Detection and source identification of faecal pollution in non-sewered catchment by means of host-specific molecular markers

W. Ahmed, D. Powell, A. Goonetilleke and T. Gardner

ABSTRACT

Multiple host-specific molecular markers were used to detect the sources of faecal pollution in a mixed land use non-sewered catchment in Southeast Queensland, Australia. These markers included human-specific *Bacteroides* (HF183 and HF134), cattle-specific *Bacteroides* (CF128), dog-specific *Bacteroides* (BacCan) and human-specific enterococci surface protein (*esp*) markers. The sensitivity and specificity of these markers were determined by testing 197 faecal samples from 13 host groups. The overall sensitivity and specificity of these markers was high (sensitivity $\geq$ 85% and specificity $\geq$ 93%) indicating their suitability for detecting the sources of faecal pollution. Of the 16 samples collected from the study area, 14 (87%) were positive for at least one of the molecular marker tested. Amongst all the markers, cattle-specific CF128 was more prevalent than others, followed by human-specific HF183 which was consistently detected in samples collected from sites within close proximity to urban development. Significant correlations were found between *E. coli* and enterococci concentrations with the positive/negative results of human-specific *Bacteroides* HF183 ($p < 0.001$, $p < 0.0001$) and HF134 ($p < 0.001$, $p < 0.004$) markers. No correlations were found between faecal indicators (*E. coli* or enterococci) with the CF128 or BacCan markers. A significant correlation was also found between enterococci concentrations and the presence/absence of the *esp* marker ($p < 0.02$). Based on the results, it appears that the host-specific markers such as HF183 and *esp* are a sensitive measure of sources of human faecal pollution in surface waters in Southeast Queensland, Australia.

Key words | enterococci, *Escherichia coli*, faecal pollution, host-specific markers, polymerase chain reaction

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INTRODUCTION

Faecal pollution from humans and animals is one of the leading causes of the degradation of surface water quality. Traditionally, faecal indicator bacteria (FIB) such as faecal coliforms, *Escherichia coli* and enterococci have been used as surrogates for pathogenic microorganisms. However, significant shortcomings in using traditional FIB have been reported in the literature. These include–1) their inability to differentiate between human and animal sources of faecal pollution, 2) ability to grow, survive and establish populations in various natural environments and 3) poor correlation with the presence of pathogenic microorganisms (Desmarais *et al.* 2002; Horman *et al.* 2004). For this reason, a number of other indicators (*Bacteroides* spp., *Bifidobacteria* spp., F-RNA coliphage and human enteric viruses) have been suggested as alternative indicators of faecal pollution (Kreader 1995). It has to be noted that the use of these alternative indicator organisms for routine monitoring is not practical because of difficulties in

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isolation and identification. However, the recent advances in polymerase chain reaction (PCR) technology offers rapid detection and identification of these indicators.

The members of the *Bacteroides* genus hold promise as an alternative FIB of faecal pollution (Kreader 1995) due to a number of advantages including their short survival rates outside the hosts, thought to be exclusive to the gut of warm-blooded animals and comprising a larger portion of faecal bacteria compared to faecal coliforms or enterococci (Sghir *et al.* 2000). It has been reported that some species in the genus *Bacteroides* could be host-specific (Allsop & Stickler 1985). A recent study reported the identification of human- and bovine-specific *Bacteroides-Prevotella* 16 S rRNA gene markers by using length heterogeneity (LH) PCR and terminal restriction fragment length polymorphisms (T-RFLP). It was concluded that these markers could be used to detect human or bovine origin faecal pollution (Bernhard & Field 2000*a*,*b*). In view of this, other researchers have developed PCR primers to detect faecal pollution from animal host groups such as pigs, horses (Dick *et al.* 2005) and dogs (Kildare *et al.* 2007). Another study reported the enterococci surface protein (*esp*) gene (i.e. a putative virulence factor) found in *E. faecium* strains as a potential marker for the identification of human faecal pollution (Scott *et al.* 2005). This marker was found to be widely distributed in sewage in the USA and reported to be host-specific. PCR detection of host-specific markers is rapid, sensitive and some of them have been shown to be accurate when evaluated against blind test samples (Griffith *et al.* 2003). Consequently, PCR assay of host-specific markers has emerged as a potential tool for faecal pollution tracking studies in the USA, Europe and Japan (Bernhard *et al.* 2003; Okabe *et al.* 2006; Seurinck *et al.* 2006; Gourmelon *et al.* 2007).

The primary objective of this study was to validate the previously published host-specific PCR markers (i.e. HF183, HF134, CF128, BacCan and *esp*) for the detection of sources of faecal pollution by testing a large number of faecal samples from 13 host groups in Southeast Queensland, Australia. In addition, water samples were also collected from a mixed landuse catchment and were tested for the presence of PCR markers along with the enumeration of FIB. The results of the specificity and sensitivity of these markers in host groups along with the positive/negative PCR results of water samples were then used to identify the most likely sources of faecal pollution in the study area.

METHODS

Study area and water samples

Ningi Creek catchment was chosen for this study. This catchment is located in Caboolture Shire in the Southeast Queensland region and is characterized by mixed landuse including urban, agriculture and forestry. The catchment is serviced by septic systems and is experiencing significant urban development. The effect of this development has become a major concern for the local authorities in terms of faecal pollution in Ningi Creek. A recent study used an antibiotic resistance analysis (ARA) library of *E. coli* and reported the presence of animal faecal pollution in agricultural areas and increased human faecal pollution in close proximity to urban developments (Carroll *et al.* 2007). Eight sampling sites (i.e. NC1-NC2) were chosen along the length of the creek for water sampling (Figure 1). In all, 16 grab samples were collected on 2 occasions during low tide after rainfall events. Samples were collected in 1 L sterile bottles after storm events and were transported to the laboratory and tested within 6–8 h. The membrane filtration method was used to process water samples. The procedures for the isolation, confirmation and enumeration of *E. coli* and enterococci were described elsewhere (Ahmed *et al.* 2007). The detection of general *Bacteroides*, host-specific *Bacteroides* and *esp* marker was undertaken according to the published methods with a few changes. Briefly, 500 mL of water samples were filtered through 0.45 μm membranes. The filters were suspended in GITC buffer overnight at −80°C (Bernhard *et al.* 2003) and the DNA was extracted using DNA tissue kit (Qiagen Inc. Valencia, CA, USA). For *esp*, the same volume of water used for DNA extraction but a cultural enrichment step was performed prior to DNA extraction (Scott *et al.* 2005).

Origin of faecal samples

In 2006, host-specific PCR markers were used to detect human faecal pollution in 3 non-sewered catchments in

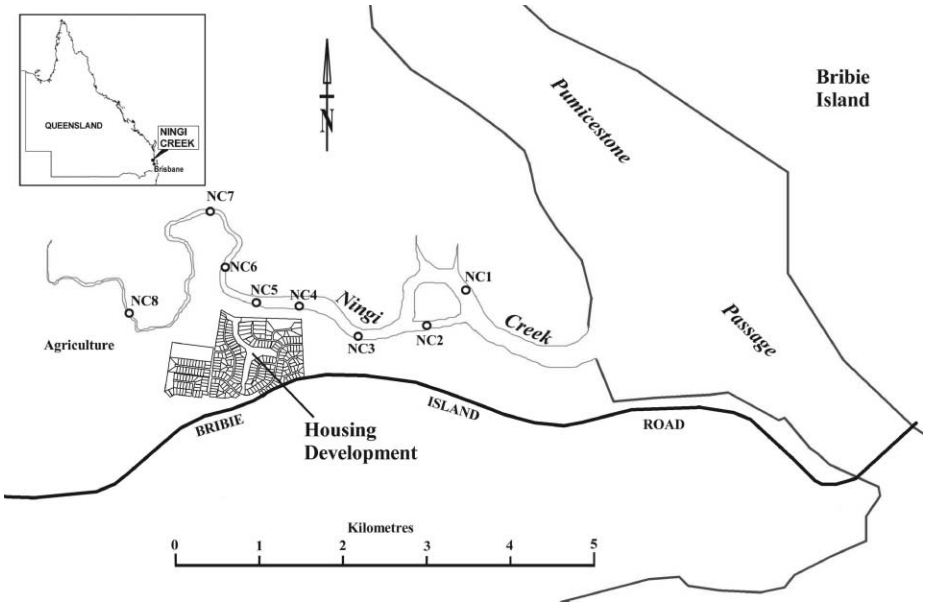

Figure 1 | Sampling sites in Ningi Creek in Caboolture Shire, Southeast Queensland, Australia.

Southeast Queensland region (Ahmed *et al.* 2007). To determine the specificity and sensitivity of the markers, 197 faecal samples (approximately 1 gm from each individual) were collected from various host groups. DNA was extracted by using DNA stool kit (Qiagen) and stored at −20°C.

PCR assay

The primers (Bernhard & Field 2000*a*,*b*; Scott *et al.* 2005; Kildare *et al.* 2007) used to amplify host-specific markers in faecal and water samples are given in Table 1. For all markers PCR was carried out in a volume of 50 μl reaction mixture containing 45 μl platinum blue supermix (Invitrogen, Carlsbad, CA), 0.3 μM of each primer and 2 μl of template DNA. PCR cycling parameters were 15 min at 95°C for initial denaturation and 35 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. For human-specific *Bacteroides*, positive controls (i.e. DNA from sewage or animal species) specific to each marker was included in each assay. For the *esp* marker, 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) was included in each assay. Samples were recorded positive when the PCR product was equal in size to the positive control band (Table 1). DNA cloning and sequencing was performed for the verification of the PCR amplified products.

Statistical analysis

Sensitivity and specificity are commonly used parameters for the validation of host-specific markers (Bernhard & Field 2000*b*; Scott *et al.* 2005). The sensitivity and specificity of host-specific 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 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 marker of another species) (Gourmelon *et al.* 2007). Binary logistic regressions were also performed in order to obtain correlation between the host-specific markers (positive/negative) and FIB concentrations for water samples. The significance level was set to 0.05.

Table 1 | Primers used for PCR assay

Primers	Target species	Oligonucleotide sequence (5′–3′)	Size of product (base pairs)
Bac32F	General *Bacteroides*	AAC GCT AGC TAC AGC CTT	700
Bac708		CAA TCG GAG TTC TTC GTG	
HF183	Human-specific *Bacteroides*	ATC ATG AGT TCA CAT GTC CCG	520
Bac708		CAA TCG GAG TTC TTC GTG	
HF134	Human-specific *Bacteroides*	ATC ATG AGT TCA CAT GTC CCG	570
Bac708		CAA TCG GAG TTC TTC GTG	
CF128F	Cattle-specific *Bacteroides*	CAA ACY TTC CCG WTA ACT	580
Bac708		CAA TCG GAG TTC TTC GTG	
BacCanF	Dog-specific *Bacteroides*	GGA GCG CAG ACG GGT TTT	145
BacUni690R		CAA TCG GAG TTC TTC GTG ATA TCTA	
Esp F	Human-specific *E. faecium*	TAT GAA AGC AAC AGC ACA AGTT	680
Esp R		ACG TCG AAA GTT CGA TTT CC	

RESULTS AND DISCUSSION

PCR positive results for host groups

In all, 197 faecal samples were tested from 13 host groups (Table 2). All faecal samples (100%) were positive for general *Bacteroides*. Of the 42 (i.e. 30 sewage and 12 septic samples) sewage/septic samples tested, all were positive for the human-specific HF183 and HF134 *Bacteroides* markers. The HF183 marker could not be detected in any faecal samples from animal host groups suggesting that the suitability of this marker to detect human faecal pollution. In contrast, the HF134 marker was detected in 7 (35%) samples from dogs. The presence of this marker in dogs could be due to the transfer of faecal bacteria between human and their companion pets (Dick *et al.* 2005). Of the 20 cattle faecal samples tested, 19 (95%) were positive for the CF128 marker. In addition, this marker was also detected in ruminants such as deer, goats and sheep and

Table 2 | PCR positive results for host-groups

		Percentage of positive samples					
Host groups	No of samples tested	Bac32F	HF183	HF134	CF128	BacCan	*esp*
Sewage/septic	42	100	100	100	0	9.5	90.5
Cattle	20	100	0	0	95	0	0
Chickens	15	100	0	0	20	13	0
Deer	10	100	0	0	20	0	0
Dogs	20	100	0	35	0	85	0
Ducks	15	100	0	0	0	0	0
Goats	10	100	0	0	30	0	0
Horses	14	100	0	0	0	0	0
Kangaroos	15	100	0	0	0	0	0
Pelicans	10	100	0	0	0	0	0
Pigs	6	100	0	0	0	17	0
Sheep	10	100	0	0	40	0	0
Wild birds	10	100	0	0	0	0	0

as well as non-ruminant (i.e. chickens) (Table 2). The study by Bernhard & Field (2000*a*) also reported the presence of cattle marker in other ruminants and concluded that the CF128 marker should be considered as ruminant marker rather than cattle marker. A recent study reported the presence of CF128 in pigs in France (Gourmelon *et al.* 2007). However, our data indicate that the CF128 marker is mainly present in ruminants and as well as in some non-ruminant animals such as chickens. However, the bands obtained from chicken faecal samples were very weak indicating a low level of prevalence and for this reason, the CF128 marker could potentially still be considered as a ruminant marker in this geographical area. Of the 20 faecal samples tested from dogs, the BacCan marker (i.e. dog marker) was detected in 17 (85%) samples. However, this marker was also detected in samples from sewage/septic, chickens and pigs. Similar findings were reported in a recent study by Kildare *et al.* (2007). Nonetheless, this marker was not detected in any samples from ruminants and therefore could potentially be used to distinguish between ruminants and non-ruminant sources of faecal pollution. Of the 42 sewage/septic samples tested 38 were positive for the *esp* marker. The marker was absent in only 4 samples from septic tanks and the level of enterococci in these samples was quite low (i.e. (1.5×10^1 CFU/100 mL). However, this marker was not detected in any faecal samples from animal host groups indicating its potential for detecting human faecal pollution.

Specificity and sensitivity of the host-specific primers

The overall sensitivity of the HF183 and HF134 primers to detect human-specific HF183 and HF134 markers in sewage/septic was 100%. The HF183 marker was found to be more sensitive than the HF134 in the USA and European studies (Bernhard & Field 2000*a*; Gourmelon *et al.* 2007). The overall specificity of these markers to differentiate between sewage/septic and animal host groups was 100% (for HF183) and 95.5% (for HF134). The CF128 marker also showed high sensitivity (95%) and high specificity (93%). The DogCan primer showed the lowest sensitivity (85%) among all *Bacteroides* markers. The overall sensitivity of the *esp* marker was low (90%) compared to HF183 or HF134 *Bacteroides* markers. This was expected as the prevalence of *Bacteroides* spp. is much higher in sewage/septic compared to enterococci. Nonetheless, the *esp* marker showed 100% specificity. Although, it has to be noted that bacterial virulence genes (such as *esp* or *E. coli* virulence genes) have been reported to be associated with pathogenicity islands which have been demonstrated to undergo horizontal transfers occurring in humans and animals (Leavis *et al.* 2004).

Source identification of faecal pollution

The concentrations of FIB in the water samples ranged between 9.1×10^2 and 1.2×10^4 CFU/100 mL (for *E. coli*) and 1.2×10^2 and 5.6×10^4 CFU/100 mL (for enterococci) (Table 3). Samples from sites NC1-NC3 were highly polluted in terms of FIB concentrations compared to upstream sites (NC7-NC8). General *Bacteroides* were detected in all water samples indicating recent faecal pollution in the creek. The water samples were collected after storm events when a large number of bacteria are known to be washed into the creek due to surface runoff from point and non-point sources (Ahmed *et al.* 2006). At least one host-specific marker was detected in 14 (87%) out of 16 samples and the number of *E. coli* and enterococci in these samples was above 1×10^3 CFU/100 mL. Human-specific *Bacteroides* HF183 and HF134 markers were detected in 9 (56%) and 6 (37%) samples respectively. This figure for human-specific *esp* marker was also 6 (37%). Cattle-specific marker CF128 was detected in 11 (69%) samples whereas dog-specific marker BacCan was detected in 5 (31%) samples. The HF183 marker was consistently detected in samples from sites NC2-NC5 which are experiencing urban development and serviced by septic systems. Samples from these sites were also positive for human-specific HF134 and *esp* markers indicating the presence of human faecal pollution. It has to be noted that the HF134 marker could be found in dogs and therefore, their presence in water samples not only indicate faecal pollution from humans but also from dogs. Based on the study outcomes, the use of the HF134 marker alone to detect human faecal pollution cannot be recommended. However, this marker could be used in combination with either HF183 or *esp* markers for confirmatory results where necessary. Similarly, the *esp* marker alone may not be

Table 3 | Detection of faecal pollution in water samples from Ningi Creek catchment

	Number of indicators (CFU/100 mL)		PCR results (+/−)					
Water samples	***E. coli***	**Enterococci**	**Bac32**	**HF183**	**HF134**	**CF128**	**BacCan**	***esp***
Occasion 1								
NC1	2.1×10^3	4.1×10^3	+	+	−	−	−	+
NC2	3.6×10^3	3.2×10^3	+	+	−	+	+	−
NC3	4.9×10^3	1.3×10^4	+	+	+	+	+	+
NC4	4.1×10^3	1.9×10^4	+	+	+	−	+	+
NC5	1.2×10^4	4.3×10^4	+	+	+	+	−	−
NC6	3.9×10^3	2.8×10^4	+	+	−	+	−	+
NC7	3.1×10^3	3.9×10^3	+	−	−	+	−	−
NC8	3.4×10^3	1.4×10^3	+	−	−	+	−	−
Occasion 2								
NC1	3.1×10^3	3.7×10^3	+	−	−	+	−	−
NC2	9.1×10^2	1.0×10^2	+	+	−	−	−	−
NC3	4.9×10^4	3.9×10^3	+	+	+	−	+	−
NC4	4.4×10^4	5.6×10^4	+	+	+	+	+	+
NC5	4.2×10^4	3.9×10^4	+	+	+	+	−	+
NC6	1.1×10^3	2.1×10^3	+	−	−	+	−	−
NC7	1.6×10^3	3.1×10^2	+	−	−	+	−	−
NC8	2.1×10^3	1.2×10^2	+	−	−	−	−	−

adequate to detect human faecal pollution due to low prevalence of the *esp* gene in enterococci. For example, water sample NC5 (occasion 1) was negative for the *esp* marker but was positive for both human-specific *Bacteroides* markers. The source of faecal pollution would not have been identified as human in this sample if only the *esp* marker was used. This outlines the importance of the enrichment step in the extraction method for this marker. In addition to enrichment, a large volume of water (i.e. >1 L) could be processed to improve the detection of the *esp* gene. The CF128 marker was found throughout the creek and was more prevalent than other host-specific markers. The high prevalence could be due to the fact that this marker was not only found in cattle but also in other ruminants (i.e. sheep, goats and deer). The upstream of the catchment is extensively used for agricultural practices and cattle farming. Runoff from cattle farms may have introduced this marker to the creek. The dog marker BacCan was also found in sites NC2-NC4. However, the results should be interpreted with caution as other host groups such as sewage/septic, pigs and chickens could not be ruled out as contributors of this marker. Nonetheless, the presence of dog marker in water samples indicates the presence of non-ruminant faecal pollution in these sites.

Binary logistic regressions were used to identify whether any correlation exists between the concentrations of FIB (*E. coli* and enterococci) and the positive/negative results host-specific markers in water samples ($n = 16$) as shown in Table 3. Significant correlations were found between *E. coli* and human-specific *Bacteroides* markers (HF183, $p < 0.001$; HF134, $p < 0.001$). Similar results were obtained for enterococci (HF183, $p < 0.0001$; HF134, $p < 0.004$). No correlations were found between both FIB and CF128 or BacCan markers. A significant correlation was also found between enterococci and the *esp* marker ($p < 0.02$) compared to *E. coli* ($p > 0.30$). Such inconsistency between FIB and host-specific markers has been previously reported (Gourmelon *et al.* 2007). This could be due to the fact that faecal indicators and markers have different survival rates in the environment. Little is known regarding the persistence of host-specific markers in the environment compared to traditional FIB (Dick *et al.* 2005; Field & Samadpour 2007).

Therefore, consistent correlation cannot be expected from environmental samples.

CONCLUSIONS

In conclusion, host-specific PCR markers were shown to be reliable to detect faecal pollution from humans and animal sources in the Southeast Queensland region. Among all markers, *Bacteroides* HF183 performed well in identifying the sources of human faecal pollution. However, combination of multiple human-specific markers provides greater reliability regarding the presence/absence of human faecal pollution when one marker is not sufficient enough to identify human faecal pollution. The CF128 marker also performed well in identifying ruminant faecal pollution. Such information would be vitally important to water quality managers who are charged with protecting water quality. To our knowledge, this is the first study in Australia that assessed the specificity and sensitivity of multiple host-specific markers followed by testing water samples to detect faecal pollution. PCR detection is rapid as thousands of bacteria can be screened without cultivation and also appears to be adequately sensitive to detect faecal pollution. However, some of these methods are not quantitative and can only be used to detect faecal pollution from humans and certain animal species. In addition, the correlation between traditional FIB and host-specific markers is not well understood and warrants further investigation. As part of the on-going research program, a real-time PCR assay for human-specific markers as well as multiplex PCR assays to detect faecal pollution from human and animal host groups simultaneously is being undertaken.

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