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Evaluation of multiple sewage-associated *Bacteroides* PCR markers for sewage pollution tracking

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ABSTRACT

The host specificity of the five published sewage-associated *Bacteroides* markers (i.e., HF183, BacHum, HuBac, BacH and Human-Bac) was evaluated in Southeast Queensland, Australia by testing fecal DNA samples ($n = 186$) from 11 animal species including human fecal samples collected via influent to a sewage treatment plant (STP). All human fecal samples ($n = 50$) were positive for all five markers indicating 100% sensitivity of these markers. The overall specificity of the HF183 markers to differentiate between humans and animals was 99%. The specificities of the BacHum and BacH markers were > 94%, suggesting that these markers are suitable for the detection of sewage pollution in environmental waters in Australia. The HuBac (i.e., 63%) and Human-Bac (i.e., 79% specificity) markers performed poorly in distinguishing between the sources of human and animal fecal samples. It is recommended that the specificity of the sewage-associated markers must be rigorously tested prior to its application to identify the sources of fecal pollution in environmental waters.

1. Introduction

Host-specific molecular markers have gained popularity for the detection of human/animal fecal pollution in environmental waters. Most commonly used markers are sewage- and animal-associated *Bacteroides* (Bernhard and Field, 2000), human-specific *Enterococci faecium esp* (Scott et al., 2005), animal-specific *E. coli* toxin gene markers (Khatib et al., 2002), and sewage- and animal-associated viruses (Fong et al., 2005; McQuaig et al., 2006; Maluquer de Motes et al., 2004). Among these markers, host-specific *Bacteroides* markers have been widely used for MST field studies in countries such as the USA (Bernhard et al., 2003), France (Gourmelon et al., 2007), Belgium (Seurinck et al., 2006), Japan (Okabe et al., 2007), Austria (Reischer et al., 2007) and Australia (Ahmed et al., 2008).

The advantages of using these markers include short survival rates in the external environments due to their strict growth requirements, exclusivity to the gut of warm-blooded animals and being constituents of a larger portion of fecal bacteria when compared to fecal coliforms and enterococci (Sghir et al., 2000). It has been reported that some species in the genus *Bacteroides* could be host-specific (Allsop and Stickler, 1985). As a result, for example PCR assays have been developed to identify host-specific *Bacteroides-Prevotella* 16S rDNA and rRNA gene markers in humans and animals (Bernhard and Field, 2000; Kildare et al., 2007; Layton et al., 2006; Okabe et al., 2007; Reischer et al., 2007). Traditional (Bernhard et al., 2003) and quantitative PCR (qPCR) (Kildare et al., 2007; Reischer et al., 2007; Seurinck et al., 2005) based methods have been developed for the quantitative detection of these markers in environmental samples.

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Specificity is the most commonly used parameter for the performance evaluation of host-specific PCR markers. Specificity is the ability to detect a source when it is not immediately evident and is determined by dividing the number of true-negative results by the number of samples that should not contain the target (Stoeckel and Harwood, 2007). Several studies have reported high specificities of sewage-associated markers and concluded that they are suitable for distinguishing between human and animal sources of fecal pollution (Ahmed et al., 2008; Seurinck et al., 2005; Bernhard and Field, 2000, Reischer et al., 2007). In contrast, sewage-associated markers have also been detected in a small number of non-target samples. Table 1 shows the specificity results for sewage-associated *Bacteroides* markers given in the research literature.

Sewage-associated *Bacteroides* markers that are used to detect human fecal pollution include human-specific HF183 (Bernhard and Field, 2000), BacHum (Kildare et al., 2007), HuBac (Layton et al., 2006), BacH (Reischer et al., 2007), and Human-Bac (Okabe et al., 2007). The specificity of the HF183 markers has been tested in several countries (Bernhard and Field, 2000, Ahmed et al., 2008; Gawler et al., 2007; Seurinck et al., 2005; Gourmelon et al., 2007). However, little is known regarding the specificity of the BacHum, HuBac, BacH and Human-Bac markers in countries other than where they were developed. The primary aim of the study discussed in the paper was to evaluate the specificity of five sewage-associated *Bacteroides* PCR markers in fecal samples from 11 host groups collected from Southeast Queensland, Australia and to determine which marker is the best to identify the sources of sewage fecal pollution in environmental waters.

Table 1 – Specificity of sewage-associated *Bacteroides* markers in various geographical regions.

Sewage-associated markers	Geographical region	Number of target and non-target samples	Specificity	Reference
HF183	Australia	52[a], 155[b]	100	Ahmed et al., 2008
HF183	France	44[b], 92[b]	94	Gourmelon et al., 2007
HF183	France	25[b], 45[b]	91	Gawler et al., 2007
HF183	Ireland	25[b], 45[b]	100	Gawler et al., 2007
HF183	Portugal	29[b], 43[b]	96	Gawler et al., 2007
HF183	UK	25[b], 45[b]	100	Gawler et al., 2007
HF183	Belgium	4[a], 19[b]	100	Seurinck et al., 2005
HF183	Belgium	7[b], 19[b]	100	Seurinck et al., 2005
HF183	USA	25[a], 73[b]	85	Carson et al., 2005
HF183	USA	14[a,b], 46[b]	100	Bernhard and Field, 2000
BacHum	USA	32[a,b], 41[b]	98	Kildare et al., 2007
HuBac	USA	3[b], 18[b]	33	Layton et al., 2006
BacH	Austria	41[a,b], 302[b]	99	Reischer et al., 2007

a wastewater.
b individual fecal samples.

2. Materials and methods

2.1. Host group sampling and DNA extraction

To determine the specificity of the sewage-associated markers, 196 fecal samples were collected from 11 animal species. Samples (i.e., 10 ml) from human fecal sources ($n = 50$) were collected via influent to a STP. Individual fecal samples of cattle ($n = 25$), pigs, ($n = 13$), sheep ($n = 17$), goat ($n = 4$), horses ($n = 9$), and chickens ($n = 10$) were collected from an abattoir and various farms within the study region. Dog fecal samples ($n = 33$) were collected from two dog parks. Duck ($n = 20$) and pelican ($n = 5$) fecal samples were collected from Brisbane City Botanical Gardens Pond and various lakes located in Brisbane and Maroochy River located 100 km from Brisbane. Kangaroo fecal samples ($n = 10$) were collected from University of the Sunshine Coast (USC) where a large numbers of kangaroos roam. A fresh fecal sample (approximately 0.5 – 1.0 g) was collected from 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. To prevent sample cross contamination, animal fecal samples were processed first, followed by sewage samples. This is particularly important to prevent false positive results when assessing host specificity of molecular markers. DNA was extracted from sewage (i.e., 10 ml) after concentrating the samples using a centrifuge (10,000 g for 15 mins), and fresh feces (i.e., 150 – 200 mg) from each individual animal by using QIAamp DNA stool kit (Qiagen, Valencia, CA, USA). Each DNA sample was serially diluted and stored at −80 °C.

2.2. PCR positive controls

The GenBac, HF183, BacHum, HuBac, BacH, and Human-Bac positive controls were isolated from sewage. The 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, IPTG, and X-gal 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).

2.3. PCR analysis

PCR detection of sewage-associated markers was done using previously published primers and PCR assays.

The primer sequence and annealing temperature for corresponding targets are shown in Table 2. PCR analyses were performed using a Rotor-Gene 6000 real-time cycler (Corbett

Table 2 – Primer sequence used for the detection of general and sewage-associated *Bacteroides* markers.

Human-specific markers	Primer sequence (5′- 3′)	Amplicon size (bp)	Reference
GenBac	F – AAC GCT AGC TAC AGC CTT R – CAA TCG GAG TTC TTC GTG	700	Bernhard and Field, 2000
HF183	F – ATC ATG AGT TCA CAT GTC CG R – GCC GTC TACT CT TGG CC	570	Bernhard and Field, 2000
BacHum	F – TGAGTTCACATGTCCGCATGA R – CGTTACCCCGCCTACTATCTAATG	81	Kildare et al., 2007
HuBac	F – GGGTTTAAAGGGAGCGTAGG R – CTACACCACGAATTCCGCCT	116	Layton et al., 2006
BacH	F – CTTGGCCAGCCTTCTGAAAG R – CCCCATCGTCTACCGAAAATAC	93	Reischer et al., 2007
Human-Bac	F – GTTGTGAAAGTTTGCGGCTCA R – CAATCGGAGTTCTTCGTGATATCTA	125	Okabe et al., 2007

Research, Mortlake, Australia). Amplification was performed in either 25 μl reaction mixtures 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 or 50 μl reaction mixtures containing 45 μl of platinum blue SuperMix (Invitrogen), 200–400 nM of each primer, and 2 μl of template DNA. The SYBR Green assay was used for the HF183 assay as this assay has been established in our laboratory for the quantitative detection of the HF183 markers. For the GenBac, BacHum, HuBac, BacH, and Human-Bac assays, conventional PCR were used as the aim was to obtain information on the host-specificity of the markers. For the HF183, the PCR analysis consisted of 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 (Bernhard and Field, 2000). For the GenBac, the PCR analysis consisted of 15 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 60 s at 53 °C and 2 min at 72 °C, and a final extension of 6 min at 72 °C (Bernhard and Field, 2000). For the BacHum, the PCR consisted of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C, and a final extension of 5 min at 72 °C (Kildare et al., 2006). For the HuBac, the PCR analysis consisted of 10 min at 95 °C followed by 45 cycles of 30 s at 95 °C, 45 s at 60 °C, and a final extension of 10 min at 72 °C (Layton et al., 2006). For BacH, the PCR analysis consisted of 3 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s at 61 °C, 45 s at 72 °C and a final extension of 5 min at 72 °C (Reischer et al., 2007). For the Human-Bac, the PCR analysis consisted of 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C, 60 s at 62 °C and a final extension of 5 min at 72 °C (Okabe et al., 2007).

2.4. Quality control

For each PCR experiment, corresponding positive (i.e., plasmid 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, DNA melting curve analysis was performed for SYBR Green assay. During the melting curve analysis, the temperature was increased from 53 to 95 °C at approximately 2 °C/min. Amplified products were also visualized by electrophoresis through 2% E-gels (Invitrogen), and exposure to UV light for further confirmation (where necessary). 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.

2.5. PCR detection limit

To determine the PCR detection limits, plasmid DNA were quantified using a spectrophotometer. 10-fold serial dilutions were made for each target and several replicates ($n = 10$) were tested with the SYBR Green and conventional PCR. The lowest concentration of gene copies detected consistently in replicate assays was considered as the PCR detection limit.

2.6. Testing for 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 PCR inhibitory substances in fecal samples collected from animals for specificity assay. Animal fecal samples which gave PCR negative results were chosen. DNA was extracted from 1 L of ultrapure DNase- and RNase-free sterile distilled water (Invitrogen) after concentrating the sample using a centrifuge. A representative number of pooled animal fecal DNA ($n = 5$) were serially diluted (i.e., 10- and 100-fold). Undiluted, 10- and 100-fold dilutions of DNA were spiked with a known amount of copies of the HF183 marker. 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 HF183 marker. One-way ANOVA was performed to determine the differences between the C_T values obtained for distilled water and those obtained for fecal samples.

2.7. DNA sequencing

To verify the identity of the PCR products obtained from animal fecal samples, up to two PCR-amplified products from each target were purified using the QIAquick PCR purification kit (Qiagen) as recommended by the manufacturer's instructions (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).

3. Results

3.1. PCR detection limit

The PCR detection limits were determined by analysing purified plasmid DNA for each sewage-associated marker. The PCR detection limits were as low as one gene copy for the HF183 and BacHum markers. For HuBac, BacH and Human-Bac, the detection limits were seven, five and three gene copies, respectively. Lower levels (i.e., one copy) were also detected for these markers, but the results were not reproducible for all replicates.

3.2. PCR inhibitory substances

For the spiked distilled water, the mean C_T value for the HF183 marker is shown in Table 3. For animal fecal samples, the mean C_T values were 21 ± 0.1 (for dogs), 22 ± 0.7 (for pigs), 22 ± 0.1 (for sheep), 21 ± 0.1 (for ducks), and 22 ± 0.4 (for cattle) when undiluted DNA was tested. For 10-fold and 100-fold dilutions of DNA, the C_T values were similar to undiluted DNA. No significant ($P > 0.05$) differences were observed between the C_T values for spiked distilled water, undiluted and diluted DNA (i.e., 10- and 100-fold) from fecal samples, indicating that the undiluted DNA extracted from feces did not contain PCR inhibitory substances.

3.3. PCR results for host groups

In all, 186 fecal samples were tested from 11 host groups including humans via influent to a STP. All fecal samples were positive for general *Bacteroides* (Table 4). All STP influent samples were positive for all five sewage-associated markers tested in the study. In the 136 animal fecal samples tested, only one sheep fecal sample was positive for the HF183 marker. Of the 136 samples, six samples (from pigs, sheep, horses and dogs) were positive for the BacHum marker. The HuBac marker was detected more frequently in fecal samples from animals compared to others. For the fecal samples tested, 50 were positive for this marker. This marker was highly prevalent in samples from dogs (22 samples were positive out of 33 samples) and cattle (eight were positive out of 22 samples). The BacH marker was also detected in a small number of fecal samples from sheep, goats and dogs. In the 33 dog samples tested, six were positive for these markers. For the 136 samples tested, 29 were positive for the Human-Bac marker with the highest positive detected in samples from dogs (14 samples were positive out of 33). Up to two amplicons (i.e., amplified from fecal samples) for each marker were sequenced and verified to ensure that they were > 96% identical to the published sequence.

Table 3 – Determination of PCR inhibitors on the PCR detection of spiked sewage-specific HF183 markers in animal fecal 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
DNA for HS-HF183 assays			
Distilled water	21 ± 0.3	–	–
Dog[a]	21 ± 0.1	21 ± 1.2	21 ± 0.7
Pig[a]	22 ± 0.7	22 ± 0.1	23 ± 1.2
Sheep[a]	22 ± 0.1	22 ± 0.4	22 ± 0.9
Duck[a]	21 ± 2.0	22 ± 0.3	22 ± 0.2
Cattle[a]	22 ± 0.4	21 ± 7.0	21 ± 1.0

a Pooled fecal samples.

3.4. Specificity and sensitivity of the human-specific Bacteroides markers

Only one fecal sample from a sheep was found to be positive for the HF183 marker. However, the PCR band was very faint on the gel analysis. Nonetheless, the sequenced PCR product was identical to the HF183 sequence. The overall specificity of the HF183 markers to differentiate between humans and animals was 0.99. Similarly, the overall specificity of the BacHum, HuBac, BacH and Human-Bac markers were 0.96, 0.63, 0.94 and 0.79, respectively. The overall sensitivity of all the sewage-associated markers was 1.0.

4. Discussion

In the current study, the host specificity of the sewage-associated *Bacteroides* markers was assessed by testing fecal DNA samples from animal species. In our previous study, the host specificity of the HF183 marker was evaluated by testing more than 150 animal fecal samples and the results showed 100% specificity. (Ahmed et al., 2008) In a recent study, the specificity of the HF183 marker was further evaluated along with the *esp* and sewage-associated viral markers (i.e., adenoviruses and polyomaviruses) (manuscript under review). The assay showed 98% specificity and only a dog sample was positive for this marker. In the current study, the HF183 marker showed 99% specificity and only a sheep fecal sample was positive. Over the last 2 years, more than 330 fecal samples were screened from 12 animal species. This marker was detected only in two fecal samples, suggesting the consistent specificity of the HF183 marker to differentiate between human and animal fecal pollution in Southeast Queensland, Australia. The high specificity of this marker has also been reported by other research studies around the world (Bernhard and Field, 2000, Gawler et al., 2007; Gourmelon et al., 2007; Seurinck et al., 2005). The research data obtained in this study and others suggest that the HF183 marker is indeed specific to sewage and could be used as a tracer for sewage fecal pollution tracking over large geographical areas.

To-date, the specificity of the BacHum marker was only tested in the USA (Kildare et al., 2007). This marker showed

Table 4 – Polymerase chain reaction positive results for sewage-associated *Bacteroides* HF183, BacHum, HuBac, BacH and Human-Bac in fecal samples collected from animals.

Host groups	PCR positive results/No. of samples tested					
	UniBac	HF183	BacHum	HuBac	BacH	Human-Bac
STP (influent)	50/50	50/50	50/50	50/50	50/50	50/50
Cattle	20/20	0/20	0/20	8/20	0/20	4/20
Pigs	8/8	0/8	1/8	4/8	0/8	0/8
Sheep	17/17	1/17	2/17	5/17	1/17	4/17
Goat	4/4	0/4	0/4	0/4	1/4	0/4
Horses	9/9	0/9	1/9	3/9	0/9	4/9
Chickens	10/10	0/10	0/10	0/10	0/10	0/10
Dogs	33/33	0/33	2/33	22/33	6/33	14/33
Ducks	20/20	0/20	0/20	4/20	0/20	0/20
Pelicans	5/5	0/5	0/5	0/5	0/5	0/5
Kangaroos	10/10	0/10	0/10	4/10	0/10	3/10

98% specificity in the USA and was only detected in dog fecal samples. However, in this present study, the BacHum marker was detected in a small number of pig (1 sample), sheep (2 samples), horse (1 sample) and dog (2 samples) fecal samples. Despite that, this marker displayed 0.96 specificity. To our knowledge, this is the first study that reports the high specificity of the BacHum marker in Australia, and it appears that BacHum marker could potentially be used for tracking human fecal pollution in Australian waters.

The specificity of the HuBac marker was tested in the USA (Layton et al., 2007) and the marker was detected in a significant number of animal fecal samples (12 animal fecal samples out of 18 were positive for this marker). In this study, this marker was detected from a wide range of animal fecal samples, and most (22 out of 33) of the dog fecal samples were positive for this marker. Based on our data, it appears that the HuBac marker may not be suitable for sewage fecal pollution tracking in mixed landuse catchments where multiple sources of fecal pollution may be present. This marker requires further validation in terms of their specificity in different geographical areas. It is recommended that this marker should not be used alone to identify the sources of sewage fecal pollution in environmental waters. The results obtained for environmental waters needs to be validated using other marker(s).

The specificity of the BacH marker was tested in Austria (Reischer et al., 2007). For the 300 animal fecal samples tested, only one cat fecal sample was positive for this marker. The overall specificity of this marker was 99%. To our knowledge, the specificity of the BacH marker has not been reported from outside Austria. In this study, the BacH marker was detected in a small number of fecal samples similar to BacHum marker, and showed 94% specificity. The specificity of the Human-Bac assay has not been reported before (Okabe et al., 2007). In this study, this marker displayed 79% specificity and were detected in dogs, cattle, horses, sheep, and kangaroos.

Therefore, care must be taken if this marker is used in Australian waters for sewage pollution tracking. More validation is required regarding the specificity of this marker in other geographical areas.

The presence of sewage-associated markers in small numbers of animals has been explained by the fact that horizontal transfer of fecal bacteria may occur among species in close contact such as humans and their pets (Dick et al., 2005). This is not a problem as long as any host specific markers exhibit > 95% specificity. However, for prior application of these markers to environmental waters, the specificity must be rigorously tested. A recent review recommended testing the specificity of the host-specific *Bacteroides* markers prior to its application for MST field study (Field and Samadpour, 2007). Before field application, a sanitary survey could be performed to identify the likely sources of fecal pollution. On the basis of the survey, the host groups can be targeted for specificity assay. In this study, HuBac and Human-Bac markers did not perform well and were present in a large number of animal fecal samples. This is not coincidental and could be explained by the fact that this assay probably amplifies a number of species and sequences in the genus *Bacteroides*.

5. Conclusions

- For the 5 sewage-associated markers tested in this study, the HF183 marker performed better than others. This marker showed 99% specificity to distinguish between the sources of human and animal fecal pollution. The performance of the five markers in terms of specificity was HF183 > BacHum > BacH > Human-Bac > HuBac.
- The BacHum and BacH markers showed 96% and 94% specificity suggesting that they could potentially be used to detect the sources of sewage pollution in environmental waters.
- HuBac marker was frequently detected in samples from animals, especially dogs followed by the Human-Bac marker which was also detected in a number of animal fecal samples including dogs. This suggests that these markers may not be suitable for the detection of sewage fecal pollution in Australian waters.
- Based on our data, it is recommended that the specificity of the sewage-associated markers must be tested prior to its application to identify the sources of fecal pollution in environmental waters.

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