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# Use of multiplex PCR assay for detection of diarrheagenic *Escherichia coli* in street vended food items

**Nishat Sarker[1], Sharmin Islam[1], Mehedi Hasan[1], Farzana Kabir[1], Md. Aftab Uddin[2], Rashed Noor[2, *]**

[1]Centre for Communicable Diseases, International Centre for Diarrhoeal Disease Research, Bangladesh (icddr, b), Dhaka, Bangladesh
[2]Department of Microbiology, Stamford University Bangladesh, 51 Siddeswari Road, Dhaka 1217, Bangladesh

**Email address:**
noor.rashed@yahoo.com (R. Noor)

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**Abstract:** Pathogenic forms of *Escherichia coli* are commonly known to cause a variety of diarrheal diseases in hosts. Present study was designed to identify such pathogenic *E. coli* isolates from 7 different groups of street vended foods including cream, egg, non fried dry food and fried dry food, salad, cooked and non cooked items. Among 400 samples studied, almost all were found to be contaminated with an array of bacteria ranging between $3.24\times10^2$ -$6.5\times10^9$ cfu/g. Results of multiplex polymerase chain reaction (PCR) assay revealed that out of 130 isolates of *E. coli* screened from all categories of food items, 52 (13%) were found to be enterotoxigenic *E. coli*, 32 (8%) were the Shiga toxin producing *E. coli* isolates, 14 (3.5%) were enteropathogenic and 6 (1.5%) were found to be enteroinvasive isolates. Results of plasmid profiling of the isolates was further in agreement to the presence of different pathogenic *E. coli* strains.

**Keywords:** Street Vended Foods, Microbiological Spoilage, *Escherichia coli*, Antibiotic Resistance, Multiplex Polymerase Chain Reaction (PCR)

## 1. Introduction

People of Bangladesh and of other countries are gradually getting habituated in consuming foods available around the street side because of their ease of availability, convenience, simplicity of handling, low-cost and some of the appealing factors making street foods popular [1]. However, the microbial hygiene in such practice may underlie within the quality of food items consumed and the frequency of microbial shedding from the food handlers as well their style of handling, preparing and serving food [2-5]. Street vended foods such as poultry, beef, fish, water, juice and rice, which are sold in the street, are frequently identified as vehicles of disease transmission especially in the developing countries with high density of population [6-9]. Therefore, it is necessary to characterize the target microbial pathogen(s) in order to develop a suitable diagnostic test to detect them.

The common food borne enteric bacterial population is known to be largely dominated by the diarrheagenic *Escherichia coli* (DEC) [10, 11]. Commonly isolated diarrheagenic *E. coli* are the enteropathogenic (EPEC) strains, with an increased expression of *eae* gene [12] encoding proteins involved in the formation of attaching and effacing (A/E) lesions on host intestinal cells. Enterohemorrhagic *E. coli* (EHEC) or Shiga toxin producing *E. coli* (STEC) is the cause of hemolytic uremic syndrome, with an increased expression of *stx*1 and *stx*2 genes. The *ipaH* gene in enteroinvasive *E. coli* (EIEC) strains is similar to *Shigella* spp., responsible for inducing dysenteric enteritis in human [13, 14].

In general the diagnosis of *E. coli* demands a number of different selective media and biochemical reagents with an increased test frequencies. Also, the traditional techniques don't allow the detection of virulent genes thereby tending the method to be less specific regarding public health measure. Alternatively, a more specific and sensitive method using the multiplex polymerase chain reaction (PCR) system might be used to reduce the number of tests needed for diagnosis of DEC strains in the traditional procedure [15,16]. Followed by such rationalization, present study thus employed multiplex PCR assay in addition to the conventional methods to identify pathogenic *E. coli* strains from different types of common food items.

collected from street vendor in Dhaka Metropolis.

# 2. Materials and Methods

### *2.1. Sample Collection and Processing*

A total of 400 food samples of 7 categories were randomly collected (Table 1) aseptically, kept in the ice box maintaining 4 °C during transportation, and were analyzed within 2 hours of collection. Ten grams of each sample was transferred to 90 ml of sterile Ringer solution and was homogenized. For microbiological assay, the homogenized suspensions were subject to serial dilutions up to $10^{-6}$.

### *2.2. Microbiological Examination of Collected Samples*

One ml from each diluted tube was transferred into sterile Plate Count Agar (PCA) media through pour plate method for the enumeration of total heterotrophic bacteria. All PCA plates were incubated into 37 °C for 24 hours. Rose Bengal Chloramphenicol Agar (RBA) media, facilitating the growth of fungi, was inoculated with 0.2 ml of samples from each diluted tubes and spread. Following incubation of 3-5 days at 25 °C, all the plates were examined.

### *2.3. Detection and Confirmation of E. coli Isolates*

Detection of *E. coli* was performed by enrichment technique into *E. coli* (EC) broth followed by incubation at 45 °C and by the most probable number (MPN) method. Twenty five gram (25gm) samples were transferred to EC broth and incubated for 24 hours at 45 °C. Following enrichment into EC broth, a loopful inoculum was transferred into Methylene Blue (EMB) agar plates, and incubated for 18-24 hours at 37 °C. Typical colonies with green metallic sheen on EMB Agar media was presumptively considered as *E. coli*. For MPN method, Lauryl Sulfate Broth (LST broth) & Brilliant Green 2% bile broth were used. Media was prepared in McCartney tube with inverted Durham's tube into it and gas production in the tube indicated the positive result. Samples were inoculated into LST broth and incubated for 24 hours at 37 °C. Upon gas formation, a loopful inoculum was transferred to Brilliant Green bile broth and incubated for 24 hours at 37 °C. Numbers of gas forming tubes were then recorded and compared with the standard chart [17]. The morphological and confirmative biochemical tests were further carried out using standard protocol [18].

### *2.4. Determination of Antimicrobial Susceptibility by Kirby-Bauer Method*

A uniform lawn of bacterial growth was prepared on Mueller Hinton agar plate. Antibiotic discs were applied aseptically of the surface of the plates at appropriate spatial arrangements. Plates were then incubated at 37 °C for 24 hours. Commercially available antibiotic discs (Oxoid, Hampshire, UK) were used for the test: ampicillin, (AMP 10µg), amikacin (AMK 10µg), bacitracin (BAC 10µg), ciprofloxacin (CIP 5µg), chloramphenicol (CHL 10µg), ceftriaxone (CEF 30µg), erythromycin (ERT 15µg), gentamicin (GEN 10µg), imipenem (IMP 30µg), kanamycin (KAN 10µg), nalidixic acid (NAL 30µg), nitrofurantoin (NTF 10µg), penicillin (PEN 10µg), piperacillin (PIP 10µg), streptomycin (STR 10µg), tetracycline (TE 30µg), trimethoprim- sulfamethoxazole (TMP-SFM 25µg) and vancomycin (30µg). Susceptibility of organism to antibiotic was interpreted by the presence of clear zone around the disc [16, 19, 20].

***Table 1.*** *Food samples with number and category*

| Groups | Food Sample Name | Street food category | Number of samples |
|---|---|---|---|
| Group-1 | Meonise, Cream Cake, Butter cake, Cream roll, Pasty Cake | Cream based food | 52 |
| Group-2 | Pudding, Cake, Egg chop, | Egg based food | 35 |
| Group-3 | Toast, Biscuit, Dry Cake, Dry Pitha, | Non fried dry food | 35 |
| Group-4 | Singara, Somucha, Potato chop, Beguni, Piaju | Fried dry food | 80 |
| Group-5 | Normal salad, Mix Vegetable Salad, Chicken salad, Chicken macaroni salad, special fruit salad | Salad | 48 |
| Group-6 | Chotpoti, Fuchka, Chola, Belpuri, Noodles | Cooked food | 80 |
| Group-7 | Chicken nugget, Meat Ball, Chicken sausage, Chicken Sandwich slice, chicken wings, chicken kivs, Fish finger. | Non cooked processed food | 70 |

### *2.5. Plasmid Profiling of Selected Isolates*

Plasmid extraction was done according to some modification of alkaline lysis method as described previously [21, 22]. Isolates were grown overnight in LB broth (HiMedia, India) (with 2% NaCl) and cell pellet was collected by centrifugation in 13000 rpm for 5 minutes, which was resuspended in 20 µl of TE buffer (50mM Tris, 1 mM EDTA, pH 8.0). Cells were lysed by adding 100 µl of

lysis buffer (50 mMTris, 3% SDS, pH 12.6) to each tube, gently mixed and incubated at 56 °C for 30 minutes followed by the addition of 100 µl chilled Phenol: chloroform: iso-amyl alcohol (25:24:1). After shaking the homogenous mixture was centrifuged for 15 min at 13000 rpm. Forty (40) µl from the upper aqueous phase (consisting of plasmid) was removed to a clean Eppendorf tube, and electrophoresed on 0.8% agarose gel for 1 h at 90V followed by ethidium bromide (0.5µg/ml) staining. The gel was then visualized on a UV trans-illuminator (Bio-Rad, 170-8195). The size of plasmid was measured by comparing the band with a 1kb ladder band and reference strains (Table 2).

### *2.6. Identification of E. coli by Multiplex PCR Assay*

Selected isolates were overnight grown in 2 ml Luria-Bertani (LB) broth at 37 °C with shaking. Thirty-six µl of culture was added to 4 µl of 10× Tris-EDTA buffer, and 60 µl of 2× proteinase K buffer. After incubation at 56 °C for 90 min and 10 min at 95 °C, the sample was centrifuged at 10,000g for 1 min, and the supernatant was used as DNA template. The reference strains and primers used in this study are listed in Table 2.

PCR was conducted using standard protocol [14]. The optimized procedure was carried out with a 50-µl mixture containing Tris-HCl (10 mM, pH 8.3), KCl (50 mM), $MgCl_2$ (2 mM), gelatin (100 µg/mL), glycerol (5% v/v), dNTP (200 µM), Taq polymerase (0.5 U/23 µL); 0.25 µM of each primer and 5 µl of the DNA template. The PCR program was 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min, for 30 cycles, and 72 °C for 10 min. PCR products were then electrophoresed on a 2.5% agarose gel (Takara; Bio-Rad Laboratories), stained with ethidium bromide, and visualized by UV trans-illuminator.

***Table 2.*** *Primers used for multiplex PCR assays*

| Strain | Locus | Primer sequence | Size (bp) |
|---|---|---|---|
| ETEC | *lt* | F:5′GGC GAC AGA TTA TAC CGT GC3′<br>R:5′CGG TCT CTA TAT TCC CTG TT3′ | 440 |
| ETEC | *st* | F:5′ATT TTT CTT TCT GTA TTG TCT T3′<br>R:5′CAC CCG GTA CAA GCA GGA TT3′ | 191 |
| EPEC | *bfp*A | F:5′AAT GGT GCT TGC GCT TGC TGC3′<br>R:5′ GCC GCT TTA TCC AAC CTG GTA3 | 324 |
| EPEC | *eae*A | F:5′ GAC CCG GCA CAA GCA TAA GC3′<br>R:5′CCA CCT GCA GCA ACA AGA GG3′ | 384 |
| STEC | *stx*1 | F:5′CTG GAT TTA ATG TCG CAT AGT G3′<br>R:5′AGA ACG CCC ACT GAG ATC ATC3 | 150 |
| STEC | *stx*2 | F:5′GGC ACT GTC TGA AAC TGC TCC3′<br>R:5′TCG CCA GTT ATC TGA CAT TCT G3 | 255 |
| EIEC | *ial* | F:5′GGT ATG ATGATGATG AGT CCA 3<br>′R:5′ GGA GGC CAA CAA TTA TTT CC 3′ | 650 |

# 3. Results and Discussion

### *3.1. Prevalence of Microorganisms in the Food Samples*

*E. coli* strains are the principal pathogens resulting in food borne disease outbreaks [22-26]. In the current study, A huge array of microorganisms was observed (Table 3). The lowest bacterial count was found in Group 7 and the highest was in Group 6 (Table 3). The highest fungal load was observed in Group 6, i. e., in the cooked food samples. The huge microbial contamination in the samples revealed that the food items sold in roadside might encounter the environmental factors promoting microbial access.

***Table 3****. Total viable bacterial and fungal load.*

| Sample groups | Total bacterial count (cfu/g) | Total fungal count (cfu/g) |
|---|---|---|
| Group 1 | $2.6x10^4$- $6.52x10^7$ | $2.32\ x10^2$-$2.3\ x10^4$ |
| Group 2 | $5.72\ x10^4$-$9.15\ x10^7$ | $2.13\ x10^2$-$4.9\ x10^5$ |
| Group 3 | $9.6\ x10^3$-$4.52\ x10^8$ | $1.52\ x10^2$-$4.32x10^4$ |
| Group 4 | $4.62\ x10^5$-$7.62\ x10^8$ | $2.54x10^2$-$3.21\ x10^5$ |
| Group 5 | $5.72\ x10^4$-$1.52\ x10^8$ | $2.51x10^2$-$8.15\ x10^5$ |
| Group 6 | $3.94\ x10^5$-$6.5\ x10^9$ | $2.32\ x10^2$-$9.6\ x10^5$ |
| Group 7 | $3.24\ x10^2$-$3.24\ x10^6$ | $5.15\ x10^2$ -$7.73\ x10^4$ |

### *3.2. Prevalence of E. coli*

As per our current study, presence of *E. coli* as well as the presence of coliforms in the food samples studied may indicate fecal contamination which might be due to insufficient cooking, use of raw vegetables, cross contamination between raw and cooked food and contaminated ingredients [24]. The highest number of *Escherichia* isolates was found in Group 6 referring to the cooked food items. Considering the amount of samples per group the highest prevalence was observed in Group 5 (salad items). The presence of *E. coli* in salad items is consistent to our previous study [27]. However, the lowest prevalence of *E. coli* was found in Group 7 (non cooked processed foods).

### *3.3. Drug-Resistance Traits of the Isolates*

The isolates were found to exhibit 100% sensitivity against imipenem (IMP), 95% against amikacin (AMK), 94% against kanamycin (KAN) and 90% against nalidixic acid (NAL) (Figure 1). Isolates showed 16% resistance against trimethoprim-sulfamethoxazole (TMP-SFM), 18% against chloramphenicol (CHL), 28% against tetracycline (TE). Since these drugs are known to be highly effective against *E. coli* [28], our results are assumptive of the probable presence of resistance gene [29]. Due to uncontrolled use of antimicrobial agents, such high incidence of multi-drug resistance may apparently prevail which may ultimately replace the drug sensitive microorganisms from antibiotic saturated environment [30].

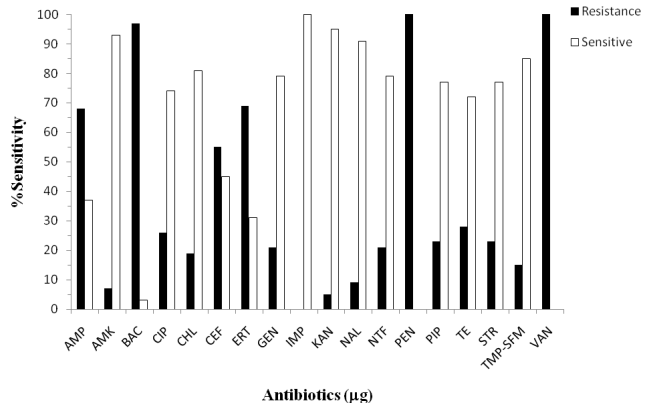


***Figure 1.*** *Antibiotic susceptibility pattern of the isolated E. coli (n=130) against different antibiotic which were measured in vitro through Kirby-Bauer method. Commercially available antibiotics used for the assay as mentioned in Materials and Method section.*

### *3.4. Demonstration Drug-Resistance through Plasmid Profiling*

Plasmid profile analysis has been widely used in epidemiological investigations [11]. In our study, the plasmid profiling of antibiotic resistant *E. coli* isolates revealed that the isolates consisted of plasmids of various sizes such as 1200bp, 1700bp, 2500bp, 3800bp, 5000bp, 7000bp and 8000bp (Figure 2). Several isolates showed very similar plasmid profiles. However, isolates that showed multiple drug resistance were also found to harbor plasmids with sizes ranging from 1kb to 8 kb. Although some isolates exhibited different antibiotic resistance patterns, some of their plasmids showed similar pattern of migration; for example, band with the size of 5000 bp was almost common in all lanes (Figure 2).

### *3.5. Multiplex PCR Assay for Identification of Specific Group of E. coli*

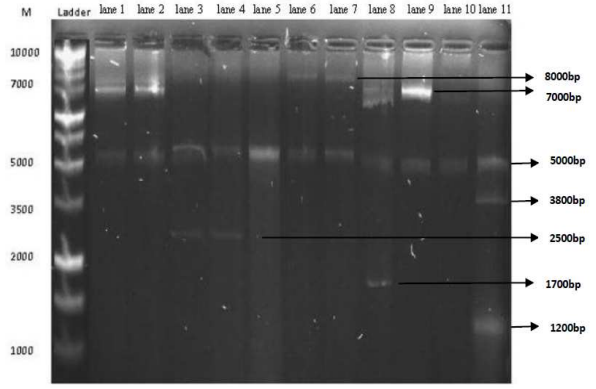


***Figure 2.*** *Plasmid profile of E. coli isolates. Lane 1 indicates the Ladder DNA. Lane 2 to lane 10: E. coli isolates isolated from food samples. All of the isolates showed bands at around 5000 bp.*

In this study for detection of four different categories of *E. coli* concurrently, a mixture of seven primer pairs were used for multiplex PCR assays which showed 100% specificity in identifying the reference strains. PCR products of the multiplex PCR assays derived from the pure cultures of reference strains of EPEC, EIEC, STEC, ETEC and clinical isolates. For the final confirmation of *E. coli* presence and their type, Multiplex PCR has been carried out. Form our study; the PCR assays detected 52 (13%) ETEC isolates, 32 (8%) STEC isolates, 14 (3.5%) EPEC isolates and 6 (1.5%) EIEC isolates.

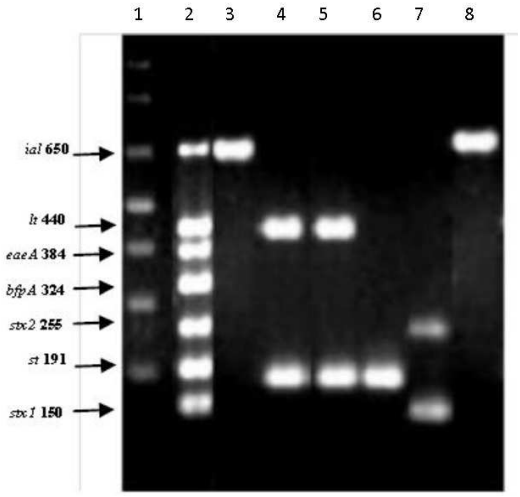


***Figure 3.*** *Multiplex PCR of reference strains and food samples. Lane 1: PCR products of each locus in base pairs, obtained from DNA mix of the four reference strains and the primer mix., Lane 2: 1 kb molecular weight marker in base pairs; Lanes 3-8: PCR products of isolates. In lane 3: ial (650bp) which was indicative of EIEC; lane 4, 5 and 6 was same: lt (440bp) and st (191bp) indicative of ETEC; lane 7 stx1(150bp) and stx2 (255bp) indicates STEC.*

As depicted from Figure 3, the highest prevalence was recorded for the ETEC isolates. The virulence gene of EPEC was *bfp*A (324bp) and *eae*A *(*384bp), ETEC was *lt* (440bp) and *st* (191bp), EIEC was *ial* (650bp), *stx*1 (150bp) and *stx*2 (255bp). Several recent studies have reported an increase in prevalence of atypical ETEC strains as has also been found in our study [31]. However, the prevalence of EPEC (3.6% in the diarrhea group) was lower in our study than in some previous studies [32].

The limitations associated with traditional diagnostic techniques can be overcome by PCR which is a sensitive, specific and rapid method for diagnosis. This study showed that multiplex amplification of nucleic acid can be used as a replacement for conventional method in detection of diarrheagenic *E. coli* strains. Conventional method requires multiple test and approximately 7 days are required to get confirmatory results whereas multiplex PCR assay requires only 3 days.

## 4. Conclusions

The presence of *E. coli* in food items as found in our study is assumptive of the probable presence of other pathogenic microorganisms which may generate serious food borne complications. Further research on drinking water associated with the food items studied would expand the total prevalence of the pathogenic *E. coli* and other

microorganisms. Another important aspect of our findings lies over the detection of virulent genes which is not only confirmative of the study appropriateness, but also makes a way to endorse the rapid molecular diagnosis over the conventional cultural and biochemical methods. Finally, the results presented here could be sufficient to comprehend the unhygienic state of street foods and hence safety measures are to be initiated in order to control food spoiling microorganisms.

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