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A real-time polymerase chain reaction assay for quantitative detection of the human-specific enterococci surface protein marker in sewage and environmental waters

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Summary

A real-time polymerase chain reaction (PCR) assay using SYBR Green I dye was developed to quantify the *Enterococcus faecium* enterococci surface protein (*esp*) marker in sewage ($n = 16$) and environmental waters ($n = 16$). The concentration of culturable enterococci in raw sewage samples ranged between 1.3×10^5 and 5.6×10^5 colony-forming units (cfu) per 100 ml. The real-time PCR detected 9.8×10^3–3.8×10^4 gene copies of the *esp* marker per 100 ml of sewage. However, the concentration of culturable enterococci and the *esp* marker in secondary effluent was two orders of magnitude lower than raw sewage. Surface water samples were collected from a non-sewered catchment after storm events and the real-time PCR was applied to quantify the *esp* marker. Of the 16 samples tested, 6 (38%) were PCR-positive and the concentration of the *esp* marker ranged between 1.1×10^2 and 5.3×10^2 gene copies per 100 ml of water samples. The newly developed real-time PCR method was successfully used to quantify the *esp* marker in samples collected from sewage and environmental waters. The presence of the *esp* marker in water samples immediately after storm events not only indicated human faecal pollution but also provided evidence of the degree of human faecal pollution. To our knowledge, this is the first study that reports the use of a real-time PCR assay to quantify the *esp* marker in sewage and surface waters. Such study would provide valuable information for managers for the improved management of water quality.

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Introduction

Over the last decade, researchers have developed microbial source tracking (MST) methods to discriminate between the sources of faecal pollution in surface waters. The underlying assumption of MST is that certain bacterial strains are host-specific (i.e. unique to a particular host group) (Scott *et al*., 2002; Field and Samadpour, 2007). Therefore, phenotypic or genotypic patterns of such bacterial strains from host group(s) could be stored in a library for comparison with the patterns of unknown environmental strains to identify their most likely sources. The most commonly used library-based methods include antibiotic resistance analysis (ARA) (Wiggins *et al*., 2003), nutrient source utilization (Hagedorn *et al*., 2003; Ahmed *et al*., 2005), ribotyping (Parveen *et al*., 1999) and repetitive extragenic palindromic polymerase chain reaction (PCR) (Dombek *et al*., 2000). However, it has to be noted that the library size is the most crucial factor for successful application of library-based method and it is yet not known what constitutes a representative library (Dickerson *et al*., 2007). The size of the library would also vary from catchment to catchment depending on land use patterns and sources of faecal pollution (Stoeckel and Harwood, 2007). In addition, the suitability of faecal indicators, such as *Escherichia coli* and enterococci for MST field application, has recently been questioned because of lack of host specificity, temporal stability and geographical stability (Field and Samadpour, 2007).

To overcome these problems, non-library-based methods can be used for source tracking. These methods are mainly PCR-based, and the most commonly used assays include host-specific *Bacteroides* markers (Bernhard and Field, 2000; Kildare *et al*., 2007), toxin/virulence gene markers (Khatib *et al*., 2003; Scott *et al*., 2005), host-specific viruses (Fong *et al*., 2005; McQuaig *et al*., 2006) including F+ RNA coliphages (Cole *et al*., 2003; Love and Sobsey, 2007). The advantage of such approach is that in most cases, these methods circumvent the need for growing microorganisms. In addition, some of these methods are rapid, sensitive and have shown higher accuracy in identifying the sources of faecal

pollution in blind water samples (Griffith *et al*., 2003). However, little is known regarding the host specificity, geographical stability and temporal stability of host-specific markers. A recent review paper recommended that the host specificity and geographical stability of the markers need to be evaluated in time and space (Stoeckel and Harwood, 2007). In addition, their correlation with indicator bacteria and pathogenic microorganism is not well documented (Field and Samadpour, 2007; Santo Domingo *et al*., 2007). Real-time PCR methods have also been developed for certain host-specific *Bacteroides* markers to quantify faecal pollution in surface waters (Okabe *et al*., 2006; Seurinck *et al*., 2006).

A recent study reported the enterococcal surface protein (*esp*) gene associated with *Enterococcus faecium* strains as a potential marker for the detection of human faecal 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 frequently detected in samples from sewage in North America (Scott *et al*., 2005), and was also reported to be present in environmental waters polluted with sewage (McQuaig *et al*., 2006).

We recently used this marker to detect human faecal pollution in non-sewered catchments in South-east Queensland, Australia (Ahmed *et al*., 2007). The initial data were promising, and suggested that the *E. faecium esp* marker has a significant association with sewage. The specificity of the marker was assessed by testing faecal samples from 13 host groups (Ahmed *et al*., 2008b). The overall specificity of this marker to distinguish between human and animal faecal pollution was 100%. However, to date little is known regarding the concentration of the *esp* marker in sewage and environmental waters, and therefore, the degree of faecal pollution could not be assessed. In view of this, we developed a real-time PCR assay using SYBR Green I dye to quantify the human-specific *esp* marker in sewage and environmental waters.

Results

Host specificity of the esp *marker*

In all, 197 faecal samples were tested and up to 5×10^4 enterococci colonies were screened from each sample for the presence of the *esp* marker. The marker was detected in all sewage samples ($n = 30$). However, of the 12 septic system samples tested, 8 (67%) were positive for the marker. The level of enterococci was quite low (i.e. $< 1.5 \times 10^1$ cfu/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 faecal samples ($n = 155$) from the animals tested. Therefore, the overall specificity of this marker to distinguish between sewage and animal faecal pollution was 100%.

Real-time PCR standards

DNA from 10-fold dilutions of quantified *E. faecium* C68 strain were analysed in order to determine the reaction efficiencies. The standard curves had a linear range of quantification from 10^6 to 10^1 *esp* markers μl^{-1} of DNA extract (Fig. 1A). The amplification efficiencies were between 90–96% for each PCR as determined by the Rotor-Gene software (Corbett Research). The correlation coefficient (r^2) was higher than 0.98 (Fig. 1B). The amplification of the correct PCR products was verified by analysing the melting curves, which showed a peak at melting temperature 78.4 $\pm$ 0.2°C, indicating a positive and correct amplification (Fig. 1C).

Real-time PCR reproducibility and limit of detection

The reproducibility of the real-time PCR was determined by assessing intra-assay and inter-assay Coefficient of Variation (CV) of the standards. These values were less than 1% and 4% respectively, indicating high reproducibility (Table 1). The limit of detection assay was performed by analysing standards (i.e. 10^3–10^1 gene copies) generated from pure culture of *E. faecium* C68 strain containing the *esp* gene. To determine the reproducibility of the assay, several replicates ($n = 6$) were tested. The real-time PCR detection limit was as low as 10 gene copies for all replicates tested. Lower level (i.e. one copy) was tested but the results were not reproducible for all replicates. To determine the limit of detection of the real-time PCR assay in freshwater samples, 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} or all samples. The number of culturable enterococci at this dilution was $4.8 \times 10^1 \pm 7.0 \times 10^0$.

Detection efficiency

The detection efficiency was determined by spiking autoclaved freshwater with known concentration of *E. faecium* C68 cells. The estimated detection efficiency in freshwater samples ranged between 77% and 51% with the greatest variability occurring at lower cell counts (Table 2). The mean detection efficiency was 65% $\pm$ 13%.

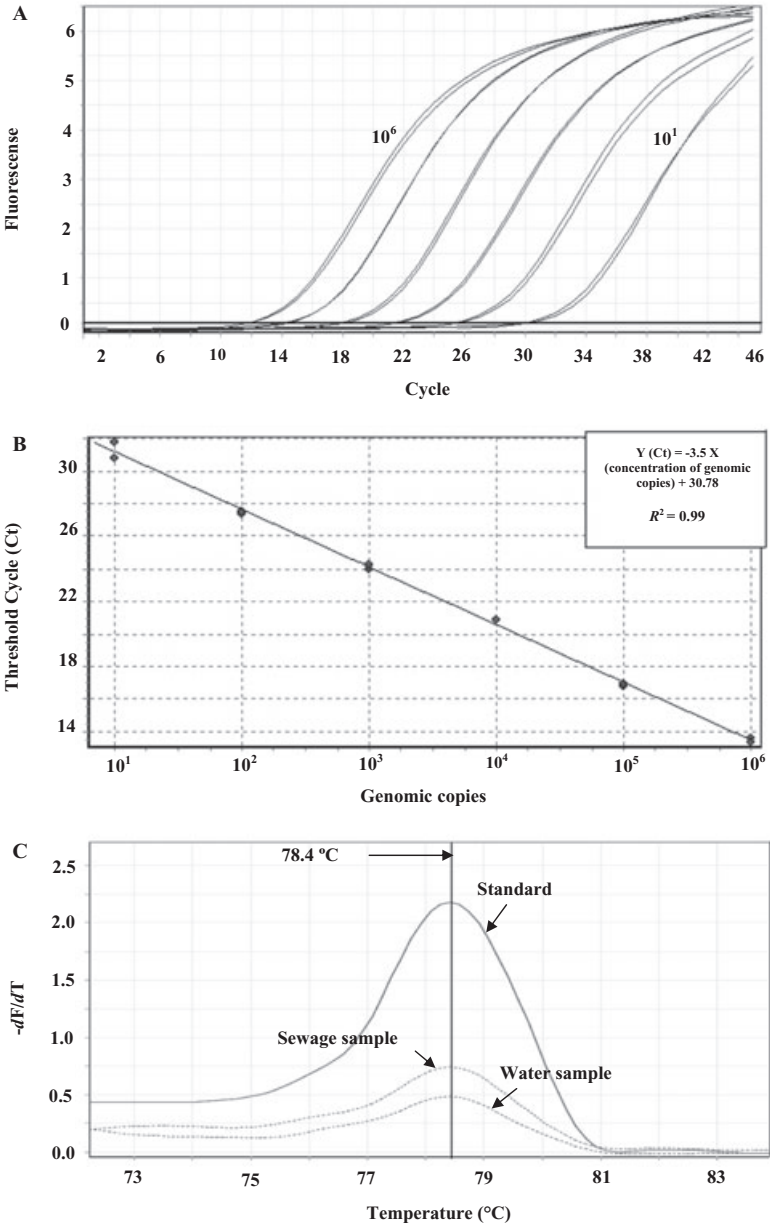

Fig. 1. A. Amplification curves of the real-time PCR standards ranging from 10^6 to 10^1 *E. faecium esp* marker μl^{-1} of DNA extract.
B. Standard curves generated using the *E. faecium* C68 DNA for the *esp* marker. The DNA concentrations are plotted against Ct values. The Ct is the cycle number at which the fluorescence signal increased above the defined threshold value, calculated by the real-time PCR software.
C. Melting curves analysis of sewage and environmental DNA compared with the standard of 10^6 *E. faecium esp* markers μl^{-1} of DNA extract. A peak at (78.4 ± 0.2)°C was indicative for positive and correct.

Table 1. The intra-assay and inter-assay CV for the real-time PCR assay within the range of 10^6–10^2 human-specific *E. faecium esp* markers μl^{-1} of DNA extract.

Concentration of gene copies μl^{-1} of DNA extract	CV (%)	
	Intra-assay	Inter-assay
10^6	0.86	2.20
10^5	0.85	2.64
10^4	0.91	2.92
10^3	0.80	3.56
10^2	0.94	3.93

Table 2. Detection efficiency with the real-time PCR assay for autoclaved freshwater samples spiked with known concentration of *E. faecium* C68 cells.

Spiked cells per 500 ml of water	Detection efficiency ± SD (%)
$1.6 \times 10^7 \pm 0.6 \times 10^7$	77 ± 3
$1.6 \times 10^6 \pm 0.6 \times 10^6$	69 ± 4
$1.6 \times 10^5 \pm 0.6 \times 10^5$	51 ± 7

Table 3. The real-time PCR results of the human-specific *E. faecium esp* markers in samples from raw sewage and secondary effluent.

Sewage samples	Number of enterococci (cfu per 100 ml)	Real-time PCR results (gene copies per 100 ml)
Raw sewage		
RS1-STP1	3.9×10^5	3.8×10^4
RS2-STP1	1.3×10^5	1.5×10^4
RS3-STP1	4.3×10^5	1.3×10^4
RS4-STP1	5.6×10^5	2.3×10^4
RS5-STP2	1.6×10^5	1.1×10^4
RS6-STP2	2.3×10^5	2.0×10^4
RS7-STP2	2.1×10^5	9.8×10^3
RS8-STP2	2.9×10^5	1.0×10^4
Geometric mean	2.6×10^5	1.6×10^4
Secondary effluent		
SE1-STP1	8.8×10^3	1.9×10^2
SE2-STP1	3.0×10^3	4.4×10^2
SE3-STP1	6.0×10^3	2.5×10^2
SE4-STP1	5.0×10^3	3.5×10^2
SE5-STP2	8.0×10^3	9.5×10^1
SE6-STP2	4.9×10^3	1.7×10^2
SE7-STP2	2.1×10^3	1.8×10^2
SE8-STP2	9.1×10^3	6.0×10^2
Geometric mean	5.2×10^3	2.4×10^2

Concentration of the culturable enterococci and the E. faecium esp *marker in sewage and environmental waters*

The real-time PCR method was used to estimate the concentration of the *esp* marker in samples from raw sewage ($n = 8$), secondary effluent ($n = 8$) and environmental waters ($n = 16$). The concentration of culturable enterococci in raw sewage samples ranging between 1.3×10^5 and 5.6×10^5 colony-forming units (cfu) per 100 ml. The real-time PCR detected 9.8×10^3–3.8×10^4 gene copies per 100 ml of raw sewage (Table 3). However, the concentration of enterococci, and the *esp* marker in samples from secondary effluent was two orders of magnitude lower than raw sewage. The concentration of culturable enterococci in water samples collected from Ningi Creek ranged between 9.1×10^2 and 4.3×10^4 cfu per 100 ml (Table 4). Of the 16 samples tested, 6 (38%) were positive for the *esp* marker using both binary and the real-time PCR. For the ease of data interpretation, the number of gene copies found in 500 ml of water samples was converted to 100 ml. Based on this conversion, the real-time PCR detected between 1.1×10^2 (site NC1) and 5.3×10^2 (site NC4) gene copies per 100 ml of water sample (Table 4).

Water sample from site NC4 on event 1 had the highest number (5.3×10^2 per 100 ml) of gene copies followed by site NC6 (i.e. 5.2×10^2 per 100 ml). Both these sites were located downstream the urban residential areas. Site NC1 that was located at the mouth of the tidal creek had the lowest number of gene copies (1.1×10^2 per 100 ml). Of the eight sites, five showed the presence of the *esp* marker. In contrast, the marker could not be detected in samples from upstream sites (i.e. NC7 and NC8) on both events. The PCR-amplified sequences of the human-specific *esp* marker found in sewage and environmental waters were verified as 95% identical to the *esp* gene sequence reported previously (Leavis *et al*., 2004).

Discussion

Previous studies used binary PCR for the detection of the *esp* marker in environmental waters (McQuaig *et al*., 2006; Ahmed *et al*., 2007). However, it has to be noted that a cultural enrichment step was performed before PCR to promote growth of injured and stressed cells. This technique is often used to detect pathogenic bacteria that generally occur at low concentration in environmental waters (Olga *et al*., 2007). A major limitation of the binary PCR is that the results are expressed as presence or absence. Therefore, such data provide little or no information regarding the degree of faecal pollution in environmental waters. In view of this, we developed a real-time PCR assay using SYBR Green I dye to quantify the *esp* marker in sewage and environmental waters. The major advantage of the SYBR Green I system is that the already designed/used primers for binary PCR detection could readily be transferred to the real-time assay without designing new primers and probes. In addition, SYBR Green I is more tolerant of polymorphic targets compared with the probe system, and as a result, it could be beneficial for environmental samples in which the target may show

Table 4. Binary PCR and the real-time PCR results of the human-specific *E. faecium esp* marker in water samples colleted from Ningi Creek on two events.

Water samples	Number of enterococci (cfu per 100 ml)	Real-time PCR results (gene copies per 100 ml)[a]
Event 1		
NC1	4.1×10^3	1.1×10^2
NC2	3.2×10^3	–
NC3	1.3×10^4	1.6×10^2
NC4	1.9×10^4	5.3×10^2
NC5	4.3×10^4	–
NC6	2.8×10^4	5.2×10^2
NC7	3.9×10^3	–
NC8	1.4×10^3	–
Event 2		
NC1	3.7×10^3	–
NC2	1.0×10^2	–
NC3	3.9×10^3	–
NC4	5.6×10^4	4.3×10^2
NC5	3.9×10^4	3.1×10^2
NC6	2.1×10^3	–
NC7	9.1×10^2	–
NC8	9.2×10^2	–

a. The number of gene copies found in 500 ml of water samples was converted to 100 ml.

slight genetic variation (Audemard *et al*., 2004). However, a notable limitation of the SYBR Green dye is that it may bind to non-specific dsDNA (Zhou *et al*., 2007). In order to reduce the level of primer dimer and other non-specific products, we optimized the PCR condition for annealing temperature and primer concentration. A melting curve analysis was performed for each real-time PCR run to check the specificity. Positive samples showed a peak at appropriate temperature, indicating correct amplification. In our study, non-specific products were not observed in melting curve analysis and the PCR products of the expected size were found in gel analyses (data not shown).

One limitation of the host-specific molecular markers is that the specificity must be evaluated prior to application in a new geographical area (Field and Samadpour, 2007). In a previous study, we assessed the specificity of the *esp* marker by testing 197 faecal samples from 13 host groups using binary PCR (Ahmed *et al*., 2008b). The *esp* marker was detected only in samples from sewage and on-site wastewater treatment systems (OWTSs), and could not be detected in faecal samples ($n = 155$) from 12 animal species, although the sensitivity of the PCR detection was increased by screening thousands of enterococci isolates from each faecal sample following a cultural enrichment step. The specificity of the *esp* marker was retested using the new real-time PCR assay. However, no discrepancies were observed between the results of the binary and real-time PCR assays. A recent study reported the presence of the *E. faecium esp* marker in dog faeces (9 out of 43 samples were positive) and gull faeces (2 out of 34 were positive) (Whitman *et al*., 2007). The presence of this marker in dogs could be attributed to horizontal transfer of faecal bacteria between human and companion pets such as dogs (Harada *et al*., 2005; Field and Samadpour, 2007). Such results have also been reported for human-specific *Bacteroides* markers (Gourmelon *et al*., 2007; Ahmed *et al*., 2008a). It is possible that the presence of the human-specific markers in animals could be incidental, and warrants more rigorous investigation.

The quantification ranges of the real-time assay were 10^6–10^1 copies μl^{-1} of DNA extract. Therefore, the sensitivity of our real-time PCR assay is comparable with the values reported in the literature (Seurinck *et al*., 2005; Okabe *et al*., 2006). The estimated mean detection efficiency in freshwater samples was 65% $\pm$ 13%. It has to be noted that the efficiency assay was performed without enrichment step commonly used for the detection of pathogens (Cudjoe *et al*., 1994). Nonetheless, the detection efficiency obtained in this study was also comparable with those obtained by others (Yáňez *et al*., 2005; Thomson *et al*., 2006).

The *esp* gene is chromosomally encoded putative virulence factor and assumed to be a single copy gene (L. Rice and R. Willems, pers. comm.), which allows the conversion of the fluorescent signal (i.e. gene copies) into cell counts. Therefore, the mean ratios of culturable enterococci and *E. faecium esp* gene in raw sewage and secondary effluent were calculated to be 16:1 and 21:1 respectively. These values were within the range of previous findings where the *esp* marker was detectable in samples from primary influent where the concentration of culturable enterococci ranged between 34 and 82 cfu per sample (Scott *et al*., 2005).

We were able to identify the sites impacted with human faecal pollution in Ningi Creek. However, the frequency of detection varied among the sampling sites. Of the 16 samples tested, 6 were positively identified by PCR. The melting curve analysis and sequence data indicated correct amplification. All negative samples were tested for the potential presence of PCR inhibitors as the concentration of inhibitors in storm water has been reported to be high (Choi and Jiang, 2005). All negatively identified samples were spiked with 10^3–10^2 gene copies of C68 DNA and tested with the real-time PCR. The real-time PCR threshold cycle (Ct) values were compared with those obtained from the same concentration of DNA that was spiked into 500 ml of distilled water. No significant differences were observed in Ct values between spiked distilled water and environmental water samples, indicating that the PCR-negative samples were free of inhibitors (data not shown).

The highest concentration of the *esp* marker was found in sample from site NC4 followed by site NC6 on first event (see Table 4). During the first sampling event, the catchment experienced 106 mm rainfall and the marker was detected in water samples from four sites. On the second event (i.e. 2 days after the first event), the marker was detected in water samples from only two sites (i.e. NC4 and NC5). During the second sampling event, the catchment had experienced a further 60 mm rainfall. Therefore, dilution associated with more water flow may have masked the detection in certain samples. During both events, higher concentration of culturable enterococci was found in water samples. It is known that, rainfall events are the major causes of poor water quality (Morrison *et al*., 2003). The presence of high concentration of enterococci along with the *esp* marker indicated not only faecal pollution, but also that a portion could be of human source. The high concentration of enterococci in the absence of the marker possibly indicated non-human sources of faecal pollution (Weaver *et al*., 2005). It has to be noted that the absence of the *esp* marker does not rule out the presence of human faecal pollution. The discrepancies in terms of detecting human faecal pollution using different bacterial markers have been reported (McQuaig *et al*., 2006; Ahmed *et al*., 2007). However, testing multiple markers simultaneously provides more information regarding the sources of faecal pollution and should be

used for environmental samples where possible (Ahmed *et al*., 2007).

Interestingly, all *esp*-positive samples were collected in close proximity to the urban residential areas where the density of OWTSs is high. A recent study used ARA of *E. coli* isolates in the same creek and reported the presence of human faecal signature in close proximity to the residential areas (Carroll *et al*., 2007). Another study reported the presence of identical biochemical fingerprints of *E. coli* and enterococci in OWTSs and nearby surface waters in South-east Queensland (Ahmed *et al*., 2005). Based on our real-time PCR data, it was possible to estimate the relative contribution of sewage in environmental water samples. We converted the number of gene copies found in 500 ml of water samples to 100 ml for the comparison with those found in sewage. On average, 100 ml of environmental samples had 2.9×10^2 gene copies of the *esp* marker, which is equivalent to 1.8% of 100 ml of raw sewage. It has to be noted that this estimation is based on the assumption that the survival/persistence of the *esp* marker is proportional to faecal indicators. It is acknowledged that the concentration of the *esp* marker in OWTSs was not determined due to access restriction in the study area. However, it could be reasonably argued that the concentration would be lower than raw sewage because an OWTS receives faecal matters from a single household compared with raw sewage from a centralized sewage treatment plant (STP) that receives faecal matters from a large population (Whitman *et al*., 2007). Furthermore, the absence of the *esp* marker in certain samples from OWTSs has been reported (Scott *et al*., 2005; Whitman *et al*., 2007).

In conclusion, the *E. faecium esp* marker appears to be host-specific and promising for human faecal pollution tracking in environmental waters in South-east Queensland. We successfully demonstrated the application of a real-time PCR assay to quantify the marker in sewage and environmental waters. The presence of the marker in environmental samples collected immediately after storm events indicated the presence of human faecal pollution. This assay could also provide additional information for quantitative microbial risk assessment as suggested by World Health Organization. To our knowledge, this is the first assay that can provide quantitative data of the *esp* marker in environmental waters. The main advantage of the real-time PCR assay is that it is rapid – results can be obtained in less than 6 h compared with days using the conventional culture-based methods. Overall, the presence of high numbers of enterococci and the quantitative data on the human-specific *esp* marker provided evidence of the extent of faecal/human faecal pollution in an urban mixed land use catchment. Such study would provide valuable information for managers who are in charge of protecting water quality.

Experimental procedures

Specificity of the PCR primers and the esp *marker*

For the real-time PCR, we used previously published primers. The forward primer specific for *E. faecium esp* gene was designed on the basis of the unique differences between the *Enterococcus faecalis* and *E. faecium esp* genes (Scott *et al*., 2005). The forward primer (5′-TAT GAA AGC AAC AGC ACA AGT T-3′) was used along with a conserved reverse primer (5′-ACG TCG AAA GTT CGA TTT CC-3′) (Hammerum and Jensen, 2002). The cross-reactivity of the primer set has been assessed by testing six species of enterococci and three species of *Enterobacteriaceae*. The primers did not amplify any PCR products (Scott *et al*., 2005). We previously reported the high specificity of the *esp* marker by testing 197 faecal samples collected from humans via sewage ($n = 30$) and septage ($n = 12$), ducks ($n = 15$), kangaroos ($n = 15$), cattle ($n = 20$), horses ($n = 14$), dogs ($n = 20$), chickens ($n = 15$), pigs ($n = 6$), pelicans ($n = 10$), goats ($n = 10$), deer ($n = 10$), wild birds ($n = 10$) and sheep ($n = 10$) using binary PCR (Ahmed *et al*., 2008b). The membrane filtration method was used to concentrate and enumerate enterococci from host groups. In brief, approximately 500 mg of each animal faecal sample and 100 ml of each human (via sewage and septage) 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 enriched in tryptic soy broth (TSB) (Oxoid, London, UK) at 41°C for 3 h (Scott *et al*., 2005). DNA was extracted from 2 ml enriched culture using QIAamp stool DNA kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. For this study, the specificity of the *esp* marker was retested using the newly developed real-time PCR assay.

Sources of samples

In all, 16 sewage samples (i.e. raw sewage and secondary effluent) were collected from two STPs for the quantitative detection of the *E. faecium esp* marker. To determine the detection efficiency with the real-time PCR assay, water samples ($n = 3$) were collected from a freshwater creek. To validate the applicability of the real-time PCR assay, environmental water samples ($n = 16$) were collected from Ningi Creek catchment in South-east Queensland. The catchment is characterized by mixed land uses, including urban, agriculture and forestry. The effect of urban development has become a major concern for the local authorities in terms of microbial water quality. A recent study used an ARA library of *E. coli* and reported the presence of sewage pollution in close proximity to urban developments (Carroll *et al*., 2007). Eight sampling sites (i.e. NC1–NC8) were chosen between upstream and downstream the creek (Fig. 2) and 16 grab samples were collected immediately after storm events. Samples were collected in 2 l sterile bottles and were transported to the laboratory, and tested within 6–8 h.

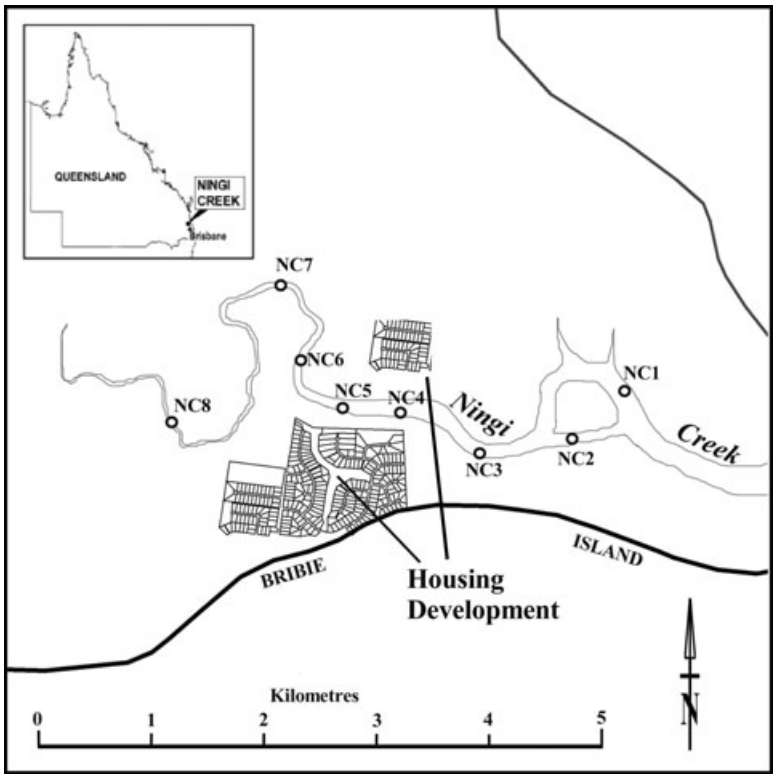

Fig. 2. Sampling sites in Ningi Creek in Caboolture Shire South-east Queensland, Australia.

Enumeration of enterococci and DNA extraction

The membrane filtration method was used to process STP samples for the enumeration (cfu per 100 ml) of culturable enterococci. In brief, serial dilutions were made, 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, USA) and incubated at 41°C for 48 h. For the real-time PCR, a second set of sewage sample (i.e. 100 ml) was filtered through the membranes (Advantec). The membranes were immediately transferred into 15 ml tube containing 10 ml of sterile STE buffer (0.1 M NaCl, 10 mM Tris and 1 mM EDTA pH 7.6). The tubes were vortexed vigorously to detach the bacteria from the membranes followed by centrifugation at 8000 *g* for 30 min at 4°C. The supernatant was discarded, and the pellet was re-suspended in 2 ml of distilled water. DNA was extracted using DNeasy blood and tissue kit (Qiagen, Valencia, CA). The membrane filtration method was also used to process environmental water samples for the enumeration of culturable enterococci. However, for PCR analysis, two sets of DNA (one set for binary PCR and the other set for the real-time PCR) were extracted from each water sample. This was done to investigate discrepancies between binary and the real-time PCR in terms of detection sensitivity. For binary PCR, 500 ml of each water sample was filtered through the membranes, and enriched in tryptic soy broth (Oxoid, London, UK) for 3 h at 41°C before DNA extraction. For the real-time PCR, 500 ml of water was used without the enrichment step. The membranes were washed into the 10 ml sterile STE buffer, and centrifuged to obtain a cell pellet followed by DNA extraction using DNeasy blood and tissue kit (Qiagen).

PCR assays

The method for the detection of the *esp* marker in environmental waters using binary PCR has been described previously except in this study the sample volume was increased to 500 ml from 300 ml (Ahmed *et al*., 2007). For the real-time PCR, amplification was performed in 25 μl of reaction mixtures using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA). The PCR mixture contained 12.5 μl of SuperMix, 300 mM of each primer and 1 μl of template DNA. The reactions were performed in Rotor-Gene 6000 real-time cycler (Corbett Research, Mortlake, Australia). The PCR consisted of 2 min at 50°C, 10 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. Fluorescence data were acquired at the end of the extension step of each cycle. To separate the specific product from non-specific products, including primer dimers, melting curve analysis was performed for each PCR run. During melting curve analysis, temperature was increased from 60°C to 95°C at approximately 2°C min^{-1}. To detect amplified products, a 10 μl aliquot of the PCR product was visualized by electrophoresis through 2% E-gel (Invitrogen), and exposure to UV light. Samples were recorded positive when the visible band was the same as the *E. faecium* C68 *esp*-positive control strain (i.e. 680 bp). To minimize PCR contamination, DNA extraction, PCR set-up and gel electrophoresis were performed in separate laboratories.

Checks for PCR inhibitors were performed for environmental water samples by spiking known concentration of *E. faecium* C68 genomic DNA.

Real-time PCR standards

The standard was prepared from the genomic DNA of *E. faecium* C68 strain. The C68 colony was inoculated into brain heart infusion broth (Oxoid, London, UK), and incubated at 37°C overnight. DNA was extracted using DNeasy blood and tissue kit (Qiagen). The concentration of genomic DNA was determined by measuring the absorbance at A_{260} using Beckman Coulter DU 730 spectrophotometer. The gene copies were calculated using the mean mass of the *E. faecium* genome that was assumed to be approximately 3 Mb (Oana *et al*., 2002). A 10-fold dilution was prepared from the genomic DNA, ranging from 10^6 to 10^1 copies μl^{-1} of DNA extract, and stored at –20°C until use. For each standard, the concentration was plotted against the cycle number at which the fluorescence signal increased above the Ct value. The amplification efficiency (E) was determined by running the standards, and was estimated from the slope of the standard curve by the formula $E = (10^{-1/\text{slope}}) - 1$. A reaction with 100% efficiency generates a slope of –3.32.

Real-time PCR reproducibility and limit of detection

The reproducibility of the real-time PCR was assessed by determining intra-assay repeatability and inter-assay reproducibility. The CV was calculated using five dilutions (10^6–10^2 gene copies) of the C68 genomic DNA. Each dilution was quantified in triplicate. The CV for evaluation of intra-assay repeatability was calculated based on the Ct value by testing the five dilutions six times in the same experiment. The CV for inter-assay reproducibility was calculated based on the Ct value of five dilutions on 10 different days. To determine the lower limits of the real-time PCR detection, genomic DNA of C68 were quantified using a spectrophotometer. Ten-fold serial dilutions were made, and tested with the real-time PCR.

Real-time PCR limit of detection in freshwater samples

To determine the limit of detection of the real-time PCR assay in freshwater samples, three raw sewage samples were collected from one STP and enterococci were enumerated in each dilution. Raw sewage samples were suspended in autoclaved water samples ($n = 3$) to a final concentration of 100 ml/l of water. Serial dilution (10^{-1} to 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 with the real-time PCR. 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.

Detection efficiency of the real-time PCR

The detection efficiency was determined by spiking freshwater with known concentration of *E. faecium* C68 cells. Water samples were first autoclaved to destroy all existing flora. The *E. faecium* C68 strain was grown overnight, and the total cell number was enumerated using epifluorescence microscope after the cells were stained with 6-diamidino-2-phenylindole (DAPI). The enumeration was performed at 10 different microscopic fields. Ten-fold serial dilutions (1.6×10^7, 1.6×10^6 and 1.6×10^5 cells per 500 ml) were made, and were filtered through the membranes. DNA extraction was performed according to the method described above. All samples were tested in triplicate, and the detection efficiency (%) was calculated according to the formula described elsewhere (Seurinck *et al*., 2006).

DNA sequencing

To verify the identity of the PCR products obtained from sewage and environmental waters, the PCR-amplified products were purified using the QIAquick PCR purification kit (Qiagen) as recommended by the manufacturer's instruction (Qiagen). DNA sequences were aligned and compared using Bioware Jellyfish Software.

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