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Studies on Biochemical Characterization, Antibiotics Sensitivity and *in-silico* Validation of a Real-Time PCR Array for Detection of *Escherichia coli* in Street Vended Food of Jaipur

H. KOTADIYA
M. SHARMA
H. SINGH
A. N. PATHAK
A. JYOTI[1]
AMITY Institute of Biotechnology
AMITY University, Rajasthan, Jaipur
India

Abstract:

Street vended foods are important source of microbial contamination and cause food poisoning. The study was aimed to biochemically characterize Escherichia coli (E. coli) a usual contaminant of street food using Gram's staining, endospore staining, catalase, urease, sugar fermentation and IMViC test; to determine their antibiotic sensitivity pattern and design an array for rapid detection of pathogen using in-silico real-time PCR of virulence gene sequences of E. coli. Eighteen different samples were collected from different sources in Jaipur for analyzing the microbial load. It was observed that total E. coli count was nearly equal or more than 10^3cfu/ gm or cfu/ ml. The study clearly indicated that consumption of street food samples from vendors can be a potential risk for food borne outbreaks because of their contamination level by E. coli. Also the degree of susceptibility of these isolates was evaluated against six different antibiotics that is, Chloramphenicol, Co-trimoxazole, Norfloxacin, Amikacin, Piperacillin and Tetracycline using disc diffusion method. E. coli had shown variable susceptibility and resistance patterns in response to different antibiotics used in the study. The classical methods used for contamination assessment are

[1] Corresponding author: ajyoti@jpr.amity.edu, anupamjyoti@rediffmail.com

tedious and time consuming. Real-time polymerase chain reaction (RT-PCR) can be a powerful molecular technique for rapid, sensitive and specific detection. However, validation of primers/ probes in real-life sample is very time consuming and expensive. Hence, in this study the potential of in-silico amplification of the virulence genes of E. coli prior to bench optimization for quantitative enumeration of E. coli in street foods was determined.

Key words: Antibiotics sensitivity, Detection, *Escherichia coli,* In silico PCR, Street food

1. Introduction

Infection of microorganism in street vended food is common these days. Pathogenic organisms can enter fruits and vegetables through damaged surfaces, such as punctures, wounds, cuts and splits that occur during growing or harvesting. Contamination may be present in raw materials or through equipments and also by avoidance of the vendors. Street foods prove to be a public health threat in view of their ready consumption, quick methods of cleaning utensils, handling and extraction. Hence, food borne illnesses of microbial origin are a major health problem associated with street foods. Public possess a high risk of food borne illness due to microbial contamination, adding to which improper use of food additives, adulteration and environmental contamination too. Also the use of artificial colours, like metanil yellow, is the cause of serious health hazards. Most of the foods are not well protected from flies, which may carry food borne pathogens. Safe food storage temperatures are rarely applied to street foods.

Microbiological studies from many developing countries, carried out on street vended food articles have revealed presence of pathogenic *E. coli* strains and high bacteria count. The high prevalence of diarrhea diseases among children and

infants can be traced to the use of unsafe street vended food and unhygienic practices. The consumers who depend on such food are more interested in its convenience than in question of its safety, quality and hygiene (Nicolas *et al*. 2007). World Health Organization (WHO) has developed five main keys to safer food, which include keeping clean, separating raw and cooked food, cooking thoroughly, keeping food at safe temperatures, and using safe water and raw materials (WHO 2007).

Bacterial pathogenic islands harbor a large number of virulence factors, which are essential for the survival and pathogenicity (Dobrindt *et al*. 2004). These essential genes are more evolutionary conserved than non-essential genes in bacteria (Jordan *et al*. 2002). Pathogenicity of enterotoxigenic *E. coli* (ETEC) is directly related to the LT1 gene expressing the enterotoxin LT (Nataro *et al*. 1998). Presence of stx1 and stx2 gene in E. coli isolates from human and cattle stool samples have been reported (Khan *et al*. 2002; Wani *et al*. 2006). eaeA gene, responsible for expression of the full virulence of Shiga toxin producing *E. coli* (STEC) in humans causes hemorrhagic colitis and hemolytic uremic syndrome (Boerlin *et al*. 1999).

"Fruit Chats" are the abundant sources of contamination depending on the conditions in which they are sold, therefore, significantly contribute to food borne ailments (Kumar *et al*. 2006). Several ailments caused by bacterial pathogens are cured by using different groups of antibiotics that are special class of chemotherapeutic agents obtained from living organisms. Resistance to antibiotics in food borne pathogens may create problems for disease or illness treatment while antibiotic susceptibility leads to healing of the illness which the organism(s) caused. Traveler's diarrhea is a major inconvenience to visitors arriving in developing countries like India from more industrialized areas. However, development of resistance in almost all bacterial species to different classes of

antibiotics poses a major and global healthcare problem. Several mechanisms are known to induce antibiotic resistance in bacteria, but the most common type of resistance develops and transmits horizontally via conjugation of a plasmid.

Conventional methods for identification of pathogenic bacteria are laborious, less sensitive, time taking and having many drawbacks. Real-Time Polymerase Chain Reaction (RT-PCR), with the use of specific primers can prove as a powerful technique for rapid, sensitive and specific detection of pathogen. However, the use of primers on bench in real life samples is time taking and expensive. Hence in the present study, alternative to this is used by performing *in-silico* amplification of the targeted gene which is necessary prior to bench optimization of the same. The array designed can detect *E. coli* through amplification of their housekeeping genes with the use of highly specific computed primers. The authenticity of RT-PCR primers was checked on BLAST results against known bacterial genomes.

2. Materials and Methods

2.1 Study sites description and sample collection

Eighteen different street food samples (liquid as well as solid) were collected from five different public sites (Table 1). The samples were stored in a sterile polyethylene bags, sealed and were analyzed within 6 hours.

S. No	Sample	Sites	Sample code
1	Strawberry shake	AMITY University Rajasthan	HS1
2	Pineapple juice	AMITY University Rajasthan	HS2
3	Paani puri Paani	Achrol Market	HS3
4	Sugarcane juice	AMITY University Rajasthan	HS4
5	Paani puri - puri	Achrol Market	HS5
6	Wood apple juice	Achrol Market	HS6
7	Mango juice	Achrol Market	HS7

8	Papaya juice	NIMS University	HS8
9	Banana juice	NIMS University	HS9
10	Pineapple juice	NIMS University	HS10
11	Maggie	NIMS University	HS11
12	Black gram sprouts	Railway Station	HS12
13	Bhelpuri	Achrol Market	HS13
14	Meethi chatni	Achrol Market	HS14
15	Fried rice	Sindhi camp Bus Stand	HS15
16	Chowmein	Sindhi camp Bus Stand	HS16
17	Salad	Railway station	HS17
18	Aaloo Chaat	Railway station	HS18

Table 1– Details of collected street food samples (solid and liquid) along with their sites and sample codes

2.2 Isolation and identification of Escherichia coli on EMB plates

1ml of liquid street food samples and 1gm of solid food sample were serially diluted in sterile PBS in 10^2, 10^4, 10^6 and 10^8 dilutions followed by spreading on Levine eosin methylene blue (EMB) agar plate and incubated at 37^0C overnight. The colonies showing blue-black colony with metallic sheen were counted at each dilution and isolates count was expressed as number of colony forming unit (CFU)/ml for liquid street food and CFU/ gm for solid street food sample. For further study, blue-black colonies with metallic sheen on EMB agar plates were randomly selected from each sample and they were characterized morphologically (Gram's and endospore stain), and biochemically [IMViC (Indole, Methyl Red, Voges Proskauer and Citrate Utilization), Sugar fermentation, Catalase, Urease and Starch hydrolysis]. Isolates confirmed as *E. coli* were maintained at -70^0C in LB broth supplemented with 15% (v/v) glycerol.

2.3 Determination of susceptibility to antimicrobial agents

The sensitivity of the 86colonies isolates to antimicrobials was determined by disc diffusion method test using antimicrobial

agents impregnated paper discs (Hi-Media Ltd, Mumbai, India) as described by Clinical and Laboratory Standards Institute: CLSI (CLSI 2005). The 6 antimicrobials (µg per disc) tested were; Aminoglycosides: amikacin, Ak (10); Phenicol: chloramphenicol, C (10); Folate inhibitor: co-trimoxazole, Co (25); Quinolone and Fluoroquinolones: norfloxacin, Nx (10); β-lactams: piperacillin, Pc (100) and Tetracyclines: tetracycline, T (30). In this study, data for antimicrobial agent resistance of each bacterial isolate has been reported as resistant (R), isolates with reduced susceptibility (RS or intermediates) or sensitive (S) based on Clinical and Laboratory Standards Institute (CLSI) break points (Table 2).

S. No.	Antimicrobial agent	Concentration (µg)	Zone of inhibition (mm)		
			Resistance (R)	Reduced susceptibility (RS)	Sensitive (S)
1	Chloramphenicol	30	≤ 12	13-17	≥ 18
2	Co-trimoxazole	25	≤ 10	11-15	≥ 16
3	Norfloxacine	10	≤ 12	13-16	≥ 17
4	Amikacin	30	≤ 14	15-16	≥ 17
5	Tetracyclin	30	≤ 14	15-18	≥ 19
6	Piperacillin	100	≤ 17	18-20	≥ 21

Table 2 -Zone size break-points described by CLSI used to interpret data in agar diffusion test performed through antimicrobial agent-impregnated paper discs

For this purpose the *E. coli* culture was inoculated in nutrient broth and were kept for overnight incubation at 37^0C. After overnight growth of bacteria in nutrient broth, 100µl of each sample was spread on Muller Hinton agar plates. For each sample two Muller Hinton agar plates were prepared. On each plate commercially prepared discs, each of which are pre-impregnated with a standard concentration of a particular antibiotic are then evenly kept and lightly pressed onto the agar surface followed by incubation at 37^0C for 16 hours. After t incubation, the bacterial growth around each disc is observed

and the zone of inhibition was measured using scale in millimeters. If the test isolate is susceptible to a particular antibiotic, a clear area of "no growth" will be observed around that particular disc. Otherwise if the test isolates is resistance to a particular antibiotic, than no or very small zone of inhibition is present around particular disc.

2.4 Retrieval of gene sequences from database

According to the objective of the study *E. coli* was selected to design a real time PCR assay for their detection in street food items (solid as well as liquid). Nucleotide sequences of virulent genes *E. coli* were retrieved from the GenBank (www.ncbi.nlm.nih.gov/genbank), National Centre for Biotechnology Information, Bethesda, MD, USA (Table 3). The target genes of *Escherichia coli* were virulent genes-Shiga-like toxin 2 (stx2), Intimin (eae A), a-Haemolysin A (hlyA), Heat-labile toxin 1 (LT1) were targeted (Ram et al., 2008).

Escherichia coli genes	Accession number of gene sequence
stx2	FR850038, FR850037, FR850036
eaeA	AJ715405, AJ715404
hlyA	EU713852, EU713851, EU627773
LT1	AY342056, FJ200361

Table 3 - GenBank sequence identification numbers of bacterial genes used for BLAST analysis to determine conserved sequences for strain specific primers

2.5 Multiple Sequence Alignment

Multiple sequence alignments of retrieved sequences of genes selected for the present study were carried out using web based software Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo) to find out conserved contigs (Fig. 1). The nucleotide sequences of selected genes were aligned separately in themselves to create the conserved sequences of these. The computed primers were highly specific towards their respective targets as confirmed from the BLAST.

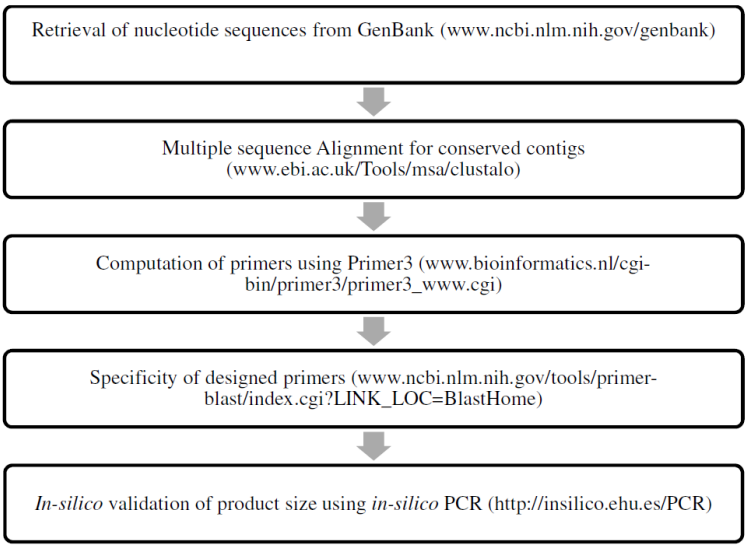

Fig. 1 - Schematic representation of real time PCR primer designing steps - Strategy for computation of primers for real-time PCR / *in silico* PCR prior to laboratory validation of a real–time PCR array developed for detection and quantification of *E. coli*.

2.6 Primer designing

The conserved regions, unique to a target pathogen, obtained from Clustal Omega were used to design primers using dedicated web based software Primer3 (www.bioinformatics.nl/cgi-bin/primer3/primer3_www.cgi). The primer designing parameters such as melting temperature, GC content, amplicon length, etc were taken into consideration.

2.7 Specificity of designed primers

The specificity of computed primers were determined against the known microbial genomes and sequences by Primer-BLAST (Basic Local Alignment Search Tool) programme of NCBI (www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK_LOC= BlastHome) to ensure no homology found in other genera or species.

2.8 In-Silico analysis of primers

The primer-pairs were used for in-silico analysis using, in-silico PCR (www.insilico.ehu.es/PCR) to ensure that each primer pair amplified the targeted sequences of the genes in the conserved region of the selected gene.

3. Results

3.1 Quantitative enumeration of blue-black colony with metallic green sheen colonies

It was found that all the samples from selected sites exhibited Enterobacteriaceae member contamination (specifically blue-black colony with metallic green sheen). The standard plate count on EMB agar was nearly equal or more than 10^3 cfu/ gm or cfu/ ml of food sample collected from different sites (Table 4).

S. No	Sample	Sites	Bacterial count (cfu/ gm or ml)
1	Strawberry shake	AMITY University Rajasthan (HS1)	20 X 10^3
2	Pineapple juice	AMITY University Rajasthan (HS2)	18 X 10^3
3	Paani puri Paani	Achrol Market (HS3)	13 X 10^3
4	Sugarcane juice	AMITY University Rajasthan (HS4)	30 X 10^3
5	Paani puri - puri	Achrol Market (HS5)	14X 10^3
6	Wood apple juice	Achrol Market (HS6)	34X 10^3
7	Mango juice	Achrol Market (HS7)	26X 10^3
8	Papaya juice	NIMS University (HS8)	24X 10^3
9	Banana juice	NIMS University (HS9)	40X 10^3
10	Pineapple juice	NIMS University (HS10)	34X 10^3
11	Maggie	NIMS University (HS11)	22X 10^3
12	Black gram sprouts	Railway Station (HS12)	25X 10^3
13	Bhelpuri	Achrol Market (HS13)	27X 10^3
14	Meethi chatni	Achrol Market (HS14)	28X 10^3
15	Fried rice	Sindhi camp Bus Stand (HS15)	17X 10^3
16	Chowmein	Sindhi camp Bus Stand (HS16)	21X 10^3
17	Salad	Railway station (HS17)	16X 10^3
18	Aaloo Chaat	Railway station (HS18)	32X 10^3

Table 4 - Quantitative enumeration of blue-black colonies with metallic green sheen in the street food samples

3.2 Biochemical characterization

The isolates were studied for their biochemical features by performing IMViC test (Fig. 2A-D), sugar fermentation test (Fig. 2E-G), urease test (Fig. 2H), catalase and starch hydrolysis. Results were compared with standard microbial identification chart and microbes were identified as *E. coli* (Supplementary Table 1).

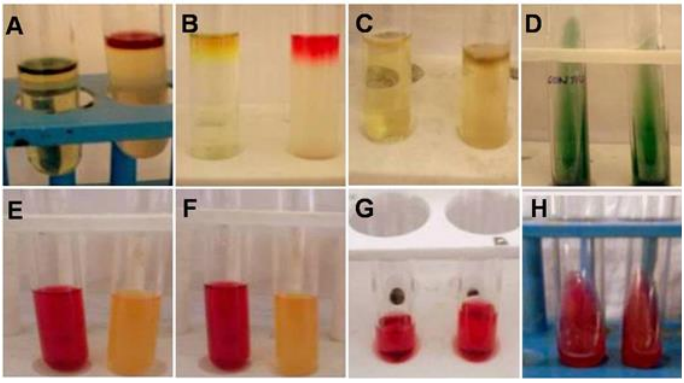

Fig. 2 - IMViC test, sugar fermentation and urease test of isolates obtained from street food sample. Control uninoculated sample tube has been shown in left tube in each figure. It shows results of Indole test (A), Methyl red Test (B), Voges Proskauer Test (C), Simmon citrate Test (D), Dextrose fermentation test (E), Lactose fermentation test (F), Sucrose fermentation test (G) and Urease test (H).

3.3 Susceptibility to antimicrobial agents

Antibiotic sensitivity assay of all the isolates for Aminoglycosides: amikacin, Ak (10); Phenicol: chloramphenicol, C (10); Folate inhibitor: co-trimoxazole, Co (25); Quinolone and Fluoroquinolones: norfloxacin, Nx (10); ß-lactams: piperacillin, Pc (100).and Tetracyclines: tetracycline, T (30) was performed on Muller Hinton agar by disc diffusion method and zone of inhibition was measured. This zone of inhibition was compared with standard CLSI table and isolates were characterized as resistant (R), Reduced susceptible (RS) and sensitive (S)

(Supplementary Table 2). Data suggested the percentage of resistant, reduced susceptible and sensitive Escherichia coli isolates from all the sites to different antibiotics (Fig. 3) Chloramphenicol (Fig. 3A); Co-trimoxazole (Fig. 3B); Norfloxacin (Fig. 3C); Amikacin (Fig. 3D); Tetracycline (Fig. 3E); Piperacillin (Fig. 3F).

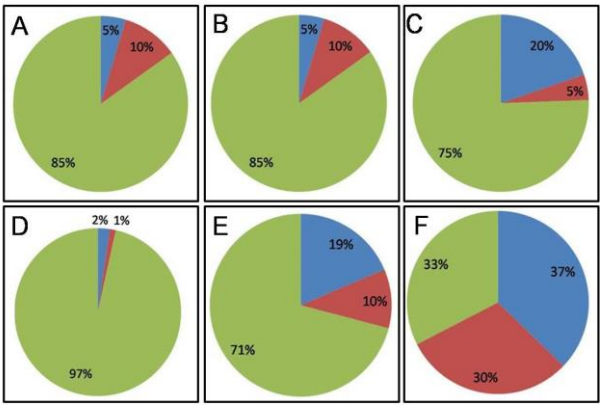

Fig. 3 - The percentage of resistant (blue), reduced susceptible (red) and sensitive (green) *E. coli* isolates against Chloramphenicol (A), Co-trimoxazole (B), Norfloxacin (C), Amikacin (D), Tetracycline (E) Piperacillin (F).

3.4 Computation and specificity of primers for detection *E. coli*

Highly specific real-time PCR primers exhibiting melting temperature 56.03^{0}C - 60.33^{0}C and amplicon size 158-249 bp were computed using dedicated web based primer designing tool, Primer 3 (Table 5).

Genes	Primer sequence (5'-3')	Length (bp)	Tm (^{0}C)	GC (%)	Product size (bp)
stx2	**F:** CGACAGGCCCGTTATAAAAA	20	56.14	45.00	249
	R: TTTTCCGGCCACTTTTACTG	20	56.14	45.00	
eaeA	**F:** AAGGCAAATGGTTCTGATGC	20	56.96	45.00	196

	R: CAACAGCATTACCTGCAACG	20	56.03	50.00	
hlyA	**F:** TGGATGGAGGGGAAGGTAAT	20	57.39	50.00	237
	R: TCCCATTGACATCATTTGACTC	22	56.41	40.91	
LT1	**F:** GATGGCAGGCAAAAGAGAAA	20	60.33	45.00	158
	R: CAATTTTGGTCTCGGTCAGA	20	58.69	45.00	

Table 5 - Primers used for *in-silico* real-time PCR assay

The specificity of computed oligonucleotides was determined against the known microbial genomes by Primer-BLAST (NCBI). The BLAST analysis showed no homology in other genera or species. Hence, the computed primers were highly specific towards their respective targets.

3.5 In-Silico validation of computed primers

In-silico PCR amplification of the genes selected for Escherichia coli were performed using the respective computed primers. In the case of the selected signature virulence gene sequences, in-silico PCR product of 249 bp for stx2 (Fig. 4); 196 bp for eaeA (Fig. 5); 237 bp for hlyA (Fig. 6); 158 bp for LT1 (Fig. 7) were observed.

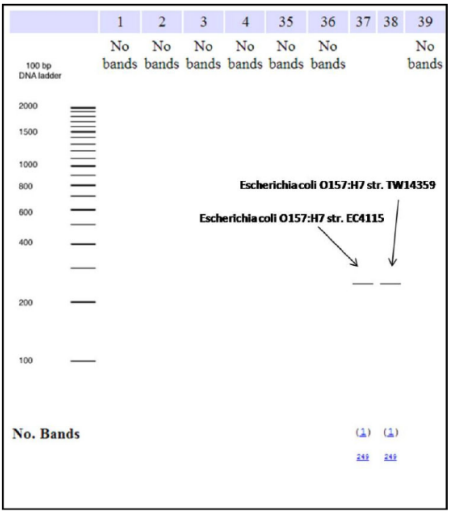

Fig. 4 - Graphical representation of *in silico* PCR amplification results targeting stx2 gene in genome of *E. coli*

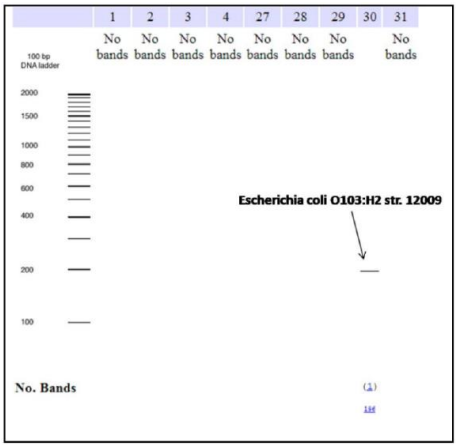

Fig. 5 - Graphical representation of *in silico* PCR amplification results targeting eaeA gene in genome of *E. coli.*

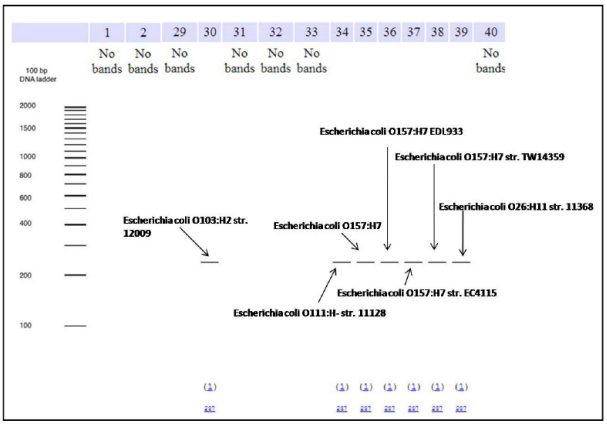

Fig. 6 - Graphical representation of *in silico* PCR amplification results targeting hlyA gene in genome of *E. coli*

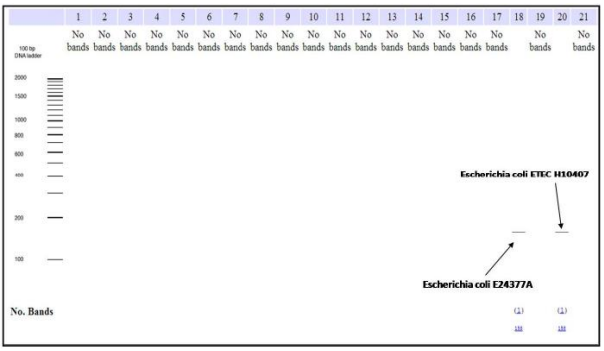

Fig. 7 - Graphical representation of *in silico* PCR amplification results targeting LT1 gene in genome of *E. coli.*

4. Discussion

Pathogen detection has been used in food production process involving tracking of contamination sources, and monitoring regulatory compliance as a means of quality control, Diarrheagenic *E. coli* are the most common bacterial pathogens implicated in diarrhea worldwide. A vast majority of diseases including hemorrhagic colitis, and diarrhea are attributable to contaminated food, lack of sanitation, and hygiene (WHO 2002). In the present study, we found street vended food samples to be contaminated by *E. coli*. Although, the level of *E. coli* should be zero in a food sample (WHO 2002) however we found significantly higher level in the collected food sample (Table 4).

In the present study, we found resistance to multiple antimicrobials in isolated *E. coli*. Multiple antimicrobial resistances have also been found in EHEC/ STEC isolates from humans, surface waters, cattle, and food (Cergole-Novella *et al*. 2006; Manna *et al*. 2006; Ram and Shanker 2005). Furthermore, Webster *et al*. (2004) reported that *E. coli* isolates from urban areas sources have resistance to more antimicrobials than rural source isolates, possibly because of greater exposure to antimicrobials. In the present study, a number of isolates exhibited resistance to Norfloxacin,

Tetracycline and Piperacillin. The resistance to these specific antimicrobials is sometimes encoded by plasmids, which may distribute resistance in susceptible bacteria through horizontal gene transfer (Hall and Barlow 2004; Sayah *et al*. 2005). Diarrhoea, water borne disease is treated by an inadequate quantity of antimicrobials, without identifying a pathogen in many places could be an important factor for multiple antimicrobial resistances in potential EHEC isolates observed in the present study. Furthermore Ram *et al* (2007) have been reported *E. coli* having reduced susceptibility (intermediates) for multiple antimicrobials in surface water. The development of resistance and decreasing levels of susceptibility (intermediates) of *E. coli* to a wide spectrum of antimicrobials is a matter of concern as it may limit the availability of antimicrobials for clinical management of waterborne outbreaks in the future.

The need for rapid detection of pathogenic organisms requires development of methods that are sensitive and highly specific. Real Time PCR has revolutionized PCR technique by application of specific chemistries and instrumentation. The real-time PCR methods are potential tools for diagnostics, environmental monitoring and risk assessment of the microbiological quality of water due to increased specificity, enhanced sensitivity and reduction in analysis time (Kubista M *et al*. 2006; Rompre A, 2002). To provide higher specificity to the assay, signature virulence genes of Enterotoxigenic *E. coli* were chosen.

The computed primers were highly specific towards their respective targets as confirmed from the BLAST results. Several parameters including max score (maximum) and query coverage (maximum) were analyzed for the specificity of oligonucleotides. The performance of computed primers was evaluated by web based *in silico* PCR programme. The computed simulation showed the length and the electrophoretic

mobility of amplicon. This approach helped in selection of the desired primer pairs minimizing possible non-targeted products. This not only improves the specificity but also minimizes time needed for *in vitro* verification of work. Therefore, the computed primers of selected genes would serve as the signature for bacterial identification.

The results of the present study emphasize the human health risk associated with exposure to contaminated street food due to the presence of multi-antimicrobial-resistant *E. coli* exhibiting virulence genes. Therefore, the presence of potential *E. coli* in street food of developing nations requires increased surveillance for risk assessment and prevention strategies to protect public health.

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