

Rapid Culture-Independent Quantitative Detection of Enterotoxigenic *Escherichia coli* in Surface Waters by Real-Time PCR with Molecular Beacon

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Rapid and reliable detection of enterotoxigenic *Escherichia coli* (ETEC) is critical for the management of the waterborne diseases threatening human lives worldwide. In this study, a culture-independent real-time PCR assay, in molecular beacon format, was designed and validated for detection and quantitative enumeration of ETEC harboring *LT1* gene (encoding heat labile toxin) in surface waters contaminated by fecal pollutants of human and animal origin. It was observed that the assay was able to detect 2 CFU/mL of ETEC ($r = 0.997$; PCR efficiency = 99.8%) from water samples spiked by a reference organism (*E. coli* MTCC 723). In the presence of 10^6 CFU/mL of nonpathogenic *E. coli* (*E. coli* DH5 α), the lowest detection limit from spiked water samples was 4 CFU/mL. The assay was 500 times more sensitive than conventional PCR using the same oligomers (Student's *t* test $p < 0.05$). The assay could specifically detect and quantify ETEC (1.2×10^3 to 1.4×10^6 CFU/100 mL) in polluted surface waters of river Gomti. The rapid culture-independent assay developed in this study for detection and quantitative enumeration of ETEC can be used for preliminary monitoring of surface waters to prevent waterborne outbreaks.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is regarded as a major cause of *E. coli* mediated diarrhea worldwide in humans, affecting mainly children and travelers (1–3). ETEC also has important implications for the farming industry where it is a major pathogen of cattle and weaning piglets (1, 4). The contamination of drinking or recreational waters with ETEC has been associated with waterborne disease outbreaks (2, 5, 6). In the developing world, an estimated 650 million cases of ETEC infection occur each year, resulting in ~800,000 deaths, mostly in young children (7). ETEC strains from humans cause mild or severe watery diarrhea by producing a heat-labile enterotoxin (LTI), similar in structure to cholera toxin and heat-stable enterotoxins (ST Ia and/or ST Ib) (2, 3). The heat-labile enterotoxins of *E. coli* are oligomeric toxins with two major serogroups LTI and LTII. LTI is expressed by *E. coli* strains that are pathogenic for both humans and animals.

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The occurrence of potential ETEC exhibiting *LT1/ST1* or both *LT1* and *ST1* genes in extensively used water resources in the developing world is an important health concern as a large population depends on both processed and unprocessed surface waters for drinking and domestic purposes (8, 9). Despite the potential public health threat from waterborne ETEC, there are no accepted methods for the rapid, accurate detection of ETEC in surface waters. Current measures of microbial water quality rely exclusively on “indicators” of fecal pollution (e.g., fecal coliform bacteria or generic *E. coli*). However, there are no established correlations between the prevalence or concentration of these “indicators” and specific pathogens, including diarrheagenic *E. coli* (10). Hence, there is a need for adequate monitoring technologies targeting representative pathogenic microorganisms like ETEC at low levels within hours to prevent mortality and morbidity caused by waterborne outbreaks. Conventional detection of ETEC strains could be accomplished by animal assays, cell culture techniques, enzyme-linked immunosorbent assays, and membrane-based DNA hybridization assays (11). All these methods are time-consuming and fail to detect nonculturable but viable strains and pathogens present in low concentrations (12, 13). The quantitative real-time PCR through use of fluorescent detection strategies (TaqMan, FRET and molecular beacon probes) enables much more rapid detection and enumeration of water quality indicator bacteria including pathogenic bacteria in a few hours (3 or less) without post-PCR analysis (10, 14, 15). At present, the information on application of fluorescent probes based real-time PCR targeting virulence genes of *E. coli* for pre-emptive monitoring and water quality management is meager (10, 16–18). A fluorescence resonance energy transfer (FRET) based real-time assay designed for detection of ETEC in clinical samples could detect 10^5 CFU/g from spiked animal feces without enrichment and 100 CFU/g after enrichment (11). However, no study reports the use of molecular beacon (MB) or any other fluorescent probe (FRET, TaqMan, and Scorpion) for detection of ETEC in water or food samples. The enterotoxin gene *LT1* commonly present in strains associated with human illness has been observed abundantly in ETEC recovered from surface waters (8, 9, 11, 19). Therefore, in the present study, a molecular beacon for specific detection of ETEC harboring *LT1* gene in surface water samples has been designed, validated for its sensitivity, and used for detection of *LT1* gene in real-life surface water samples.

Materials and Methods

Designing of Primers and Probes. A set of primers (*LT1* 'F': 5'- GGCAGGCAAAGAGAAATGG -3' and *LT1* 'R': 5'- TTG-GTCTCGGTTCAGATATGTG -3') and a fluorescent probe (molecular beacon: 5'-CACGCCCGG GACTT CGACCTGAAA TGTTGGCGTG-3') were designed in B subunit of *LT1* gene (Supporting Information S2, Table S1) for culture independent detection and quantitative enumeration of ETEC in surface waters. The probe and primers were synthesized from Metabion (GmbH, Germany).

Generation of Standard Curve. To establish a quantitative PCR for detection of *LT1* gene, a standard curve of serial 10-fold dilutions of positive control (*E. coli* MTCC 723) was constructed. In brief, for preparation of standard curve *E. coli* MTCC 723 was grown in Luria–Bertani broth (LB) broth for 12 h at 37 ± 1 °C to approximate optical density of 0.8 at 600 nm and serially diluted 10-fold in phosphate-buffered saline to yield 2×10^6 down to 2×10^{-1} CFU/mL as estimated by standard plate count method. DNA template was prepared

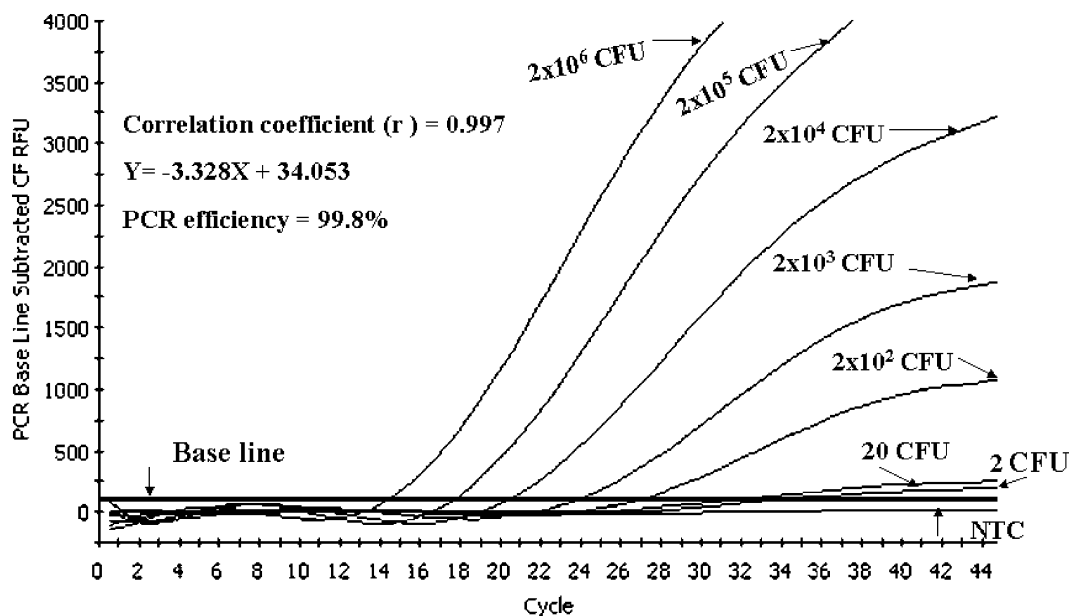


FIGURE 1. Standard curve generated from 10-fold serial dilution (from 2×10^6 down to 2×10^{-1} CFU/mL) of *E. coli* MTCC 723 grown in Luria–Bertani broth (LB) broth for 12 h at 37 ± 1 °C. NTC: no template control; Baseline: threshold cycle baseline.

from each dilution (Supporting Information, S3). Real-time PCR assays were performed using an iCycler (BIO-RAD, USA) real-time PCR instrument and Jumpstart TaqRead Mix (Sigma, St. Louis, MO). Briefly, the reaction mixture contained deoxynucleoside triphosphates (0.4 mM), Taq DNA polymerase (3 units) and reaction buffer, $MgCl_2$ (6.0 mM), primers (0.4 μ M, each), probe (0.2 μ M), and 5 μ L of DNA template (30–50 ng DNA) in a final volume of 50 μ L. The PCR program was as follows: initial denaturation at 95 °C for 3 min and then 45 cycles at 95 °C for 20 s, 55.8 °C for 30 s, and 72 °C for 30 s. Detection of the PCR product was performed in real time by measuring the fluorescent signal emitted by the MB when it hybridized to its target at the end of each annealing step. A mixture of all PCR reagents containing 5 μ L of sterile Milli-Q water instead of DNA template served as a negative control. All the real-time assays were performed in triplicate. The standard curve was automatically generated by the iCycler system software by plotting the cycle number (C_T) at which the threshold fluorescence was reached, versus the logarithmic concentration of positive control DNA. Sample concentrations were calculated from this standard curve. The sample was considered negative if the fluorescent signal did not increase within 45 cycles.

Sensitivity and Specificity of the Real-Time PCR Assay for ETEC. A total of 20 strains containing *LT1* gene (19 *E. coli* isolates recovered from surface water and one reference strain: *E. coli* MTCC 723 procured from Microbial type Culture Collection at Institute of Microbial Technology (IMTECH, Chandigarh, India), two strains of *Vibrio cholerae* exhibiting *ctx* gene, and five bacterial strains negative for *LT1* gene were used for evaluating the specificity of real-time PCR primers and molecular beacon designed in this study (Table S2, Supporting Information). All the bacterial strains were grown in LB broth (Hi Media, India) for 12 h at 37 ± 1 °C (optical density 0.8 at 600 nm). DNA template was prepared from bacterial cultures diluted 3-fold (1×10^3 CFU/mL) and real-time PCR was performed as described above to test the specificity of the assay (Supporting Information, S4).

To assess the sensitivity of the PCR assays for detection of *LT1* gene in ETEC contaminated surface waters, *E. coli* (MTCC 723) was grown in LB broth for 12 h at 37 ± 1 °C (optical density 0.8 at 600 nm). A serial 2-fold dilution from 500 down to 1 CFU/mL was spiked, in triplicate, to 10 mL of sterile Milli-Q water. DNA template was prepared from

spiked samples, and bacterial recovery from each spiked sample was assayed by real-time PCR as described above. Further, to assess the sensitivity of the real-time PCR assay targeting *LT1* gene in presence of background of DNA of nonpathogenic *E. coli*, water samples spiked with 10^6 CFU/mL of *E. coli* DH5 α were spiked with 2-fold serially diluted cultures of *E. coli* MTCC 723 (500 down to 1 CFU/mL). DNA templates were prepared and recovery of bacteria exhibiting target gene was assayed by real-time PCR as described earlier.

Comparison of Real-Time and Conventional PCR Assays. The specificity of real-time PCR assay designed for detection of ETEC in this study was compared with conventional PCR using the same primer set and a PTC-150 minicycler (MJ Research, USA). In brief, the reaction mixture in a final volume of 50 μ L comprising dNTP (0.2 mM), Taq DNA polymerase (1.5 units), $10\times$ reaction buffer (5 μ L), $MgCl_2$ (1.5 mM), primers (0.4 μ M, each), DNA template (5 μ L) from spiked water samples (sterilized water spiked by serial 10-fold dilutions from 2×10^6 down to 2×10^{-1} CFU/mL of *E. coli* MTCC 723 in presence or absence of 10^6 CFU/mL of *E. coli* DH5 α) as described earlier. The PCR program was as follows: initial denaturation at 95 °C for 3 min and then 45 cycles at 95 °C for 20 s, 55.8 °C for 30 s, and 72 °C for 30 s. All the assays were done in triplicate.

Quantitative Enumeration of ETEC in Surface waters by Molecular Beacon Based Real-Time Assay. To test the applicability of the assay, water samples from river Gomti, a highly polluted tributary of the river Ganga (21, 22), in northern India were analyzed. The water samples (1 L) were collected in sterilized bottles at four locations (Gandhi Setu, Nishant Ganj Bridge, Chinhat, and Gaughat) in the vicinity of Lucknow city, transported on ice, and analyzed immediately after arrival in laboratory. A 500 mL aliquot of the water sample from each site was concentrated to 500 μ L by repeated centrifugation at 18 000g for 10 min (4 °C). Finally, DNA template was prepared by boiling the 500 μ L of concentrated water and removing the debris by centrifugation at 16 000g for 5 min at 4 °C. DNA was precipitated from supernatant using an equal volume of ice cold ethanol. The precipitated DNA was pelleted by centrifugation at 12 000g for 5 min. The DNA pellet was washed thrice with 70% ethanol and finally dissolved in 250 μ L of TE (pH 8.0). DNA (5 μ L) was used as template in real-time PCR assays as described above. Quantitative enumeration of ETEC at each sampling location

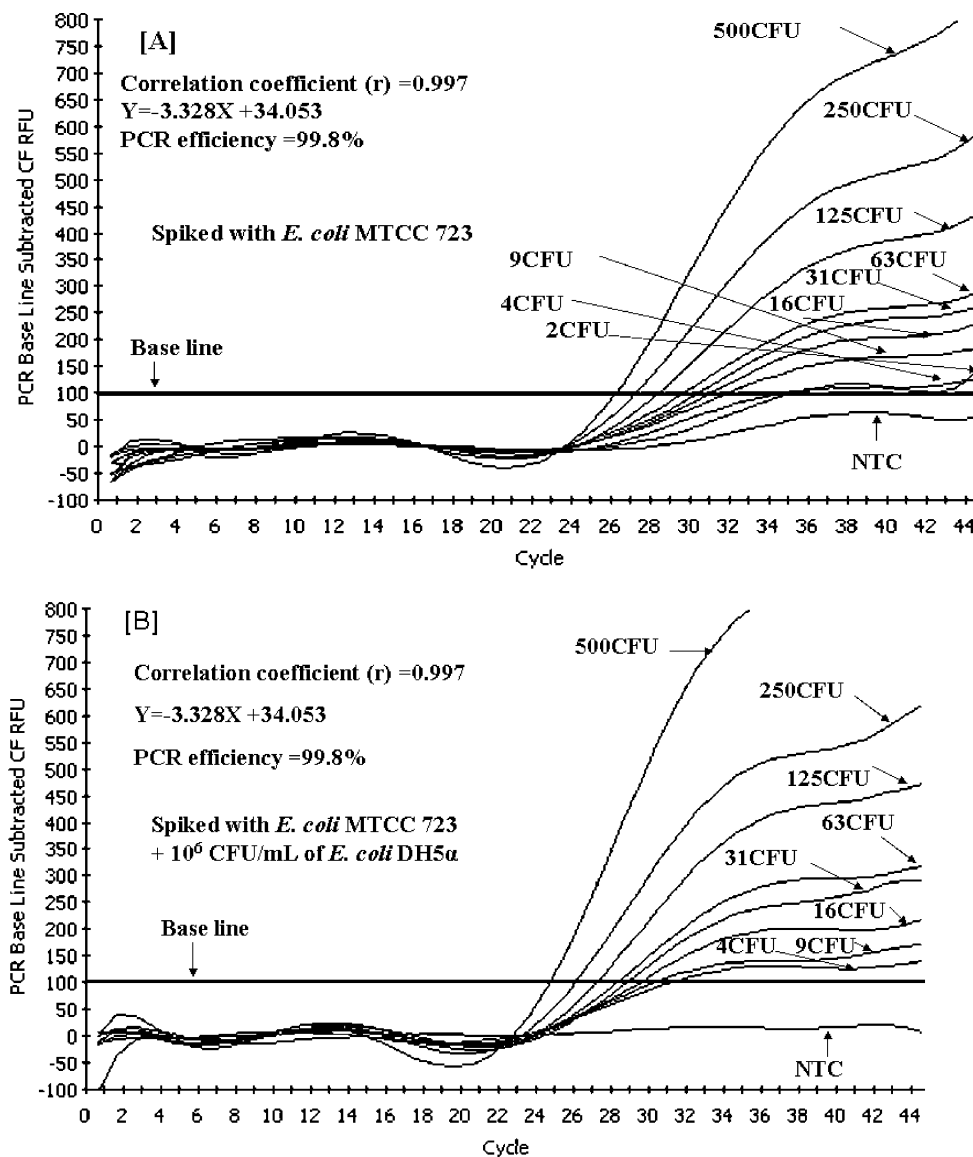


FIGURE 2. Sensitivity of quantitative detection of ETEC in water samples spiked by 2-fold serially diluted (500 down to 1 CFU/mL) culture of *E. coli* MTCC 723 (A) only by *E. coli* MTCC 723; (B) in presence of 10^6 CFU/mL of *E. coli* DH5 α through molecular beacon based real-time PCR assay.

of river Gomti was carried out using a standard curve prepared by 10-fold dilution of *E. coli* MTCC 723 culture (from 2×10^6 down to 2×10^{-1} CFU/mL).

Statistical Analyses. For comparison of PCR amplification efficiencies and detection sensitivities among different experiments, slopes of the standard curves were calculated by performing a correlation and regression analysis through iCycle iQ Real-Time Detection System Software Version 3.0A. Amplification efficiency (E) was estimated by using the slope of the standard curve and the formula $E = (10^{-1/\text{slope}}) - 1$. A reaction with theoretical 100% efficiency will generate a slope of -3.322 . The comparison of pollution levels of four sites in river Gomti in terms of ETEC load was performed using one-way analysis of variance (23). Data obtained from conventional and real-time PCR were compared by Student's t test (23).

Results

Sensitivity and Specificity of the Real-Time Assay. To establish a quantitative PCR for detection of ETEC exhibiting *LT1* gene, a standard curve of serial 10-fold dilutions (from 2×10^6 down to 2×10^{-1} CFU/mL) of the positive control (*E. coli* MTCC 723) was constructed. The lowest detection

limit of the quantitative real-time PCR using molecular beacon was 2 CFU/mL (Figure 1, Figure S1, Supporting Information).

All twenty strains of *E. coli* exhibiting *LT1* gene were positive in both conventional and real-time PCR assays (Table S2, Supporting Information). However, no amplification of the target gene was observed in *E. coli* strains reported to be negative for the target gene such as *Vibrio cholerae* and other bacterial strains used in the study (Table S2, Supporting Information).

The real-time PCR in MB format was 500 times more sensitive than conventional PCR (Student's t test $p < 0.05$) using the same oligomers (Figure 2A). The conventional PCR was able to detect 10^3 CFU/mL of *E. coli* MTCC 723 in spiked water samples. However, the presence of a nonpathogenic *E. coli* DH5 α (10^6 CFU/mL) in spiked water samples reduced the sensitivity of conventional PCR to 10^5 CFU/mL of *E. coli* MTCC 723 (Figure S2, Supporting Information). The assay was capable of rapidly detecting 2 CFU/mL ($r = 0.997$; PCR efficiency = 99.8%) in water samples spiked by the reference organism (*E. coli* MTCC 723). However, in the presence of 10^6 CFU/mL of nonpathogenic *E. coli* (*E. coli* DH5 α), the

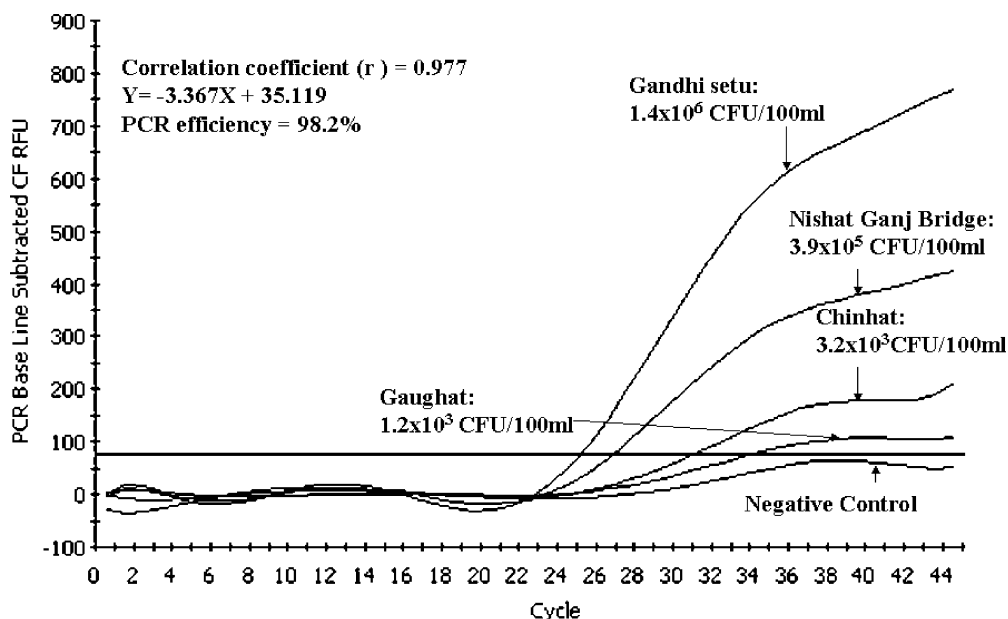


FIGURE 3. Quantitative detection of ETEC in river Gomti water at four sites through molecular beacon based real-time PCR assay. Results are mean of three replicates.

lowest detection limit from spiked water samples was 4 CFU/mL (Figure 2B).

Culture-Independent Quantitative Enumeration of ETEC in Surface Water Samples. The real-time PCR probe could quantitatively detect ETEC in the surface water samples collected from four sites of river Gomti in the vicinity of Lucknow city (Figure 3). All four sites varied significantly in terms of ETEC load (one way ANOVA $p < 0.05$).

The maximum concentration of the ETEC exhibiting target gene was recorded at Gandhi Setu (1.4×10^6 CFU/100 mL) followed by Nishatganj Bridge (3.9×10^5 CFU/100 mL), Chintahat (3.2×10^3 CFU/100 mL) and Gaughat (1.2×10^3 CFU/100 mL).

Discussion

This study has led to the first development and application of a quantitative culture independent PCR assay based on molecular beacon for detection and quantification of total ETEC bacterial cells exhibiting *LT1* gene in ambient water samples. The assay developed in this study is more accurate and 50,000-fold more sensitive than previously reported real-time assay (in FRET format) for ETEC in clinical samples targeting *LT1* gene. The earlier assay could detect 10^5 CFU/g stool samples without enrichment and 100 CFU/g after enrichment (11).

A comparison of molecular beacon based quantitative PCR with conventional PCR using the same set of primers revealed that the present assay is superior to conventional PCR in terms of sensitivity. The lowest detection limit for ETEC harboring *LT1* gene observed in this study for molecular beacon based assay and conventional PCR were 2 and 10^3 CFU/mL, respectively. This is in agreement with earlier studies which report that real-time fluorescent assays for detection of ETEC are more rapid and sensitive in comparison to conventional PCR (11, 24). Further, no amplification was observed in real-time assays conducted with *E. coli* and other bacterial strains lacking target gene including *Vibrio cholerae* (exhibiting *ctx* gene with high resemblance to *LT1* gene). These observations validate the high specificity of molecular beacon assay.

Khan et al. (13) reported a SYBR green based assay targeting the distal and proximal conserved flanking regions of the 16S rRNA gene, the internal transcribed spacer region

(ITS) region, and the 23S rRNA gene that could detect *E. coli* (10 CFU/mL) in agricultural watersheds. However, the assay could not differentiate between the pathogenic and non-pathogenic *E. coli* in real water samples. Recently, Lothigius et al. (24) reported a SYBR green based assay which could detect 5×10^2 *E. coli* exhibiting *LT1* gene, using boiled bacterial cell lysate as template. In the present study, we could quantify as low as 2 CFU/mL of ETEC in Milli-Q water samples spiked by 2×10^6 down to 2×10^{-1} CFU/mL of *E. coli* MTCC 723. However, presence of a high concentration of DNA from nonpathogenic *E. coli* reduced the detection limit 2-fold. Khan et al. (13) observed a 10-fold reduction in sensitivity of real-time PCR assay based on detection and quantification of *E. coli* in agricultural watersheds in presence of nonspecific DNA. Therefore, molecular beacon based assay developed in the present study is more sensitive and specific than SYBR green based assays reported earlier in quantitative detection of pathogenic or nonpathogenic *E. coli* in surface water samples.

In developing countries, a large population depends on untreated water from rivers, lakes, wells, and other surface water resources for drinking, bathing, laundry, recreation, and other domestic purposes (2, 25). Surface water resources have emerged as reservoirs of fecal coliforms including diarrheagenic forms of *E. coli* (enterotoxin and shiga toxin producing *E. coli*: ETEC and STEC) exhibiting virulence genes and resistance to multiple antimicrobials agents due to addition of municipal sewage and wastes from animal production industries and hospitals (9, 26, 27). We determined the applicability of the developed real-time PCR assay to surface water samples collected from a highly polluted Indian river, the Gomti. The assay has the potential to enumerate the comparative load of ETEC between the sites of polluted surface waters against high background of both nonpathogenic and pathogenic *E. coli*. The occurrence of high concentration of ETEC (2×10^3 to 1.4×10^6 CFU/100 mL) in the river Gomti might be due to addition of 450 million liters per day of untreated domestic wastewater generated by the 3.5 million population of Lucknow city (21). In addition, sampling sites receive slum wastewaters, hospital wastes, and carcasses due to inadequate cremation practices (22). The river Gomti water is a source for drinking water and domestic supply for residents of Lucknow city and sur-

rounding ecozones. Hence, a large population is at high risk of waterborne diarrheal diseases.

Diarrheal disease caused by ETEC is the main cause of death in infants and small children in developing countries. Therefore, in the case of widespread diarrheal epidemics, rapid identification of causative agents is critical for early intervention. The present assay overcomes the limitations of the culture-based assays that include length of time required (18–96 h), potential for false positive and negative results, loss of viability of bacteria between the time of the sample collection and enumeration, and lack of growth of viable but nonculturable bacteria (13). The real-time PCR method presented here may be preferable to traditional culture-based methods, conventional PCR, and previously reported real-time PCR assays using FRET and SYBER green I for detection of ETEC because of its ability to detect and quantify low numbers of ETEC in 2 h (including DNA extraction and quantitative PCR amplification) without any post-PCR handling including agarose gel electrophoresis. In addition, the present assay validated a centrifugation concentration and ethanol precipitation procedure for purification of DNA directly from surface waters which makes the protocol simple and cost-effective. The real-time PCR assay developed here also accounts for nonculturable but viable forms which escape conventional culture-based methods (14).

Thus, the unique quantitative culture independent real-time PCR developed in this study is an extremely rapid procedure with high specificity and sensitivity that requires only 2 h to detect ETEC in water samples which can be a valuable tool for rapid detection of ETEC in surface waters to prevent waterborne outbreaks of diarrheal diseases. Further, this assay has high applicability to test the pathogen level in surface waters and waters in sewage treatment plants prior to their disposal in rivers and lakes.

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Supporting Information Available

Methodology for PCR primer and molecular beacon design, preparation of template DNA, the basic real-time program, optimization of molecular beacon assay, specificity of the assay for detection of ETEC isolates, graph showing relationship between log cell concentration and threshold cycles for standard curve generated with 10-fold dilution of *E. coli* MTCC 723, and gel pictures of conventional PCR. This information is available free of charge via the Internet at <http://pubs.acs.org>.

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