ORIGINAL ARTICLE

Evaluation of *Bacteroides* markers for the detection of human faecal pollution

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Abstract

Aims: This paper reports on the results of a study aimed at evaluating the specificity and sensitivity of human-specific HF183 and HF134 *Bacteroides* markers in various host groups and their utility to detect human faecal pollution in storm water samples collected from nonsewered catchments in Southeast Queensland, Australia.

Methods and Results: The specificity and sensitivity of the HF183 and HF134 *Bacteroides* markers was evaluated by testing 207 faecal samples from 13 host groups, including 52 samples from human sources (via sewage and septic tanks). Polymerase chain reaction analysis of these samples revealed the presence/absence of HF183 and HF134 across these host groups, demonstrating their suitability for distinguishing between human and animal faecal pollution. The HF183 marker was found to be more reliable than that of HF134, which was also found in dogs.

Conclusions: Based on our data, it appears that the HF183 marker is specific to sewage and is a reliable marker for detecting human faecal pollution, while the use of HF134 marker alone may not be sufficient enough to provide the evidence of human faecal pollution.

Significance and Impact of the Study: This is the first study in Australia that rigorously evaluated the specificity and sensitivity of *Bacteroides* markers. Based on our findings, we suggest that the HF183 marker could reliably be used to detect the sources of human faecal pollution in Southeast Queensland region.

Introduction

The microbiological quality of water is generally assessed by monitoring faecal indicator bacteria (*Escherichia coli*, enterococci and *Clostridium perfringens*). However, it has to be noted that the sole presence of these bacteria in surface waters does not provide definitive information regarding their possible sources. Recently, microbial source tracking (MST) methods have been developed to identify, and in some cases, quantify the sources of faecal pollution in environmental waters so that it can be corrected. Many commonly used methods require the development of a library of *E. coli* or enterococci from known host groups based on their genotypic or phenotypic attributes. The library is then compared with unknown environmental isolates to identify their most likely sources. The library size is one of the most crucial factors and it is yet not known what constitutes a representative library (Stoeckel *et al.* 2004). In addition, *Escherichia coli* and enterococci appear not to be host-specific for MST studies. For example, the genetic diversity in *E. coli* measured by multi-locus enzyme electrophoresis revealed limited host-specificity (Gordon *et al.* 2002).

It has been proposed that the members of the *Bacteroides* genus hold promise as alternative indicators of faecal pollution (Kreader 1995) owing to a number of advantages, including short survival rates outside the hosts, exclusivity to the gut of warm-blooded animals and constituents of a larger portion of faecal bacteria compared with faecal coliforms or enterococci (Sghir *et al.*

2000). The use of these organisms for routine monitoring is limited because of difficulty of cultivating; however, the recent advances in PCR technology results in rapid detection and identification of these anaerobes (Field and Samadpour 2007).

It has been reported that some species in the genus *Bacteroides* could be host-specific (Allsop and Stickler 1985). A recent study reported the identification of human- and bovine-specific *Bacteroides-Prevotella* 16S rRNA gene markers by using length heterogeneity (LH) PCR and terminal restriction fragment length polymorphism (T-RFLP), and concluded that these markers could be used to detect human or bovine origin faecal pollution (Bernhard and Field 2000). This method is rapid, sensitive and has been shown to be accurate when evaluated against blind test samples in a method comparison study (Griffith *et al.* 2003). Because of these, PCR detection of *Bacteroides* markers has emerged as a potential tool for MST field studies in the USA (Bernhard *et al.* 2003), France (Gourmelon *et al.* 2007), United Kingdom, Portugal, Ireland (Gawler *et al.* 2007), Belgium (Seurinck *et al.* 2006) and Japan (Okabe *et al.* 2006).

The primary objective of this study was to evaluate the specificity and sensitivity of human-specific HF183 and HF134 *Bacteroides* markers to detect human faecal pollution by testing a large number of faecal samples from 13 host groups [including humans *via* septic tanks and sewage treatment plants (STP)] in Southeast Queensland, Australia. In addition to the testing of specific host groups, stormwater samples from three nonsewered catchments were also tested for the PCR markers. The results of the specificity and sensitivity tests along with the positive/negative PCR results of environmental water samples were then used to support the presence of human-sourced faecal matters during storm events.

Materials and Methods

Host-group sampling

Altogether, 207 faecal samples were collected from 13 host groups (Table 1). Human faecal samples were collected *via* three STP ($n = 40$) and septic systems ($n = 12$), whereas animal faecal samples were collected from 12 animal species ($n = 155$) using aseptic technique. All samples were transported to the laboratory, stored at 4°C and processed within 6–8 h.

Enumeration of faecal indicators

The membrane filtration method was used to process sewage, septic and water samples for bacterial enumeration. The isolation, identification and confirmation of *E. coli* and enterococci were performed according to the methods described elsewhere (Ahmed *et al.* 2006).

Table 1 Polymerase chain reaction-positive/negative results for human-specific *Bacteroides* HF183 and HF134 markers in host groups in Southeast Queensland, Australia

Host groups	No. samples	Human-specific *Bacteroides* HF183 marker		Human-specific *Bacteroides* HF134 marker	
		Positive	Negative	Positive	Negative
Humans					
Septic systems	12	12	0	12	0
STP (primary influent)	15	15	0	15	0
STP (secondary effluent)	15	15	0	15	0
STP (treated effluent)	10	10	0	9	1
Total sewage samples	52	52	0	51	1
Animals					
Ducks	15	0	15	0	15
Kangaroos	15	0	15	0	15
Cattle	20	0	20	0	20
Horses	14	0	14	0	14
Dogs	20	0	20	7	13
Chickens	15	0	15	0	15
Pigs	6	0	6	0	6
Pelican	10	0	10	0	10
Goat	10	0	10	0	10
Deer	10	0	10	0	10
Wild birds	10	0	10	0	10
Sheep	10	0	10	0	10
Total animal samples	155	0	155	7	148

STP, sewage treatment plants.

DNA extraction

DNA was extracted from sewage and septic samples (i.e. 100 ml) and fresh faeces (i.e. 200 mg) from each individual animal by using QIAmp stool DNA kit (Qiagen, Valencia, CA, USA). Water samples were processed according to published methods (Bernhard and Field 2000). Briefly, 300 ml of water samples were filtered through 0·45 μm pore size membranes (Advantec, Tokyo, Japan). The filters were transferred in sterile 1·5-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 by using QIAamp stool DNA kit (Qiagen).

PCR analysis

Human-specific *Bacteroides* markers were amplified with forward primers (5′-ATC ATG AGT TCA CAT GTC CG-3′) for HF183 and (5′-GCC GTC TAC TCT TGG CC-3′) for HF134 coupled with the Bac708 reverse primer (5′-CAA TCG GAG TTC TTC GTG-3′) (Bernhard and Field

2000). For both markers, PCR was carried out in a volume of 50-μl reaction mixture containing 45-μl platinum blue supermix (Invitrogen, Carlsbad, CA, USA), 0·3 μmol l^{-1} of each primer and 2 μl of template DNA. PCR was optimized and performed using a mastercycler gradient (Eppendorf, Hamburg, Germany). For both markers, the cycling parameters were 15 min at 95°C for initial denaturation and 30 cycles of 94°C for 30 s, 59°C for 1 min for annealing and 72°C for 10 min. To detect amplified products, 5 μl aliquot of the PCR product was visualized by electrophoresis through 2% E-Gel® (Invitrogen) and exposure to UV light. The positive control (i.e. DNA from sewage samples), specific to each marker and negative control (no DNA), were included in each assay. Samples were recorded as positive when the visible band was the same size as the positive control (i.e. 520 bp for HF183 and 570 bp for HF134).

Limit of detection (LOD) and environmental samples

To determine the LOD of the PCR assay for freshwater samples, three raw sewage samples were collected and the numbers of *E. coli* and enterococci were enumerated. Sewage samples were suspended in freshwater samples ($n = 3$) collected from a freshwater lake to a final concentration of 100 ml l^{-1} of water. The serial dilution (10^{-1}–10^{-9}) was made for each sample and the numbers of *E. coli* and enterococci were enumerated for each dilution. DNA extraction was performed for each dilution and tested for PCR as described earlier. Total culturable *E. coli* and enterococci colonies were compared with the positive and negative results of PCR to estimate the minimum colony forming units (CFU) and the amount of sewage that must be present in a sample for the detection of these markers.

Environmental samples were collected from three non-sewered catchments – Bergin Creek, Four Mile Creek and River Oaks Creek in Pine Rivers Shire, Queensland. The catchments were selected *via* geographical information system (GIS) analysis on the basis of high density of septic systems in close proximity to drainage lines (Stewart *et al.* 2006). A total of 10 stormwater samples were collected between November 2006 and March 2007 representing three storm events per catchment. The samples were collected in sterilized 2·5-l containers and transported to the laboratory on ice and processed within 6–8 h.

DNA cloning and sequencing

To verify the identity of the PCR product obtained using human-specific HF183 and HF134 *Bacteroides* primers, the PCR-amplified sequences from the HF183 and HF134 primer sets were purified using the QIAquick PCR purification kit (Qiagen), and cloned, in duplicate, into the pGEM®-T Easy Vector system (Promega, Madison, WI, USA) as recommended by the manufacturer. Plasmids were extracted using the QIAprep Spin® Miniprep kit (Qiagen). Bidirectional sequences were obtained using T7 and SP6 long sequencing primer targeting sites on either side of the insert. DNA sequencing was carried out at the Australian genome Research Facility (St Lucia, Queensland, Australia). The sequences were analysed using Bioware Jellyfish Software.

Data analysis

The sensitivity and specificity of these markers were determined as: sensitivity = $a/(a + c)$ and specificity = $d/(b + d)$, where '*a*' is true positive (samples were positive for the marker of its own species), '*b*' is false positive (samples positive for the PCR marker of another species), '*c*' is false negative (samples were negative for the marker of its own species), '*d*' is true negative (samples were negative for the PCR marker of another species) (Gawler *et al.* 2007).

Results

Sensitivity and specificity

The HF183 marker was detected in 52 (100%) sewage and septic samples and could not be detected in any of the 155 samples from the 12 animal species (Table 1). Similarly, HF134 was also detected in 51 (97·3%) of the septic and sewage samples (only one sample from treated effluent was negative) and was not detected in 148 (95·5%) of the animal faecal samples. The only animal host group that was found to contain this marker was dogs. In all, seven (35%) samples were positive for this marker. The overall sensitivity of the HF183 primer to detect human-specific HF183 marker in sewage and septic was 100%. For HF134, this figure was 97·3%. The overall specificity of these markers to differentiate between sewage and animals was 100% (HF183) and 95·5% (HF134), respectively. The PCR-amplified sequences of the human-specific HF183 and HF134 *Bacteroides* 16S rRNA genetic markers found in the sewage samples were verified as ≥98·7% identical to the marker sequences described by Bernhard and Field (2000).

LOD and sourcing human faecal pollution

The number of faecal indicators in 100-ml raw sewage samples ranged between $1{\cdot}2 \times 10^6$ and $5{\cdot}1 \times 10^6$ (for *E. coli*) and $4{\cdot}5 \times 10^5$ and $5{\cdot}6 \times 10^5$ (for enterococci). The LOD assay resulted in the detection of HF183 marker up

Table 2 The number of *Escherichia* coli and enterococci and polymerase chain reaction (PCR)-positive/negative results of human-specific *Bacteroides* markers in water samples collected from the Bergin Creek, Four Mile Creek and River Oaks Catchment in Southeast Queensland, Australia

		Number of indicators (CFU 100 ml^{-1})		PCR results (positive/negative)	
Catchments	Samples	*E. coli*	Enterococci	HF183	HF134
Bergin Creek Catchment	Event 1	$2{\cdot}6 \times 10^3$	$2{\cdot}7 \times 10^3$	+	+
	Event 1*	$3{\cdot}9 \times 10^3$	$4{\cdot}3 \times 10^3$	+	+
	Event 2	$4{\cdot}0 \times 10^3$	$3{\cdot}1 \times 10^3$	+	+
	Event 3	$4{\cdot}1 \times 10^3$	$3{\cdot}4 \times 10^3$	–	+
Four Mile Creek Catchment	Event 1	$1{\cdot}4 \times 10^3$	$1{\cdot}8 \times 10^3$	–	+
	Event 2	$9{\cdot}6 \times 10^3$	$8{\cdot}5 \times 10^3$	+	+
	Event 3	$2{\cdot}6 \times 10^3$	$2{\cdot}5 \times 10^3$	–	–
River Oaks Catchment	Event 1	$2{\cdot}7 \times 10^3$	$2{\cdot}4 \times 10^3$	–	+
	Event 2	$2{\cdot}1 \times 10^3$	$1{\cdot}8 \times 10^3$	–	–
	Event 3	$1{\cdot}6 \times 10^3$	$1{\cdot}4 \times 10^3$	–	–

*Rising stage sample.

to dilution 1×10^{-7} with the PCR for all three samples. Similarly, HF134 marker was detected up to dilution 1×10^{-7}, but for only one sample. However, at dilution 1×10^{-6}, all three samples were positive for the HF134 marker. At this dilution (i.e. 1×10^{-6}), no culturable *E. coli* and enterococci were found. The number of *E. coli* and enterococci in storm water samples colleted from the three catchments is shown in Table 2. Of the four samples tested from the Bergin Creek, three (including a rising stage sample) were positive for both the markers (events 1 and 2). Of the three samples tested from the Four Mile Creek, two were positive for either HF134 (events 1 and 2) or both the markers (event 2). However, none of these markers could be detected in sample from event 3. Only one sample (event 1) from the River Oaks was positive for the HF134 marker. Eight of the 10 samples tested were in agreement (i.e. either all detected or all nondetected). Two samples [Four Mile Creek (event 1) and River Oaks (event 1)] were only positive for the HF134 marker.

Discussion

Little is known regarding the geographical distribution and host-specificity of the human-specific *Bacteroides* markers (namely HF183 and HF134) in Australia. In this paper, the utility of these markers to detect human faecal pollution was rigorously evaluated by testing 13 host groups from Southeast Queensland. Both the markers were consistently detected in wastewater samples (≥98%), indicating high sensitivity of the primers to detect these markers in sewage and septic samples. None of the samples tested from animal species were positive for the HF183 marker indicating its high (100%) specificity. Although, the HF134 marker displayed high (overall 95·5%) specificity, this marker was detected in seven (35%) samples from dogs. The presence of this marker in dogs could be attributed to horizontal transfer of faecal bacteria between human and their companion pets (Dick *et al.* 2005).

The LOD of the PCR assay to detect these markers in freshwater samples was determined. It was possible to detect the HF183 marker up to dilution 1×10^{-7} for all three samples. This figure for the HF134 marker was up to dilution 1×10^{-6}. At such dilution, no culturable *E. coli* or enterococci were found, indicating the high sensitivity of the PCR assay to detect human faecal pollution. Both these markers were field tested to identify whether human faecal pollution is being transported to catchment outlets during storm events. The studied catchments are characterized by a high density of septic systems and a limited number of other nonpoint sources of faecal pollution (dogs, cattle, horses and wild birds). The concentrations of *E. coli* and enterococci in all storm water samples exceeded relevant water quality guidelines [Australian and New Zealand Environment and Conservations Council (ANZECC) 2000]. This is because, water samples were collected immediately after storm events when a large number of bacteria are generally washed into the creek *via* surface runoff. Both markers were detected in six environmental samples, indicating that human faecal pollution and septic systems were the most likely sources. Four samples were negative, although the number of faecal indicators was high in these samples. To obtain confirmatory results, the specificity of the PCR assay was increased by optimizing PCR conditions and we were still unable to amplify any products. Therefore, this finding suggests the presence of animal and/or diffuse sources of faecal pollution in these catchments. As only human-specific markers were used in this study, it was not possible to identify the other sources of faecal pollution.

Before field testing, the specificity of these markers must be tested in various host groups from the study catchment to eliminate false-positive results. However, in this study, it was not possible to test septic tanks and dogs found in the study catchments owing to access restriction. Instead, the samples from the host groups were collected from multiple catchments within this region. Based on the results, we recommend that the source prediction results using HF134 marker should be interpreted with care because of its presence in dogs. For example, the positive HF134 results for two samples from the Four Mile Creek and River Oaks Creek may have originated from dogs. Nonetheless, the remaining four samples were positive for both markers, indicating human

faecal pollution. One major limitation of the presence/absence of PCR is that it does not provide information regarding the magnitude of faecal pollution. However, real-time PCR methods have been developed and used to quantify the human-specific *Bacteroides* markers in environmental samples (Okabe *et al.* 2006; Seurinck *et al.* 2006). Based on our data, it can be concluded that the human-specific *Bacteroides* markers are a sensitive measure of human faecal pollution in environmental water samples and could be used for routine monitoring to identify human faecal pollution in Southeast Queensland, Australia. Such information would be important to water-quality managers, who are charged with protecting water quality.

To the best of our knowledge, this is the first study in Australia that rigorously evaluated the specificity and sensitivity of the human-specific *Bacteroides* markers. Based on our data, it appears that the HF183 marker is specific to human sewage and is a reliable marker for detecting human faecal pollution in Southeast Queensland, while the use of HF134 marker alone may not be sufficient enough to provide evidence of human faecal pollution. A major limitation of this method is that markers are not available for all host groups (Field and Samadpour 2007). In addition, the correlation between these markers and faecal indicators/pathogens is not well understood. Therefore, future research should extensively focus on investigating the relationship between host-specific markers, faecal indicators and pathogens. As part of the on-going research programme, a real-time PCR assay for human-specific *Bacteroides* markers and the validation of animal-specific markers to detect animal faecal pollution simultaneously is being undertaken.

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