Supporting Information

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

Siya Ram, Poornima Vajpayee and Rishi Shanker

Industrial Toxicology Research Center, P. O. Box 80, Mahatma Gandhi Marg, Lucknow-226 001, U. P., India

**Corresponding author:* Dr Rishi Shanker

Phone: 91+ 0522 –2613786/2614118/2627586 Extn.237,

Fax : 91+ 0522-2611547,

e-mail :rishishanker1@rediffmail.com.

Materials and Methods

Designing of primers and probes. To design a molecular beacon for specific detection of ETEC harboring *LT1* gene, the complete coding sequences of *LT1* gene (S60731, AB011677) were retrieved from NCBI GenBank database (http://www.ncbi.nlm.nih.gov) and alignments were created using Clustal W programme (www.ebi.ac.uk/clustalW) to determine conserved sequences. A primer set (LT1'F': 5'-GGCAGGCAAAAGAGAA ATGG -3' and LT1 'R': 5'- TTGGTCTCGGTCAG ATATGTG -3') was first generated in B sub unit of the *LT1* gene against sequences that were conserved for the target gene and were divergent from the analogous region of the cholera toxin gene from *Vibrio cholerae* using Beacon Designer 5.0 (Premier Biosoft International) to design real - time assay. The specificity of primers was determined against known microbial genomes and sequences by BLAST (Basic Local Alignment Search Tool) programme (http://www.ncbi. nlm. nih. gov/ BLAST/) to ensure no homology was observed with known gene sequences of other water-borne pathogens. Further, specificity of the primer set was validated against reference strain *E. coli* MTCC 723. Designing of the probe was carried out by selecting conserved 22 mer sequences within the primer regions. A set of two 6 mer arms was added to the probe sequence based on DNA melting temperature predictions to ensure a higher melting temperature for probe-target hybrid compared to primer- target hybrid.. The resulting molecular beacon (5'-CACGCCCGGGACTTCGACCTGAAATGTTGGCGTG-3') was checked in mFold server (http://www.bioinfo. rpi.edu/ applications/mfold/old/dna/) to ensure the correct stem loop configuration and no secondary structures. The reaction conditions used for calculation of secondary structure formed by MB were: 0.25 mM

nucleic acid, 50 mM monovalent ion, 3 mM free Mg++ ion and 269 mM total Na+ equivalent. The free energy calculation temperature was 55^0C for MB and 25^0C for primer pair.

TABLE S1. Nucleotide Sequences of Candidate Oligomers and Molecular Beacon for *LT1* Gene of Enterotoxigenic *E. coli*

Primer/ **Probe** (5'-3')	**Product length/** position of primers & probes	*T*m of primers and probes	Self dimer ΔG (I)	Hairpin ΔG (I)	Cross Dimer Δ (I)
GGCAGGCAAAAGAGAAATGG	**150**	54.5	0	-0.7	N.A.
TTGGTCTCGGTCAGATATGTG	111-260	54.4	0	-0.7	N.A.
CACGCCCGGGACTTCGACCT GAAATGTTGGCGTG	177	58.3	-1.1	0	-0.9

Finally, a fluorophore (FAM: 6- carboxy – fluorescein) was used as fluorescent reporter dye and conjugated to 5' end of the probe to detect amplification product specific for *LT1*. The quenching dye attached to 3' end of the probe was DABCYL. Probes and primers were synthesized from Metabion (Gmbh, Germany).

Preparation of Template DNA. Template DNA from bacterial cultures was prepared as previously described (*1*). Briefly, an aliquot (1.5 mL) of bacterial culture was centrifuged at 16000 x g for 10 min to pellet the bacterial cells. The cells were resuspended in 500 μL sterile Milli-Q® water, boiled at 99.9 ^{o}C for 10 min to lyse the

bacterial cells and centrifuged at 16000 x g for 5 min at 4 ^{o}C to sediment the debris. DNA quality and quantity was measured using ND 1000 spectrophotometer Nanodrop (NanoDrop Technologies, Inc. U.S.A.).

The Basic Real - time PCR Program. The basic real-time PCR program for optimization and sensitivity assays was as follows: initial denaturation at 95°C for 3 min and then 45cycles at 95°C for 20 s, 55.8°C for 30 s, and 72°C for 30 s. Detection of PCR product was performed in real- time by measuring the fluorescent signal emitted by the MB when it hybridizes to its target at the end of each annealing step. Negative control contain 5 µl sterilized sterile Milli-Q® water instead of DNA template. All the real-time optimization assays were performed in triplicates. If the fluorescent signal did not increase within 45 cycles, the sample was considered negative.

Optimization of Molecular Beacon Assay. For optimization assays, template DNA was prepared from *E. coli* MTCC 723 cultures grown for 12 h at 37 ^{o}C to optical density of 0.8 at 600 nm and diluted to three fold (1x 10^{3} CFU/mL). The first stage of optimization involved performing a gradient PCR with temperature and Mg2+ concentrations between 55and 65 ◦C, and 3.5 and 7.5 mM, respectively. On determining the temperature/Mg2+ combination a primer matrix was carried out. This involved varying the forward and reverse primers in all combinations of 0.1, 0.2, 0.4, 0.6 and 0.8 µM concentrations. Finally a variety of beacon concentrations from 0.1 to 0.8 µM were tested with the optimal annealing temperature (55.8 ^{o}C), Mg2+ (6.0 mM and primer concentrations (0.4 µM) (data not given).

Table S2. Specificity of Molecular Beacon Based Real-time Assay to Detect Enterotoxigenic *Escherichia coli* Exhibiting *LT1* Gene.

Isolate/strain identity	Source (Origin)	Detection of *LT1* gene	
[a]ETEC		Conventional PCR (1x 10^3 CFU)	Real-time PCR (1x 10^3 CFU)
E. coli MTCC 723	[b]MTCC, Chandigarh	+	+
R2A	[c]I.T.R.C. (River Ganga)	+	+
M2E	[c]I.T.R.C. (River Ganga)	+	+
L2C	[c]I.T.R.C. (River Ganga)	+	+
R3D	[c]I.T.R.C. (River Ganga)	+	+
M3D	[c]I.T.R.C. (River Ganga)	+	+
M3E	[c]I.T.R.C. (River Ganga)	+	+
L3D	[c]I.T.R.C. (River Ganga)	+	+
L1A	[c]I.T.R.C. (River Ganga)	+	+
L1B	[c]I.T.R.C. (River Ganga)	+	+
L1C	[c]I.T.R.C. (River Ganga)	+	+
L1D	[c]I.T.R.C. (River Ganga)	+	+
M5C	[c]I.T.R.C. (River Ganga)	+	+
L5A	[c]I.T.R.C. (River Ganga)	+	+
L6A	[c]I.T.R.C. (River Ganga)	+	+

L6B	[c]I.T.R.C. (River Ganga)	+	+
1	[c]I.T.R.C. (River Gomti)	+	+
2	[c]I.T.R.C. (River Gomti)	+	+
26	[c]I.T.R.C. (River Gomti)	+	+
30	[c]I.T.R.C. (River Gomti)	+	+
31	[c]I.T.R.C. (River Gomti)	+	+
38	[c]I.T.R.C. (River Gomti)	+	+
50	[c]I.T.R.C. (River Gomti)	+	+
11b	[c]I.T.R.C. (River Gomti)	+	+
68	[c]I.T.R.C. (River Gomti)	+	+
69	[c]I.T.R.C. (River Gomti)	+	+
75	[c]I.T.R.C. (River Gomti)	+	+
77	[c]I.T.R.C. (River Gomti)	+	+
78	[c]I.T.R.C. (River Gomti)	+	+
80a	[c]I.T.R.C. (River Gomti)	+	+
86	[c]I.T.R.C. (River Gomti)	+	+
89	[c]I.T.R.C. (River Gomti)	+	+
129	[c]I.T.R.C. (River Gomti)	+	+
133	[c]I.T.R.C. (River Gomti)	+	+
Non Enterotoxigenic *Escherichia coli* isolates			
97	[c]I.T.R.C. (River Gomti)	-	-
98	[c]I.T.R.C. (River Gomti)	-	-
99	[c]I.T.R.C. (River Gomti)	-	-

M5E	[c]I.T.R.C. (River Ganga)	-	-
R6D	[c]I.T.R.C. (River Ganga)	-	-
Other bacteria			
Enterococcus faecalis	[d]ATCC 51299	-	-
Enterococcus durans	[b]MTCC 3031		
Enterococcus faecium	[d]ATCC 51559	-	-
Vibrio cholerae	[d]ATCC 51394	-	-
Salmonella typhimurium	[d]ATCC 13311	-	-
Salmonella typhimurium	[d]ATCC14028	-	-

[a]ETEC: Enterotoxigenic *Escherichia coli* ; [b]MTCC: Microbial Type Culture Collection at Institute of Microbial Technology (IMTECH), Chandigarh, India ; [c]I.T.R.C. : Industrial Toxicology Research Centre, [d]ATCC: American Type Culture Collection, USA

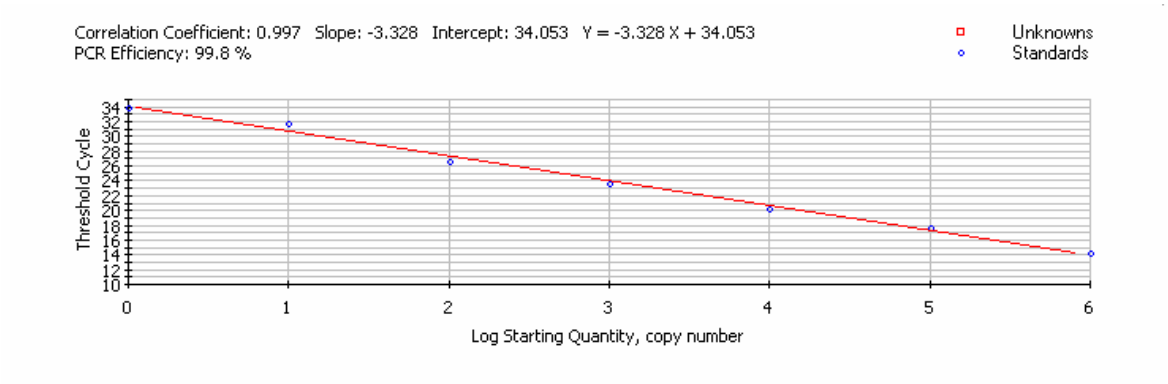

FIGURE S1. Standard curve for molecular beacon assay generated from 10- fold serial dilution (From $2x10^{6}$ down to $2x10^{-1}$ CFU/mL) of *E. coli* MTCC 723.

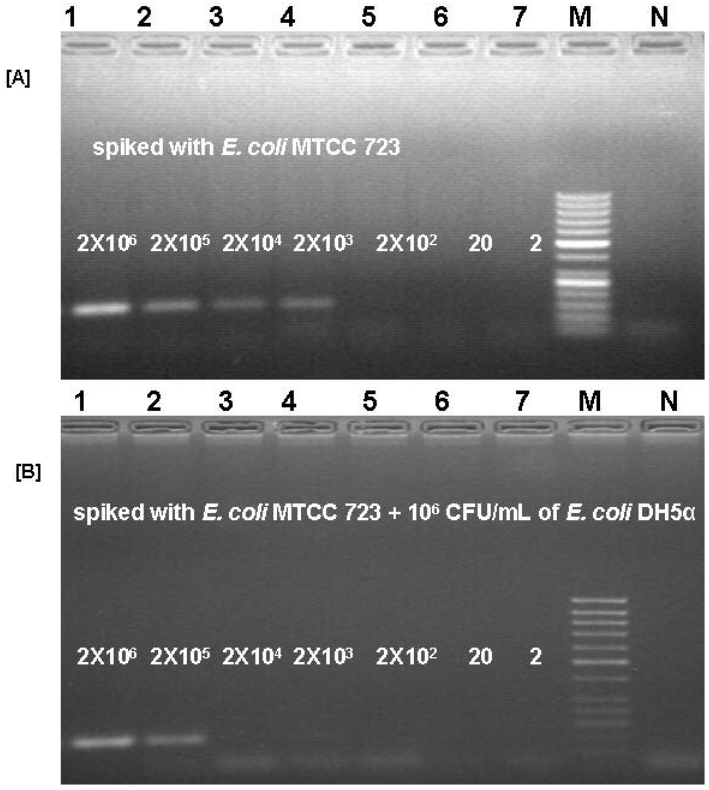

FIGURE S2. Amplification of *LT1* gene (150bp) by conventional PCR from waters samples spiked by 10- fold serially diluted ($2x10^{6}$ CFU/mL down to $2x10^{-1}$ CFU/ mL) culture of *E. coli* MTCC 723 (A : only with *E. coli* MTCC 723 (Lane 1,2, 3 and 4 : a PCR amplicon of 150bp, Lane M : MBI Fermentas 50bp DNA ladder), B : in presence

of 10^6 CFU/mL of *E. coli* DH5α , Lane 1,2, 3 and 4 : a PCR amplicon of 150bp, Lane M : MBI Fermentas 100bp DNA ladder).

Literature Cited

(1) Ram , S.; Vajpayee , P.; Shanker R. Prevalence of multi-antimicrobial-agent resistant, shiga toxin and enterotoxin producing *Escherichia coli* in surface waters of river Ganga. *Environ. Sci. Technol.* **2007**, *41*, 7383-7388.