



**Original article**

## Detection of diarrhoeagenic *Escherichia coli* virulence genes by multiplex-PCR method and their antibiotic susceptibility profile

Z. Nazari<sup>a,\*</sup>, K. Amini<sup>b</sup>, A. Mokhtari<sup>c</sup>

<sup>a</sup>Department of Microbiology, Saveh Branch, Islamic Azad University, Saveh, Iran.

<sup>b</sup>Assistant Professor, Department of Microbiology, Saveh Branch, Islamic Azad University, Saveh, Iran.

<sup>c</sup>Department of Microbiology, Science and research Branch, Islamic Azad University, Tehran, Iran.

\*Corresponding author; Department of Microbiology, Saveh Branch, Islamic Azad University, Saveh, Iran.

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### ABSTRACT

Acute diarrhoeal disease is a public health problem and an important cause of morbidity and mortality, particularly in developing countries. The etiology is varied, and the diarrhoeagenic *Escherichiacolipathotypes* are most important. Our objectives were detection of Diarrhoeagenic *E. coli* virulence genes by Multiplex-PCR method and their antimicrobial susceptibility profile. Fifty five *E. Coli* strains were isolated from 150 fresh fecal samples. Multiplex PCR was performed to characterize the diarrhoeagenic *E. coli* strains. Antibiotic susceptibility patterns were determined by the disc-diffusion method. The highest and lowest frequency of DEC isolates were 12.7% and 1.8% for EHEC and EIEC, respectively. *bfp* and *stx1* genes were not carried out by DEC strains. Thirty six isolates were negative for any gene amplification. The highest rate of resistance among DEC strains were related to Imipenem with 100% frequency. All DEC isolates (100%) were susceptible to ciprofloxacin. Differentiation between the diarrhoeagenic *E. coli* pathotypes are of great importance since they are complicated in acute diarrheal illnesses and may need specific antimicrobial therapy. The high antimicrobial resistance raises a broad discussion on the indiscriminate or improper use of antimicrobials, besides the risks of self-medication.

## 1. Introduction

Diarrhoeal diseases are a global public health problem, causing significant morbidity and mortality among infants and children under five years old particularly in the developing countries (Onanuga et al., 2014). Approximately 2.6 million deaths occur each year, especially among children (Bryce et al. 2005). Person to person transmission by direct or indirect contact, consumption of contaminated foods, drinking sewage-contaminated water and unpasteurized juice are the most modes of obtaining the infections (M Ali et al. 2014). Many cases are not identified, because of, they are minor and self-limiting, in which the patient does not seek medical care, or because, particularly in developing countries, the medical and laboratory resources are not accessible (Toma et al., 2003). Despite the various accessibility microbiological test, approximately half of the cases of diarrhea have no clear etiology, which confuses the application of strategies for mapping and monitoring endemic regions of the incidence of such pathogens (Garcia et al., 2011). *Escherichia Coli* (*E. coli*) is one of the most significant etiological agent of acute diarrhea in Iran (Alikhani et al., 2007) and other developing countries (Nweze, 2010). Six different patho types of diarrheagenic *E. coli* (DEC strains) are well identified according to their patterns of gastrointestinal illness and virulence determinants: Enteropathogenic *E. Coli* (EPEC), Enterotoxigenic *E. Coli* (ETEC), Shiga toxin-producing *E. Coli* (STEC) or Enterohaemorrhagic *E. Coli* (EHEC), Enteroaggregative *E. Coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. Coli* (DAEC) (Croxen et al., 2010). The DEC strains pathogenesis mechanisms are genetically coded by chromosome, plasmid, and phage and contain heat-labile (LTI, LTIIa, and LTIIb) and heat-stable (STI, STIIa and STIIb) toxins, verotoxin or shiga-like toxin types 1, 2, and 2e (VT1(SLT1), VT2(SLT2), and VT2e(SLT2e), respectively), cytotoxic necrotizing factors (CNF1 and CNF2), attaching and effacing mechanisms (*eaeA*), enteroaggregative (*Eagg*), and enteroinvasive (*Einv*) mechanisms (Kaper et al., 2004). The detection of DEC pathotypes is via the use of molecular approaches for recognition of the genes responsible for pathogenicity (Paton et al. 2002). This is necessary for identification and classification of DEC and is according to the presence of various chromosomal and/or plasmid-encoded virulence elements that are absent in commensal *E. Coli* (Pass et al. 2000). Furthermore, the frequency and other epidemiological features of the pathogens as causative agents of diarrhoea differ from one area to the other, and even between and within one area in the same geographical region (Al-Dulaimi et al., 2015). Reliable and accurate identification of diarrheagenic *E.coli* can't be accomplished only by microbiological, biochemical, serotyping routine tests, since they are indiscernible from the non-pathogenic *E. coli* that frequently found in human feces (Nataro et al., 1998). So, only DNA based method, for example, polymerase chain reaction (PCR) assay and sequencing can be used for rapid and reliable identification, and which has a high sensitivity and specificity for their recognition (Vu Nguyen et al. 2005).

Diarrhoeal disease is self-limiting and responsive to oral rehydration therapy. Nevertheless, antibiotic therapy is suggested for critically ill patients and for those with risk factors for invasive infection such as AIDS, cancer, neonatal and hematologic malignancies (Hohmann et al., 2001). High frequency of resistance to antimicrobial agents among DEC strains has been described from several developing countries. In the recent years, antibiotic resistance has extensively increased, which has led to wide defeat in antibiotic therapy, mostly in gram negative infectious disease. Misuse, abuse and overuse of antimicrobial agents are the chief reasons to appear resistant bacteria in clinic. In some countries, arbitrary use of antimicrobial agents and food additives in livestock, poultry and household cleaners can cause mutations and selection pressure in organisms that cause the creation of resistant strains (Nguyen et al. 2005). So, the aim of this study was the detection of the targets selected for each category were *eae* for EPEC, *stx* for STEC, *e/t* and *est* for ETEC, *ipaH* for EIEC and *bfpA* for EPEC by multiplex-PCR assay. Therefore we survey of antibiotic susceptibility profiles in the DEC isolates.

## 2. Materials and methods

### 2.1. Sample collection and bacterial isolation

This descriptive-cross sectional study was performed in a period of 5 months in 2014. A total of 150 stool specimens collected from children younger than 5 years old with complaints of diarrhoea (with or without fever or other accompanying signs) admitted to the different hospitals in Tehran, Iran. Information about some epidemiological data was collected through questionnaires. The data comprised results of the doctor's physical examination and clinical signs, counting fever, abdominal pain, vomiting, convulsions, dysentery, and dehydration using standard methods. Feeding performs (breastfeeding, artificial supplements, or both [mixed]), history of drug use, source of drinking water (treated or untreated), and history of travel abroad in the one month prior to stool collection were also recorded. Inclusion criteria in the study were patients with diarrhea, characterized by the occurrence of three or more, loose, liquid, or watery stools or at least one bloody loose stool in a 24-h period. The majority of children specimens were from low-income families. Stool samples from enrolled children were collected using a wide-mouthed sterile plastic containers and transported within two hours of collection to the microbiology laboratory for analysis. All samples were cultured on the Mac Conkey agar (Difco, Detroit, MI, USA) for isolation of *E. Coli*. After incubation at 37°C for 24h, three lactose-fermenting colonies and a demonstrative non-lactose fermenting colony with a various morphological appearance were picked, and their identities were evaluated using a panel of microbiological and biochemical routine tests interpreted as previously reported [7] and the API 20E system (bioMerieux, Marcy l'Etoile, France).

### 2.2. Antibiotic susceptibility test

Antibiotic susceptibility test was accomplished by disk diffusion method agreeing with Clinical and Laboratory Standards Institute (CLSI, 2011) guidelines (7). A loopful of bacterial colony from each sample was suspended in 200µl sterile saline solution in eppendorf tubes and turbidity adjusted to 0.5 McFarland standard. Then, samples cultured on Mueller Hinton agar (Merck Co., Germany) media, and antibiotic disks placed in standard distances on the plates and incubated in 37 °C and for 24h. The following antibiotics were examined using a disk diffusion method; imipenem (30 µg), tetracycline (30 µg), ceftriaxone (2 µg), ofloxacin (5 µg), ceftazidime (5 µg), gentamicin (5 µg), ciprofloxacin (2 µg) which prepared for HIMEDIA company (Himedia Laboratories Pvt. Limited-INDIA). Also, the DEC reference strain of ATCC 25922 was used as a positive control for evaluating quality of procedures.

### 2.3. Multiplex-PCR

Genomic DNA from each confirmed *E. coli* isolate was extracted from the organism after a 24h incubation period on the nutrient agar plates (Merck Co., Germany) by the Cinnagen extraction kit (Cinna Pure DNA KIT-PR881613). The oligoneucleotide primer sequences are listed in table I. The Multiplex-PCRs were performed with a 25µl reaction mixture containing 3µl of template DNA, 2µl of 10×PCR buffer II, 0.5 µl of a 1.25 mM mixture of deoxynucleoside triphosphates, 0.7 µl of 25 mM MgCl<sub>2</sub>, 0.1 µl of 5U of AmpliTaq Gold DNA polymerase (Perkin-Elmer) and a 0.6 µl concentration of each primer. The thermocycling conditions with a Gene Amp PCR system 9700 (AB Applied Biosystem) were as follows: 95°C for 5 min, 94°C for 20 s, 55°C for 20 s, and 72°C for 10 s for 35 cycles, with a final 7-min extension at 72°C. PCR products were evaluated with a 1% (wt/vol) agarose gel (Gibco Life Technologies, Paisley, United Kingdom) at 120 mV for 30 min.

### 2.4. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, ver. 19 (IBM) for descriptive statistical tests.

### 3. Results

#### 3.1. Population study, bacterial isolates and antibiotic susceptibility profile

Ninety three (62%) patients were male and 57 (38%) patients were female. Out of 150 stool samples, 55(36.6%) were identified as *E. Coli* strains. The antimicrobial susceptibility testing by agar disk diffusion method among *E.coli* isolates determined that the percentage of resistance to gentamicin, Imipenem, ciprofloxacin, tetracycline, Ceftriaxone, Ceftazidime and Ofloxacin were 3.4%, 100%, 0%, 73.4%, 56.6%, 53.3% and 16.7%, respectively (Table II). The highest rate of resistance among Diarrhoeagenic *E. Coli* was related to Imipenem with 100% frequency. All DEC isolates (100%) were susceptible to ciprofloxacin.

#### 3.2. Multiplex-PCR amplification test

The minimum criteria for identification of diarrheagenic *E. coli* were defined as follows: the presence of *eltB* and/or *estA* for ETEC, the presence of *stx1* and/or *stx2* for EHEC (the additional presence of *eaeA* confirms the detection of a typical EHEC isolate), the presence of *bfpA* and *eaeA* for typical EPEC (but the presence of only *eaeA* for atypical EPEC), the presence of *ipaH* for EIEC and the presence of *Pcvd<sub>432</sub>* for EAEC. The highest and lowest frequency of DEC isolates were 12.7% and 1.8% for EHEC and EIEC, respectively. *bfp* and *stx1* genes were not carried out by DEC strains. Thirty six (65.4%) isolates were negative for any gene amplification, therefore, these strains were not categorizable. The distribution of the DEC strains were shown in the Table III. The multiplex-PCR-amplified DNA products of these genes are shown in Figures.

### 4. Discussion

Diarrhoeagenic *Escherichia coli* (DEC) strains are pathogens of global public health importance affecting both adults and children worldwide. Most diarrhoeal cases in children under 5 years old are due to diarrhoeagenic *E. coli* infection and EAEC, ETEC and EPEC pathotypes are the most dominant in the developing countries (Kaper et al., 2004).

Out of 150 stool samples, 53(35.3%) were DEC strains. This significant association of DEC with diarrhoea is consistent with other previous studies carried out in another area such as Ghana (Addy et al., 2004), Brazil (Garcia et al., 2011) and Nigeria (Nweze, 2010). Parallel results have also been stated from the range between 21.4% in India (Nair et al. 2010) and 36.8% in Brazil (Garcia et al., 2011). The low rate of DEC among the diarrhoeal cases also proposes that other causative agents (such as protozoan *Giardia*, *rotavirus*, *Shigella* and *Salmonella*, *Campylobacter*, *Yersinia* spp.) that were not examined in the study might be other reasons of diarrhoea (Dans and Martinez, 2006; Navaneethan and Giannella, 2008). Five common pathotypes of *E.coli* which contain ETEC, EPEC, STEC, EAEC and EIEC were distinguished in this study.

EPEC is presently categorized into two subcategories, that is, typical and atypical EPEC. Whereas typical EPEC is well-known pathogen, the pathogenicity of atypical EPEC is still a subject of argument (Swimm and Kalman, 2008). The EPEC strains detected in this study were all atypical EPEC. The results of the current study showed out of 55 *E.coli* strains, 3 (5.4%) isolates belonging to atypical EPEC pathotype (containing *eae* gene without the presence of *bfp* gene). Borjian in the 1998 (Borjian, 1998), and Asadi in the 2010 (Asadi et al., 2010) showed the prevalence of EPEC strains in diarrheal children were 5.4% and 5.3%, respectively. This data have agreed with our study, and support the finding of previous studies that there is a significant association between EPEC pathotype and diarrhoeal infection (Moyo et al., 2007). So, Animals act as a reservoir of atypical EPEC strains and removing of atypical strains in comparison with typical isolates (which their reservoir is human) may be more complicated (Trabulsi et al., 2002; Paula and Marin, 2008). Atypical strains are more common in industrialized countries due to the processes of industrialization in such countries (Orlandi et al., 2001). Climate and weather conditions, food hygiene, lack of hygiene, sanitation and low socio-economic status communities could consider as another predisposing factor for higher prevalence of atypical strains, and also contact with livestock by children under 5 years could play roles in transferring this group of *E.coli* (Ali et al., 2003, Feachem and Koblinsky, 1984).

EAEC strains were the lowest recovered DEC pathotypes in this study. Out of 55 isolates, the frequency of EAEC pathotype was 3.6% (N; 2). All of EAEC strains were positive for *CVD<sub>432</sub>* gene, therefore detected as

EAEC pathotype. Onanuga et al., showed the most frequency recovered DEC pathotypes in their study (Onanuga et al., 2014). This contrast is as a result of geographical distance and level of hygiene.

Shiga toxin-producing *E. coli* (STEC) infection causes acute and bloody diarrhoea. The STEC strains isolated in this study were made up of 7 strains with *Stx2*, 0 with *Stx1* and 0 with both *Stx1* and *Stx2* genes. This observed prevalence of STEC strains disagrees with the results of preceding workers who described very low or no isolation in children with diarrhoea (Nweze, 2010; Nair et al., 2010; El Metwally et al., 2007) whereas it is similar to reports of Alikhani et al. (Alikhani et al., 2007) in Iran and Garcia et al. (Garcia et al., 2011) in Brazil who obtained STEC about 8.7% and 7.4% respectively. The reason for the isolation of this patho type within the study environment is not known and needs further study. The ETEC strains in this study were made up of 13 strains carrying out genes encoding LT, 6 strains with ST and 1 strain with both LT and ST enterotoxins. This higher frequency of LT-ETEC over ST-ETEC has been formerly reported by Valentiner-Branth et al. (Valentiner-Branth et al., 2003) in Guinea and El Metwally et al. (El Metwally et al., 2007) in Egypt while the recognition of ETEC strains producing the LT and ST enterotoxins has been described by Okeke (Okeke et al., 2000) and El Metwally et al. (El Metwally et al., 2007). The high prevalence of ETEC diarrhoea may be due to the fact that the older children lose the immunity conferred on them through the antibodies delivered to them from the breast milk of their mothers.

The isolation of 3(5.4%) EIEC isolates in this study agrees with earlier studies in the recognition of very low rate of this pathotype that is known to cause diarrhoea signs related to shigellosis in adults and children (Garcia et al.2011 & El Metwally et al. 2007). The lack of epidemiological attention to EIEC is associated with the low frequency of this pathogen as a reason for diarrhoea when compared to other pathotypes of DEC. As pointed out by Vierra et al. (Vieira et al., 2007), investigators have not informed the isolation of these organisms from patients with diarrhoea. One of the causes why the isolation rate of this type of DEC is low is connected to the circumstance that they are missed when only lactose fermentation is used as an initial screening tool for diarrhoeagenic *E. Coli* since over 70% *E. Coli* in this group do not lactose fermentation. In the current study, 5 strains were positive both *est* and *stx2* genes, 4 strains were harboring for *est*, *stx2* and *lpaH* genes, 4 strains were positive both *elt* and *stx2* genes and one strain was positive only *elt* genes.

Aranda et al explored the pathotypes of typical and atypical EPEC, EAEC, ETEC, EIEC, STEC and *Shigella* spp., in acute bloody diarrhea patients (Aranda et al. 2004). The incidence of typical EPEC, atypical EPEC, EAEC, EIEC, *Shigella* spp., and STEC strains were 6%, 4.7%, 2%, 2%, 0.7% and 0.7%, respectively (Aranda et al. 2004). These conflicting results can be in a result of differences in health standards of geographic area. In similar study in Tanzania (Moyo et al., 2007), out of 22.9% of *E. coli* strains, 14.6%, 92.3% and 3.6% were EAEC, EPEC and ETEC patho type, respectively. The genes identified in the study were included; *agg Randaa* genes for EAEC, *eae* and *bfpA* genes for EPEC and *Stlaor Stlb* genes for ETEC. EIECpathotype not founded in any of the samples (Moyo et al. 2007). The Study showed typical EAEC and typical EPE Care the main causes of bloody diarrhea in Tanzania and the results have a contrast with the present study. This disagreement may be related to food hygiene and the appropriate handling of foods, particularly cooking using safe, diet and people's lifestyle.

Delay in treatment of infectious illnesses could lead to increase the mortality and morbidity in patients with serious infections, particularly those caused by antibiotic resistant bacteria (Ibrahim et al. 2012). Tankhiwale et al, reported 82%, 79.9%, 38% and 41.3% *E. coli* strains were resistance to cotrimoxazole, ampicillin, nitrofurantoin and Ceftizoxime, respectively (Tankhiwale et al., 2004). In the Bouzari et al study, the highest susceptibility was related to nalidixic acid, gentamicin and ciprofloxacin (Bouzari et al. 2001). In the present study, prevalence of multi-drug resistance (MDR) strains were 60%. Our results showed 100% and 73.4% of isolates were resistant to imipenem and tetracycline which is inconsistent with the results from the other researchers around the world. Therefore, these antibiotics would not be appropriate drugs for treatment of patients with diarrheagenic *E. coli*.

## 5. Conclusions

Atypical EPEC is common causes of diarrhea in children under 5 years and being detected more frequently in worldwide. Multiplex PCR is a rapid, affordable, specific and sensitive method for identification of virulence genes in a short period of time. However, considering the difficulty of accomplishment phenotypic

method in some laboratories, the multiplex PCR presented here is a practical and rapid diagnostic tool for recognition of diarrheagenic *E. Coli* in a single reaction tube. The authors declare no conflict of interest.

**Table 1**

Primers used in the multiplex PCR.

Target gene	Primer sequence (5' → 3')	Amplicon size(bp)
eae	F= 5'-CTGAACGGCGATTACGCGAA-3'	917
	R=5'-CCAGACGATACGATCCAG-3'	
bfPA	F=5'-AATGGTGCTTGCCTTGTCTGC-3'	326
	R=5'-GCCGCTTTATCCAACCTGGTA-3'	
CVD432	F=5'-CTGGCGAAAGACTGTATCAT-3'	630
	R=5'-CAATGTATAGAAATCCGCTGT-3'	
LT	F=5'-GGCGACAGATTATACCGTGC-3'	450
	R=5'-CGGTCTCTATATCCCTGTT-3'	
ST	F=5'-ATTTTTMTTCTGTATRTCTT-3'	190
	R=5'-CACCCGGTACARGCAGGATT-3'	
IpaH	F=5'-GTTCTTGACCGCCTTCCGATACCGTC-3'	600
	R=5'-GCCGGTCAGCCACCCTCTGAGAGTAC-3'	
Stx1	F=5'-ATAAATCGCCATTCGTTGACTAC-3'	180
	R=5'-AGAACGCCACTGAGATCATC-3'	
Stx2	F=5'-GGCACTGTCTGAAACTGCTCC-3'	255
	R=5'-TCGCCAGTTATCTGACATTCTG-3'	

**Table 2**

Antimicrobial susceptibility profile.

Interpretation	GM	IMP	CIP	TET	CF	CAZ	OFX
% of Sensitivity	96.6(53)	0	100(55)	26.6(15)	43.4(24)	43.4(24)	66.6(37)
% of Intermediate	0	0	0	0	0	3.3(2)	16.7(9)
% of Resistance	3.4(2)	100(55)	0	73.4(35)	56.6(31)	53.3(29)	16.7(9)

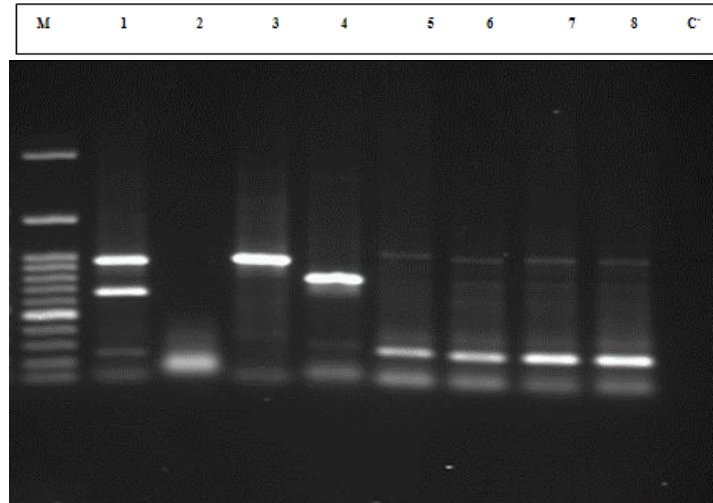
GM: Gentamicin, IMP: Imipenem, CIP: Ciprofloxacin, TET: Tetracycline, CF: Ceftriaxone, CAZ: Ceftazidime, OFX: Ofloxacin.

**Table 3**

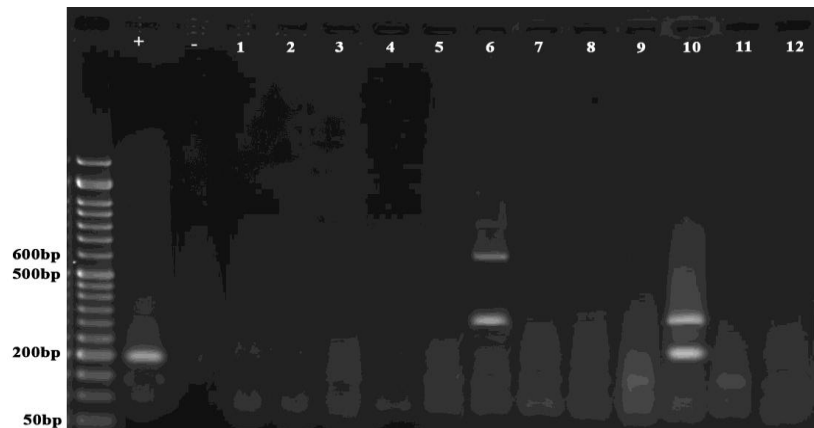
Distribution of the selected target genes in the DEC strains.

Category	Gene	No (%). of positive by M-PCR
EPEC	<i>bfp</i>	0(0.0)
	<i>eae</i>	3(5.4)
STEC/VTEC/EHEC	<i>stx1</i>	0(0.0)
	<i>stx2</i>	7(12.7)
ETEC	<i>elt</i>	4(7.2)
	<i>est</i>	2(3.6)
EAEC	<i>Pcvd<sub>432</sub></i>	2(3.6)
EIEC	<i>ipaH</i>	1(1.8)
None	-	36(65.4)





**Fig. 1.** Agarose gel electrophoresis of products from multiplex PCR.M; 100bp DNA ladder, 917 bp (*eaeA* gene) and 326bp (*bfpA* gene); the presence of *bfpA* and *eaeA* for typical EPEC (but the presence of only *eaeA* for atypical EPEC), 630 bp (*Pcvd<sub>432</sub>* gene for EAEC), C<sup>-</sup>; negative control (*Klebsiellapneumoniae* subsp. *pneumoniae* ATCC 13883).



**Fig. 2.** Agarose gel electrophoresis of products from multiplex PCR.M; 50bp DNA ladder, C<sup>+</sup>; positive control (*Escherichiacoli* ATCC 25922), C<sup>-</sup>; negative control (*Klebsiellapneumoniae* subsp. *pneumoniae* ATCC 13883); 450 bp (*elt* gene) and 190bp (*est* gene) for ETEC; 600 bp (*ipaH*gene for EIEC); 255 bp (*stx<sub>2</sub>* gene for STEC).

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