Evaluation of the Host-Specificity and Prevalence of Enterococci Surface Protein (*esp*) Marker in Sewage and its Application for Sourcing Human Fecal Pollution

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The suitability of the enterococci surface protein (*esp*) marker to detect human fecal pollution was evaluated by testing 197 fecal samples from 13 host groups in Southeast Queensland, Australia. Overall, this marker was detected in 90.5% of sewage and septic system samples and could not be detected in any fecal samples from 12 animal host groups. The sensitivity of the *esp* primer to detect the human-specific *esp* marker in sewage and septic samples was 100 and 67%, respectively. The overall specificity of this marker to distinguish between human and animal fecal pollution was 100%. Its prevalence in sewage was also determined by testing samples from the raw sewage, secondary effluent, and treated effluent of a sewage treatment plant (STP) over five consecutive days. Of the 15 samples tested, 12 (80%) were found to be positive for this marker. In contrast, it was not found in three samples from the treated effluent and these samples did not contain any culturable enterococci. The PCR limit of detection of this marker in freshwater samples was up to dilution 1×10^{-4} and the number of culturable enterococci at this dilution was $4.8 \times 10^{1} \pm 7.0 \times 10^{0}$ colony forming unit (CFU). The utility of this marker was evaluated by testing water samples from three non-sewered catchments in Pine Rivers in Southeast Queensland. Of the 13 samples tested, eight were positive for this marker with the number of enterococci ranging between 1.8×10^{3} to 8.5×10^{3} CFU per 100 mL of water. Based on the results, it can be concluded that the *esp* marker appears to be sewage specific and could be used as a reliable marker to detect human fecal pollution in surface waters in Southeast Queensland, Australia.

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For the past decade, considerable efforts have been made to develop fecal source tracking (FST) or microbial source tracking (MST) methods to identify and in some cases quantify the likely source(s) of fecal pollution in surface waters. These methods could be categorized into two groups: (i) library-dependent methods (LDMs) and (ii) library-independent methods (LIMs). Library-dependent methods as implied require the development of a library of indicator(s) from host groups using phenotypic or genotypic fingerprinting methods. The phenotypes and genotypes of these target strains are then compared to the unknown environmental strains to predict their most likely source(s) (Scott et al., 2002; Field and Samadpour, 2007; Stoeckel and Harwood, 2007). On the other hand, LIMs rely on detecting host-specific gene biomarkers/microorganisms in a given sample by PCR assay and the results are generally expressed as positive or negative (Bernhard and Field, 2000; Khatib et al., 2002; Fong et al., 2005; McQuaig et al., 2006).

The application of LDMs could be limited by: (i) the size and representativeness of the library, (ii) temporal and geographical variability of the indicators, (iii) host specificity, (iv) statistical analyses, and (v) accuracy for predicting polluting sources (Gordon, 2001; Harwood et al., 2003; Ritter et al., 2003; Wiggins et al., 2003; Stoeckel et al., 2004). In contrast, LIMs offer several advantages over LDMs such as: these methods are rapid, sensitive, and comparatively cheaper and some have shown higher accuracy in method comparison studies (Griffith et al., 2003). For this reason, PCR detection of the host-specific biomarkers has recently emerged as a potential MST tool and gained popularity in field studies in the USA (Bernhard et al., 2003; Lamendella et al., 2006; McQuaig et al., 2006; Shanks et al., 2006), Europe (Seurinck et al., 2006; Gourmelon et al., 2007), Japan (Okabe et al., 2006), and Australia (Ahmed et al., 2007).

A previous study reported sewage-associated enterococci surface protein (*esp*) gene found in *Enterococci faecium* strains as a potential marker for the identification of human fecal pollution (Scott et al., 2005). The *esp* gene has been identified as a putative virulence factor and is reported to be associated with nosocomial outbreaks of *E. faecium* (Leavis et al., 2004). This marker was found to be widely distributed in sewage in the USA and reported to be host specific

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Table 1. Specificity and sensitivity of enterococci surface protein (*esp*) marker in various host groups.

Host groups	No. of samples	Enterococci surface protein (*esp*) marker			
		Positive	Negative	Specificity	Sensitivity
				%	
Humans					
Sewage	30	30	0	–	100
Septic	12	8	4‡	–	67
Total human	42	38†	4	–	90.5
Animals					
Cow	20	0	20	100	–
Chickens	15	0	15	100	–
Deer	10	0	10	100	–
Dogs	20	0	20	100	–
Ducks	15	0	15	100	–
Goats	10	0	10	100	–
Horses	14	0	14	100	–
Kangaroos	15	0	15	100	–
Pelicans	10	0	10	100	–
Pigs	6	0	6	100	–
Sheep	10	0	10	100	–
Wild birds	10	0	10	100	–
Total animals	155	0†	155	100	–

† Statistically significant ($p < 0.001$) as shown by chi-square test.

‡ Level of enterococci < 1.5×10^1 CFU 100 mL^{-1}.

(Scott et al., 2005). However, little is known regarding the host specificity and prevalence of this marker in sewage in Australia.

The aim of this study was to determine the host specificity and prevalence of the *esp* marker and its utility for the detection of human fecal pollution in Southeast Queensland, Australia. The host specificity of this marker was validated by testing 13 host groups followed by field testing to identify human fecal pollution in three non-sewered catchments.

Materials and Methods

Source Sampling

To determine host specificity, 197 fecal samples were collected from 13 host groups between October and December 2006 (Table 1). Samples from human (*Homo sapiens*) fecal sources (i.e., 42) were collected via septic system outlets ($n = 12$) and three sewerage treatment plants (STPs) ($n = 30$). Cow (*Bos taurus*), chicken (*Gallus domesticus*), goat (*Capra hircus*), horse (*Eqqus caballus*), pig (*Artiodactyla suidae*), and sheep (*Ovis aries*) fecal samples were collected from various farms within the region. Dog (*Cannis familiaris*) fecal samples were collected from three dog parks. Deer (*Artiodactyl cervidae*) and kangaroo (*Macropus macropodidae*) fecal samples were collected respectively from a deer sanctuary and University of the Sunshine Coast (USC) where a large number of kangaroos roam. Duck (*Anas platyrhynchos*), pelican (*Pelecanidae pelecaniformes*), and wild bird fecal samples were collected from three parks and the Maroochy River. A fresh fecal sample (approximately 1.0–1.5 g) was collected from each individual 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 determine the prevalence of the *esp* marker in sewage, 100-mL samples were collected on five consecutive days from raw sewage, secondary effluent, and treated effluent (i.e., chlorinated) of a STP in addition to the samples tested for the host specificity assay.

DNA Extraction

The membrane filtration method was used to concentrate and enumerate enterococci from host groups and water. In brief, approximately 500 mg of each animal fecal sample and 100 mL of each human wastewater (via STP and septic tanks) sample was suspended in 200 to 300 mL of sterile phosphate buffer saline (PBS) solution. Appropriate serial dilutions were made from each suspension and filtered through 0.45-µm pore size (47 mm diameter) nitrocellulose membranes (Advantec, Tokyo, Japan). The membranes were then transferred to mEI agar (Difco, Detroit, MI) and incubated at 41°C for 48 h. After incubation, the colonies were enumerated and 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). For water samples, 300 mL of each sample was filtered through a 0.45-µm membrane and enterococci were concentrated as described above. DNA was extracted from 2 mL of enriched culture (for both host groups and water samples) by using either QIAamp stool DNA kit or DNA blood and tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

PCR Analysis

The *esp* marker was amplified by using forward primer (5′TAT GAA AGC AAC AGC ACA AGT T-3′) specific for *E. faecium* (Scott et al., 2005) and an enterococcus reverse primer (5′-ACG TCG AAA GTT CGA TTT CC-3′) (Hammerum and Jensen, 2002). PCR was performed in a 50-µL reaction containing 45 µL Platinum Blue Supermix (invitrogen, Carlsbad, CA), 0.3 µmol L^{-1} of forward and reverse primers, and 2 µL of template DNA. 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 1 min, followed by a final extension step of 72°C for 10 min. To detect amplified products, a 5-µL aliquot of the PCR product was visualized by electrophoresis through 2% E-gel (invitrogen) and exposure to UV light. A positive control (*E. faecium* C68 strain provided by Dr. Louis B. Rice of Cleveland Veterans Affairs Medical Center, OH), and a negative control (only sterile H_2O) were included in each assay. Samples were recorded positive when the visible band was the same size as the positive control (i.e., 680 bp). To minimize PCR contamination, dedicated equipment was used, and DNA extraction, PCR setup, and gel electrophoresis were performed in separate laboratories. Checks for PCR inhibitors were performed for each water sample by spiking a portion of the sample with positive control genomic DNA of the C68 strain. No evidence of inhibition was detected.

Evaluation of Specificity and Sensitivity, and the Limit of Detection in Freshwater Samples

Specificity and sensitivity are commonly used parameters for the validation of host-specific markers (Bernhard and Field, 2000). Specificity and sensitivity of the *esp* marker were determined according to Gawler et al. (2007). To determine the limit of detection (LOD) of the PCR assay for freshwater samples, three raw sewage samples were collected from one STP and enterococci were enumerated in each dilution. Raw sewage samples were suspended

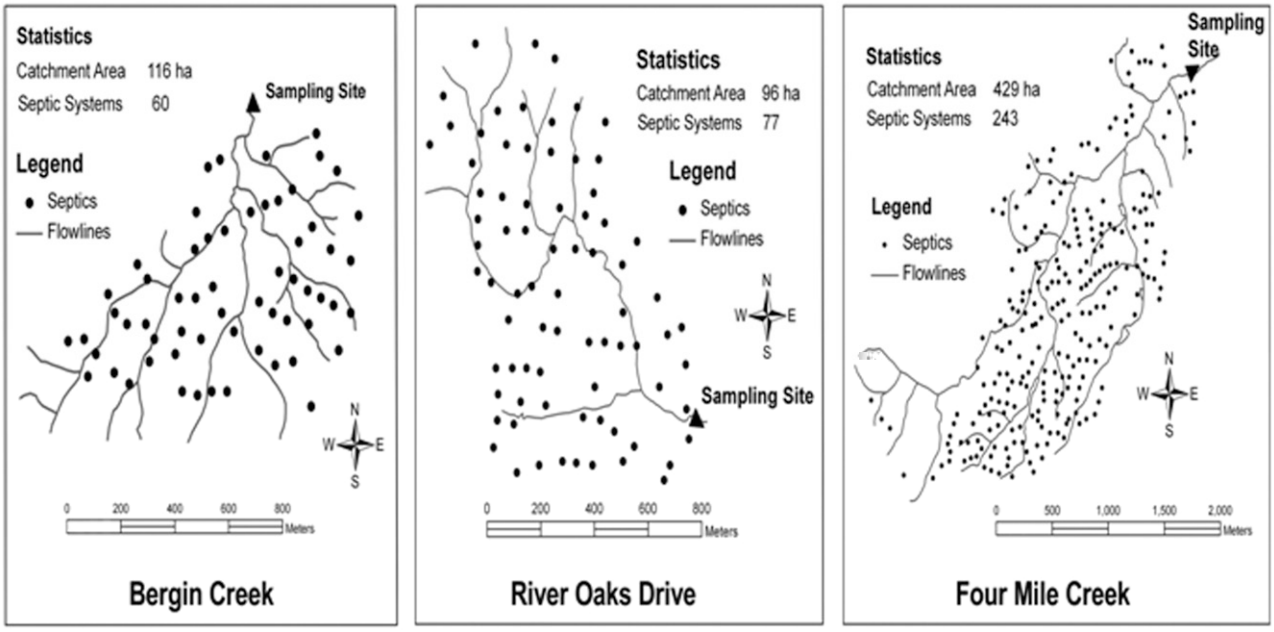

Fig. 1. Map of the Bergin Creek, River Oaks Drive and Four Mile Creek catchments showing sampling sites (▲) and the septic systems (●).

in autoclaved water samples (n = 3) (collected from a lake at USC) to a final concentration of 100 mL per 1 L of water. Serial dilution (10^{-1}–10^{-6}) was made for each sample and the number of enterococci was enumerated for each dilution. DNA extraction was performed for each dilution and tested for PCR as described above. Total culturable enterococci colonies were compared to the PCR positive and negative results to estimate the minimum colony forming units (CFU) and the amount of sewage that must be present in a sample for the detection of the marker.

Environmental Water Samples

Water samples were collected from three non-sewered catchments- Bergin Creek, Four Mile Creek, and River Oaks Creek in Pine Rivers Shire, Queensland (Fig. 1). The catchments were selected on the basis of high density of septic systems. The selected catchments are generally small in size, ranging between 96 and 429 ha and containing between 60 and 243 septic systems. The drainage systems in these areas discharge during storm events and the possible sources of fecal pollution included defective septic systems, horses, dogs, and wild animals. Thirteen water samples (twelve were hydrograph recession grab samples and one was a rising stage sample) were collected between November 2006 and August 2007, representing four storm events. Samples were collected in sterilized 2.5-L containers and transported to the USC laboratory on ice and processed within 6 to 8 h.

Statistical Analysis

Chi-squared test was performed using the statistical package SPSS 13 (SPSS Inc., Chicago, IL). The sensitivity and specificity of the *esp* marker 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), and 'd' is true negative (samples were negative for the PCR marker of another species) (Gawler et al., 2007).

Results

Specificity and Sensitivity

In all, 197 fecal samples were tested and up to 5×10^4 enterococci colonies were screened from each sample. The *esp* marker was detected in all sewage samples (n = 30) (Table 1). However, of the 12 septic system samples tested, eight (67%) were positive and the level of enterococci in these samples ranged between 1×10^3 and 3×10^3 CFU per 100 mL. The level of enterococci was quite low (i.e., $< 1.5 \times 10^1$ CFU per 100 mL) in the remaining four samples from septic tanks where this marker could not be detected. Overall, the *esp* marker was detected in 90.5% of combined sewage and septic tank samples and was not detected in any of the fecal samples from the animals tested. A chi-square test indicated that total human *esp* positive samples differed significantly ($p < 0.001$) compared to animal *esp* positive samples. The overall sensitivity of the *esp* primer being able to detect human-specific *esp* marker in sewage was 100%, while this figure for septic samples was 67%. The overall specificity of this marker to distinguish between sewage and animal fecal pollution was 100%.

Prevalence and Limit of Detection

The level of enterococci on five consecutive days in raw sewage from the STP ranged from 3.9×10^5 to 5.6×10^5 CFU per 100 mL and the marker was consistently detected in all five samples (Table 2). Samples from the secondary effluent were also positive and the number of enterococci ranged from 5.0×10^2 to 8.8×10^3 CFU per 100 mL. Among the five samples tested from the treated effluent, two samples (i.e., Day 1 and 3) were positive and the level of enterococci was low (2.0×10^1 and 4.0×10^1 CFU per 100 mL,

Table 2. Prevalence of enterococci surface protein (*esp*) marker in samples from raw sewage, secondary effluent, and treated effluent of a STP over 5 d.

Sewage samples	No. of enterococci colonies (CFU per 100 mL of wastewater)	PCR results of the *esp* marker (±)
Raw sewage		
Day 1	3.9×10^5	+
Day 2	1.3×10^5	+
Day 3	4.5×10^5	+
Day 4	4.3×10^5	+
Day 5	5.6×10^5	+
Secondary effluent		
Day 1	8.8×10^3	+
Day 2	3.0×10^3	+
Day 3	1.0×10^3	+
Day 4	6.0×10^2	+
Day 5	5.0×10^2	+
Treated effluent		
Day 1	2.0×10^1	+
Day 2	ND†	–
Day 3	4.0×10^1	+
Day 4	ND	–
Day 5	ND	–

† ND: Not detected.

respectively). The *esp* marker could not be detected in treated effluent samples from Day 2, 4, and 5 (none of these samples contained any culturable enterococci). To determine the LOD, raw sewage samples were suspended in autoclaved freshwater samples. The number of enterococci in 100 mL raw sewage samples ranged between 4.5×10^5 to 5.6×10^5. Serial dilutions resulted in the detection of human-specific *esp* marker up to dilution 1×10^{-4} for all three samples (data not shown). The number of culturable enterococci at this dilution was $4.8 \times 10^1 \pm 7.0 \times 10^0$.

Human Fecal Pollution

The number of enterococci in all water samples was high, ranging from 1.4×10^3 to 8.5×10^3 CFU per 100 mL (Table 3). Of the five samples tested from Bergin Creek, four were positive and the marker was detected in both base flow and rising stage samples (first storm event). The number of enterococci in this catchment ranged from 2.7×10^3 to 4.3×10^3 CFU per 100 mL of water. Of the four samples tested from Four Mile Creek, three were positive and the number of enterococci ranged between 1.8×10^3 to 8.5×10^3 CFU per 100 mL. Only one sample from River Oaks Creek was positive and the number of enterococci ranged between 1.4×10^3 to 2.4×10^3 CFU per 100 mL.

Table 3. Detection of human fecal pollution in three catchments up to three storm events using human-specific enterococci surface protein (*esp*) marker.

Subcatchments	Storm events	No. of enterococci (CFU per 100 mL)	Human-specific *esp* marker
Bergin Creek	1 (Nov. 2006)	2.7×10^3	+
	1 (Nov. 2006)†	4.3×10^3	+
	2 (Nov. 2006)	3.1×10^3	–
	3 (Mar. 2007)	3.4×10^3	+
	4 (Aug. 2007)	4.1×10^3	+
Four Mile Creek	1 (Nov. 2006)	1.8×10^3	+
	2 (Nov. 2006)	8.5×10^3	+
	3 (Mar. 2007)	2.5×10^3	–
	4 (Aug. 2007)	3.9×10^3	+
River Oaks Creek	1 (Nov. 2006)	2.4×10^3	+
	2 (Nov. 2006)	1.8×10^3	–
	3 (Mar. 2007)	1.4×10^3	–
	4 (Aug. 2007)	2.1×10^3	–

† Rising stage sample.

Discussion

The *esp* marker found in *E. faecium* has been reported to be associated with sewage and has previously been used to identify human fecal pollution in Florida (McQuaig et al., 2006). However, little is known regarding the geographical distribution and host specificity of this marker outside the USA. In this paper, the utility of this marker to detect human fecal pollution was rigorously evaluated by testing 13 host groups from Southeast Queensland, Australia. In all, 30 (100%) sewage samples and eight (67%) septic system samples were positive for this marker. However, it could not be detected in 155 fecal samples from 12 animal species, although the sensitivity of the PCR detection was increased by screening thousands of enterococci from each fecal sample following a cultural enrichment procedure. However, a recent study reported the presence of the *E. faecium esp* marker in dog feces (9 out of 43 samples were positive) and gulls feces (2 out of 34 were positive) (Whitman et al., 2007). The presence of this marker in dogs could be attributed to horizontal transfer of fecal bacteria between human and companion pets such as dogs (Dick et al., 2005). Such results have also been reported for human-specific *Bacteroides* markers (Gourmelon et al., 2007). It is possible that the presence of the human-specific markers in other animals could be incidental and warrants more rigorous investigation.

For MST applicability, target biomarkers should display high specificity and sensitivity to detect fecal pollution. The high specificity (100%) and sensitivity (90.5%) of the *esp* marker as reported in this study indeed indicate its suitability for detecting human fecal pollution. The *esp* marker has also been shown to be sewage-specific in Florida (Scott et al., 2005), Georgia (McDonald et al., 2006), and Louisiana (LaGier et al., 2007) in the USA. Therefore, it appears that this marker could potentially be used for detecting human fecal pollution over a broader geographical area.

Of the 12 septic tanks tested, eight (67%) were positive for this marker. Similarly, Scott et al. (2005) reported *esp* positive results for 80% of septic systems. The rarity of the *esp* marker in *E. faecium* strains from healthy humans has been reported (Leavis et al., 2004; Lund et al., 2006). It has to be noted that, in this and other studies (Scott et al., 2005), the number of culturable enterococci was quite low in samples from septic tanks and probably because of that, the marker could not be detected. The presence of the *esp* marker is associated with the number of culturable enterococci, especially *E. faecium*. If a sample contains low levels of enterococci, then the chance of finding this marker would also be low. The number of fecal indicator bacteria may vary in septic tanks and a general consensus is that not all of the indicator bacteria survive when introduced into the tank (Ahmed et al., 2005). It is possible that some of the *E. faecium* strains carrying the *esp* gene died off quickly and therefore, could not be detected. Another possible explanation is that septic tanks receiving wastewater from healthy humans

may not have this marker. Similar findings have been reported for human-specific *Bacteroides* markers (Seurinck et al., 2005).

The prevalence of the marker was assessed by testing samples from a STP. It is acknowledged that the samples were collected from only one STP due to difficulty in obtaining access. Nonetheless, the marker was consistently detected in samples from the raw sewage and secondary effluent. Two samples from the treated effluent (Day 1 and 3) were also found to be positive for the marker, although the level of enterococci was low (see Table 2). However, three treated effluent samples (Day 2, 4, and 5) neither contained this marker nor any culturable enterococci. We also determined the LOD of the PCR assay to detect this marker in freshwater samples by spiking raw sewage. It was possible to detect this marker up to dilution 1×10^{-4} mL of sewage for all three samples and the level of culturable enterococci at this dilution was $4.8 \times 10^{1} \pm 7.0 \times 10^{\circ}$. Therefore, $4.8 \times 10^{1} \pm 7.0 \times 10^{\circ}$ sewage origin enterococci colonies must be present in a water sample for the detection of the marker.

The *esp* marker was field tested to identify whether human fecal pollution is being transported to catchment outlets during storm events. The number of enterococci in all storm water samples exceeded relevant water quality guidelines (ANZECC, 2000). This is not surprising as water samples were collected immediately after storm events when a large number of bacteria are washed into the creek via surface runoff. The *esp* marker was detected in eight environmental samples collected from the outlet of the catchments. It is also acknowledged that the number of samples tested was low ($n = 13$) due to a limited number of storm events in this region. Nonetheless, the high specificity and sensitivity of the marker, and its presence in water samples indicated the presence of human fecal pollution after storm events. In a recent study, the *esp* marker was also used to identify the sources of fecal pollution in a mixed land use catchment along with *Bacteroides* host-specific markers. The human markers were consistently detected in samples collected from the sites within close proximity to residential areas serviced by septic systems as opposed to upstream sites characterized by agricultural and farming practices.

Five samples were negative, although the number of enterococci was high in those samples. To obtain confirmatory results, the specificity of the PCR assay was increased by optimizing PCR conditions (by increasing cycles and performing nested PCR) and we were still unable to amplify any products. The negative results could also be associated with factors such as dilution or inactivation rate of indicator bacteria in the environment (Stoeckel and Harwood, 2007). The survival of the *E. faecium* strain carrying the *esp* gene was assessed under laboratory conditions. The *E. faecium* strain survived up to 10 d (Scott et al., 2005). Environmental factors such as radiation, temperature, salinity, and predators could accelerate the inactivation rate of fecal indicators in the environment.

None of the MST methods is superior to others or provide a complete picture about the polluting sources, and the *esp* marker is no exception. However, a combination of methods should be used (where possible) to obtain additional information and confirmatory results. Human-specific *Bacteroides* markers (HF183 and HF134) could complement the *esp* marker in environmental studies. Another limitation is that the presence of *E. faecium* carrying the *esp* gene in environmental samples could be low (because of its rarity and dilution) and because of that, a cultural enrichment step or nested PCR could be required. If an enrichment step is used, then the method is no longer culture independent. A larger volume (>1 L) of water sample can be processed to overcome these shortcomings. Moreover, virulence gene markers such as *esp* and *E. coli* toxin genes may lack stability due to horizontal transfer of genes. Bacterial virulence genes are carried in pathogenicity islands which could undergo horizontal transfers, and can occur between humans and animals (Leavis et al., 2004).

In conclusion, the *esp* marker was shown to be a reliable marker for detecting human fecal pollution in Southeast Queensland, Australia. To our knowledge, this is the first study that assessed the specificity, sensitivity, and geographical distribution of this marker outside the USA. However, this method is not quantitative and can only be used to detect human fecal pollution. Our future aim is to develop a multiplex PCR assay for several enterococcus virulence genes which could provide valuable information in the field of MST.

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