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Quantitative Analysis of Human- and Cow-Specific 16S rRNA Gene Markers for Assessment of Fecal Pollution in River Waters by Real-Time PCR

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The base sequences representing human- and cow-specific 16S rRNA gene markers identified in a T-RFLP analysis were recovered from clone libraries. The human- and cow-specific primers were designed from these sequences and their specificities were analyzed with fecal DNAs from human, cow, and pig. The AllBac primer set showed positive results for all human, cow, and pig samples, whereas the human-specific primer set showed positive result only for the human sample but not for the cow or pig samples. Likewise, the cow-specific primer set showed positive results only for the cow sample but not for the human or pig samples. Real-time PCR assay with these primers was developed for the identification and quantification of fecal pollution in the river water. The human- and cow-specific markers were detected in the order of 9 $\log_{10}$ copies per gram wet feces, which were two orders of magnitude lower than those of total *Bacteroidales*. For the river water samples, the human-specific marker was detected in 1.7–6.2 $\log_{10}$ copies/100 ml water, which was 2.4–4.9 orders of magnitude lower than those of total *Bacteroidales*. There was no significant correlation between total *Bacteroidales* and conventional fecal indicators, but there was a high correlation between *Bacteroidales* and the human-specific marker. This assay could reliably identify and quantify the fecal pollution sources, enabling effective measures in the watersheds and facilitating water quality management.

Keywords: 16S rRNA gene markers, *Bacteroidales*, fecal pollution, real-time PCR

The water quality of rivers, lakes, and reservoirs could be deteriorated as a result of fecal pollution. The fecal pollution mainly originates from point sources such as untreated sewage, combined sewer overflows, and effluents from wastewater treatment plants. In addition, nonpoint source discharges from agriculture and wildlife contribute to fecal contamination of natural waters.

The standard testing methods of fecal pollutions using fecal indicators, such as total coliforms, fecal coliforms, and *E. coli*, cannot distinguish their origins owing to the presence of the indicators in feces of humans and animals. Thus, it is necessary to develop a reliable method to identify and quantify the fecal pollution sources. If the source of the fecal pollution can be correctly identified and quantified, it would allow implementation of target-oriented measures in the watershed, such as installation of treatment plants and restoring of septic systems or sewers, and thus facilitating watershed management and helping securing drinking water sources [14].

Kreader [8] found that some species of *Bacteroides* were present only in the human, and designed primers to distinguish human from nonhuman fecal pollution sources. Bernhard and Field [2] developed host-specific fecal DNA markers for human and cow using terminal restriction fragment length polymorphism (T-RFLP). Qualitative and quantitative methods for detecting host-specific markers by conventional and quantitative PCR (QPCR) using TaqMan or SYBR Green were developed in previous works [3, 10, 13]. Human-specific primers were also developed in some studies [4, 6, 10, 13, 14]. The HF183 marker, identified by Bernhard and Field [2], has been widely used as a human-specific marker in real-time assay [12, 17, 20]. HuBac primers developed by Layton *et al.* [10] amplified 90% of the human fecal samples, but also detected significant percentages of animal fecal samples. The human-Bac1 primer set designed by Okabe *et al.* [13] also detected human-specific marker's from cow and pig feces, although it was two orders of magnitude lower than for human fecal

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DNA. The BacHum-UCD primer set developed by Kildare *et al.* [6] detected 67% of human and 13% of dog fecal samples tested, but was not tested against swine feces. Reischer *et al.* [14, 15] developed BacH and BacR primer sets for the quantitative detection of human- and ruminant-specific fecal genetic markers by aligning the published 16S rRNA gene. However, most of the previously developed molecular markers were not useful in distinguishing fecal pollution sources in Korea.

In our previous study [5], new host-specific 16S rRNA genetic markers for human and cow were identified in a T-RFLP analysis, and their existence in the river water was tested. However, there have not been any researches on the quantification of host-specific DNA markers with real-time assay. The objectives of this study were (1) to recover the clones that have host-specific DNA markers identified in the T-RFLP analysis of our previous study, (2) to design the host-specific primer sets from recovered sequences for development of a real-time PCR procedure, and (3) to demonstrate that the real-time PCR assay can be used to identify and quantify the fecal pollution sources in natural water bodies.

Materials and Methods

Fecal Samples

Fecal samples were collected from 10 humans (including 8 adults and 2 children) 10 cows, and 5 pigs from livestock farms in Icheon City, Gyeonggi-do, Korea. River water samples were taken from 11 sites of four rivers two times (April and May 2009). The four rivers were Anseong, Jinwi, Osan, and Hwangguji, which comprise the Anseong river basin and are national water quality monitoring sites located in the southern part of Gyeonggi-do (Fig. 1). Two sites were selected from the Anseong river, and three sites, upstream, midstream, and downstream, were chosen for the others. The Anseong river basin has approximately over 2 million population, 100,000 cows, and 500,000 pigs. There are many small-scale livestock farms around the basin. Streams in this area are heavily polluted, except upstream of Jinwi stream (J1). The fecal pollution sources of these rivers were likely to be mainly human and partly cow or pig.

DNA Extraction

Fecal DNA was extracted from approximately 200 mg of fecal sample using the QIAamp stool DNA mini kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's recommendation. To extract DNA from the river water samples, about 200 ml of water was filtered using a 0.22-μm pore size Durapore membrane filter (Millipore, County Cork, Ireland). After filtration of the samples, DNA was extracted by using the xanthogenate–sodium dodecyl sulfate [1% potassium ethyl xanthogenate, 100 mM Tris-HCl (pH 7.4), 20 mM EDTA (pH 8.0), 1% sodium dodecyl sulfate, and 800 mM ammonium acetate] method [19]. The filter was cut in half, put into a 14-ml tube and 2.0 ml of buffer was added. Then, the tube was incubated at 70°C for 120 min. After incubation, the filter was vortexed briefly. The lysate was transferred into a 2-ml tube, placed on ice for 30 min, and then centrifuged at 20,000 ×*g* for 15 min. Part of the supernatant (750 μl) was transferred into a 1.5-ml tube and mixed with an equal volume of isopropanol. After incubation at room temperature for 10 min, the precipitated DNA was pelleted by centrifuging at 20,000 ×*g* for 20 min. The pellet was washed with 70% ethanol, dried down using a MicroVac concentrator, and resuspended in 100 μl of water.

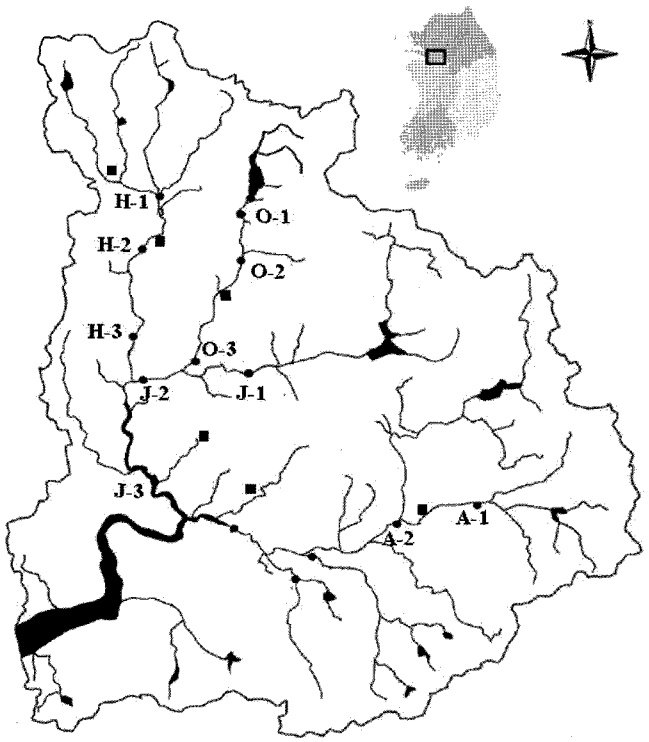

Fig. 1. Location of sampling sites in the Anseong river basin.
A, Anseong; J, Jinwi; O, Osan; H, Hwangguji River. 1, Upstream; 2, midstream; 3, downstream. ●, Sampling site; ■, Wastewater treatment plant.

Cloning, Sequencing of 16S rRNA Gene, and Phylogenetic Analysis

Equal portions of fecal DNAs from fecal sources were pooled to make samples representing humans, cows, and pigs. PCR reactions were performed to amplify the 16S rRNA gene of *Bacteroidales* from pooled fecal and water samples with Bac32F and Bac708R primers [2], yielding approximately 690-bp products.

The PCR product was cloned into competent TOP10F' One Shot *E. coli* cells (Invitrogen, Carlsbad, CA, U.S.A.) using a pGEM-T Easy Vector system (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions. Positive colonies were selected and the inserted region was amplified with M13F and M13R primers. The clones were analyzed with an ABI 3730 DNA sequencer using ABI prism BigDye Terminator Cycle sequencing V2.0 (Applied Biosystems, Foster city, CA, U.S.A.).

The sequences were edited with the Bioedit program and analyzed to eliminate chimeric sequences using the MALLARD software [1]. After aligning by MAFFT (version 6), the distance matrix was calculated by DNADIST (version 3.5c). This matrix was used to allocate the sequences operational taxonomic units (OTUs) by the DOTUR software [16], assigning >98% similarity to the same OTUs. Each representative sequence was compared with the GenBank database

using the NCBI-BLAST to determine their phylogenetic affiliation. Multiple alignments were performed using the ClustalW program and a tree was built by MEGA4 [18] using neighbor-joining algorithms.

Design of 16S rRNA Gene Host-Specific Primers

Restriction sites were searched to find clone sequences that had host-specific 16S rDNA markers identified in the T-RFLP analysis of our previous study [5]. The clone sequences representing human- and cow-specific markers were recovered from the clone libraries generated from Bac32F and Bac708R primers [2]. Each set of host-specific primer for human and cow was designed for the SYBR Green real-time PCR assay with the NCBI Primer-BLAST program (Table 1). The specificity of primer sets was verified with the Probe Match program of Ribosomal Database Project (RDP) II. We also confirmed the specificity by conventional PCR on fecal DNAs from humans, cows, and pigs. To detect total *Bacteroidales*, the primer set of Layton *et al.* [10] that was found to amplify all of the clone sequences in this study was selected and used in the SYBR Green assay.

Real-Time PCR Analysis

All the real-time assays, including for total *Bacteroidales*, were performed using the SYBR Green assay. In the real-time PCR assay, each reaction (20 μl) contained 10 μl of Lightcycler 480 SYBR Green master mix (Roche, Mannheim, Germany), 150 nM of each forward and reverse primer, and 5 μl of template DNA. Sample without any target DNA was used as a negative control for each assay. All real-time PCR assays were performed in Lightcycler 480 multiwell plates with Lightcycler 480 (Roche, Mannheim, Germany). The reaction was performed by incubation for 5 min at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 62°C (AllBac primer set), 63°C (human- and cow-specific primer sets) for 20 s, and extension at 72°C for 30 s. A melting curve analysis was performed after amplification to distinguish the target product from the nonspecific product.

Plasmid DNAs that have host-specific target sequences were extracted with the Accuprep plasmid extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer's instruction. The plasmid DNA concentrations were determined by a biophotometer (Eppendorf, Hamburg, Germany) and then used to generate standard curves for real-time PCR. For the quantitative analysis of fecal DNA markers, standard curves were generated from serial dilution of a known concentration of plasmid DNAs. Standard curves were plotted as threshold cycles against target 16S rRNA gene copy number.

Enumeration of Conventional Fecal Indicator Bacteria

To find the relationship between cell copy numbers obtained from real-time PCR and conventional fecal pollution indicators, the numbers of total and fecal coliform bacteria (MPN/100 ml) were determined by multiple tube fermentation technique (Standard Method)

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences determined in this study were deposited into GenBank under accession numbers from GQ921848 to GQ921913.

RESULTS

Phylogenetic Analysis of *Bacteroidales* 16S rDNA from Human, Cow, Pig, and River Water Samples

Bacteroidales 16S rDNA clone libraries were constructed for human, cow, and pig feces, as well as for river water samples. A total of 169 clone sequences obtained in this study (67 humans, 40 cows, 24 pigs, 38 river water samples, respectively), 22 environmental sequences, and 10 pure-culture sequences within the *Bacteroidales* order were used to generate a phylogenetic tree (Fig. 2). All of the sequences were found in *Bacteroides*-like or *Prevotella*-like groups. Chao1 richness estimates were 54, 58, and 20 for human, cow, and pig feces sequences, respectively, at the 2% cutoff, which demonstrated the high diversity of human and cow fecal sequences compared with pig fecal sequences. Phylogenetic analysis demonstrated that sequences obtained from human feces were mostly grouped into the Human cluster (59.7%) and two minor clusters, the Human-Cow (3.0%) and Human-Pig (34.3%) clusters. The Human cluster included several pure-culture *Bacteroides* such as *B. uniformis*, *B. ovatus*, *B. acidofaciens*, *B. salyersiae*, *B. thetaiotaomicron*, *B. vulgatus*, and *B. coprosuis*, whereas the Human-Pig cluster contained several *Prevotella* strains such as *P. bryantii*, *P. enoeca*, and *P. pallens*. Sequences from cow feces were also grouped into three distinct clusters, but different from those of human fecal sequences: the Human-Cow (67.5%), the Cow 1 (10.0%), and the Cow 2 (7.5%) clusters. Unlike sequences from human or cow feces, pig fecal sequences were mainly found in the Human-Pig cluster (87.5%) and did not constitute a separate pig-specific cluster. River water sequences were mostly found in the Human (63.2%), the Human-Pig (31.6%), and Cow 1 (5.2%) clusters, demonstrating that the river waters were contaminated with different sources including human, pig, and cow feces.

Table 1. Primers used in this study for real-time PCR.

Target	Name	Sequence	Reference
All *Bacteroidales*	AllBac296F	GAGAGGAAGGTCCCCCAC	[10]
	AllBac412R	CGCTACTTGGCTGGTTCAG	
Human-specific	YHF67F	GGGGCAGCATACTTAGCTTG	This study
	YHF210R	ATCATGTGAACATGCGGACT	
Cow-specific	YCF79F	GAGTGCTTGCACTTCTGTCG	This study
	YCF168R	GAGGTTTCCCTCGCTTATCC	

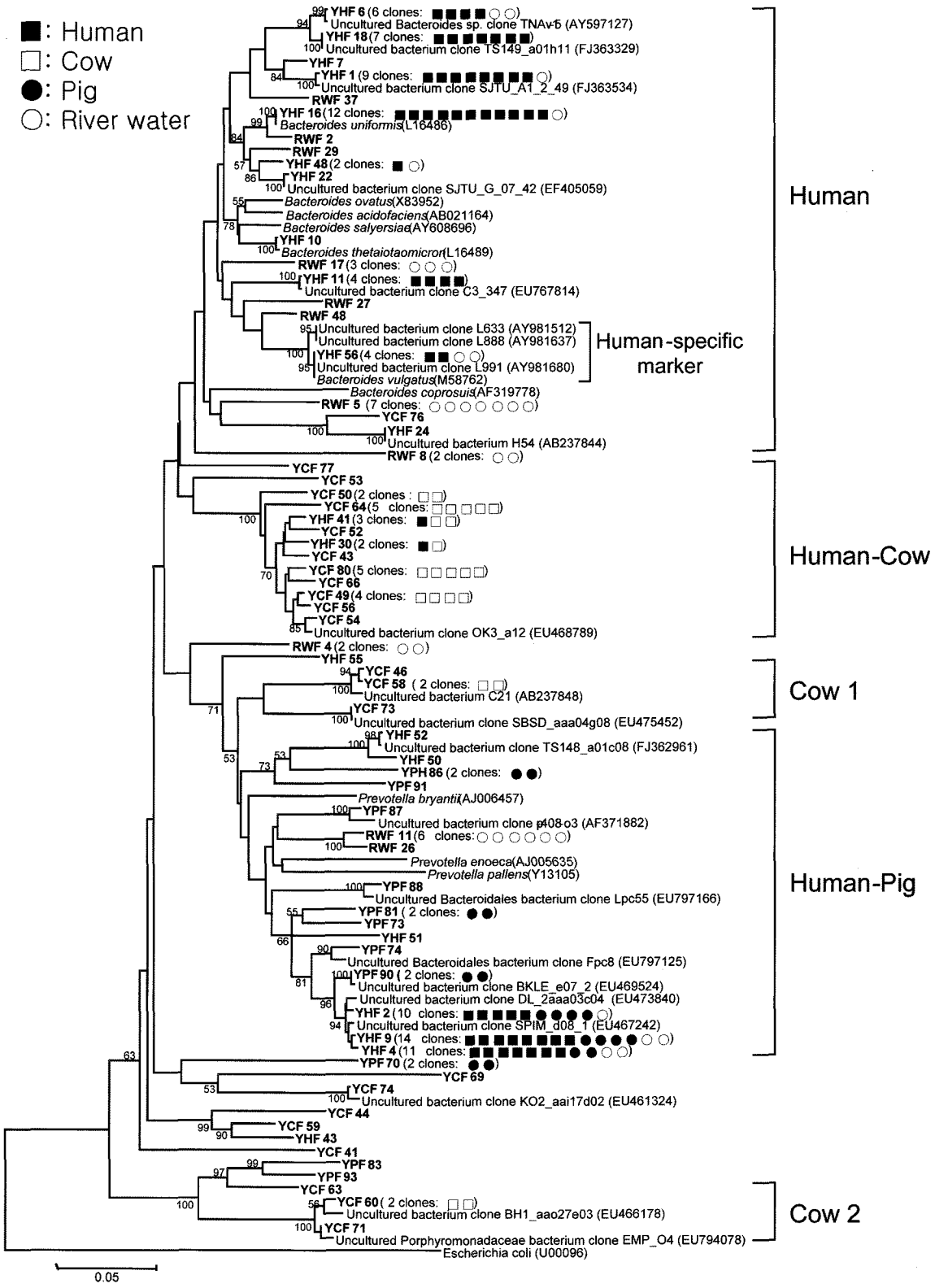

Fig. 2. Phylogenetic relationship of 66 OTUs among 16S rRNA gene sequences amplified using Bac32F and Bac708R primers [2] from fecal samples of humans (YHF plus clone number), cows (YCF plus clone number), pigs (YPF plus clone number), and river water (RWF plus clone number).

An OTU was defined by assigning 16S rRNA gene sequences of >98% similarity to the same species. Bootstrap values less than 50% were not included. The tree was built by using the neighbor-joining method in the MEGA4 program and *E. coli* was used to root the tree.

Design of Real-Time PCR Assay

The identification of human- and cow-specific *Bacteroidales* genetic markers from the clone libraries was based on our previous study [5], in which the restriction enzymes *Hae*III and *Rsa*I generated a 60-bp human-specific terminal fragment (t-RF) and 290-bp cow-specific t-RF, respectively, for the amplicons generated with the primer set Bac32F and Bac708R [2]. With *in silico* analysis, the YHF 56 clone was observed to have a specific 60-bp *Hae*III t-RF that belonged to the Human cluster (Fig. 2). YHF 56 clustered with *B. vulgatus* (M58762) and other uncultured bacterium clones (AY981512, AY981637, and AY981680), which were all recovered from human fecal samples and generated a 60-bp fragment for *Hae*III digestion. Thus, for this cluster, a PCR primer set (*i.e.*, YHF67F and YHF210R) was developed for the human-specific *Bacteroidales* genetic marker. Likewise, the YCF 71 clone was selected for the cow-specific *Bacteroidales* genetic marker within the Cow 2 cluster (Fig. 2) and a PCR primer set (*i.e.*, YCF79F and YCF168R) was developed. These primer sets yielded 149-bp and 89-bp PCR products for the human- and cow-specific *Bacteroidales* 16S rRNA gene, respectively. The sequence information is provided in Table 1. The specificity of primer sets was confirmed by the Probe Match program in RDP II (http://rdp.cme.msu.edu/probematch/search.jsp).

The sensitivity of the developed host-specific primer sets was evaluated from the plots of C_t versus gene copy numbers. As shown in Fig. 3, the linear range of quantification for SYBR Green real-time assay for AllBac, human-specific, and cow-specific primer sets were $50\sim10^7$, $10\sim10^7$, and $50\sim10^6$ copies per PCR, indicating the detection limit of 50, 10, and 50 target DNA copies, respectively. The results on detection limit suggested that the developed real-time assay was very sensitive. The slopes of standard curves varied from 3.25 to 3.35 for all real-time PCR reactions, and the correlation coefficients (R^2) were always higher than 0.99 (Fig. 3).

The specificity of the host-specific primer sets was confirmed with real-time assay and with conventional PCR of fecal DNAs extracted from target and non-target feces. As shown in Fig. 4, the AllBac primer set showed positive results for all human, cow, and pig samples, whereas the human-specific primer set showed positive result only for the human sample but not for the cow or pig samples, demonstrating that the developed human-specific primer had high specificity. Likewise, the cow-specific primer set showed positive results only for the cow sample but not for the human or pig samples. The AllBac primer set that covers all *Bacteroidales* quantified 11.9 ± 0.77 $\log_{10}$ copies per gram of human feces, 11.6 ± 0.32 $\log_{10}$ copies per gram of cow feces, and 11.4 ± 0.36 $\log_{10}$ copies per gram of pig feces. The human-specific primer set detected 9.7 ± 0.82 $\log_{10}$ copies per gram of human feces, which was two orders of magnitude lower than that of total *Bacteroidales* determined with the AllBac primer set. The cow-specific primer set detected 9.3 ± 0.48 $\log_{10}$ copies per gram of cow feces, which was also two orders of magnitude lower than that of total *Bacteroidales* determined with the AllBac primer set (Fig. 4).

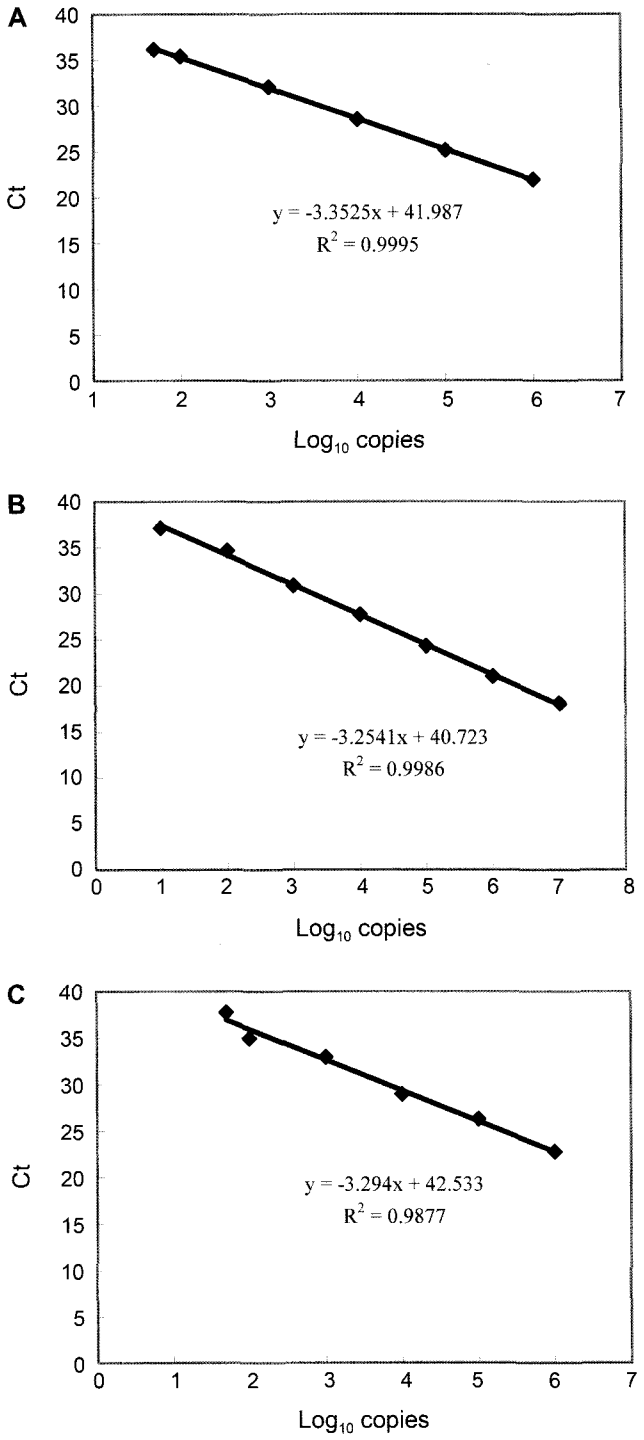

Fig. 3. C_t values plotted against known copy number of 16S rDNA. The standard curves for (**A**) total *Bacteroidales*, (**B**) human, and (**C**) cow were prepared from duplicated sets of serial dilution of plasmid DNA containing the target sequence.

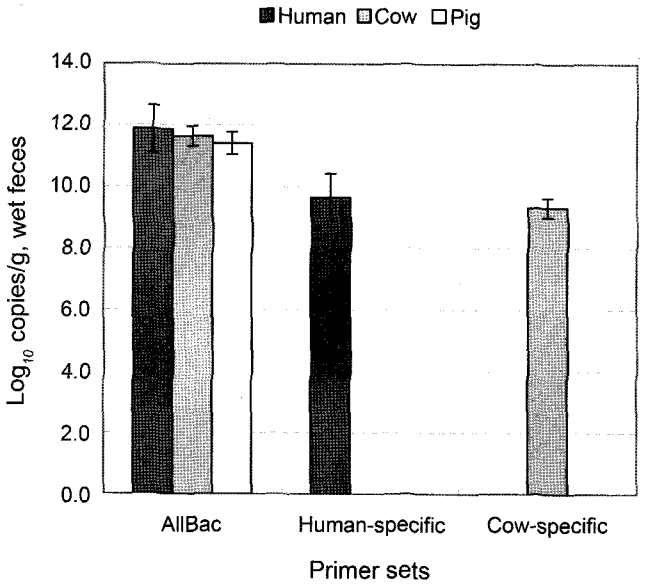

Fig. 4. Average number of copies $\log_{10}$ per gram of wet human (n=5), cow (n=5), and pig (n=5) feces as determined by SYBR Green real-time PCR assays with AllBac and newly designed host-specific primer sets.
The error bars represent standard deviations.

Quantification of Host-Specific Markers in Water Samples

Real-time PCR assay using total, and human- and cow-specific *Bacteroidales* primer sets developed in this study was carried out for the river water samples collected at 11 sites from the Anseong (O), Jinwi (J), Osan (O), and Hwangguji (H) rivers (Fig. 5). The total and human-specific *Bacteroidales* markers were detected in all water samples and showed high correlation between them (Fig. 6A). The average counts of conventional fecal indicators (total and fecal coliforms) and the average copy numbers of each total and host-specific *Bacteroidales* markers are shown in Fig. 5. For the water samples of April, total and fecal coliforms were 3.2–5.4 and 2.3–3.9 $\log_{10}$ MPN/100 ml, respectively. Total and human-specific *Bacteroidales* markers were 5.8–9.0 and 3.1–5.2 $\log_{10}$ copies/100 ml, respectively. The cow-specific *Bacteroidales* marker was detected in the downstream of Osan (O-3 site) and in the Hwangguji River (H-1, H-2, and H-3 sites), ranging 1.7–3.4 $\log_{10}$ copies/100 ml. For the water samples of May, total and fecal coliforms were 3.5–6.2 and 2.3–5.5 $\log_{10}$ MPN/100 ml, respectively. Total and human-specific *Bacteroidales* markers were 5.9–8.7 and 1.7–6.2 $\log_{10}$ copies/100 ml, respectively. The cow-specific marker was detected in the upstream (H-1 site) and downstream (H-3 site) of the Hwangguji River, ranging 1.7~2.8 $\log_{10}$ copies/100 ml. In the water samples, the human-specific *Bacteroidales* marker copy numbers were 2.4–4.9 orders of magnitude lower than the total *Bacteroidales*, the difference of which was higher compared with that (two orders of magnitude) in the fecal samples.

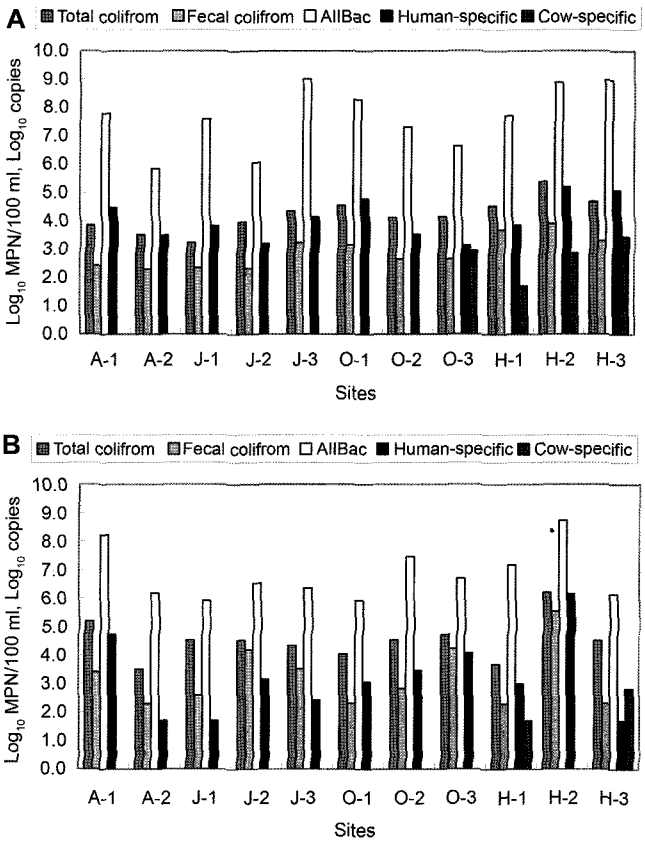

Fig. 5. Concentrations of total coliforms, fecal coliforms, total *Bacteroidales*, and human- and cow-specific 16S rDNA markers in the river waters collected from 11 sites in (**A**) April and (**B**) May.

Correlation Between *Bacteroidales* 16S rRNA Genetic Markers and Conventional Fecal Indicators

The correlation between the quantification of fecal pollution by the real-time assay developed in this study and by conventional fecal indicators was evaluated for the river water samples. As shown in Fig. 6, the total *Bacteroidales* 16S rRNA gene marker showed relatively low levels of correlation with total (R^2=0.26) and fecal (R^2=0.24) coliforms, whereas the human-specific *Bacteroidales* 16S rRNA genetic marker showed relatively high levels of correlation with total (R^2=0.32) and fecal (R^2=0.37) coliforms.

Discussion

In this study, we recovered *Bacteroidales* 16S rRNA genes from human, cow, and pig fecal samples, respectively, and designed human- and cow-specific *Bacteroidales* genetic markers based on the sequences. Then, we developed a real-time PCR assay based on SYBR Green chemistry to identify and quantify human and cow fecal pollutions in water samples. We were not successful in designing pig-

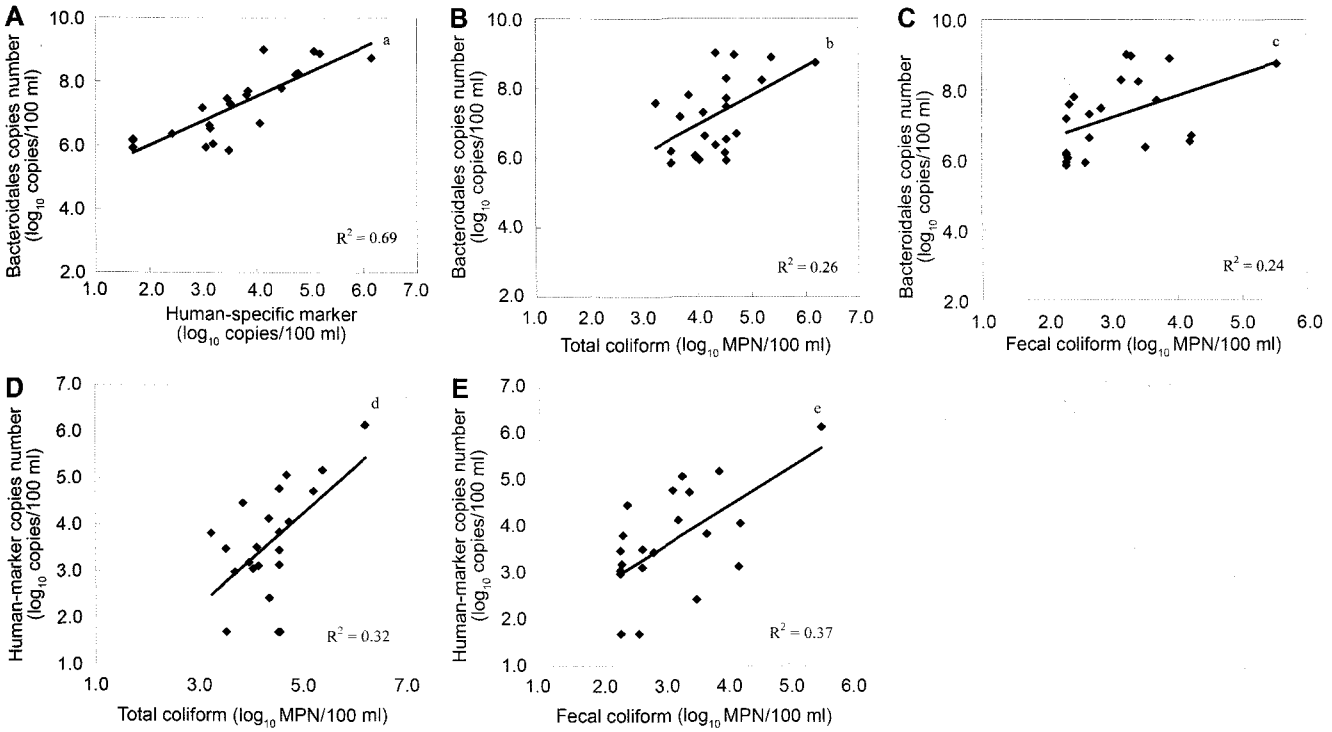

Fig. 6. Correlation between *Bacteroidales* 16S rDNA markers and conventional fecal indicators in the river waters.
(A) Total *Bacteroidales* marker vs. human-specific marker, **(B)** Total *Bacteroidales* marker vs. total coliform, **(C)** Total *Bacteroidales* marker vs. fecal coliform, **(D)** Human-specific marker vs. total coliform, **(E)** Human-specific marker vs. fecal coliform.

specific *Bacteroidales* genetic marker, mainly because sequences from the pig samples were clustered together with sequences from the human samples (the Human–Pig cluster in Fig. 2) and did not constitute a separate pig-specific cluster.

The AllBac primer set (*i.e.*, AllBac296F and AllBac412R) used in this study was originally developed by Layton and coworkers [10] for the quantification of total *Bacteroidales*. With *in silico* analysis, the primer sequences were observed to be perfectly matched with all the clone sequences recovered in this study, whereas the BacPRe1 primer set developed by Okabe and coworkers [13] did not cover all the clone sequences of this study.

Generally host-specific primers were designed from host-specific clone sequences recovered in each study. Several research groups introduced human-specific *Bacteroidales* genetic markers [3, 4, 6, 10, 13, 14]. However, the *in silico* analysis demonstrated that none of the genetic markers were perfect to distinguish human-specific markers in environmental samples. Some of the human-specific *Bacteroidales* genetic markers [3, 4] did not match any clone sequences recovered in this study, and others [6, 10, 13, 14] were found to amplify the non-target host. We also tested HF183F (human-specific primer), CF128F (cow-specific primer), and CF193F (cow-specific primer) developed by Bernhard and Field [3] with conventional PCR, but we did not get any specific PCR products. These results suggested that the host-specific primers developed previously were not very discriminative, except for a primer for total *Bacteroidales* (*i.e.*, AllBac [10]).

We designed and tested several primer sets with broader detection ranges. However, we did not find a host-specific primer set with a high specificity and broad detection range simultaneously. In specificity tests, HuBac [10] and Human-Bac1 [13] primers detected the majority of *Bacteroidales* microorganisms in human fecal samples. However, the Human-Bac1 primer set captured non-target pig and cow feces, although the magnitudes were 2–3 orders of magnitude less than target (*i.e.*, human feces) samples. The HuBac primer set detected significant percentages of animal fecal samples. One possible reason for the imperfect specificity of the host-specific genetic markers is that they include a relatively large phylogenic group, which increases the possibility of including non-target sequences. Thus, we designed the host-specific genetic markers by narrowing the range of target sequences to minimize nonspecific amplification

The human- and cow-specific primers developed in this study did not show any positive signals in non-target fecal samples (Fig. 4). However, the strategy for increasing specificity seemed to have sacrificed some of the recovery of target 16S rRNA gene copies. The levels of target

copies of human- or cow-specific *Bacteroidales* were two orders of magnitude lower than those of total *Bacteroidales* in the target fecal samples, occupying 0.62% and 0.29% of total *Bacteroidales* for human and cow feces, respectively. In this study, the human-specific marker was detected as 9.7± 0.8 $\log_{10}$ copies per gram of human feces compared with 9.9±0.3 and 11.3±0.7 $\log_{10}$ copies per gram of human feces reported by previous studies [11, 13]. This is probably because the human-specific marker copies per gram of human feces were varied among individuals, depending on the selected target sequences [11].

The real-time assay developed in this study showed the detection limits ranged 50~10^7, 10~10^7, and 50~10^6 copies per PCR for total *Bacteroidales*, human, and cow, respectively. The sensitivity of the assay is comparable with published ones [10, 13, 17], in which the detection limits ranged 4.3 to 200 copies per reaction.

It is reported that *Bacteroidales* have a different 16S rRNA gene copy number from species to species [7], which makes it difficult to estimate the exact copy numbers of *Bacteroidales*. Assuming the average copy number of the 16S rRNA gene of *Bacteroidales* is the same as that of *B. fragilis* (*i.e.*, six), the ranges of quantification were 8.3~1.7×10^6, 1.7~1.7×10^6, and 8.3~1.7×10^5 cells per PCR for total *Bacteroidales*, and human- and cow-specific markers, respectively. This demonstrated that the detection limits for river waters were from 8.5×10 to 4.2×10^2 cells per 100 ml of river water samples. However, the detection limit would in reality be changeable depending on many factors, such as filtering volume, DNA extraction, and PCR efficiency [13].

The *Bacteroidales* 16S rRNA gene markers showed a low level of correlation with conventional fecal indicators (R^2=0.26 with total and 0.24 with fecal coliforms). The human-specific marker demonstrated a relatively higher correlation than those of total *Bacteroidales* and fecal indicators (R^2=0.32 with total and 0.37 with fecal coliforms). Although a study showed a high correlation (R^2=0.85) between total *Bacteroidales* marker and *E. coli* [10], there seemed to be no significant correlation between *E. coli* [12] or coliform bacteria [13] concentration with total *Bacteroidales* and host-specific makers. This was probably because the river waters were contaminated with multiple fecal sources rather than a single source, even though the correlation between total *Bacteroidales* and human-specific markers is relatively high (R^2=0.69). This could also be explained by the fact that the concentration of human-specific marker was on average 3.6 orders of magnitude lower than that of total *Bacteroidales*, compared with 2 orders of magnitude lower than in the fecal samples. The real-time assay detected *Bacteroidales* 16S rRNA gene markers from live and dead microorganisms, whereas conventional fecal indicators were counted by a culture-dependent method, which could detect only culturable cells. Moreover, the survival of *Bacteroidales* is influenced by many factors, including dissolved oxygen, water temperature, sunlight, and presence of predators [9].

In summary, our real-time PCR assay with newly designed human- and cow-specific primer sets was successfully developed and could reliably discriminate and quantify human- and cow-specific fecal pollution in river waters. For future application, it will be necessary to estimate the occurrence of the markers in sediment and to develop a pig-specific primer set. After additional assessment, this assay could allow specific allocation of fecal pollution, enabling cost-effective actions in the watersheds and thus facilitating water quality management.

Acknowledgment

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