markers prior to its application for MST field studies (Field and Samadpour, 2007).

The HS-*esp* markers were not detected in any animal fecal samples further confirming the high specificity of this marker to distinguish between human and animal sources of fecal pollution. A recent study reported the presence of HS-*esp* markers in dog and gull fecal samples in the USA (Whitman et al., 2007). This could be explained by the fact that horizontal transfer of genes or cells is possible between humans and animals (van den Boggard et al., 2002; Oancea et al., 2004). The HS-AVs and HS-PVs markers were not detected in any fecal or wastewater samples from the animals tested. The specificity of these

markers has not been tested outside the USA and Spain where human-specific adenoviruses were shown to be highly sewage specific (Maluquer de Motes et al., 2004).

The HS-PVs can be shed in urine from a healthy individual and highly prevalent in sewage (McQuaig et al., 2006). However, limited data is available on the host-specificity of HS-PVs. In view of this, wastewater samples (i.e., mixture of urine and feces) were tested for host-specificity assay. To our knowledge, this is the first study that reports the high host-specificity of HS-AVs and HS-PVs in sewage in Australia. All the markers tested in this study were present in all target samples except one sample which was negative for the HS-AVs marker. A limitation of the HS-*esp*, and viral markers is that their concentrations may be low in target samples and may not be present in fecal samples from all individuals (Scott et al., 2005; Field and Samadpour, 2007). Due to low concentrations, bacterial enrichment (for HS-*esp*), and nested real-time PCR protocols (for HS-AVs and HS-PVs) needs to be performed for the detection of these markers.

PCR is highly prone to contamination and special care must be taken during PCR and post PCR analysis. To prevent sample and real-time PCR cross contamination, animal fecal samples were processed first, followed by sewage samples. The real-time PCR detection was chosen over conventional PCR to avoid gel analysis and to prevent any carry over amplification. This is particularly important when assessing host-specificity and sensitivity of molecular markers to prevent false positive and negative results. Environmental and fecal samples contain numerous organic and inorganic substances with the potential to inhibit PCRs (Wilson 1997). To check the effects of real-time PCR inhibitory substances in the real-time PCR detection of human-specific markers, animal fecal DNA samples were spiked with known gene copies of the HS-HF183 markers, and STP samples were spiked with known gene copies of the CS-CF128 markers (Bernhard and Field, 2000). The threshold cycle (C_t) values of these spiked DNA samples were compared to those of the DNA samples from distilled water spiked with the same concentration of the HS-HF183 and CS-CF128 markers. The results indicated that fecal DNA extracted for the HS-HF183 host-specificity and sensitivity assay contained PCR inhibitory substances. A 10-fold dilution of DNA was required to remove PCR inhibitory effects. The DNA extracted for the HS-*esp*, and viral markers assays tested were PCR inhibitor free. For viral DNA/RNA extraction, previously published method was used which involves concentrations and purification and desalting of

nucleic acid before extraction. Therefore, DNA/RNA extracted using this method should not contain any PCR inhibitory substances (Haramoto et al., 2005).

E. coli and enterococci were detected up to dilution 10^{-6} (contains 0.06 µl sewage) and 10^{-5} (contains 0.6 µl of sewage), respectively for freshwater, seawater, and distilled water samples spiked with sewage. However, HS-*esp*, HS-AVs, HS-PVs, and enteric viruses (i.e., SVs and TVs) were detected up to dilution 10^{-4} (contains 6.25 µl of sewage). EVs were detected up to dilution 10^{-5} due to the fact that this group of viruses consisted of polioviruses, coxsackieviruses and echoviruses, and therefore their concentrations are expected to be higher in sewage than other viruses tested. *C. perfringens* spores were detected up to dilution 10^{-3} (contains 62.5 µl of sewage). The low concentration of this indicator suggests that it may not be suitable to identify public health risks associated with sewage pollution in environmental waters.

The HS-HF183 markers were detected up to dilution 10^{-9} (contains 0.001 µl of sewage). However, at this dilution no culturable fecal indicators were detected. The number of HS-HF183F *Bacteroides* markers has been reported to be high, ranging from $2.0 \pm 0.2 \text{ X } 10^{8}$ to $3.1 \pm 0.3 \text{ X } 10^{9}$ per 100 ml raw sewage (Seurinck et al., 2005). Therefore, the real-time PCR minimum detection limit of these markers is expected to be high compared to the HS-*esp* and viral markers, as well as traditional fecal indicators. The HS-HF183 marker was detected in dilutions 3 to 4 orders of magnitude higher than those of enteric viruses implying that the concentration of this marker is much higher than enteric viruses in fresh sewage samples. From a public health point of view, such data needs to be interpreted with caution as a positive signal of the HS-HF183 marker in environmental waters may not always indicate the presence of enteric viruses or other pathogens. However, quantitative PCR could be used to quantify the HS-HF183 and enteric viruses in sewage samples to establish a relation between the concentration of the HS-HF183 and the occurrence of enteric viruses. For environmental samples, the HS-HF183 and HS-*esp* real-time PCR negative results do not rule out the presence of potential enteric pathogens and especially viruses which could have a different survival rate than these anaerobic and toxin gene markers.

It has to be noted that in this study, different DNA extraction methods and real-time PCR cycling parameters were used for different human-specific markers and enteric viruses. Therefore, it is possible that the real-time PCR may have underestimated the minimum limit

of detection assay for certain markers and/or enteric viruses. Nonetheless, the results at least indicate each published protocol's ability to detect fresh sewage in various matrices of water. The real-time PCR minimum detection limit of the HS-HF183, HS-*esp*, HS-AVs, HS-PVs and enteric viruses for different water matrices were similar. No discrepancies were observed probably due to the fact that a fixed volume of fresh sewage was added into freshwater, seawater and distilled water matrices in the same manner and the spiked water samples were processed at the same time, and tested with the real-time PCR. The real-time PCR would detect target from both viable and non-viable cells. Therefore, the matrices did not have any effects on the real-time PCR minimum detection limit of the host-specific markers and enteric viruses.

Limited data is available on the correlation between human-specific markers (i.e., *Bacteroides* and toxin gene) and enteric pathogens in environmental waters. A recent study reported the positive correlation between the HS-HF183 markers and bacterial zoonotic pathogens in environmental waters in the USA (Walters et al., 2005). Quantitative data on these markers would be required to obtain information regarding the likelihood of the presence of enteric viruses or other pathogens in environmental waters. Recently, quantitative real-time PCR methods have been developed to quantify human-specific *Bacteroides* and toxin gene markers in sewage and environmental waters (Ahmed et al., 2008c; Layton et al., 2006; Reischer et al., 2006; Seurinck et al., 2005). Consequently, these methods have the potential to provide information regarding the concentration of these markers, and the occurrence of pathogens in sewage and environmental waters.

The HS-*esp*, HS-AVs, and HS-PVs markers were detected up to dilution 10^{-4}, and the concentrations of *E. coli* and enterococci at this dilution were $3.9 \pm 0.8 \times 10^{2}$ and $8.1 \pm 6.0 \times 10^{1}$ CFUs respectively. It has to be noted that, at this dilution all the enteric viruses were also detected. The most important feature of the toxin gene and viral markers is that they provide direct information on pathogen status in environmental waters which could not be obtained using anaerobic markers or traditional fecal indicators. One potential drawback of toxin gene and viral markers is that their concentrations could be relatively low in environmental waters due to dilution and therefore, it may not be always possible to detect these markers. In this scenario, it is recommended that multiple markers (i.e., bacterial and viral) should be used in MST field studies to provide multiple lines of evidence of sewage pollution and public health risks.

5. Conclusions

- Human-specific bacterial and viral molecular markers tested in this study are highly sewage specific. To our knowledge this is the first study that evaluated the real-time PCR minimum detection limit of four human-specific markers to detect fresh sewage in freshwater, seawater and distilled water matrices.
- The minimum detection limit of the HS-HF183 marker was 3 to 4 orders of magnitude higher than that of the HS-*esp* and viral markers. Based on our data, the HS-HF183 marker appears to be the most sensitive to detect fresh sewage pollution. However, its presence in environmental waters may not necessarily indicate the presence of enteric viruses due to their high abundance in sewage compared to enteric viruses.
- Minimum detection limit of the human-specific markers and enteric viruses for freshwater, seawater and distilled water matrices were similar suggesting that the matrices did not have any effects on the real-time PCR minimum detection limit of fresh sewage.
- More research is required on the persistency of these markers in environmental water samples in relation to traditional fecal indicators and pathogenic microorganisms. In addition, quantitative real-time PCR data would be required to assess the magnitude of fecal pollution and associated public health risks. Our future research would focus on evaluating the survival of these markers in various environmental waters along with the traditional fecal indicators and pathogens.

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Table 1 – Primer sequence used for the detection of host-specific molecular markers and enteric viruses

Targets	Primer sequence (5´- 3´)	Amplicon size (bp)	Reference
Human-specific *Bacteroides* HF183 (HS-HF183)	F – ATC ATG AGT TCA CAT GTC CG	520	Bernhard and Field, 2000
	R – GCC GTC TACT CT TGG CC		
Human-specific enterococci surface protein (*esp*) marker	F – TAT GAA AGC AAC AGC ACA AGT T	680	Scott et al., 2005
(HS-*esp*)	R – ACG TCG AAA GTT CGA TTT CC		
Human-specific adenoviruses (HS-AVs)	F – GCC GCA GTG GTC TTA CAT GCA CAT C	300	Fong et al., 2005a
	R – CAG CAC GCC GCG GAT GTC AAA GT		
	F – GCC ACC GAG ACG TAC TTC AGC CTG	143	
	R – TTG TAC GAG TAC GCG GTA TCC TCG CGG TC		
Human-specific polyomaviruses (HS-PVs)	F – AGT CTT TAG GGT CTT CTA CC	172	McQuaig et al., 2006
	R – GGT GCC AAC CTA TGG AAC AG		
Torquetenovirues (TVs)	F – CGG GTG CCG DAG GTG AGT TTA CAC	79	Haramoto et al., 2005
	R – GAG CCT TGC CCA TRG CCC GGC CAG		
Enteroviruses (EVs)	F – CCT CCG GCC CCT GAA TG	196	Haramoto et al., 2005
	R – ACC GGA TGG CCA ATC CAA		
Sapovirus (SVs)	F1 – CCA GGC TCT CGC CAC CTAC	196	Haramoto et al., 2008
	F2 – CCA GGC TCT CGC TAC CTAC		
	F3 – TTT GGC CCT CGC CAC CTAC		
	R1 – GCC CTC CAT CTC AAA CAC TAT TTTG		
	R2 – GCC CTC CAT TTC AAA CAC TAA TTTG		

Table 2 - Effects of PCR inhibitors on the real-time PCR detection of spiked human-specific HS-HF183 and CS-CF128 markers in animal fecal and sewage 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
HS-HF183 host-specificity assay			
Distilled water	23 ± 0.3	-	-
STP (primary influent)	28 ± 0.5	23 ± 0.7	23 ± 0.5
Distilled water	21 ± 0.3	-	-
Cattle	25 ± 0.1	21 ± 1.2	21 ± 0.7
Pig	27 ± 0.7	22 ± 0.1	23 ± 1.2
Sheep	26 ± 0.1	22 ± 0.4	22 ± 0.9
Dog	24 ± 2.0	22 ± 0.3	22 ± 0.2
Duck	26 ± 0.4	21 ± 7.0	21 ± 1.0
HS-*esp* host-specificity assay			
Distilled water	23 ± 0.7	-	-
STP (primary influent)	24 ± 0.2	23 ± 0.9	-
Distilled water	22 ± 0.6	-	-
Cattle	23 ± 0.4	22 ± 0.9	-
Pig	23 ± 0.9	23 ± 0.9	-
Sheep	22 ± 1.2	22 ± 0.8	-
Dog	23 ± 0.6	23 ± 0.9	-
Duck	24 ± 0.2	23 ± 0.7	-
HS-AVs and HS-PVs host-specificity assays			
Distilled water	26 ± 0.3	-	-
STP (primary influent)	26 ± 0.9	26 ± 0.6	-
Distilled water	25 ± 0.4	-	-
Cattle	26 ± 0.7	26 ± 0.4	-
Pigs	26 ± 0.3	25 ± 0.8	-
Sheep	25 ± 0.9	25 ± 0.3	-
Dogs	27 ± 0.3	26 ± 0.7	-
Ducks	26 ± 0.6	25 ± 0.2	-

Table 3 – Polymerase chain reaction positive results for human-specific *Bacteroides* HF183 (HS-HF183), human-specific *Enterococci faecium* enterococci surface protein markers (HS-*esp*), human-specific adenoviruses (HS-AVs) and human-specific polyomaviruses (HS-PVs) in host groups in Southeast Queensland, Australia.

Host groups	No. of samples tested/real-time positive results			
	HS-HF183	HS-*esp*	HS-AVs	HS-PVs
STP (primary influent)	32/32	32/32	32/31	32/32
Cattle	10/0	10/0	10/0	10/0 [a]
Pigs	10/0	10/0	10/0	10/0 [a]
Sheep	10/0	10/0	10/0	10/0 [a]
Dogs	10/1	10/0	10/0	10/0
Ducks	10/0	10/0	10/0	10/0

[a] composite wastewater samples

Table 4 – Real-time PCR limit of detection (LOD) for host-specific molecular markers and enteric viruses.

Targets	No. of gene copies tested	No. of replicates	No. of positive (%)
Human-specific *Bacteroides* HF183 (HS-HF183) [a]	$10^3 - 10^2 - 10^1 - 10^0$	10	10 (100%) – 10 (100%) – 10 (100%) – 10 (100%)
Human-specific enterococci surface protein (*esp*) marker (HS-*esp*)	$10^3 - 10^2 - 10^1 - 10^0$	10	10 (100%) – 10 (100%) – 10(100%) – 9 (90%)
Human-specific adenoviruses (HS-AVs) [a]	$10^3 - 10^2 - 10^1 - 10^0$	10	10 (100%) – 10 (100%) – 10(100%) – 7 (70%)
Human-specific polyomaviruses (HS-PVs) [a]	$10^3 - 10^2 - 10^1 - 10^0$	10	10 (100%) – 10 (100%) – 10(100%) – 3 (30%)
Torquetenovirues (TVs) [a]	$10^3 - 10^2 - 10^1 - 10^0$	10	10 (100%) – 10 (100%) – 10(100%) – 5 (50%)
Enteroviruses (EVs) [a]	$10^3 - 10^2 - 10^1 - 10^0$	10	10 (100%) – 10 (100%) – 10(100%) – 1 (10%)
Sapovirus (SVs) [a]	$10^3 - 10^2 - 10^1 - 10^0$	10	10 (100%) – 10 (100%) – 10(100%) – 3 (30%)

[a]: Plasmid DNA

Table 5 – Concentrations of fecal indicators and occurrence of host-specific molecular markers and enteric viruses in sewage spiked freshwater, seawater and distilled water samples

Source water	Dilution	Concentrations of fecal indicators			No. of samples tested/real-time PCR positive results						
		E. coli	Enterococci	*C. perfringens*	HS-HF183	HS-*esp*	HS-AVs	HS-PVs	TVs	EVs	SVs
Freshwater	10^{-1}	$4.3 \pm 0.3 \times 10^{5}$	$9.6 \pm 0.2 \times 10^{4}$	$5.0 \pm 1.0 \times 10^{2}$	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	10^{-2}	$4.7 \pm 0.4 \times 10^{4}$	$8.3 \pm 0.6 \times 10^{3}$	$4.8 \pm 2.0 \times 10^{1}$	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	10^{-3}	$4.1 \pm 0.6 \times 10^{3}$	$9.3 \pm 1.0 \times 10^{2}$	$5.1 \pm 3.0 \times 10^{0}$	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	10^{-4}	$3.9 \pm 0.8 \times 10^{2}$	$8.1 \pm 6.0 \times 10^{1}$	ND	3/3	3/3	3/3	3/1	3/3	3/3	3/3
	10^{-5}	$4.0 \pm 1.0 \times 10^{1}$	$7.1 \pm 3.0 \times 10^{0}$	ND	3/3	ND	ND	ND	ND	3/2	ND
	10^{-6}	$3.0 \pm 2.0 \times 10^{0}$	ND	ND	3/3	ND	ND	ND	ND	ND	ND
	10^{-7}	ND	ND	ND	3/3	ND	ND	ND	ND	ND	ND
	10^{-8}	ND	ND	ND	3/2	ND	ND	ND	ND	ND	ND
	10^{-9}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	10^{-10}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Seawater	10^{-1}	$4.1 \pm 0.1 \times 10^{5}$	$9.8 \pm 0.4 \times 10^{4}$	$5.2 \pm 2.0 \times 10^{2}$	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	10^{-2}	$4.3 \pm 0.5 \times 10^{4}$	$9.1 \pm 0.3 \times 10^{3}$	$4.6 \pm 1.0 \times 10^{1}$	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	10^{-3}	$4.0 \pm 0.8 \times 10^{3}$	$9.4 \pm 2.0 \times 10^{2}$	$8.1 \pm 6.0 \times 10^{0}$	3/3	3/3	3/3	3/2	3/3	3/3	3/3
	10^{-4}	$3.6 \pm 0.7 \times 10^{2}$	$7.1 \pm 9.0 \times 10^{1}$	ND	3/3	3/3	3/2	3/1	3/3	3/3	3/3
	10^{-5}	$3.8 \pm 0.9 \times 10^{1}$	$6.1 \pm 6.0 \times 10^{0}$	ND	3/3	ND	ND	ND	ND	ND	ND
	10^{-6}	$7.0 \pm 3.0 \times 10^{0}$	ND	ND	3/3	ND	ND	ND	ND	ND	ND
	10^{-7}	ND	ND	ND	3/3	ND	ND	ND	ND	ND	ND
	10^{-8}	ND	ND	ND	3/1	ND	ND	ND	ND	ND	ND
	10^{-9}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	10^{-10}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Distilled water	10^{-1}	$4.5 \pm 0.2 \times 10^{5}$	$9.9 \pm 0.2 \times 10^{4}$	$4.9 \pm 0.8 \times 10^{2}$	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	10^{-2}	$4.5 \pm 0.3 \times 10^{4}$	$8.8 \pm 0.6 \times 10^{3}$	$5.0 \pm 1.0 \times 10^{1}$	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	10^{-2}	$4.3 \pm 0.2 \times 10^{3}$	$9.1 \pm 3.0 \times 10^{2}$	$9.0 \pm 4.0 \times 10^{0}$	3/3	3/3	3/2	3/3	3/3	3/3	3/3

10^{-4}	$4.1 \pm 0.5 \times 10^{2}$	$8.9 \pm 7.0 \times 10^{1}$	ND	3/3	3/3	3/1	3/2	3/3	3/3	3/3
10^{-5}	$3.6 \pm 0.7 \times 10^{1}$	$8.1 \pm 4.0 \times 10^{0}$	ND	3/3	ND	ND	ND	ND	ND	ND
10^{-6}	$6.0 \pm 4.0 \times 10^{0}$	ND	ND	3/3	ND	ND	ND	ND	ND	ND
10^{-7}	ND	ND	ND	3/3	ND	ND	ND	ND	ND	ND
10^{-8}	ND	ND	ND	3/3	ND	ND	ND	ND	ND	ND
10^{-9}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
10^{-10}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND