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Rapid Estimation of Numbers of Fecal *Bacteroidetes* by Use of a Quantitative PCR Assay for 16S rRNA Genes

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Assessment of health risk associated with fecal pollution requires a reliable fecal indicator and a rapid quantification method. We report the development of a *Taq* nuclease assay for enumeration of 16S rRNA genes of *Bacteroidetes*. Sensitivity and correlation with standard fecal indicators provide experimental evidence for application of the assay in monitoring fecal pollution.

The enumeration of fecal indicators is the cornerstone of testing for fecal pollution in recreational, potable, and shellfish water. There is currently no single bacterial indicator used in all water systems. Total coliforms are the U.S. Environmental Protection Agency (EPA) standard indicators of pollution for drinking water (14), *Escherichia coli* and enterococci are approved for freshwater (12), enterococci are recommended for marine water (12), and fecal coliforms are used for shellfish waters (15). Traditional membrane filtration and most probable number (MPN) methods require 24 to 74 h for enumeration. Recent updates in methods include the Colilert-18 test (Idexx Laboratories, Westbrook, Maine), which has received U.S. EPA approval for use in ambient water testing (13). Colilert-18 uses a defined substrate medium to test colorimetrically for *E. coli* and total coliforms within 18 h. *E. coli* is considered a more specific fecal indicator than total or fecal coliforms, which are found in ambient water in the absence of fecal pollution (11). Epidemiological studies have established a correlation between standard fecal indicators and associated human health risks (5, 7, 12).

Fecal members of the class *Bacteroidetes* have distinct advantages over coliforms and *E. coli* as fecal indicators. They are more abundant in the feces of warm-blooded animals than *E. coli* (8). They are likely to predict recent fecal contamination because they are obligate anaerobes and are unlikely to survive long outside the intestinal tract (1, 8). Enterococci and *E. coli* are facultative anaerobes, and they can proliferate in soil, sand, and sediments (6, 10, 16, 17).

Bernhard and Field (3, 4) developed 16S rRNA gene (rDNA) markers from *Bacteroidetes* to detect fecal pollution and to distinguish between human and ruminant sources by PCR. Markers for additional host sources have been recently developed (L. K. Dick, A. E. Bernhard, T. J. Brodeur, J. W. Santo Domingo, J. M. Simpson, S. P. Walters, and K. G. Field, unpublished data). PCR source identification is rapid, specific, and sensitive, and it does not require maintenance of databases or libraries of bacterial isolates.

Here we report a quantitative *Taq* nuclease assay (TNA) (2, 9) for general fecal pollution using a *Bacteroidetes* 16S rDNA marker. The TNA was compared with the Colilert-18 system for accuracy, range, and limits of quantification in serial dilutions of primary sewage influent.

A fluorogenic probe and primer set was designed for *Bacteroidetes* 16S rDNA by using the Primer Design function in the ARB software program (Ludwig and Strunk, Munich, Germany). The sequences were verified for use in a TNA with Primer Express software (PE Applied Biosystems, Foster City, Calif.). The primers did not bind to fecal bacteria outside the class *Bacteroidetes* when up to five mismatches were chosen by the ARB Probe Match program. They did amplify 16S rDNA of *Bacteroidetes* from human, cow, dog, cat, pig, elk, deer, and gull feces. Sequences used were AACGCTAGCTACAGGCT TAACA (3), ACGCTACTTGGCTGGTTCA (this study), and CAATATTCCTCACTGCTGCCTCCCGTA (this study), for the forward primer, reverse primer, and probe, respectively.

We collected 1 liter of primary influent from the Corvallis Wastewater Reclamation plant in Corvallis, Oreg. It was transported and stored in a sterile polypropylene container on ice. Six separate 10-fold serial dilutions to 10^{-10} were made in 100-ml volumes in sterile glass containers with nanopure water. Colilert-18 tests were performed on three of the dilution sets. The other three sets were filtered, and the bacteria were extracted and used in the TNA. A nondiluted influent sample was not included in the experiment because it clogged the filter.

Three sets of 100-ml primary influent serial dilutions were filtered through 47-mm-diameter, 0.2-μm-pore-size filters (Supor-200 membrane disk filters; Pall Gelman Laboratory, Ann Arbor, Mich). The glass filtration apparatus was heat sterilized prior to use and was soaked for 3 min in 20% bleach and rinsed under distilled water between filtrations. The filters were placed in 500 μl of guanidine isothiocyanate buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH 8], 0.5% Sarkosyl) in 15-ml polypropylene tubes. DNA was extracted by using the DNeasy tissue kit (QIAGEN, Valencia, Calif.) with a slightly modified protocol. We omitted the proteinase K digestion, and 500 μl of QIAGEN AL buffer was added to the guanidine isothiocyanate filter and vortexed for 1 min. A second wash step was used to ensure a clean product, and the DNA was eluted in 200 μl of Tris-HCl buffer.

Amplifications were run on an ABI Prism 7700 (PE Applied Biosystems). The 25-μl PCR mixtures included 1× TaqMan

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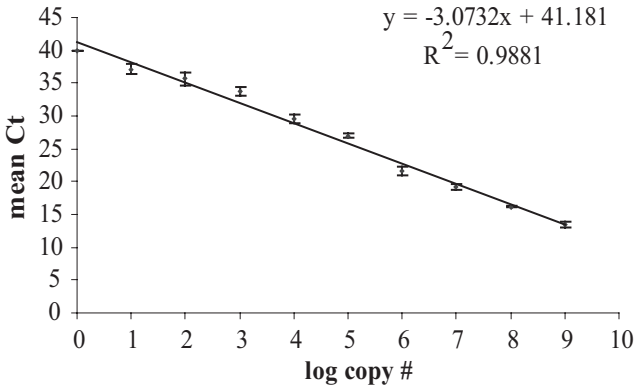

FIG. 1. TNA dynamic range for enumeration of 16S rDNA of *Bacteroidetes*. Plasmid DNAs containing known template copy numbers were added in 10-fold serial dilutions to create the standard curve. Mean threshold cycle values (Ct) are plotted against corresponding DNA log concentrations of *Bacteroidetes*. Error bars represent the standard deviations among results of triplicate PCRs.

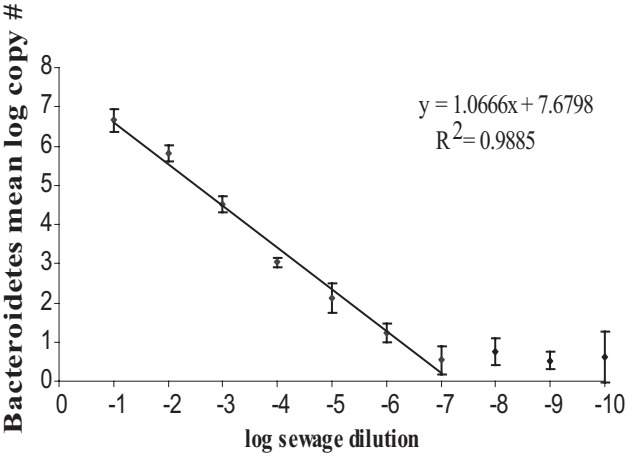

FIG. 2. TNA analysis of 16S rDNA copy numbers of *Bacteroidetes* in serial dilutions of sewage primary influent. Geometric mean copy numbers are plotted against log serial sewage dilutions. The error bars represent the standard deviations from results of three replicate serial dilutions of sewage.

buffer A (PE Applied Biosystems), 3.5 mM $MgCl_2$, 400 μM dUTP, a 200 μM concentration of each remaining deoxynucleoside triphosphate, a 0.4 μM concentration of each primer, a 0.2 μM concentration of the fluorogenic probe, 0.06% bovine serum albumin, 0.25 U of uracil-*N*-glycosylase, 0.63 U of AmpliTaq Gold, and 2 μl of template DNA. Cycling parameters were 2 min at 50°C for uracil-*N*-glycosylase activation, 10 min at 95°C for denaturation, and 40 cycles of 15 s at 95°C, followed by 1 min at 60°C for annealing and extension. All of the reaction mixtures were run in triplicate, and a standard curve was created from serial dilutions of plasmid DNA containing known copy numbers of the template.

The Colilert-18 kit was used with the manufacturer's protocol. Briefly, medium was added to each 100-ml diluted sample, mixed, and transferred to 97-well trays (Quanti-Tray 2000; Idexx Laboratories). The trays were sealed and incubated at 35°C for 18 h. Wells that turned yellow were positive for coliforms, and wells that fluoresced under UV light were positive for *E. coli*. An MPN chart provided by the manufacturer was used for enumeration.

The threshold cycle method used in a TNA allows a wide, dynamic range for the calibration of copy numbers to standards. During 40 cycles of PCR, standards were quantified over 8 orders of magnitude, down to 10 copies (Fig. 1). The primers provided specificity, while the probe was more general. Becker et al. (2) found that using primers with specificity greater than or equal to that of the probe helped to reduce the PCR bias inherent when a background of complex DNA is present.

There was a linear decrease in copy numbers of 16S rDNA of *Bacteroidetes* as a function of serially diluting the sewage influent (Fig. 2). The decrease remained linear over 7 orders of magnitude. After the assay reached its minimum detection level of about 10 copies, it became inconsistent. The linearity of the dilution curve shows that neither PCR inhibitors nor the presence of large amounts of heterologous DNA inhibited amplification, even at the highest copy numbers.

16S rDNA copy numbers of *Bacteroidetes* are compared with coliform and *E. coli* MPNs in sewage dilutions in Fig. 3. Geometric means were used, since they are the standard for coliform and *E. coli* data (12). The Colilert-18 upper limit of enumeration is 2,419 organisms for both coliforms and *E. coli* (Idexx Laboratories). This limit was reached or exceeded in the first three serial dilutions for coliforms and in the first two dilutions for *E. coli*. Copy numbers of *Bacteroidetes* were quantifiable in the first three dilutions. Lower limits of enumeration for Colilert-18 (one organism per 100 ml of dilution [Idexx Laboratories]) were reached at the 10^{-6} dilution for *E. coli* and the 10^{-7} dilution for coliforms. The limit for *Bacteroidetes* of 10 copies was also reached at the 10^{-7} dilution. Because cells of *Bacteroidetes* contain multiple copies of the 16S rRNA gene, 10 copies represents as few as one or two cells, and it suggests that the lower limits of enumeration for all three groups are approximately the same.

Simple linear correlation analysis of the 10^{-4} to 10^{-7} dilutions showed that mean concentrations of *Bacteroidetes* were highly and positively correlated with both coliform and *E. coli* mean concentrations (*r* values of 0.999 for both comparisons). This dilution range contains the U.S. EPA threshold concentration for *E. coli* in ambient water above which the health risk from waterborne illness is deemed unacceptably high (5-day geometric mean of 126 organisms/100 ml) (12).

The TNA for 16S rDNA of *Bacteroidetes* is rapid, sensitive, and reproducible in sewage dilutions. It correlates with present standard indicators but takes only 3 to 4 h to complete. Recent advances in rapid thermocycling allow 40 cycles of PCR to be completed in 30 min, potentially reducing the assay time even more. The quantitative range spans 8 orders of magnitude, compared with approximately 4 orders for *E. coli* enumeration. However, it will be necessary to validate the temporal and spatial application of the assay by obtaining data from additional sites and over several seasons.

The quantitative assay described here provides a framework for expanding the use of PCR indicators for *Bacteroidetes* beyond fecal source tracking and into a health risk-based analysis of fecal pollution.

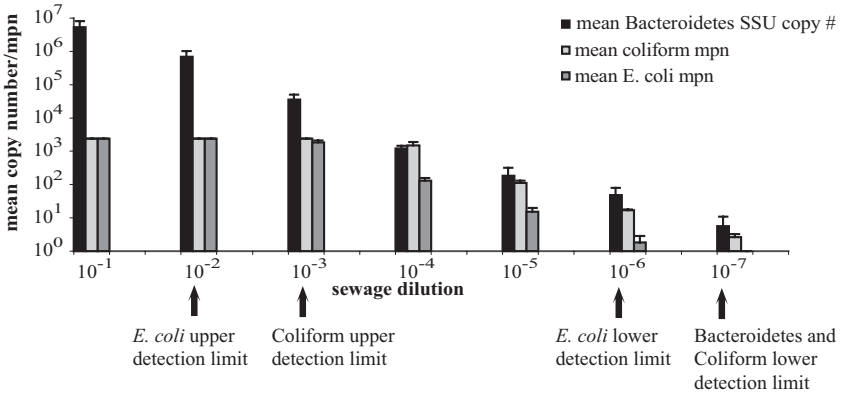

FIG. 3. Comparison of copy numbers of 16S rDNA of *Bacteroidetes* with coliform and *E. coli* MPNs in 10-fold primary-influent dilutions. Equivalent sewage volumes (100 ml) were used for the TNAs and the Colilert assays, but only 1 μl of the total filtered, extracted DNA was used in the TNA. The MPN and copy number data were log transformed. The error bars represent 1 standard deviation based on geometric means of results for triplicate sewage dilutions.

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ADDENDUM IN PROOF

When tested on seawater samples, the quantitative assay reported here amplified nonfecal *Bacteroidetes* species, including *Cytophaga fermentans*. New quantitative primers GCTCA GGATGAACGCTAGCT (forward) and CCGTCATCCTTC ACGCTACT (reverse), specific for sequences adjacent to and partially overlapping those specified by the original primers, amplified fecal *Bacteroidetes* only but otherwise had quantitative characteristics identical to those of the original primers.

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