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The evaluation of PCR-based techniques for the detection of fecal indicator organisms in environmental samples

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THE EVALUATION OF PCR-BASED TECHNIQUES FOR THE DETECTION OF FECAL INDICATOR ORGANISMS IN ENVIRONMENTAL SAMPLES

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DEDICATION

I would like to dedicate this work in honor of my family, especially my ancestors. They blazed a path so bright that all I had to do was follow. They not only gave me the opportunity to dream, but to realize those dreams. It is on their backs and because of their prayers and strength that I have arrived at this point. For them and everything I am and will be, I give all thanks and praise to God.

Momma, all that I do and that I am is a direct reflection of your love and is always dedicated to you.

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ABSTRACT

Escherichia coli is the indicator organism of choice for the detection of fecal contamination. Standard methods for detecting fecal contamination often rely on detecting fecal coliforms. While this practice if highly efficient in most applications, when used with the sediment-laden waters of Louisiana, free-living soil bacteria, such as *Klebsiella pneumoniae* were shown to inflate the fecal coliform counts. An accurate assessment of the contamination problem requires methods that detect *E. coli*. To detect *E. coli*, several of species-specific genes were tested before the *gadAB* genes were selected. The polymerase chain reaction (PCR) was chosen to detect these genes using a nested primer array. Using the nested PCR assay, *E. coli* was routinely detected in laboratory samples down to the single-cell level. This level of detection was deemed essential for a quantitative or semiquantitative assay to determine *E. coli* numbers. The most beneficial use of PCR-based techniques may be the use of specific fecal indicator organisms to differentiate the sources of fecal contamination. *E. coli* is the indicator of fecal contamination from essentially all sources posing a risk to human health. The *Bacteroides fragilis* group (BFG) demonstrates strong potential as a fecal indicator organism of human waste. While the levels of *E. coli* in humans and cattle are comparable, the levels of BFG are approximately 10^5 times greater in humans than cattle. By determining the ratio of *E. coli* to BFG in contaminated water, one should be able to distinguish fecal contamination from humans and common farm animals, such as dairy cattle. This requires the determination of the relative levels of *E. coli* and BFG and not the absolute levels in the sample. Using the nested PCR method, a region of the

16S rRNA genes specific to members of the BFG was targeted. In the laboratory, *B. fragilis* cells were detected down to the single-cell level. When environmental water samples were tested, impurities in the template preparations limited the amount of DNA template that could be added to the reactions. Within those limitations the level of detection was comparable to laboratory studies using cultured cell suspensions.

Introduction

The microbiological quality of surface water is a primary environmental concern. This concern is warranted whether it centers on the monitoring of potentially consumable water supplies or recreational water systems. While *Enterococcus faecalis* is the primary fecal contamination indicator in many European countries, *Escherichia coli* has been designated as the principle indicator organism in the monitoring of consumable as well as recreational water supplies and systems throughout the United States.

Escherichia coli belongs to the coliform group, which are identified as Gram-negative, aerobic, or facultative anaerobic non-endospore forming rods that ferment lactose with gas production at an incubation temperature of 35 ºC. Fecal coliforms, which include *E. coli*, are distinguished from coliforms by an incubation temperature 44.5 ºC. Fecal coliform determinations are commonly used as an indicator for the potential presence of pathogens in environmental samples. Fecal coliform determination from environmental sources can include bacteria of non-fecal origin, including species of *Klebsiella* and *Citrobacter* (Wright, 1982; Paille et al., 1987). Most methods used for the simultaneous detection of total coliforms, fecal coliforms, and *E. coli* in environmental samples have centered on the enzymatic hydrolysis of chromogenic and fluorogenic substrates by the enzymes *β*–D-galactosidase and *β*–D-glucuronidase (Brenner et al., 1993). These assays were predicted on the supposition that *β*–D-galactosidase and *β*–D-glucuronidase enzymatic activities indicate the presence of total coliforms and *E. coli*, respectively.

Chromogens and fluorogens produce a visual signal when cleaved by a specific enzyme that is present or is being expressed by a particular organism. Media containing the substrate *o*-nitrophenyl-β-D-galactopytanoside (ONPG) is used to demonstrate the presence of *β*-D-galactosidase, an enzyme produced by coliforms. Upon hydrolysis of the substrate ONPG, a yellow color, 0-nitrophenol, is released to indicate the presence of coliforms producing *β*-D-galactosidase activity. Media may also be supplemented with the substrate 4-methylumbelliferyl- β-D-glucuronide (MUG), which has been used to detect the presence of *E. coli* in dairy products, shellfish, water, wastewater, urine, and other clinical samples by means of *β*-D-glucuronidase enzyme production (Clark et al., 1990). Following cleavage of the fluorogenic substrate MUG, samples produce a blue fluorescence when viewed under ultraviolet light. This observation signals the production of *β*-D-glucuronidase by the organism, usually *E. coli*, under examination (Bej et al., 1991). *Shigella spp*. can also produce a positive MUG result because they also produce*β*–D-glucuronidase. Although *Shigella* tests positive for MUG, they test negative for ONPG activity and can be distinguished from *E. coli* and other coliforms.

Currently, the membrane filtration (MF) technique is the approved method to detect total and fecal coliform numbers in water samples. This technique is used in association with several different media and two different incubation temperatures. Water in volumes which range from 0.0001-100ml, depending on the source, is passed through a sterile membrane filter. The filter is then placed onto a sterile absorbent pad saturated with the enrichment medium lauryl tryptose broth (Standard Methods, 1998).

If an agar-based medium (*e.g.*, LES Endo agar) is used, the filter is separated from the pad and "rolled" onto the agar surface. If a liquid media (*e.g.*, M-Endo medium) is used, the filter is transferred to a new absorbent pad saturated with the liquid medium. The membrane-agar plates are then inverted and incubated for 22 to 24 hours at 35 °C for total coliforms or 44.5 °C for fecal coliforms. Pink to dark red colonies with a metallic sheen are enumerated as coliforms using this method (Standard Methods, 1998). The coliforms are enumerated by direct counts. The combined MF procedure, which tests for total coliforms or fecal coliforms and *E. coli* may require 28 to 72 hours to complete. The MF test, using MUG, is a confirmatory test for *E. coli* that demonstrates the presence or absence of the organism only.

The most probable number (MPN) serial tube test for fecal coliforms may also require 72 hours to complete. This method is based upon gas production by coliforms from the utilization of lactose. This statistical approach to enumerating bacteria uses selective and differential broth media along with a series of confirmatory tests. The MPN technique consists of a presumptive, confirmed, and completed test. The presumptive test demonstrates gas production through the use of lauryl tryptose broth after 48 hours of incubation. The number of positive tubes at each inoculation volume is applied to an MPN table to estimate the number of organisms in a 100 ml sample. Samples from positive tubes (showing gas production) are then transferred to tubes of brilliant green lactose bile broth (confirmed test) to "confirm" the presence of coliforms through gas production. This formulation is designed to inhibit the growth of non-coliforms. Following a 48 hours incubation, samples demonstrating gas production are then inoculated into brilliant green lactose bile broth for total coliforms and EC broth for fecal

coliforms in the completed test (Standard Methods, 1998). Each test is incubated at either 35 °C or 44.5 °C depending on the type of organism that is to be isolated.

One test approved recently for the evaluation of drinking water for the presence of *E. coli* is Colilert (IDEXX Laboratories, Inc., Westbrook, Maine). It is a liquid medium that contains the ONPG-MUG (MMO-MUG) chromogenic substrates designed to detect both coliforms and *E. coli* within 24 to 28 hours (Brenner et al., 1993). The medium is inoculated with 100ml of an environmental water sample, sealed within a multi-well tray and incubated at either 35 °C or 44.5 °C. This medium can be used in either the MPN or presence-absence format.

Some problems that may occur when utilizing rapid environmental assays are the potential for the test results to be influenced by "false-positive bacteria" (Tryland and Fiksdal, 1998), as well as *E. coli* false-negatives (Brenner et al., 1993). "False-positive bacteria" are β–D-galactosidase positive, but they are not coliforms. This group may include: Gram-negative bacteria in the *Enterobacteriaceae, Vibrionaceae, Pseudomonadaceae,* and *Neisseriaceae* families; various Gram-positive bacteria; and occasionally, yeasts, protozoa, and fungi (Tryland and Fiksdal, 1998). In addition, approximately, 30% of fecal coliforms are undetected because of "*E. coli* false-negatives" (Chang et al., 1989), which is attributed to high levels of β–D-glucuronidase negative *E. coli* in some specimens (Bej et al., 1990).

Traditional methods to identify fecal bacteria include assorted culture techniques, bacteriological isolation methods, biochemical tests, morphological examination and analysis of volatile and nonvolatile fatty acid production. There are also approaches, which use gas-liquid chromatography that profiles bacterial cellular fatty acids to identify

various species (Wang et al., 1996). Most of these methods have proven to be extremely labor-intensive, time-consuming, and expensive.

In the evaluation of molecular approaches in microbial identification, hybridization provides a view of genomic relatedness between a particular strain or species and a specific probe labeled with a radioisotope. These hybridizations can be done even for organisms that have not been fully characterized, including environmental or clinical isolates (Murray et al., 2001). The DNA relatedness of closely related organisms can also be determined through hybridization.

The introduction of polymerase chain reaction-based methods has dramatically changed the potential for commercializing DNA probe technology. This approach is rapid, convenient and cost-effective (Amhelm and Levenson, 1990). PCR-based methods amplify a "unique" target DNA sequence. This *in vitro* method of amplifying DNA sequences begins with DNA of any origin and over several hours increases the quantity of the target sequence hundreds of millions of times.

Emergence of PCR as a commercial DNA probe can be accredited to a few key points. It reduces the difficulty of isolating and manipulating specific DNA sequences. The reaction makes it possible to study biological problems without being limited by the amount of biological material available. PCR is a rapid and sensitive approach.

Employing nested PCR can enhance the sensitivity of the PCR process. Nested PCR utilizes the initial PCR product as a DNA template for a second round of amplification. This round of PCR can be used to confirm the presence of the targeted DNA sequence by amplifying a shorter region within the initial PCR product. Nested PCR is especially beneficial when the expected DNA product is obscured by artifacts

caused by an excessive number of cycles when trying to amplify a very low number of templates (Bell and DeMarini, 1991).

Recent developments in quantitative PCR technology now allow the rapid cycling of PCR combined with fluorescence-based identification and verification of PCR products using a light cycler instrument in real-time PCR (Bellin, et al., 2001). Dyes can be designed to bind nonspecifically to double-stranded DNA and hybridization probes. This allows instantaneous sequence-specific detection using fluorescence energy transfer (FRET) between two fluorophores (Livak et al, 1995). The fluorescence can then be measured in different channels. Another method to quantitate the number of PCR templates in a sample is the use of limiting dilution. The PCR is optimized to give an all-or-nothing endpoint with very few DNA targets present. The reaction yields a distribution of positive and negative tests. Quantitative results can then be obtained by consulting a most probable number table (Sykes et al., 1992)

PCR-based methods to simultaneously detect and quantify targeted DNA have also been developed. One method utilizes a competitor PCR template cloned into another organism, frequently *E. coli*, and placed into a vector (Hyman et al., 2000). The end product numbers are then estimated by comparing the ratio of products generated by the target DNA and competitor template DNA following PCR.

For food and environmental samples, reverse transcriptase PCR has emerged as a viable technique for evaluating water samples. It is relatively easy to perform and gives fast, sensitive, and specific results. The process utilizes mRNA as a template to be amplified. Using the reverse primer and reverse transcriptase, cDNA is derived, *in vitro*, of the mRNA region. The cDNA is then transferred into a standard PCR and amplified

(Hill, 1996). Reverse transcription followed by PCR (RT-PCR) can reduce the time for analysis in most cases, but the isolation and processing of samples for the detection reaction still requires a great deal of time and may rely on costly equipment. One disadvantage of PCR-based techniques is that they amplify DNA from viable and nonviable cells. Positive results may be obtained from sterilized materials. In environmental samples the persistence of intact "naked" DNA is possible but rare. Bare DNA that is not intact is quickly degraded.

The key to using PCR-based techniques to detect fecal coliforms, specifically *E. coli*, in environmental water samples is to identify a genomic target that is unique to the target species. One possible target for the detection of *E. coli* is *lacZ*, which is found in all coliforms. It is the gene that encodes for the enzyme *β*-D-galactosidase, which is responsible for the organism's ability to utilize lactose. This activity is one of the main characteristics of coliforms but is not specific enough to rule out the presence of other non-coliform species that may also contain *lacZ* within their genome. Initially, using *Citrobacter* DNA as a template and an annealing temperature of 40 ºC, researchers were able to differentiate between *E. coli lacZ* and the *lacZ* gene in other organisms. The PCR product of *Citrobacter* visualized by gel electrophoresis along side the *E. coli* PCR product, revealed a larger DNA fragment from Citrobacter (Bej et al., 1990). With an increase in annealing temperature between *E. coli* and other species, differences can also be noted. Increasing the annealing temperature to 50 ºC increases the specificity of the PCR and eliminates the amplification of most non-coliforms. It also eliminates amplification of some coliforms (e.g., *Enterobacter aerogenes* and *Citrobacter freundii*).

Amplification does occur in *E. coli*, *Enterobacter cloacae*, *Shigella spp*., and *Klebsiella pneumonea* at an annealing temperature of 50 ºC. At an annealing temperature of 60 ºC and 70 ºC, *E. coli, Enterobacter cloacae* and *Shigella spp.* are still detectable at varying sizes (Bej et al., 1990).

The gene, *uidA*, which encodes for the enzyme *β*-D-glucuronidase, is found in *E. coli* and *Shigella spp*. Since *Shigella* is not a coliform, simultaneous bacteriological tests have been formulated to detect total coliforms and *E. coli*. PCR-based detection methods using primers synthesized to amplify regions of *uidA* could be used to identify *E. coli* after. Multiplex PCR that targets both the *uidA* and *lacZ* genes can be used to specifically identify *E. coli*.

Another target for amplification and detection of *E. coli* in water samples is a pair of genes *gadA/gadB*. This gene pair encodes for isoenzymes of the glutamate decarboxylase enzyme unique to *E. coli* and *Shigella spp.* (McDaniels et al., 1996). These genes offer the same species specification as the *uidA* gene, but with two genes per chromosome, *gadAB* increase the sensitivity of PCR-based detection of *E. coli*.

With the identification of indicative pollutants comes the determination of the origin of the pollutants present. Contamination by human wastes constitutes a greater health risk than waste from most animal sources. It is important to know if human waste is suspected. Some fecal coliforms can persist and even grow in a variety of environments. Various free-living coliforms may be indigenous to some tropical waters (Wright, 1982; Paille et al., 1987; Rivera, 1988). Also, the presence of *E. coli* does not identify the source of contamination since *E. coli* is present in the intestinal tract of both

humans and other warm-blooded animals. However, anaerobic bacteria belonging to the *Bacteroides fragilis* Group (BFG) including, *B. distasonis, B. fragilis, B. vulgatus, B. ovatus, B. thetaiotaomicron, B. uniformis*, and 3452 A homology group (*i.e.*, an unnamed *B. fragilis* DNA homology group closely resembling *B. distasonis*) are present in high levels in humans and domestic pets but are rare in herding animals like cattle and horses (Sutter et al., 1985).

Previous investigations have revealed that *Bacteroides thetaiotaomicron* is a candidate indicator for water contamination to distinguish human from nonhuman contamination sources (Kreader, 1995). This has also been confirmed with the detection of *B. thetaiotaomicron* (BFG) at high titers in human fecal samples but at low titers or not detectable in animal fecal samples using PCR to amplify the target DNA within environmental samples (Wang et al., 1996).

The need to maintain anaerobic conditions during the cultivation, isolation, and biochemical identification of BFG bacteria initially discouraged use of BFG as an indicator organism for human contamination of environmental water. However, with the invent of DNA probes, the need to cultivate these organisms was circumvented (Kreader, 1995). Also, since all species have a unique DNA sequence, the choice of the appropriate probe sequences and use of specific conditions make DNA sequence-based detection very selective. Other traits used to identify an organism, such as a specific enzyme activity or morphological feature, may or may not be expressed or present under all environmental conditions or at the time of the assay. This process would also require the cultivation of organisms that may require extensive time and effort to cultivate.

Information encoded in the DNA provides a means of detection whether or not the encoded information is expressed, phenotypically, in the organism (Kreader, 1995).

This desire to distinguish between human and nonhuman sources of fecal contamination in water samples prompted the search for a species-specific indicator since the BFG appear within the human gut at a level of $10^4 – 10^6$ times higher than in cattle (Allsop and Stickler, 1985; Kreader, 1995). BFG also serves as a suitable indicator because greater than $6x10^{10}$ BFG bacterial cells per gram of human feces may be present, whereas, *E. coli* reaches levels of ~$1.9x10^7$ cells per gram of human feces. (Fiksdal, 1985; Wang et al., 1994 and 1996). BFG are prevalent in the human intestine, and several species within the group surpass the concentration or density of *E. coli*. Since BFG are obligate anaerobes, they do not survive in oxygenated waters, and the inability to routinely culture BFG reduces the possibility of their presence being attributed to environmental sources.

Investigators have also reported that antibiotic resistance patters among fecal streptococci can be useful in the identification of contamination sources (Wiggins et al., 1999). However, prior exposure of organisms to antibiotics and persistence in the environment reduces the usefulness of fecal streptococci in determining sources of recent contamination.

The rep-PCR fingerprint technique is used to differentiate between human and animal sources of pollution. This technique focuses on PCR amplification of the DNA between adjacent repetitive extragenic elements used to obtain strain-specific DNA fingerprints, which can be analyzed with pattern recognition computer software

(Dombek et al., 2000). It has been reported to be a simple, yet precise approach to differentiate between closely related strains of bacteria.

Random amplified polymorphic DNA (RAPD) and arbitrarily primed (AP) PCR techniques use a single primer that is relatively short or a low annealing temperature. Both of these conditions allow the primer to bind to many sites. Amplification produces an array of fragments that represents some of the distances between the primer binding sequences on the template providing data on the distribution of the primer binding sequences (Welsh and McClelland, 1991). Even though RAPD analysis permits the rapid and inexpensive detection of polymorphisms, artifacts can result. Disruptive variations in the patterns can be caused by differences in primer-to-template ratio, annealing temperature, and the magnesium concentration (Ellsworth et al., 1993). While these factors also affect standard PCR amplification, the variations in RAPD results can be much more pronounced.

Restriction endonucleases may also be used to differentiate sources of contamination. These nucleases cleave DNA at specific nucleotide sequence recognition sites that generate a unique array of fragments of varying lengths. Variations in nucleotide sequences and in the distance between cleavage sites leads to variation in the size of the fragments obtained (Hill, 1996). These restriction fragment length polymorphisms (RFLP) may become useful to distinguish indicator bacteria. The combination of restriction digestion and pulsed field gel electrophoresis has been found to be a reproducible means to fingerprint closely related organisms (Harsono et al., 1993; Bannerman et al., 1995; Abika et al., 1999; Louie et al., 1999; Abika et al., 2000).

The need for a rapid method to revolutionize the simultaneous detection of total coliforms and *E. coli*, the possibility of eliminating the delay in the detection of fecally-contaminated drinking, recreational, and environmental water with currently used methods, and the potential for differentiating between human and nonhuman fecal contamination in a timely manner provided the motivation for research to monitor and improve environmental water conditions. In contrast to the other forms of identification, PCR allows the rapid and specific detection of a wide range of bacterial species and has become an extremely important procedure for detecting microorganisms.

Environmental samples commonly contain bacteria of non-fecal origin that interferes with the detection of fecal contamination using standard methods. PCR-based techniques permit one to target specific fecal indicator organisms. In addition, the specificity of this technique provides an opportunity to differentiate amongst sources of fecal contamination, which would enable researchers to identify human-borne pathogens that threaten the welfare of the human population. This work explores the use of PCR to detect *E. coli* as the standard fecal indicator and the *Bacteroides fragilis* group as an indicator of human fecal contamination.

Materials and Methods

Culturing and Preparation of Bacterial Cells

Escherichia coli DH5αMRC cells when needed were cultured from the laboratory into 2-4 ml L-Broth (10g Bacto Trytone, 5g Bacto Yeast Extact and 5g NaCl per liter distilled water, pH 7.2) and incubated with agitation at 37 ºC for at 16-14 hours. The broth was transferred to several Eppendorf tubes, and the cells were sedimented at 14,000 x g for 3 minutes at room temperature. The supernatant fluid was removed and the pellet resuspended in 200µl 0.85% NaCl. The cells were quantified by direct microscopic count using a hemocytometer and stored frozen, at –78 ºC, until needed. Cell samples were then serially diluted in 5% Chelex 100 (Bio-Rad Laboratories, Hercules, CA) in ten-fold increments ranging from 10^{-2} to 10^{7} cells per 2.5µl aliquot.

Bacteroides fragilis ATCC 25285 cells were cultured from the laboratory stock culture in thioglycollate liquid medium in screw cap tubes with no air space. The tubes were incubated for two days at 37 ºC. The medium was centrifuged at 500 x g to separate the agar from the cells suspended in the liquid medium. The cell suspension was transferred to 1.5 ml Eppendorf tubes and centrifuged at 14,000 x g at room temperature for 5 minutes to pellet the cells. The pellets were resuspended in 200 µl of distilled water. Immediately after suspension, the cells were quantified by direct microscopic count using a hemocytometer and frozen at –78 ºC. To prepare cell suspensions for PCR, the cells were serially diluted in 5% Chelex 100 to remove potentially inhibitory metal ions in ten-fold increments ranging from 10^{-2} to 10^{7} cells per 2.5µl aliquot. Each dilution in 5% Chelex 100 was heated at 95 ºC for 5 minutes followed by incubation on ice for

5 minutes to lyse the cells and remove nucleases. The heat treatment was repeated two times with vigorous mixing between each "heat-cold" incubation.

Preparation of Chromosomal DNA for PCR

Chromosomal DNA was prepared from an ONPG-positive, MUG-positive *E. coli* isolated from dairy waste. The cells were collected from a 50 ml culture grown overnight in L broth. The cells were suspended in 5 ml 100mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0) and 2 mg per ml lysozyme (Sigma Chemical Co., St. Louis, MO). After 10 minutes at room temperature, sodium dodecyl sulfate was added to a final concentration of 0.2%, and the cells were mixed until the suspension cleared. The lysed cell suspension was extracted twice with phenol saturated with TE (10 mM Tris-HCl, 1mM EDTA, pH 8.0). Following phenol extraction, sodium acetate was added to the aqueous phase to a final concentration of 0.3 M. The sample was overlaid with 10 ml 100% ethanol at –20º C. DNA was spooled onto a glass rod and suspended in 2 ml TE. The DNA was dialyzed at 4 ºC against two liters of TE for twelve hours. The dialysis was repeated three times. The DNA concentration was determined for ten-fold dilutions of the DNA from measurements of absorbance at 260 nm. Based on a genome size of 4.6×10^6 bp, the number of DNA molecules was calculated for a 2.5 µl aliquot of each dilution.

PCR Amplification of *Escherichia coli*

PCR amplification was performed by using a DNA Thermal Cycler 480 (PE Applied Biosystems Perkin-Elmer Cetus Corp., Norwalk, Conn.) with AmpliTaq Gold DNA polymerase. The PCR reactions contained PCR amplification buffer (5 mM KCl, 10 mM Tris hydrochloride [pH 8.13] and 0.01% [wt/vol] gelatin), 200µM each of dGTP, dATP, dCTP, and dTTP, 1 µM each of primer, 1.25 mM $MgCl_2$, and 0.3 U AmpliTaq

Gold DNA polymerase per 25 µl. Template DNA was initially denatured and DNA polymerase activated at 95 ºC for 8 minutes. A total of 30 cycles were run under the following conditions: denaturation at 95 ºC for 1 minute, primer annealing at 56 ºC for 2 minutes, and DNA extension at 72 ºC for 3 minutes. The time of the third step was incrementally increased by 10 seconds per cycle.

For multiplex PCR reactions, regions of *gadAB* from *E. coli* and the 16S rRNA region of *B. fragilis* were amplified simultaneously by combining primers GAL/GAR and BF410/BF800. The reaction conditions had been optimized for this combination of primers.

Design of Primers for the Detection of *Escherichia coli*

Detection of *E. coli* by PCR targeted the *gadA* and *gadB* genes. These highly homologous genes correlate with the presence of the coliform *E. coli* and the non-coliform, *Shigella spp*. (McDaniel et al., 1996; Grant et al., 2001). The DNA sequence for both genes was downloaded from the database of National Centers of Biotechnology Information (NCBI), National Institute of Health. The DNA sequence of the *gadA* and *gadB* genes were aligned and primers were designed that would amplify either gene with equal efficiency. The primers were designated GAR-720 (5'GGTATCGACATCGACATGCAC) and GAL-1337 (5'GTCTTCCAGCAACAGTTCAGC). These primers amplify a 618 bp DNA product from *gadAB*.

A set of internal primers was designed for use in a nested PCR analysis. As above, the primers will amplify from either *gadA* or *gadB*. Primers GNR-1211

(5'TACAGAACGCCTCTTACCAG) and GNL-1042 (5'ACGTTCAGAGAGGTCGTACA) amplify a 170bp DNA product from *gadAB*.

The four primers were tested for their uniqueness to the *gadAB* genes using the BLAST nucleotide sequence through NCBI, NIH. In each case the 3' end of the primer was unique to the gadAB genes. The primers were predicted to be unique to these genes.

Primer Detection of *Bacteroides fragilis* Group

The sequences of PCR primers for the detection of the Bacteroides fragilis group (BFG) were obtained from literature (Roll et al., 1996). The primers were designed from the 16S rRNA genes of members of the BFG. Primers BF410 (5'GTGAAGGATGAAGGCTCTAT) and BF800 (5'CGTTTACTGTGTGGACTACC) will amplify a 410 bp DNA product.

To permit detection of BFG using a nested PCR assay, internal primers were designed using the sequence of five 16S rRNA genes from *B. fragilis, B. ovatus*, and *B. thetaiotaomicrom*. The sequences were aligned to maximize homology and primers were designed to the conserved regions. BF548 (5'GAGCGTAGGTGGACTGGTAA) and BF702 (5'CCTTCGCAATCGGAGTTCTT) amplify a 155 bp DNA product from within the 410 bp product of the BF410 and BF800 primers. All primers were tested to insure that they were unique to the BFG 16S rRNA genes. The 3' ends of all but one primer's sequences were unique to the BFG. One primer was homologus with not only the BFG but also with a sequence from a Gram-negative anaerobic rod-shaped bacterium from the human gut. The sequence of the primer paired with it was unique.

PCR Amplification of *Bacteroides fragilis* Group Bacteria

PCR amplification was performed by using a DNA thermal cycler and PE Applied Biosystems kits with AmpliTaq Gold polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.). The PCR solution contained 10X PCR amplification buffer (contains 50 mM KCl, 100 mM Tris hydrochloride [pH 8.13] and 0.1% [wt/vol] gelatin), combined dNTPs, 0.2 to 1 uM each of primer, 25 mM $MgCl_2$, 1ug template DNA, 1.25 U AmpliTaq Gold polymerase and distilled water. Template DNA was initially denatured at 94 ºC for 8 minutes. Then a total of 30 PCR cycles were run under the following conditions: denaturation at 94 ºC for 1 minute, primer annealing at 56 ºC for 2 minutes, and DNA extension at 72 ºC for 3 minutes. Oligonucleotide primers were synthesized.

A 410 base pair (bp) region of the 16S rRNA of *Bacteroides fragilis* was amplified using the 20 mer primers was used to amplify a shorter 155 bp region of the 16S rRNA.

Detection of Amplified DNA Products

PCR-amplified DNA products were detected using gel electrophoresis. The DNA products were separated using a vertical 6% polyacrylamide gel (30:0.5, acrylamide:bis-acrylamide). These gels were run in TBE buffer (89mM Tris base, 89mM boric acid, 2.5mM disodium EDTA) at 100 volts. The gels were stained with a 0.5 µg/ml ethidium bromide solution and visualized with a still video system using UV light (312nm) Eagle Eye II (Stratagene, Cedar Creek, TX). Migration of DNA was monitored using molecular size standards (100 bp ladder, New England Biolabs, Beverly, MA).

Fecal Indicator Detection in Environmental Samples

Procedures for preparing PCR samples from various water sources were done using centrifugation. Three hundred milliliter samples were collected from lakes at a depth of 8-12 inches below the water surface using a subsurface grab sampler (Wheaton Science Products). Water samples (100 ml) from each sampling site were centrifuged at 12,000 x g for ten minutes to sediment the cells. The cells were then suspended in sterile 0.85% saline to maintain appropriate osmotic conditions while concentrating them in a 2 ml snap cap tube. The cells were suspended in 200µl 5% Chelex 100 and frozen at –20 ºC. The IDEXX Quantitray/2000 system (IDEXX Laboratories, Inc., Westbrook, Maine), was also used to analyze each of the water samples. In this assay, one hundred milliliters of water sample was combined with Colilert medium (IDEXX Laboratories, Inc., Westbrook, Maine), sealed in a multi-welled tray and incubated at 35 ºC for 24 hours. ONPG-positive wells were identified by yellow color in reference to the IDEXX QuantiTray/2000 comparator. The number of ONPG-positive wells as applied to the MPN chart to determine the total coliforms. The wells were visualized under UV light to detect the blue fluorescence indicative of MUG breakdown by *β*–glucuronidase. Using the comparator as a reference, the blue fluorescent wells were counted and the numbers of *E. coli* were estimated from the MPN table. For *E. coli* confirmation, they must be yellow under visible light and blue under UV light (*i.e.,* ONPG-positive and MUG-positive). MUG-positive wells were counted.

Results

PCR-based Detection of *Escherichia coli*

To explore the possibility of a semiquantitative detection method for *E. coli* in environmental samples using PCR, a primary set of primers (GAR-720/GAL-1337) were designed that displayed specific homology to a region within the alpha- and beta-glutamate decarboxylase genes (*gadA* and *gadB*) of *E. coli* (Table 1). The pair of primers was designed to amplify a 618 base pair region within the *E. coli* genome that confirmed the presence of the organism within environmental samples. These functionally identical isoenzymes are unique to *E. coli* and *Shigella* (McDaniels et al., 1996) and are capable of detecting *E. coli* at the single-cell level.

Initially, there were multiple possible targets to be considered before selecting a DNA-specific probe for PCR-based analysis. Previously, PCR primers were designed to detect *lacZ* and *uidA* of *E. coli*. These latter two genes were attractive because their gene products are targeted in bacteriological testing utilizing ONPG and MUG. However, sensitivity of PCR reactions targeting these genes proved to be very poor. Three separate pairs of primers were used to detect *uidA* and did not provide the desired sensitivity, detection of one cell per reaction (E. Achberger, Personal Communication). The GAR-720/GAL-1337 primers for *gadAB* provided the required sensitivity and specificity. PCR conditions were optimized for the primer pair with a 56 ºC annealing temperature and a 1.25mM $MgCl_2$ concentration per 25ul reaction. Routine detection of *gadAB* at the one cell level required the use of AmpliTaq Gold DNA polymerase (Applied Biosystems).

Table 1. *Escherichia coli* and *Bacteroides fragilis* Group PCR Primer Sequences

Organism	Primary Primers	Nested Primers
E. coli	**GAR-720** 5'GGTATCGACATCGACATGCAC **GAL-1337** 5'GTCTTCCAGCAACAGTTCAGC	**GNR-1211** 5'TACAGAACGCCTCTTACCAG **GNL-1042** 5'ACGTTCAGAGAGGTCGTACA
B. fragilis	**BF410** 5'GTGAAGGATGAAGGCTCTAT **BF800** 5'CGTTTACTGTGTGGACTACC	**BF548** 5'GAGCGTAGGTGGACTGGTAA **BF702** 5'CCTTCGCAATCGGAGTTCTT

The presence of *E. coli* was confirmed by the visualization of a PCR product 618 bp in length following polyacrylamide gel electrophoresis (PAGE) and ethidium bromide staining (Figure 1). Analysis of the GAL-1337/GAR-720 primer pair demonstrated that detection of *E. coli* DNA via resuspended bacterial cells in laboratory samples is possible at 3.2×10^{1} cells (32cells) in a single 25ul reaction.

To confirm the presence of *E. coli* and to increase the sensitivity of the assay, a set of nested primers (GNL-1042/GNR-1211) was designed to amplify a 170 bp region within the 618 bp primary PCR product. The results of restriction enzyme analysis, using *Bsr*I, of both the 618 bp product and the 170 bp nested product confirmed that they originated from the *gadAB* genes. The nested PCR assay was able to detect and confirm the presence of *E. coli* within laboratory samples down to 3.2 cells per reaction (Figure 2). In 10 independent trials, *E. coli* was detected at the 3.2 cells per reaction level in every trial. Analyses of the lowest positive sample, 3.2×10^{-1} cells per reaction, revealed that 0.32 *E. coli* cells were detected 69% of the time. Representative results of repeated nested PCR reactions at the 0.32 cell level are presented in Figure 3.

These results were confirmed by the analysis of purified DNA quantified using measurements of absorbance at 260nm. DNA dilutions in ten-fold increments were assayed under identical PCR conditions, and detection of one genome equivalent was demonstrated based on the *E. coli* genome size of 4.6×10^{6} bp.

PCR-based Detection of *Bacteroides fragilis* Group Bacteria

The possibility of differentiating human from nonhuman sources of contamination was explored. The approach utilized PCR to detect key fecal indicator organisms.

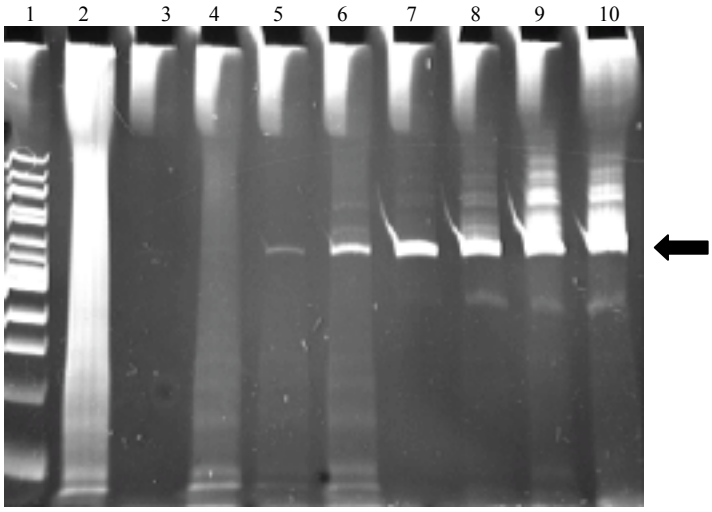


Figure 1. Polyacrylamide gel electrophoresis of products amplified from *E. coli* cells with GAL-1337/GAR-720 primers. The arrow indicates the location of the 618 bp product indicative of amplification using the GAL-1337/GAR-720 primers. Lane 1, 100 bp DNA marker; Lane 2, negative control of distilled water; Lane 3, 3.2×10^{-1} cells per reaction; Lane 4, 3.2×10^{0} cells per reaction; Lane 5, 3.2×10^{1} cells per reaction; Lane 6, 3.2×10^{2} cells per reaction; Lane 7, 3.2×10^{3} cells per reaction; Lane 8, 3.2×10^{4} cells per reaction; Lane 9, 3.2×10^{5} cells per reaction; and Lane 10, 3.2×10^{6} cells per reaction.

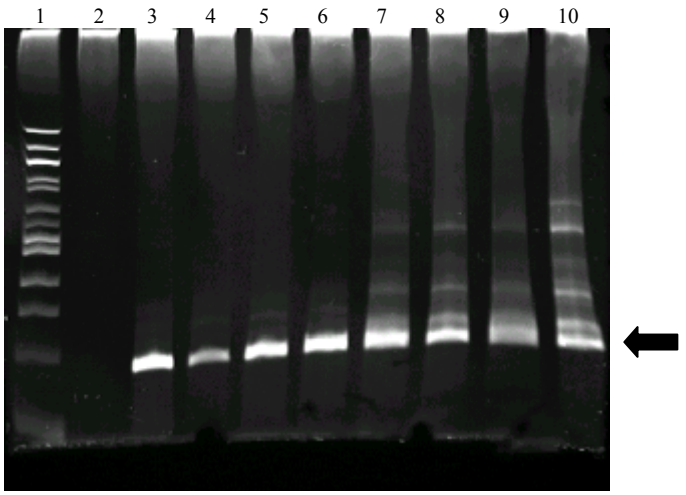


Figure 2. Polyacrylamide gel electrophoretic analysis of nested PCR of products using GNL-1042/GNR-1211 primers. The arrow indicates the location of the 170 bp product indicative of amplification using the GNL-1042/GNR-1211 primers. Lane 1, 100 bp DNA marker; Lanes 2-10 represent nested PCR reactions where the templates were the reactions listed in Figure 1. Lane 2, negative control of distilled water; Lane 3, 3.2×10^{-1} cells per reaction; Lane 4, 3.2×10^{0} cells per reaction; Lane 5, 3.2×10^{1} cells per reaction; Lane 6, 3.2×10^{2} cells per reaction; Lane 7, 3.2×10^{3} cells per reaction; Lane 8, 3.2×10^{4} cells per reaction; Lane 9, 3.2×10^{5} cells per reaction; and Lane 10, 3.2×10^{6} cells per reaction.

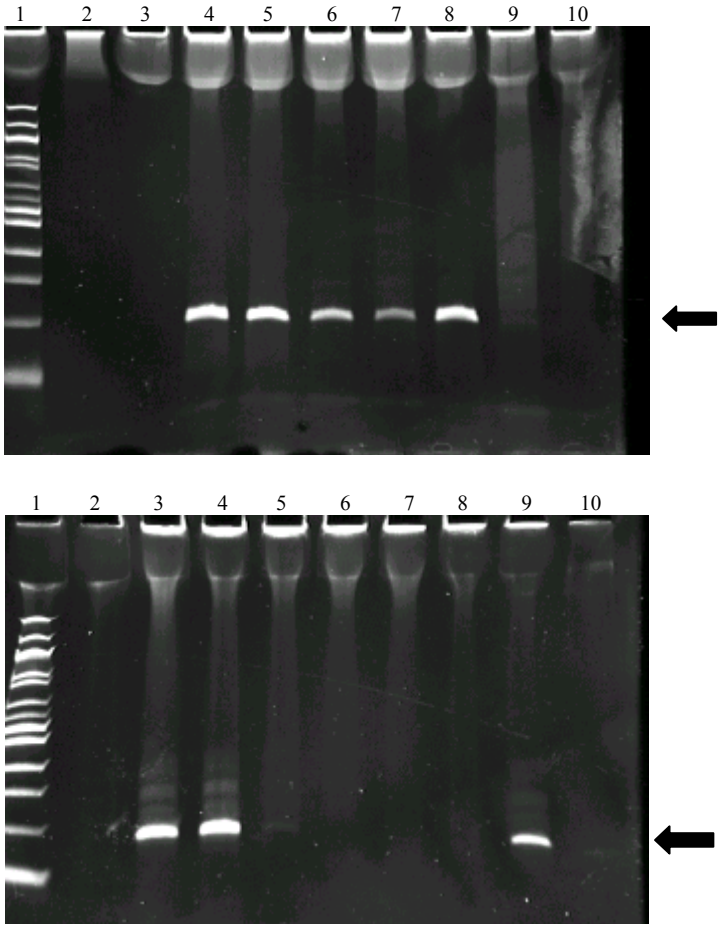


Figure 3. Nested PCR analysis of primary PCR products generated at the 0.32 cell level. Polyacrylamide gel electrophoresis of nested PCR products generated with the GNL-1042/GNR-1211 primers. The arrows indicate the location of the 170 bp product indicative of amplification using GNL/GNR. For each gel, Lane 1, 100 bp DNA marker; Lane 2, distilled water; Lanes 3 through 10 (on each gel) indicate 16 representative trials yielding positive or negative results when evaluating the *E. coli* nested primer pair at a low level of detection (*i.e.*, 0.32 cell level in the primary PCR reaction).

The *gadAB* PCR assay targets *E. coli* as an indicator of fecal contamination from either humans or animals. The *Bacteroides fragilis* group (BFG) was chosen as an indicator of contamination from human sources, not cattle or horses. It was reasoned that by determining the ratio of these two indicators in a water sample, municipal and agricultural sources of contamination could be differentiated. Reports confirm that the levels of the BFG in dairy cattle are 10^4 to 10^6 times lower than that of humans (Allsop and Stickler, 1985; Kreader, 1995). The PCR primers used to detect the presence of BFG bacterial cells, BF410/BF800 (Table 1), were described in literature (Roll et al., 1996). They permit the amplification of a 410 bp region within a 16S rRNA gene sequence that appears to be unique among bacteria within the BFG. Samples were evaluated using the optimized conditions of a 56 ºC annealing temperature and a 1.25mM $MgCl_2$ concentration. The assay reproducibly detected the target at the single-cell level when AmpliTaq Gold DNA polymerase was used.

Analysis of the BF410/BF800 primer pair allowed detection of laboratory-cultured *B. fragilis* cells down to 1.7×10^{-1} cells (0.17 cells) per reaction in approximately 60% of the 14 trials assayed. A 410 bp fragment was visible following PAGE and ethidium bromide staining (Figure 4). The identity of the PCR products was confirmed based on restriction enzyme analysis using *Eco*RI, which was predicted from the DNA sequence of the 16S rRNA gene. Nested PCR primers were designed to increase the sensitivity of the assay and to provide a routine confirmation of the presence of BFG cells. This set of primers, BF548/BF702, was designed to amplify a 155 bp region within the 410 bp primary PCR product. This nested PCR assay detected of *B. fragilis* down to 1.7×10^{-1} cells (0.17 cells) per reaction 100% of the time.

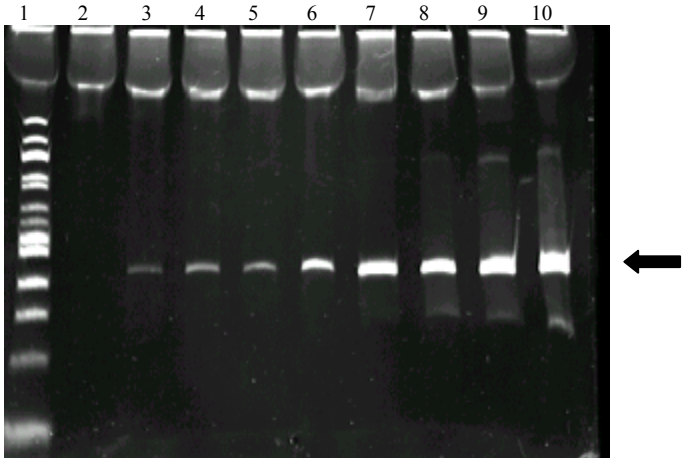


Figure 4. Polyacrylamide gel electrophoresis of products amplified from serial dilutions of *B. fragilis* cells using the BF410 /BF800 primers. The arrow indicates the location of the 410 bp product indicative of amplification using the BF410 /BF800 primers. Lane 1, 100 bp DNA marker; Lane 2, negative control of distilled water; Lane 3, 1.7×10^{-1} cells per reaction; Lane 4, 1.7×10^{0} cells per reaction; Lane 5, 1.7×10^{1} cells per reaction; Lane 6, 1.7×10^{2} cells per reaction; Lane 7, 1.7×10^{3} cells per reaction; Lane 8, 1.7×10^{4} cells per reaction; Lane 9, 1.7×10^{5} cells per reaction; and Lane 10, 1.7×10^{6} cells per reaction.

Multiplex PCR Detection of *E. coli* and *B. fragilis*

A multiplex PCR technique was established to detect the presence of *E. coli* and *B. fragilis* in a single sample reaction. The primary sets of primers for each organism were combined and used to evaluate ten-fold serial dilutions of a mixture of the laboratory-cultured *E. coli* and *B. fragilis*. The 618 bp product indicative of *E. coli* and the 410 bp BFG generated PCR products were clearly resolved by PAGE (Figure 5). Although there was amplification, these results demonstrated a decrease in the sensitivity of the primer pairs when assayed simultaneously on combined cell samples. The *E. coli* cells were detectable at 3.2×10^1 to 3.2×10^3 cells per reaction (32-3200 cells). The *B. fragilis* cells were detectable at 1.7×10^1 cells per reaction (17 cells).

To examine the loss of sensitivity observed in the multiplex PCR assay, the *E. coli* and BFG primer sets PCR series were tested using the cell mixture described above but with only one set of primers at a time (Figure 6). Results suggest that the loss of sensitivity was observed when only one set of primers was used. This suggested that the assay may be negatively affected by DNA from other organisms. Nested PCR assays of these primary PCR reactions were able to detect *E. coli* cells at the 0.32 to 3.2-cell level (Data not shown). This indicated that the nested PCR reactions could restore the levels of sensitivity expected.

PCR-based and Bacteriological Detection of *E. coli* in Environmental Samples

Through the repeated testing of laboratory-cultured cell samples, a PCR procedure for the evaluation of samples was established. The sediment-laden waters of Louisiana pose definite problems when processing environmental water samples for PCR analysis. Since silt and soil contain compounds that interfere with DNA amplification by

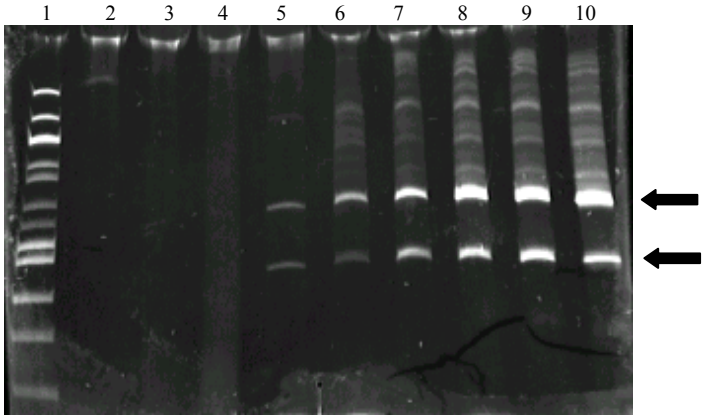
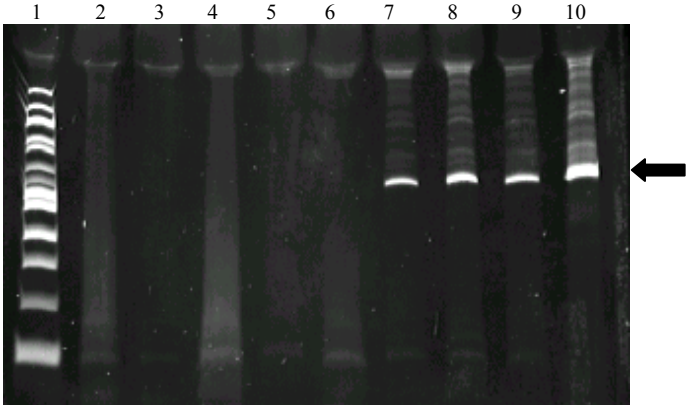


Figure 5. Polyacrylamide gel electrophoresis of products amplified in a multiplex PCR series with GAL/GAR and BF410/BF800 primers. The upper arrow indicates the location of the 618 bp product indicative of amplification using GAL/GAR. The lower arrow indicates the location of the 410 bp product indicative of amplification using BF410 /BF800. Lane 1, 100 bp DNA marker; Lane 2, negative control of distilled water; Lane 3, 10^{-1} cells per reaction; Lane 4, 10^{0} cells per reaction; Lane 5, 10^{1} cells per reaction; Lane 6, 10^{2} cells per reaction; Lane 7, 10^{3} cells per reaction; Lane 8, 10^{4} cells per reaction; Lane 9, 10^{5} cells per reaction; and Lane 10, 10^{6} cells per reaction. For each cell dilution there was 3.2 x 10^{n} *E. coli* and 1.7 x 10^{n} *B. fragilis* per reaction where *n* is the exponent listed.

A.



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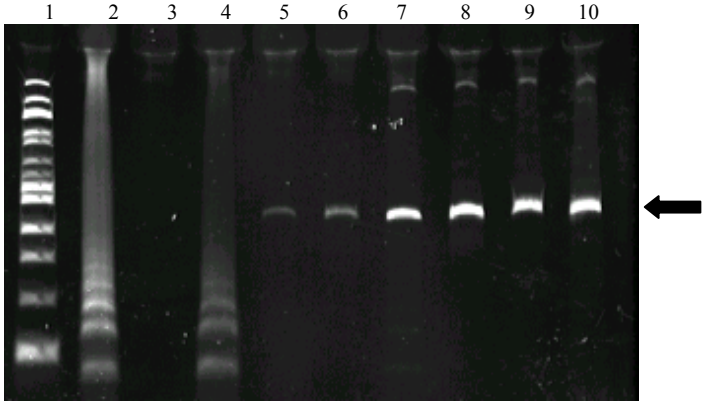

Figure 6. Polyacrylamide gel electrophoresis of PCR products amplified from a mixture of *E. coli* and *B. fragilis*. The amount of *E. coli* and *B. fragilis* cells per reaction were the same as those listed in Figure 5. Panel A.) GAL-1337/GAR-720 primers were used to amplify from the cell mixture. The arrow indicates the location of the 618 bp product indicative of amplification using the GAL-1337/GAR-720 primers.
Panel B.) BF410/BF800 primers were used to amplify from the cell mixture. The arrow indicates the location of the 410 bp product indicative of amplification using the BF410 /BF800 primers. For both panels, Lane 1, 100 bp DNA marker; Lane 2, negative control of distilled water; Lane 3, 10^{-1} cells per reaction; Lane 4, 10^{0} cells per reaction; Lane 5, 10^{1} cells per reaction; Lane 6, 10^{2} cells per reaction; Lane 7, 10^{3} cells per reaction; Lane 8, 10^{4} cells per reaction; Lane 9, 10^{5} cells per reaction; and Lane 10, 10^{6} cells per reaction.

PCR, it was of interest to remove debris from the water samples before concentrating the cells for PCR. The two methods used to concentrate cells for PCR sample preparation were centrifugation and filtration using a 13 mm, 0.45 micron teflon filter. It was not possible to collect 100 ml water samples using the teflon filters due to the high concentration of suspended particles, algae and bacteria present. Since the pellets collected by centrifugation were mostly composed of algae and sediment, a means to clarify water samples was explored.

The most promising approach to clarifying the water samples was differential centrifugation. Differential centrifugation has proven useful in separating bacterial cells and bacteriophage from sewage sludge (Staub et al., 1994) and from feces (Wang et al., 1994). Water samples with fecal contamination were collected from Campus Lake and University Lake on the LSU campus and used to evaluate the clarification techniques. For the differential centrifugation, the samples were centrifuged at 200 x g for 5 min to remove particulate matter followed by centrifugation at 9,000 x g for 3 min to sediment the bacterial cells. The MMO-MUG MPN assay was used to enumerate the total coliforms and *E. coli* before and after the low speed centrifugation step. In three independent trials, 89 ± 6 % of the coliforms and 81 ± 5% of the *E. coli* were removed by the low speed step. This loss would greatly reduce the chance of detecting low levels of fecal contamination.

Attempts to remove the particulate material by filtration produced similar results. Samples filtered through filter paper (Whatman, No. 1 Quantitative) or glass fiber filters (Whatman, GF/A) lost 60 to 95% of the coliform bacteria as determined by IDEXX QuantiTray/2000 (IDEXX Laboratories, Inc., Westbrook, Maine). In general, the more

debris removed from the sample, the greater the loss of bacterial cells, which suggested that coliform bacteria were bound to particles suspended in the water.

The decision was made to sediment material at 12,000 x g for 10 minutes to prepare the samples for PCR because the MMO-MUG MPN assay using the IDEXX QuantiTray/2000 revealed 89 ± 2% of the *E. coli* in a 100 ml sample is pelleted using this centrifugation step. Six environmental samples were collected at approximately two week intervals from three sources in and around Louisiana State University, Baton Rouge, Louisiana: College, Campus, and University Lakes (Figure 7). From each site, a 300 ml sample was collected at a depth of 8 to 12 inches by a sub-surface environmental grab sampler and analyzed within 4 hours by the MMO-MUG MPN assay with IDEXX QuantiTray/2000, a bacteriological technique that uses the MMO-MUG substrate for the enumeration of coliform bacteria and *E. coli* in 100 ml water samples. A second series of 100 ml samples was centrifuged to pellet the bacterial cells present in each environmental sample. The pelleted cells were resuspended in 200ul of 5% Chelex 100 and heat-treated at 95 ºC. Results were compiled for each sample site and are presented as *E. coli* per 100ml sample of water, and the PCR results for all sets of primers for the detection of *E. coli* and BFG bacteria (Table 2).

The pelleted bacterial cells resuspended in 5% Chelex 100 were tested with the nested PCR assay for *E. coli* as well as BFG. The ability to detect *E. coli* was tested as a function of the amount of the environmental sample added to the PCR reaction. It was found that sample volumes greater than 10% of the PCR reaction volume were inhibitory, which means only one-eightieth of the sample (*i.e.,* material pelleted from 100ml of water and suspended in 200µl) could be assayed. This is a definite disadvantage relative to the

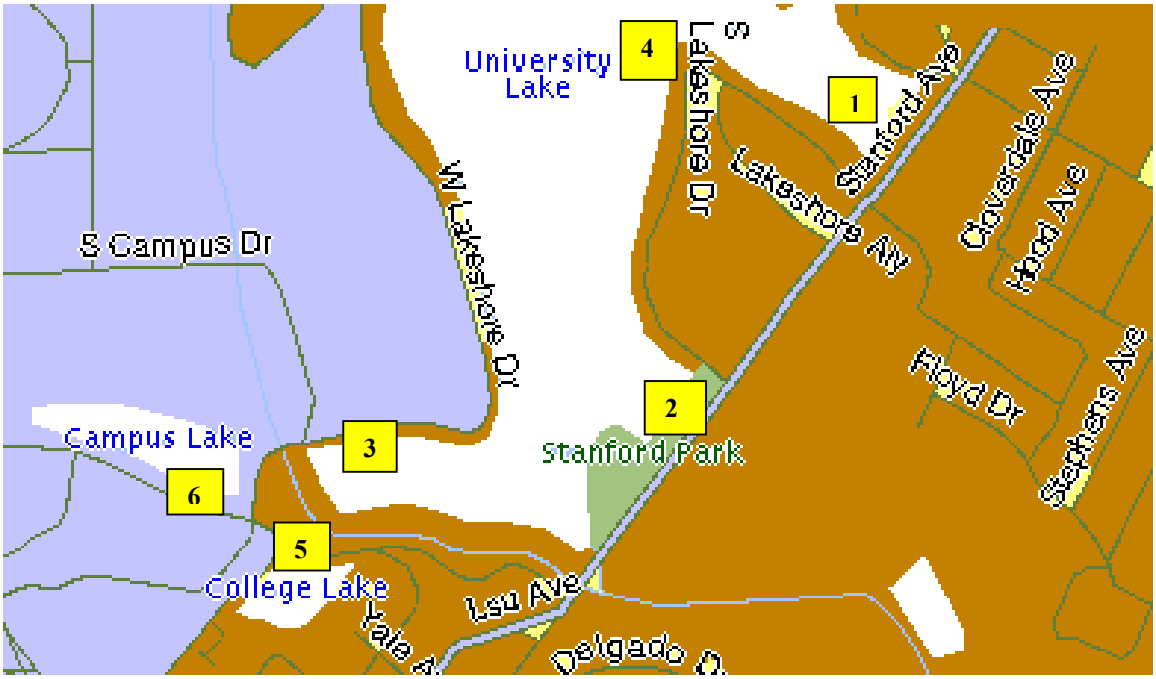

Figure 7. Map of sampling area displaying the six sampling sites tested and the approximate location of each site. Four samples were taken from University Lake (1-4), and one each from College (5) and Campus (6) Lakes.

Table 2. Lake water samples results using PCR in conjunction with IDEXX QuantiTray/2000

Environmental Samples	IDEXX QuantiTray/ 2000 *E. coli/100 ml*	*Escherichia coli* PCR Results Primary&Nested	*Bacteroides fragilis* Group PCR Results Primary & Nested
Lakes Series 1			
1	980	+ +	+ +
2	74	+ +	+ +
3	76	- +	- +
4	125	- +	+ +
5	125	- +	+ +
6	91	- +	- -
Lakes Series 2			
1	112	- -	- -
2	687	- -	- +
3	67	- -	- +
4	7	- -	- -
5	88	- +	- -
6	46	- +	+ +
Lakes Series 3			
1	141	- -	- +
2	11	- -	- +
3	7	- -	- -
4	17	- -	- +
5	102	- -	- +
6	30	- -	- -

IDEXX QuantiTray/2000, which allows 100 ml samples to be tested. In this study, the PCR assay detected *E. coli* in seven of eleven (64%) lake samples that contained greater than 74 *E. coli*/100 ml as determined by the IDEXX QuantiTray/2000 assay. Only eight of thirteen samples were positive by PCR when there was 46 or more *E. coli*/100 ml. No samples were positive by PCR when there were less than 46 *E. coli* per 100 ml. Since the PCR assay tested the equivalent of approximately one milliliter, these numbers are not unreasonable. However, tests showed that there is definite inhibition of PCR by the suspended sediment in environmental water samples. With a 1 to 10 dilution of the prepared environmental samples, detection of BFG bacteria was achieved (Figure 8), while detection of *E. coli* occurred less frequently. DNA extraction from these samples would remove the inhibiting impurities but may risk loss of DNA material and an underestimation of *E. coli* numbers.

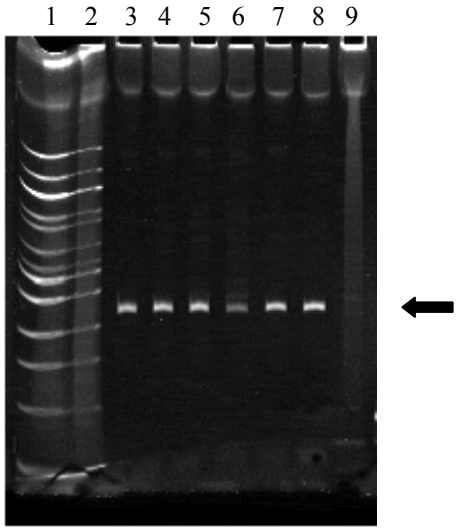

Figure 8. Detection of BFG in lake water samples. Polyacrylamide gel electrophoresis of PCR products of a 1:10 dilution of environmental lake series 2 amplified with BF410 /BF800 primers. The arrow indicates the location of the 410 bp product indicative of amplification using the BF410 /BF800 primers. Lanes 1 and 2, 100 bp DNA marker; Lane 3, site 1; Lane 4, site 2; Lane 5, site 3; Lane 6, site 4; Lane 7, site 5; Lane 8, site 6; and Lane 9; distilled water as a negative control.

Discussion

The use of PCR-based techniques to evaluate the microbiological quality of water has both advantages and disadvantages. One benefit for using a DNA-specific probe to detect these contaminants is being able to identify both viable culturable and as non-culturable organisms. With the use of PCR-based detection, even the most fastidious organism can be detected. DNA sequences can be amplified within hours as opposed to days, which could prove to be important to public health in the monitoring water systems. DNA-specific probes are also highly specific to the indicator organism targeted and under optimum conditions, can be very sensitive for that organism. The expression of enzymatic activity encoded by the target gene is not necessary when using the PCR. One disadvantage is that the PCR may detect viable as well as nonviable bacterial cells, such as may be found in a solution that has been sterilized. The process also requires the concentration of large volumes of environmental water samples to small reaction volumes for PCR in order to detect a single organism. These samples must go through a process of filtration or centrifugation that reduces 100ml of water to less than 5µl of PCR template. This restriction limited the usefulness of direct amplification by PCR.

PCR-based detection of broth-cultured *E. coli* cells proved to be sensitive and effective. *E. coli* cells were detectable down to the single-cell level on a reproducible basis when utilizing the primer pairs that targeted gadAB, GAR-720/GAL-1337 and GNR-1211/GNL-1042. The *gadAB* genes share extensive sequence homology, which made it possible to design PCR primers that detect both genes. This gave the advantage of having two template sequences per chromosome. Combined with the fact that growing *E. coli* cells have multiple chromosome equivalents, this explains why the PCR reaction

was able to detect less than one cell, routinely. The assay was positive at the 0.32 cell level 69% of the time tested.

Recently, Grant, et al., (2001) reported the use of *gadAB* genes to identify *E. coli* by PCR. Every *E. coli* strain tested contained the *gadAB* genes. Samples seeded with laboratory strains of *E. coli* were incubated in enriched broth for 24 hours. Using this enrichment study, they could seed the sample with as few as two colony-forming units. Since the *E. coli* would multiply extensively in the enrichment medium, the results of this group were not comparable with those reported in the present study. In the present study, *E. coli* was detected down to the single-cell level when assayed directly.

PCR-based detection of broth-cultured *B. fragilis* group bacterial cells also proved to be sensitive and reproducible and were detectable at the single-cell level on a consistent basis. BFG cells contain multiple copies of *rrn* operons encoding the rRNA genes. Between 4-6 *rrn* operons are found within the genome of several sequenced BFG members. The presence of multiple targets for the PCR primers on each chromosome increases the sensitivity of the assay. The ability of the nested PCR assay for the BFG bacteria to routinely detect 0.17 cells in an assay likely reflects the gene dosage.

For each of the amplified DNA products, restriction enzyme analysis correlated with the pattern predicted from the DNA sequence. This approach, in addition to the ability of the nested primers to bind and amplify the predicted products, confirmed the identity of the target DNA.

The primers were compared with known sequences using BLAST, National Center for Biotechnology Information, National Institute of Health. In each case, primer

pairs were predicted to be unique to the targeted organism. The primers for detection of *E. coli* and the BFG were specific and sensitive.

The results of multiplex PCR demonstrated that the primers for detecting *E. coli* and BFG would function under the same reaction conditions. Routinely, multiplex PCR results revealed a reduction in amplification sensitivity for the primary PCR reaction. While 0.2-0.3 cells per reaction were detected with a single DNA template, 17-32 cells per reaction were detected when using a DNA template preparation of a mixture of *E. coli* and BFG cells. Under these conditions, the nested PCR reactions were needed to restore sensitivity.

With the application of PCR-based techniques to environmental samples arose the dilemma of obtaining less than 5ul of sample per reaction from a 100 ml of water sample. Differential centrifugation removed 89±6% of coliforms and 81±5% *E. coli* at a low speed spin of 200 x g for five minutes suggesting these cells were adherent to the suspended particles within the water sample and were being pelleted along with those particles at a low speed. The filtration approach included the use of filter paper and glass fiber filters and showed a loss of 60-95% of coliforms. Again, these cells were believed to be attached to particulate matter suspended within the water sample. Adhesion of bacterial cells to surfaces including soil particles has been documented (Reviewed in Trevors and van Elsas, 1995). Ultimately, the samples were centrifuged at 12,000 x g for 10 minutes to remove all suspended particles, which pelleted 89% of *E. coli* cells from a 100ml sample. Detection of *E. coli* proved to be inconsistent in the primary PCR reaction, but nested PCR assay results demonstrated detection more frequently.

Nested PCR assays detected *E. coli* in seven of eleven (64%) lake samples that contained greater than 73 *E. coli*/100ml as determined by the IDEXX QuantiTray/2000 assay. Only eight of thirteen samples were positive by PCR when there was 46 or more *E. coli*/100 ml. No samples were positive by PCR when there was less than 46 *E. coli*/100ml. Since the PCR assay tested the equivalent of approximately one ml, these numbers are not unreasonable. BFG cells demonstrated more detection consistency using both primary and nested PCR techniques. With a 1 to 10 dilution of the prepared environmental samples, increased detection of BFG and, less frequently, *E. coli* detection was achieved suggesting that impurities associated with the suspended sediment in environmental water samples inhibit the PCR reaction.

The removal of impurities by DNA purification would alleviate interference from these compounds. However, attempts to purify chromosomal DNA from the material sedimented from water resulted in large losses of DNA, which were unacceptable for the experimental approach tested in this study. These losses would not be a major concern if the primers were adapted to real-time PCR. Quantitative PCR using this technology would determine the ratio of the two fecal indicator bacteria and allow differentiation of human from farm sources of contamination. For this approach, the absolute amount of target is not as important as an accurate ratio of the two targets.

In conclusion, the evaluation of PCR-based techniques for the detection and differentiation of human and non-human fecal indicator organisms has demonstrated that the *gadAB* genes of *E. coli* and a region within the 16S rRNA of BFG have proven to be suitable DNA targets to serve as primers for the detection and amplification of bacterial

cells in water samples. PCR is a rapid, efficient method of detection for *E. coli* and BFG, but further methods for the processing of water samples in preparation for PCR should be explored to reduce reaction-inhibiting factors within this process.

REFERENCES

Abika, M., T. Masuda, T. Sameshima, K. Katsuda, and M. Nakazwa. 1999. Molecular typing of *Escherichia coli* 0157:H7 (H-) isolates from cattle in Japan. Epidemiol. Infect. **122**:337-341.

Abika, M., T. Sameshima, and M. Nakazawa. 2000. Clonal turnover of enterohemorrhagic *Escherichia coli* 0157:H7 in experimentally infected cattle. FEMS Microbiol. Letters. **184**:79-83.

Allsop, K. , and D. J. Stickler. 1985. An assessment of *Bacteroides fragilis* group organisms as indicarors of human fecal pollution. J. Appl. Bacteriol. **58**:95-99.

Amhelm, N. and C. H. Levenson. 1990. Special Report: Polymerase Chain Reaction. C&EN. 1 Oct.:**36**-44.

Bannerman, T. L., G. A. Hancock, F. C. Tenover, and J. M. Miller. 1995. Pulsed-field electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. J. Clin. Microbiol. **33**:551-555.

Bej, A. K., R. J. Steffan, J. DiCesare, L. Haff, and R. M. Atlas. 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Appl. Environ. Microbiol. **56**:307-314.

Bej, A. K., S. C. McCarty, and R. M. Atlas. 1991. Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: comparison with defined substrate and plating methods water quality monitoring. Appl. Environ. Microbiol. **57**:2429:2432.

Bell, D. and D. M. DeMarini. 1991. Excessive cycling converts PCR products to random-length higher molecular weights fragments. Nucleic Acids Res. **19**:5079.

Bellin, T., M. Pulz, A. Matussek, H.-G., Hempen, and F. Gunzer. 2001. Rapid detection of enterohemorraghic *Escherichia coli* by real-time PCR with fluorescent hybridization probes. J. Clin. Microbiol. **39**:370-374.

Benner, K. P., C. C. Rankin, Y.R. Roybal, G. N. Stelma, Jr., P. V. Scarpino, and A. P. Dufour. 1993. New medium for the simultaneous detection of total coliforms and *Escherichia coli* in water. Appl. Environ. Microbiol. **59**:3534-3544.

Chang, G. W., J. Brill and R. Lum. 1989. Proportion of β-D-glucuronidase-negative *Escherichia coli* in human fecal samples. Appl. Eviron. Microbiol. **55**:335-339.

Clark, D., D. B. Milner, R. Wolfe, M. S. Stuart, and B. Olson. 1990. Comparative study of commercial 4-methylumbelliferyl-β-D-glucuronide preparations with the *Standard Methods* membrane filtration fecal coliform test for the detection of *Escherichia coli* in water samples. Appl. Environ. Microbiol. **57**:2443-2447.

Dombek, P. E., L. K. Johnson, S. T. Zimmerley, and M. J. Sadowsky. 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates form human and animal sources. Appl. Environ. Microbiol. **66**:2572-2577.

Ellsworth, D. L., K. D. Rittenhouse, and R. L. Honeycutt. 1993. Artificial variation in randomly amplified polymorphic DNA banding patterns. BioTechniques. **14**:214.

Fiksdal, L., J. S. Maki, S. J. LaCroix, and J. T. Staley. 1985. Survival of *Bacteroides spp*., prospective indicator bacteria. Appl. Environ. Microbiol. 49:148-150.

Grant, M. A., S. D. Weagant, and P. Feng. 2001. Glutamate decarboxylase genes as a prescreening marker for the detection of pathogenic *Escherichia coli* groups. Appl. Environ. Microbiol. **67**:3110-3114.

Harsono, K. D., C. W. Kaspar, and J. B. Luchansky. 1993. Comparison and genomic sizing of *Escherichia coli* 0157:H7 isolates by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. **59**:3141-3144.

Hill, W. 1996. The polymerase chain reaction: applications for the detection of food borne pathogens. Critical Reviews in Food Science and Nutrition. **36**:123-173.

Hyman, L.J., P. R. J. Birch, A. Dellagi, A. O. Avrova, and I. K. Toth. 2000. A competitive PCR-based method for the detection and quantitation of *Erwinia cartovora subsp. atroseptica* on potato tubers. Lett. Appl. Microbiol. **30**:330-335.

Kreader, C. 1995. Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. Appl. Environ. Microbiol. **61**:1171-1179.

Livak, K. J., S. J. Flood, J. Marmaro, W. Giusti, and K. Deetz. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. **4**:357-362.

Louie, M., S. Read, L. Louie, K. Ziebell, K. Rahn, A. Borczyk, and H. Lior. 1999. Molecular typing methods to investigate transmission of *Escherichia coli* 0157:H7 from cattle to humans. Epidemiol. Infect. **123**:17-24.

McDaniels, A., E. Rice, A. Reyes, C. Johnson, R. Haughland, and G. Stelma, Jr. 1996. Confirmational identification of *Escherichia coli*, a comparison of genotypic and phenotypic assays for glutamate decarboxylase and beta-glucuronidase. Appl. Environ. Microbiol. **62**:3350-3354.

Murray, A. E., D. Lies, G. Li, K. Nealson, J. Zhou, and J. M. Tiedje. 2001. DNA/DNA hybridization to microarrays reveals gene-specific differences between closely related microbial genomes. PNAS. **98**:9853-9858.

Pialle, D., C. Hackney, L. Reily, M. Cole, and M. Kilgen. 1987. Seasonal variation in fecal coliform population of Louisiana oysters and its relationship to microbiological quality. J. Food Protection. **50**:545-549.

Rivera, S. C., T. C. Hazen, and G. A. Toranzos. 1988. Isolation of fecal coliforms from pristine sites in a tropical rain forest. Appl. Environ. Microbiol. **54**:513-517.

Roll, B.M. A. Brown, and F. Bonilla. 1996. Development of a polymerase chain reaction detection system for the *Bacteroides fragilis* group as a sensitive indicator of sewage contamination, abstr Q-472, p.486. Abstr. 96th Annual Meeting of the American Society for Microbiology.

Standard Methods for the Examination of Water And Wastewater. 1998. 20th edition. United Book Press-Maryland. American Public Health Assoc. American Water Works Assoc. Water Environment Federation.

Staub, T. M., I. L. Pepper, and C. P. Gerba. 1994. Detection of naturally occurring enteroviruses and hepatitis A virus in undigested and anaerobically digested sludge using the polymerase chain reaction. Can. J. Microbiol. **40**:884-888.

Sutter, V. L., D. M. Citron, M. A. C. Edelstein, and S. M. Finegold. 1985. Wadsworth Anaerobic Bacteriology Manual. 4th edition. Star Publishing Company.

Sykes, P. J., S. J. Noeh, M. J. Brisco, E. Hughes, J. Condon, and A. A. Morley. 1992. Quantitation of targets for PCR by use of limiting dilution. BioTechniques. **13**:444.

Trevors, J. T. and J. D. van Elsas (Eds.). 1995. Nucleic Acids in the Environment: Methods and Applications. Springer-Verlag Berlin Heidelberg.

Tryland, I. and L Fiksdal. 1998. Enzyme characteristics of β-D-glactosidase- and β–D-glucuronidase-positive bacteria and their interference in raid methods for detection of waterborne coliforms and *Escherichia coli*. Appl. and Environ. Microbiol. **64**:1018-1023.

Wang, R.-F., W.-W. Cao, W. L. Campbell, L. Hairston, W. Franklin, and C. E. Cerniglia. 1994. The use of PCR to monitor the population abundance of six human intestinal bacterial species in an *in vitro* semicontinuous culture system. FEMS Microbiol. Lett. **124**:229-238.

Wang, R.-F., W.-W. Cao, and C. E. Cerniglia. 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol. **62**:1242-1247.

Welsh, J. and M. McClellen. 1991. Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combinations of primers. Nucleic Acids Res., **19**:5275.

Wiggins, B. A., R. W. Andrews, R. A. Conway, C. L. Corr, E. J. Dobratz, D. P. Dougherty, J. R. Eppard, S. R. Knupp, M. C. Limjoco, J. M. Mettenburg, J. M. Rinehardt, J. Sonisino, R. L. Torrijos, and M. E. Zimmerman. 1999. Use of antibiotic resistance analysis to identify nonpoint sources of fecal pollution. Appl. Environ. Microbiol. **65**: 3483-3486.

Wright, R. C. 1982. A comparison of the levels of faecal indicator bacteria in water and human faeces in a rural area of tropical developing country (Sierra Leone). Journal of Hygiene. **89**:69-78.

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