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A novel genetic marker for the rapid detection of *Bacteroides fragilis* in recreational water as a human-specific faecal indicator

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ABSTRACT

Bacteroides spp. has gained substantial interest among the suggested potential candidates for alternative faecal indicators for untreated recreational waters by the US EPA. Interest in *Bacteroides* as a faecal indicator is based upon the relative abundance of selected members of the *Bacteroides* genus in the human colon and human faeces. In this study, we developed a real-time PCR detection system based on gyrase B subunit genes (*gyrB*) specific to *Bacteroides fragilis*. The *gryB*-based method was compared with previously described 16S rRNA-based real-time qPCR methods and evaluated for specificity, sensitivity and robustness in detecting *B. fragilis* from untreated recreational water impacted by human and non-human faecal sources. The new *gyrB*-based system only detected *B. fragilis*, whereas the 16S rRNA-based methods generated cross-amplifications with other *Bacteroides* and *Prevotella* species. We used a procedure of prefiltration, filtration, sonication and DNA concentration in order to improve the DNA extraction efficiency and the sensitivity of the real-time PCR while removing interference. The amplification and sequencing of PCR products generated by the *gyrB*-based method confirmed that *gyrB*-amplified sequences only contained *B. fragilis.* This rapid method is useful for quantifying faecal contamination and may assist beach and watershed managers in elucidating possible contamination sources.

Key words | *Bacteroides*, *gyrB* gene marker, human-specific, rapid method, recreational water

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INTRODUCTION

The association between illness and recreational contact with surface waters has been well documented since the 1950s (Stevenson 1953). Associations between faecal indicators and illness have also been established for these water users (Cabelli *et al*. 1979, 1982; Prüss 1998; Wade *et al*. 2003, 2006; Colford *et al*. 2007), which includes EPA studies establishing *Escherichia coli* as a criteria indicator in freshwater (Dufour 1984). Despite associations between exposure and water quality, the minimum time to obtain an *E. coli* colony count result is 18–24 h post-sample (US Environmental Protection Agency 2006) which is inadequate for communicating same-day water quality risks to the public to prevent exposure. This warrants rapid methods to improve risk reporting. Additionally, current US recreational water quality criteria for pathogens and pathogen indicators are scheduled for revision by 15 October 2012 (*Natural Resources Defense Council v. Johnson and U.S. EPA* 2008). Emerging from this consent decree is a mandate for US EPA to consider alternative rapid indicators of recreational water quality to ensure improved protection of US recreational water users (e.g. swimmers and bathers).

With the advent of rapid molecular techniques, recent exploratory research has suggested *Clostridium*, *Bacteroides*, *Bifidobacterium* and bacteriophages as potential candidates for alternative faecal indicators in recreational waters (US EPA 2007). Among the suggested potential

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candidates for alternative indicators by US EPA, the *Bacteroides* genus has gained substantial interest, particularly because of the relative abundance of certain genera in human faeces and human-impacted waters (Holdeman *et al.* 1976; Layton *et al.* 2006; Converse *et al.* 2009), where they may outnumber faecal coliforms 1,000-fold (Fiksdal *et al.* 1985). Another advantage of the *Bacteroides* genus as an indicator of recent faecal contamination is their inability to persist or grow in the aerobic foreshore sediments or freshwater environment since they are anaerobic, unlike the more common faecal indicator, *E. coli*, which is aerobic and has been observed to persist or replicate in this environment thereby not permitting a more complete understanding of the timing of the faecal contamination (Solo-Gabriele *et al.* 2000). Thus, *Bacteroides* indicates a more recent episode of faecal contamination. Furthermore, some evidence suggests that the *Bacteroides* group is a better indicator of non-point-source human faecal pollution than *E. coli* in recreational waters (Converse *et al.* 2009).

Thus far, some epidemiological evidence from a Great Lakes point-source impacted beach demonstrates a marginally significant association between concentrations of the genus *Bacteroides* and closely related genera and gastrointestinal illness (Wade *et al.* 2006). Among the *Bacteroides* species, *Bacteroides fragilis* is a commensal organism that constitutes a significant component of the normal colonic bacterial microflora in humans (Pumbwe *et al.* 2006). Epidemiological associations specific to *B. fragilis* densities have not yet been observed owing to methodological limitations. All existing methodologies are specific to a group or class of closely related taxa merely including *B. fragilis*. Thus, a methodology specific to the quantification of only *B. fragilis* is warranted, thereby enabling the consideration of this specific species in epidemiological studies of illness risk among recreational water users.

Traditionally, *E. coli* has been used as an indicator microorganism for communicating waterborne disease risk, particularly because of their ability to be easily and safely cultured (Simpson *et al.* 2002). However, most of the bacterial species composing the mammal gut microbiota are anaerobes that are not easy to enumerate by conventional culturing techniques and the majority of that flora is not *E. coli* but of the genus *Bacteroides* (Harmsen *et al.* 2002). Many methods have recently been designed to detect members of the *Bacteroides* genus based upon their 16S rRNA gene (Bernhard & Field 2000; Matsuki *et al.* 2002, 2004; Layton *et al.* 2006; Kildare *et al.* 2007; Okabe *et al.* 2007), including a recently developed real-time PCR assay utilizing a SYBR probe and primers designed to detect the 16S rRNA gene with a limit of quantification of 4.7×10^5 gene copy per litre in environmental samples (Seurinck *et al.* 2005). A limitation of 16S rRNA genetic marker methods involves the extremely slow rate of evolution associated with 16S rRNA, which has a low interspecies polymorphism or multiple ribosomal RNA operon copies in a single bacterial genome. Recently, several housekeeping genes, such as *rpoB*, *gyrB*, elongation factor Tu (*EF-Tu*), phosphoglycerate kinase (*pgk*) and heat shock protein (*dnaK*) genes, have been reported to evolve much faster than 16S rRNA gene, thus providing better discrimination among closely related species (Volokhov *et al.* 2007).

In this study, we developed a real-time PCR assay by using a novel primers-probe set for *B. fragilis* detection based upon *gyrB* amplification and evaluated assay performance in water samples collected from untreated recreational water. *B. fragilis* is a significant component of the normal human gut microbiota as colonic commensal bacteria (Finegold 1977), and a clinically important species among the genus *Bacteroides* for its virulence in human colonic infection (Namavar *et al.* 1989; Kuwahara *et al.* 2004). *B. fragilis* is also linked to the risk of human gastrointestinal (GI) illness (Baums *et al.* 2007) and inflammatory diarrhoeal disease in both children and adults when exposed to recreational water (Sears 2009). Thus, *B. fragilis* may serve as a more human-specific faecal indicator compared with other faecal indicators.

METHODS

Bacterial strain

Bacteroides fragilis ATCC 25285^{T} was obtained from the American Type Culture Collection and propagated on *B. fragilis* bile-esculin agar (BBE) (Oxyrase, Inc. Mansfield, Ohio) plates and anaerobically incubated in an anaerobic chamber containing an AnaeroPack system (Mitsubishi

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Gas Chemical, Tokyo, Japan) for 72 h at 37 °C in order to confirm purity of *B. fragilis* colonies. BBE agar is a convenient hemin-enriched medium for the selection and presumptive identification of *Bacteroides* and related taxa based upon their hydrolysis of esculin and resistance to both bile and gentamicin (Livingston *et al.* 1978). The *B. fragilis* ATCC 25285^T was used to generate a standard curve, spike environmental samples and to compare the sensitivity and specificity of the primers from the two qPCR methods.

Environmental isolates

Three environmental strains of *B. fragilis* were isolated from field samples. Beach water from Madison Lake (Madison County, Ohio) and stream water from Glade Run (Madison County, Ohio) were collected on 10 December 2008. Three different volumes of sample (25, 50 and 100 mL) were filtered through a nylon prefilter membrane (pore size 20.0 μm, Magna, GE Water & Process Technologies, Trevose, PA, USA) in order to remove algae and other large particles while allowing passage of bacteria (Lee & Deininger 2004). After prefiltration, the prefiltered water was filtered through a 0.45-μm pore size, 47-mm diameter cellulose membrane filter (Millipore, Bedford, MA, USA) to capture bacteria present in the water samples. The filtered membranes were then placed on BBE plates and incubated in an anaerobic chamber containing an AnaeroPack system for 72 h at 37 °C. Colonies blackening BBE plates were selected and serially subcultured on pre-reduced TSA plates in an anaerobic chamber. After confirmation as a pure colony, each isolate was identified based on the analysis of the 16S rRNA sequence with an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) at the Plant and Microbes Genomics Facility of Ohio State University. Among the isolates, three strains (1AL, 1BL, 1B2) were identified as *B. fragilis* with 100% identical 16S rRNA sequences to that of *B. fragilis* ATCC 25285^T by using the NCBI (National Center for Biotechnology Information) database and the BLAST program. The strains, 1AL and IBL, were originally collected from 100 mL of beach water from Madison Lake, Ohio. The strain 1B2 originated from 25 mL of stream water from Glade Run, Ohio. These three strains were stored in 40% glycerol at −80 °C for further study. The ultimate application of these strains in this study was to enable an evaluation of the generalizability of the *gyrB* PCR assay to other recreational and environmental waters via PCR detection using positively identified *B. fragilis* from environmental sources.

Recreational water samples

Recreational water samples were collected for water matrix interference testing, identifying minimum limits of detection and confirming the *gyrB* PCR assay results from environmental samples. Interference testing and minimum detection limit determination was performed using samples collected from the Olentangy River (Franklin County, Ohio), which has multiple designated uses, including recreation. Two-litre water samples of Olentangy River water were collected in sterile bottles (Nalgene, Rochester, NY, USA) and transported to the laboratory on ice. Samples were immediately filtered through a nylon prefilter membrane (pore size 20.0 μm, Magna, GE Water & Process Technologies, Trevose, PA, USA) in order to remove algae and other large particles while allowing passage of bacteria (Lee & Deininger 2004). The prefiltered water was filtered through a 0.45-μm pore size, 47-mm diameter cellulose membrane filter (Millipore, Bedford, MA, USA) to concentrate bacteria present in the water samples. In order to test possible interference coming from the water matrix, a PCR assay was performed with filtered Olentangy River water samples that were spiked with *B. fragilis* ATCC 25285^T and three *B. fragilis* environmental isolates (1AL, 1BL, 1B2).

The Olentangy River water matrix was of interest because of its proximal location to the laboratory, recreational use designation and water quality. Presently, like many Ohio waterways, the river is defined as impaired with regards to recreational use, as it exceeds the numeric criteria for pathogens based upon the faecal coliform indicator. The Ohio Environmental Protection Agency (2007) indicates that the primary sources of this impairment in the upper portions of the river is failing home sewage treatment systems and manure from livestock operations; however, in the sample area, which is more urban, combined and sanitary sewer overflows are the primary contamination sources, with home sewage treatment systems contributing slightly.

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DNA extraction from recreational water samples

The filtered membranes from the Olentangy River samples were transferred into 15-mL sterile tubes. Two mL of sterile phosphate buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.6]) was added to the tube to soak the filter membrane, which then was rigorously vortexed. Since the improvement of DNA extraction efficiency is important for the success of PCR assays, sonication was carried out to detach cells from the filter membranes efficiently. Sonication was done for 10 min using the Fisher Scientific FS20 sonicating water bath (Fisher Scientific, Pittsburgh, PA). After sonication, the resuspended cells were centrifuged at 10,000 g for 15 min at 4 °C and the supernatant was gently removed. The harvested cells were suspended in 1 mL of ASL buffer and DNA extraction was performed with a QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instruction as performed in previous studies evaluating the occurrence and diversity of *Bacteoridales* (Jeter *et al.* 2009) and other human-associated markers (McQuaig *et al.* 2006). In order to concentrate DNA, the final elute of 200 μL during the last step of DNA extraction was evaporated under a gentle stream of air at room temperature and then reconstituted with 20 μL elution buffer. The concentration of the DNA was determined with a NanoDrop system (Nanodrop Technologies, Wilmington, DE, USA) and the purity was checked with gel electrophoresis.

Primer design

The *B. fragilis* specific *gyrB* gene-based primers and probe set was based on 748 *gyrB* genes obtained from the NCBI database. From the 748 sequences of bacterial *gyrB* genes, 322 sequences were selected and aligned by Clustal X version 2.0 (Larkin *et al.* 2007) by eliminating disqualified and duplicated sequences. Unique DNA sequence regions were chosen as forward/reverse primers and one minor groove binder (MGB) probe using Primer Express® software (version 3.0, Applied Biosystems, Foster City, CA, USA).

Comparison of the primers

The newly designed primers were compared with previously known *Bacteroides–Prevotella* group-specific primers (Bernhard & Field 2000; Okabe *et al.* 2007; Okabe & Shimazu 2007). This reference primer set was designed relying on the 16S rRNA gene to detect the *Bacteroides–Prevotella* group in a TaqMan real-time PCR system. Another reference primer set was the *B. fragilis* group-specific primer which was selective not only for *B. fragilis* but also other members of the *Bacteroides* group (e.g. *B. fragilis*, *B. ovatus*, *B. thetaiotaomicron* and *B. uniformis*) in a SYBR real-time PCR system (Matsuki *et al.* 2002, 2004).

First, we compared our *gyrB* gene-based primer set with the *Bacteroides–Prevotella* group-specific primer set using *in-silico* analysis. A sequence-similarity search was done using primer-BLAST software at the NCBI database (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Altschul *et al.* 1997) and the Probe Match application at the Ribosomal Database Project (http://rdp.cme.msu.edu). The most similar sequences to both *Bacteroides–Prevotella* and *gyrB* gene-based primer were retrieved and entered into MEGA software (http://www.megasoftware.net) in order to construct schematic phylogenetic trees. For the illustration of the differences in the primers and the related sequences, the number of nucleotide differences was added to radial neighbour-joining trees (Saitou & Nei 1987). The degree of confidence in phylogenetic branching was assessed by repeating bootstrap analyses 1000 times (Saitou & Nei 1987).

The specificities of the primers were also tested by conventional PCR. The *B. fragilis* group primer (Matsuki *et al.* 2002) was included in this comparison. For this, DNA extracts from *B. fragilis* (ATCC 25285^T) and three environmental isolates (1AL, 1B2, 1BL) were tested together with *Bacteroides ovatus* (ATCC 8483^T) and *Prevotella melaninogenica* (ATCC 25845^T) by using each *Bacteroides/Prevotella*, *gyrB* gene-based, and *B. fragilis* group-specific primer. Thermal cycles for PCR were similar except the variations in annealing temperature; incubation for all was at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at each 60 °C (*Bacteroides/Prevotella* primer), 58 °C (*gyrB* gene-based primer) and 50 °C (*B. fragilis* group primer) for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. After PCR, 5 μL aliquots of each amplification product was loaded onto 1.0% agarose slabs (13 by 5 cm) for gel electrophoresis and run in TBE (89 mM Tris-borate, 2 mM EDTA) buffer at 100 V for 30 min. The slabs were stained with ethidium

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bromide and documented with a Bio-Rad Quantity One Gel Doc system (Bio-Rad, Hercules, CA, USA) with the 1-kb DNA ladder (Invitrogen, Carlsbad, CA, USA) as a molecular weight marker.

Standard curve

After *B. fragilis* ATCC 25285^T was grown on BBE plates, two sets of serial dilution of *B. fragilis* were prepared to generate standard curves. For each set of the serial dilutions, one colony of *B. fragilis* was suspended in 1 mL of PBS and then suspension aliquots of 100 μL were serially diluted (1:10 dilution) for *B. fragilis* standard curve development. Ten microliters of the well-mixed solution of *B. fragilis* was then spread on BBE plates to enumerate *B. fragilis* as colony forming units after incubation in an anaerobic chamber for 72 h at 37 °C. At the same time, DNA was extracted from the remaining 990 μL of the same serial dilution set with a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. The DNA was regarded as being extracted from 1,000 μL of *B. fragilis* solution for convenience. The standard curve was derived by plotting cycle threshold (C_T) values against the log cell counts (CFU mL^{-1}), which were obtained from plate counts. DNA extracts were stored at 4 °C for immediate use (real-time PCR) or at –20 °C for less than a month (end-point PCR).

Real-time PCR assays

A TaqMan real-time quantitative PCR was performed with the reference (16S rRNA *Bacteroides/Prevotella*) and *gyrB* gene-based primers and probes sets by using a 48-well StepOne™ Real Time System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The amplification reactions were performed in optical microplates using a total volume of 30 μL. The reaction mixtures contained 1x TaqMan universal PCR master mix (PCR buffer, deoxynucleoside triphosphates, AmpliTaq Gold polymerase, internal reference signal 6-carboxy-x-rhodamine [ROX], Amp Erase uracil *N*-glycosylase [UNG], $MgCl_2$; Applied Biosystems), 500 nM of each oligonucleotide primer, and 250 nM of TaqMan Minor Grove Binding (MGB) probe labelled with 6-carboxy fluorescein (FAM). A mixture of all PCR reagents without template DNA was used as a negative control for each PCR reaction. For the *gyrB* gene-specific primers and probe, thermal cycling consisted of an initial cycle of 50 °C for 2 min (activation of the UNG) and 95 °C for 10 min (activation of the AmpliTaq Gold DNA polymerase), followed by 45 cycles of denaturation at 95 °C for 30 s and annealing and extension at 58 °C for 3 min. For the reference (16S rRNA *Bacteroides/Prevotella*) primers and probe, the thermal cycle condition was as follows: incubation at 50 °C for 2 min and at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 62 °C for 1 min.

Nucleotide sequences accession numbers

The DNA sequences were deposited in the GenBank database under accession numbers from GU130198 to GU130203.

RESULTS AND DISCUSSION

gyrB gene-based primer design and comparison with 16S rRNA-based primers and probe

The *gyrB* gene-specific primers and probe were designed to perform quantitative real-time PCR detection of *B. fragilis*. These are listed in Table 1 and we used the 16S rRNA-based primers and probe as a comparison group known as *Bacteroides/Prevotella* group-specific (Okabe *et al*. 2007).

Figures 1 and 2 show the cluster of target bacteria that can be amplified with *gyrB* gene-based primers. The relationships in both radial phylograms were determined by MEGA4 software with the model based on the nucleotide differences and the neighbour-joining method (1,000 replicates). The *gyrB* primers, Bf904F and Bf1272R, were used to root the trees in Figures 1 and 2, respectively. The reference forward primer, qHS601F, was generally specific for *Bacteroides* and *Prevotella*. However, there are three mismatches with *Arenibacter certesii* (NR_025747), *Arenibacter latericius* (NR_024893), *Aequorivita sublithincola* (NR_025031), *Amphibacillus tropicus* (NR_025192), *Acetobacterium paludosum* (NR_026327), which are non-*Bacteroides/Prevotella*. The reference reverse primer, qBac725R has almost the same oligonucleotide sequences as *Prevotella salivae*

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Table 1 | Nucleotide sequences of primers and probes for real-time PCR assay in this study

	Sequence (5′–3′)	Size of product (bp)	Annealing temp[c] (°C)
16S gene-based primers and probe compared in this study	qHS601F[a] GTTGTGAAAGTTTGCGGCTCA (Okabe *et al.* 2007)	150	60.0
	qHS624MGB FAM-CGTAAAATTGCAGTTGA-MGB (Okabe *et al.* 2007)		65.0
	qBac725R CAATCGGAGTTCTTCGTGATATCTA (Bernhard & Field 2000)		58.0
gyrB gene-based primers and probe developed in this study	Bf904F[b] GGCGGTCTTCCGGGTAAA	368	59.5
	Bf923MGB FAM-TGGCCGACTGCTC-MGB		69.0
	Bf1272R TGGCATATAGCGGAAGAAAAAAG		58.5

[a]The numbers correspond to numbers in the *E. coli* 16S rRNA gene.
[b]The numbers correspond to numbers of the *gyrB* gene (AB048185).
[c]Annealing temperature was determined by Primer Express® software.

(NR_024816), *Prevotella albensis* (NR_025300), *Prevotella pallens* (NR_026417), *Prevotella enoeca* (NR_025281) and *Prevotella intermedia* (NR_026119). Thus, this reverse primer may be the major factor for most cases of 16S rRNA-based amplification that detect *Bacteroides* together with *Prevotella*. From our *in-silico* analysis, three additional bacteria, *Arenibacter certesii* (NR_025747), *Campylobacter hominis* (NR_025377) and *Mesonia algae* (NR_025263), were found to possibly cross-amplify with this reverse primer (qBac725R). In contrast, the *gyrB* forward primer,

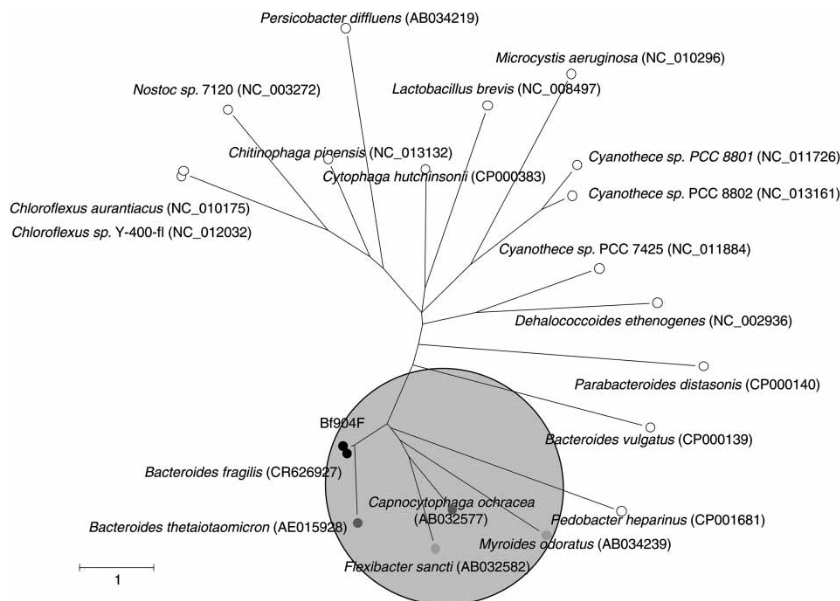

Figure 1 | Radial phylogram showing the target bacterial cluster that can be amplified with *gyrB* gene-based primer, Bf904F. The large circle shows the boundary of sequence identity within 3-bp difference. Closed black, dark grey and light grey circles represent the sequences that are the same, 1 bp- and 3 bp-different, respectively. Scale bar 1 indicates 1 bp-sequence divergence. Numbers in parenthesis indicate Genbank accession numbers.

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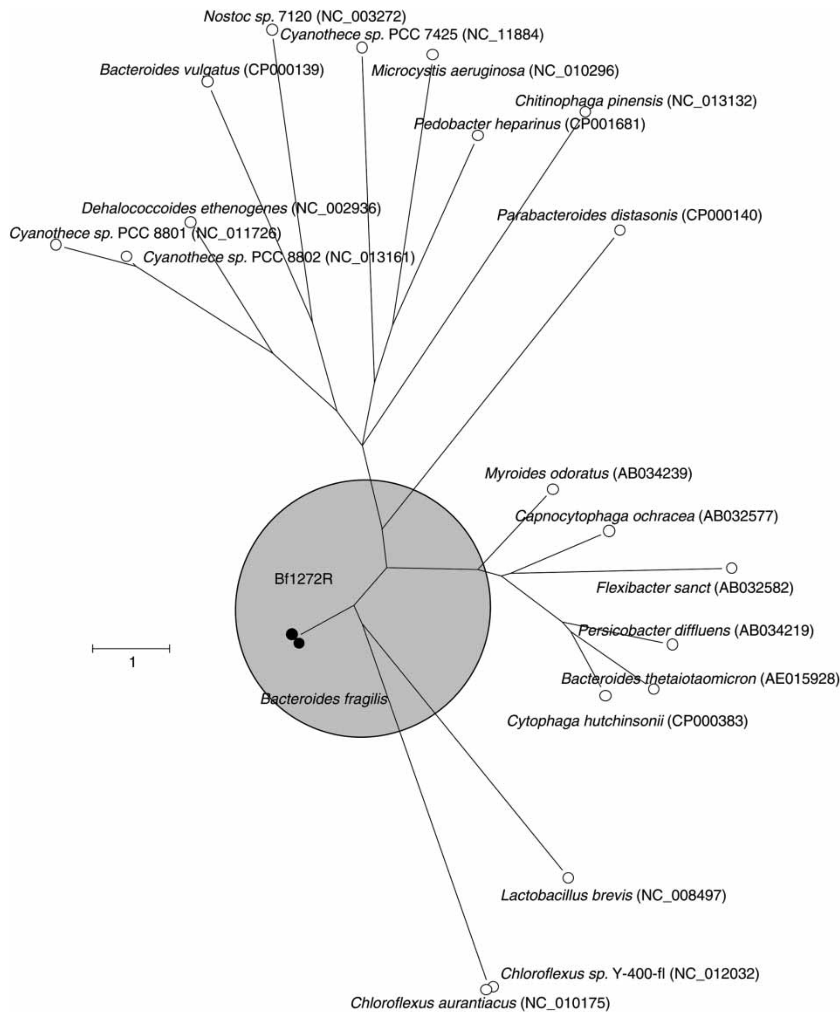

Figure 2 | Radial phylogram showing the target bacterial cluster that can be amplified with *gyrB* gene-based primer, Bf1272R. The large circle shows the boundary of sequence identity within 3-bp difference. Closed black, dark grey and light grey circles represent the sequences that are the same, 1 bp- and 3 bp-different, respectively. Scale bar 1 indicates 1 bp-sequence divergence. Numbers in parenthesis indicate Genbank accession numbers.

Bf904F, has one mismatch with *Capnocytophaga ochracea* (AB032577), three mismatches with *Flexibacter sancti* (AB032582) and *Myroides odoratus* (AB034239) (Figure 1). The *gyrB* reverse primer, Bf1272R, shows strikingly high specificity by not showing any mismatch within the 3-bp difference. It only matches with *B. fragilis* (Figure 2).

Real-time PCR detection of *B. fragilis* and standard curves

A series of experiments was conducted with *B. fragilis* to compare the results obtained from the primers and probes specific to the reference and *gyrB* gene-based approaches. Additionally, for both approaches standard curves were developed for quantitative PCR. For this, 10-fold serial dilutions of known *B. fragilis* cells were prepared in the range of 7.0×10^2 to 2×10^7 (CFU mL^{-1}) and C_T values were checked with the 48-well StepOne™ Real Time System (Applied Biosystems) using the *gyrB*-based and the reference primers and probe after DNA extraction. Data points were plotted in each standard curve and the limit of quantification was illustrated in terms of C_T values (data not shown). For application of logarithmic functions, 0 cells (CFU mL^{-1}) were temporarily regarded as 1 cell. Linear regression analyses of the results with *gyrB* and the *Bacteroides/Prevotella* 16S rRNA primers and probes produced

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standard curves with similar slopes (−3.751, −3.506) and good correlation coefficients ($R^2 = 0.986$, 0.983). However, different *y*-intercepts were observed, 48.2 for *gyrB*-based and 39.5 for 16S rRNA-based *Bacteroides/Prevotella* primer sets. This indicates that in this study the reference primers and probe had greater sensitivity for *B. fragilis* detection than the *gyrB* gene-based primers and probe.

Interference of recreational water matrix and presence of other bacteria in real-time PCR detection of *B. fragilis*

We examined the possible interference due to the natural water matrix and the presence of other bacteria in the real-time PCR detection of *B. fragilis.* For this, *B. fragilis* (ATCC 25285^T) was spiked into the recreational water sample and PBS, and their detection was compared. A variety of negative controls including sterile MilliQ water, membrane filters, andnon-*Bacteroides* (*E. coli*, *Prevotella melaninogenica*, *Pseudomonas aeruginosa*, *Salmonella enterica*), and other *Bacteroides* (*B. ovatus*)-spiked water were tested using the same condition of real-time PCR. The results demonstrate that the presence of *B. fragilis* in the recreational water and in the PBS produced very similar C_T values when detected with the *gyrB*-based primers (Figure 3). Analysis of covariance (ANCOVA) was performed with the fitted curves from the PBS and the recreational water matrices in order to determine whether the slope and intercept between these two curves are similar. The result shows that there was no statistically significant difference between the two water matrices in measuring *B. fragilis* using the *gyrB*-based real-time PCR method ($p = 0.53$). It also indicated that the entire procedure of the real-time PCR detection including DNA extraction and purification effectively removed possible interference that may be present in the natural recreational water matrix. The negative controls (sterile MilliQ water, membrane filters, non-*B. fragilis* and non-*Bacteroides*) all produced negative signals.

Comparison of real-time PCR sensitivity in detecting *B. fragilis*

In order to determine the sensitivity of the real-time PCR method in detecting *B. fragilis*, a series of samples containing different levels of *B. fragilis* were tested with the two

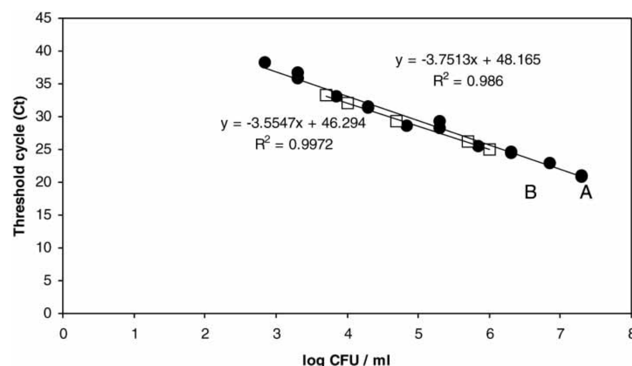

Figure 3 | Examination of interference of recreational water matrix in detecting *B. fragilis* with *gyrB*-based real-time PCR. Cultured *B. fragilis* cells were spiked into PBS solution (A) and a recreational water sample (B) and their levels were detected with real-time PCR and plate count.

sets of primers and probes (Table 2). With the real-time PCR using the *gyrB*-specific primers and probe, valid C_T values were obtained from all four samples containing 100–1,000 cells (CFU mL^{-1}), and three of four samples containing 10–100 cells. The *gyrB*-specific primers and probe demonstrated 100% accuracy in detecting the samples containing 100–1,000 cells (CFU mL^{-1}), 75% accuracy in detecting 10–100 cells. No C_T values were detected in samples when bacterial levels were less than 10 cells. In contrast, C_T values were generated from all samples in the 16S rRNA-based *Bacteroides/Prevotella* primers and probe method even when there no cells were added. In contrast, the *gyrB*-based assay did not generate C_T values when there were no bacterial cells present. It is noteworthy that, in the range of less than 10 cells, all detected C_T values using 16S rRNA-based method were above 40 (mostly 40–41). These high C_T values could possibly be interpreted as invalid since they were located beyond the valid range of the standard curve which had a *y*-intercept of 39.5.

Detection of *B. fragilis* from recreational water samples

Quantitative real-time PCR detection of *B. fragilis* was performed from the Olentangy River water samples and the C_T values from the different DNA concentration procedures were compared (Table 3). The positive-control after filtration of 250 mL shows a C_T value of 33.35 ± 0.26 and this corresponds to 3.9 ± 0.1 log CFU based upon the standard curve. The detection efficiency was enhanced by increasing

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Table 2 | Comparison of the sensitivity of real-time PCR in detecting *B. fragilis* by the two primers and probes

B. fragilis concentration (cells mL^{-1})	No. of replicates	16S rRNA primers and probe			*gyrB* specific primers and probe		
		No. positive[a]	No. negative[b]	% Positive	No. positive	No. negative	% Positive
100–1,000	4	4	0	100	4	0	100
10–100	4	4	0	100	3	1	75
1–10	4	4	0	100	0	4	0
0	4	4[c]	0	100	0[d]	4	0

The bacteria were spiked in PBS and serially diluted.
[a]C_T values were detected.
[b]C_T values were not detected.
[c]C_T values were detected after 40 cycles (40–41) and they were recorded as positive. The total cycle was 45.
[d]C_T values were not detected during the entire 45 cycles.

the water filtration volume. Improved DNA concentration was achieved by two steps: sonication and DNA post-concentration. Sonication was used for more efficient detachment of the bacterial cells from the membrane filter. DNA post-concentration was achieved by air-drying the extracted DNA and then reconstituting it with 1/10-volume of sterile MilliQ water. The lowest level of detection within the quantification range was $C_T = 37.67 \pm 0.44$, which corresponds to 63.1 ± 0.1 CFU/100 mL. This low detection limit was achieved by the combination of sonication and DNA concentration.

Environmental *B. fragilis* isolates

Environmental isolates (1AL, 1BL and 1B2) of *B. fragilis* were positively confirmed by *Bacteroides/Prevotella*, *gyrB* gene-based and *B. fragilis* group-specific primers (Table 4). The PCR products were visualized by agarose gel electrophoresis and the bands were located at the corresponding position of 150 bp (*Bacteroides/Prevotella*), 368 bp (*gyrB*) and 495 bp (*B. fragilis* group). The sequencing results showed that the three isolates amplified by all primer sets are identified as *B. fragilis* with 100% similarities. *B. fragilis* ATCC 25285^T, *B. ovatus* ATCC 8483^T and *P. melaninogenica* ATCC 25845^T were used as controls. Table 4 shows that the *gyrB* gene-based primer was the only one that targeted *B. fragilis* specifically, whereas the other two primers generated positive results for *B. ovatus* and *P. melaninogenica* as well as *B. fragilis*. These results suggest that the *gyrB* gene-based approach is a specific and suitable method for

Table 3 | Detection and quantification of *B. fragilis* in recreational water with *gyrB*-based real-time PCR

Sample	Concentration procedures: 250 mL filtering	1 L filtering	1 L filtering + sonication	1 L filtering + sonication + DNA concentration after DNA extraction
(+) Control	3.9 ± 0.1[a]	4.3 ± 0.1	5.3 ± 0.1	6.9 ± 0.0
Recreational water	ND[b]	ND	ND	2.8 ± 0.1
(−) Control	ND	ND	ND	ND

Various filtration volumes and DNA concentration procedures were compared.
[a]Unit: log CFU per each filtration volume.
[b]Not detected.

Table 4 | Comparison of the cross-amplification by end-point PCR with each primer set

Bacterial targets	End-point PCR amplification: *gyrB* marker (368 bp)	*B. fragilis*-group (495 bp) (Matsuki *et al.* 2002)	*Bacteroides/Prevotella* (150 bp) (Okabe *et al.* 2007)
B. fragilis			
ATCC 25285^T	+	+	+
Environmental isolate 1AL	+	+	+
Environmental isolate 1B2	+	+	+
Environmental isolate 1BL	+	+	+
B. ovatus			
ATCC 8434^T	−	+	+
P. melaninogenica			
ATCC 25845^T	−	−	+

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amplifying gene product from only *B. fragilis* in recreational water samples when *B. fragilis* is present.

CONCLUSIONS

The objective of this study was to develop a novel *B. fragilis*-specific primers and probe set for real-time PCR detection of human faecal contamination in recreational water. Using a TaqMan probe, a new method to rapidly detect *B. fragilis* in untreated recreational water samples was successful in achieving results within 3 h. The use of the 16S rRNA gene as a selective primer in PCR applications continues to be regarded as a common method; however, it is limited by only being able to successfully quantify the aggregate of *B. fragilis* and other closely related taxa. Indeed, our results confirmed that 16S rRNA gene-based reference methods are inherently unable to discriminate specific *Bacteroides* species from closely related taxa such as *B. ovatus* or the genus *Prevotella*. By targeting *gyrB*, specific primers for *B. fragilis* were designed with careful investigation of related sequences to avoid possible non-specific matches. The real-time PCR analysis of the *Bacteroides/Prevotella*-specific primer/probe set still provided better sensitivity than the *gyrB* gene-based primer/probe set. In contrast, the *gyrB*-based primer/probe set provided superior specificity for *B. fragilis* and generated no false-positive results with other *Bacteroides* or related *Prevotella*. Furthermore, the newly designed primers have good selectivity for detecting *B. fragilis* despite the presence of other bacteria in recreational water samples. Statistical analysis demonstrated that the recreational water matrix did not interfere with the *gyrB*-based qPCR assay.

We suggest that the *gyrB*-based assay can be considered for use in marine recreational waters. The important factors that may influence differences between freshwater and marine water are suspended solids and salinity. The majority of suspended solids can be removed during the prefiltration step. The influence of salinity in marine water on DNA extraction and the downstream PCR reaction may be negligible because of the rinsing steps involved in the initial procedure of the prefiltration, filtration and elution. The stability of the *gyrB* genetic marker in marine waters needs to be investigated, particularly because *B. fragilis* survivability may differ substantially in freshwater and marine environments. This novel rapid method can detect human-specific faecal contamination of recreational and potentially other environmental waters with better specificity than existing human-associated 16S rRNA-based methods used in microbial source tracking studies. In our previous study, we evaluated the specificity of the *gyrB*-based assay with animal and human faecal samples. Results showed superior specificity of the *gyrB*-based assay in characterizing human faecal sources (Lee & Lee 2010).

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