

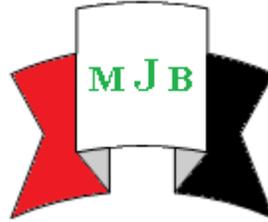
## Molecular Characterization and Antibiotic Susceptibility of Diarrhegenic *Escherichia coli* from Children

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### **Abstract**

In Thi Qar there are relatively few studies have been done to reveal and classify diarrhegenic *Escherichia coli* (DEC) strains among children with diarrhea. This study focused genetically at investigating DEC among hospitalized children under 2 years in Thi Qar city.

A total of 200 children (Below 12 years) with diarrhea and 75 without diarrhea were their stools investigated using culture on MacConkey and EMB agar, and *E. coli* isolates were examined for detection of diarrhegenic *E. coli* types, antimicrobial susceptibility pattern. Virulence genes detected using PCR. A multiplex PCR system method was used to detect a species specific gene for *E. coli* and ten different virulence genes for detection of five pathogroups of DEC namely enteroaggregative- (EAEC), enteropathogenic- (EPEC), enterotoxigenic- (ETEC), enteroinvasive- (EIEC) and enterohemorrhagic- *Escherichia coli* (EHEC).

The study has indicated that diarrhegenic *E. coli* isolates were found mostly in stools of children with diarrhea. DEC diarrhoeagenic *E. coli* was significantly detected among diarrheic children (44.8%) compared with control children (16.4%). Of the DEC pathotypes examined, EAEC was found in (64.73%), ETEC in (19.5%), EPEC in (10.5%) and EHEC in (5.27%) of diarrheic. Virulence-gene factors in DEC isolated from children with diarrhea and from controls. The distribution of virulence gene was; *pCVD432* 93.6%, 87.3%; *eaecA* 64.2%, 8.5%; *bfpA* 59.1%, 2.05%; *stx1* 22.8%, 0.8%; *stx2* 18.6%, 0%; *estA1* heat-stable (ST) 8.14%, 1.3%; *estA2-4* heat-stable (ST) 14.6%, 0.2%; *eltB* heat-labile (LT) 43.1%, 0.4% in DEC and control children respectively. This study revealed high incidence of diarrhegenic *E. coli* isolates and high prevalence of antimicrobial resistance among normal intestinal *E. coli* with typical EAEC and typical EPEC predominating. The use of primers for both variants of ST useful to detect the sensitivity for detection of ETEC strains of hospitalized children in Thi Qar.

**Key words:** Diarrhegenic, *Escherichia coli*, Virulence genes, Phylogenetic grouping, Antibiotic susceptibility, Thi Qar, DEC, EPEC, EHEC, ETEC, EAEC.

### **التوصيف الجزيئي والحساسية تجاه المضادات لبكتريا القوقون الاشريكية المسببة للإسهال في الأطفال**

#### **الخلاصة**

هناك العديد من الدراسات ذات العلاقة اجريت في ذي قار انجزت حول كشف التصنيف لسلاسل بكتريا الإشريكية القولونية *Escherichia coli* المترافقه مع حالات الاسهال (Diarrhegenic *Escherichia coli* DEC) في الاطفال المصابين بالاسهال. ركزت هذه الدراسه على التحري عن حالات DEC جينيا في الاطفال بعمر دون السننتين للراقيدين في المستشفيات.

من بين ٢٠٠ طفل مصابين بحالات الاسهال و ٧٥ طفل سليم (مجموعة سيطره) اخذت عينات الخروج وزرعت على اوساط الماكونكي و الايوسين حيث تم عزل وتشخيص *E. coli* كما تمت دراسة الحساسيه للمضادات الحيويه كما ان جينات الضراوه تم تشخيصها بواسطه تفاعل البلمره المتسلسل PCR

وذلك باستخدام بواديء متخصصه لتعيين كل جين وحسب وزنه بالنسبه الى حالات DEC حيث تم تشخيص enteroaggregative (EAEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC) enterohemorrhagic (EHEC) . تم تشخيص حالات DEC في هذه الدراسه والتي تم عزلها من خروج الاطفال الراقدين في المستشفيات حيث كانت النسب ٤٤.٨% مقارنة مع حالات السيطرة ١٦.٤%، كما ان نسب مجاميع الإشريكية القولونية *Escherichia coli* كانت كالآتي: EAEC (64.73%), ETEC (19.5%), EPEC (5.27%) and EHEC (10.5%). كانت نسب جينات الضراوه لكل من حالات DEC وجموعه السيطرة كالآتي: *pCVD432* 93.6%, 87.3%; *eeA* 64.2%, 8.5%; *bfpA* 59.1%, 2.05%; *stx1* 22.8%, 0.8%; *stx2* 18.6%, 0%; *estA1* heat-stable (ST) 8.14%, 1.3%; *estA2-4* heat-stable (ST) 14.6%, 0.2%; *eltB* heat-labile (LT) 43.1%, 0.4% على التوالي.

كشفت هذه الدراسه عن مدى تأثير عالي من حالات DEC حيث كانت نسب بكتريا *E. coli* عاليه بمختلف مجاميعها حيث كانت هناك نسبه عاليه من حالات المقاومه للمضاد الحيوي وبالاخص حالات كل من EAEC و EPEC حيث كانت هي السائده . كان لاستخدام نوعين من البواديء لتشخيص جين ST مفيدا لغرض تحديد حساسية تشخيص الجين المذكور في حالات الاسهال المرتبطة ب DEC.

**الكلمات الرئيسية:** Diarrheagenic, antimicrobial-resistance genes, *Escherichia coli*, Virulence genes, Phylogenetic grouping, Antibiotic susceptibility, Thi Qar, DEC, EPEC, EHEC, ETEC, EAEC

## Introduction

Diarrhea is one of the major causes of serious issues among children in the developing world [1]. In period from 50<sup>th</sup> to 70<sup>th</sup> of last century it was estimated that more than 4 million children died annually from diarrhea in developing world [2,3].

The major etiology of diarrhea was viruses, bacteria, and parasites. Most common bacterial causes diarrhea was diarrheagenic *Escherichia coli* (DEC) is the most important etiologic agent of childhood diarrhea and represents a major public health problem in developing countries [4]. Identification of DEC strains requires that these organisms be differentiated from non-pathogenic members that constitute normal intestinal flora. DEC strains can be divided into six main categories on the basis of distinct molecular, clinical and pathological features: enteroaggregative *E. coli* (EAEC), enterohemorrhagic (Shiga-toxin producing *E. coli* (EHEC/STEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC) [5].

*Escherichia coli* are the most important etiological agent of childhood diarrhea and represent a major public health problem in developing countries [6].

Accurate detection and identification of diarrheagenic *E. coli* can't be done only by culture, biochemical and serotyping tests, since they are indistinguishable from the non-pathogenic *E. coli* commonly found in human feces. Therefore, only DNA based method such as polymerase chain reaction (PCR) assay can be used for rapid and reliable diagnosis, and which has a high sensitivity and specificity for their detection [7, 8]. The epidemiological significance of different DEC pathotypes in childhood diarrhea varies geographically. Many studies have examined the clinical relevance of ETEC- associated diarrhea in certain area [9, 10].

Diarrheagenic *Escherichia coli* (DEC) are commonly used as marker of fecal contamination of waterways in most areas, which was naturally habitat in the intestinal tracts of warm-blooded animals [11,34], and is released into the environment through deposition of fecal material. In a typical mixed watershed, host sources of *E. coli* may be from humans, farm animals, wildlife, and pets, among others. These hosts are generally described as primary habitats, and until recently *E. coli* was believed to survive poorly in the environment, and not to grow in secondary habitats such as surface water, sediment, and soil [12,13]. However, it has been shown that *E. coli* can survive in the secondary environments for long periods of

time and grow in water, sediment, and soil even in temperate environments [14, 15].

As we mention previously DEC cannot be diagnosed adequately by routine method like culture and biochemical, identification of these strains is difficult. In contrast, polymerase chain reaction technique (PCR) used to detect many numerous genes that responsible of virulence behavior of DEC [16,17,18]. Multiplex PCR assays designed to detect EPEC, ETEC, EAEC, EHEC and EIEC virulence genes.

The objectives of our study aimed to reveal the hospitalized children incidence with DEC diarrhoeagenic *E. coli*, in addition to investigate the microbial sensitivity profiles of *E. coli* isolates. Phylogenetic isolates of the DEC was specify of virulence encoding genes in DEC diarrhoeagenic *E. coli* isolates were determined by PCR.

## **Materials and Methods**

### **Clinical Specimens**

During the period from October 2013 and October 2014, 275 (200 as patients and 75 as control) stool samples from children were investigated in Thi Qar to determine the prevalence of diarrheagenic *Escherichia*. Cases and controls were selected from patients attending the outpatient clinic of three different hospitals after receiving permission from institutional ethical committee. The sample size was determined with 85% confidence level and 80% power according to similar studies conducted earlier [19].

Children were enrolled in the study if they had diarrhea characterized by the occurrence of three or more, loose watery stool or at least one bloody loose stool in a 24 hour period. Control subjects were healthy children with no history of diarrhea for at least one month. Neither patients nor controls had been treated with antibiotics in the week preceding sampling.

Stool specimens were directly streaked onto Nutrient, blood, Eosin Methylene Blue and

MacConkey agar (HiMedia, India) for isolation of *E. coli*. After overnight incubation at 37°C, three lactose-fermenting colonies and a representative non-lactose fermenting colony with a different morphological appearance were picked, and their identities were determined using a panel of biochemical tests interpreted as previously described [20] and the API 20E system (bioMerieux, France), confirmed *E. coli* isolates were stored at - 86°C in Trypticase soy broth supplemented with 20% glycerol for further procedures.

### **Antibacterial Susceptibility**

The antibacterial susceptibility testing of the isolates was done using the Kirby-Bauer disk diffusion method following the definition of the Clinical and Laboratory Standards Institute (CLSI, 2006) using antibiotics containing discs from Oxoid. Briefly, 20 ml of Mueller- Hinton agar (Himedia medium, India) was prepared and poured into sterile plates. The agar medium was allowed to solidify at room temperature on a flat bench.

Then some few colonies of an 18 h culture of the isolates were streaked on the surfaces of the well-dried agar plates. Then some antibiotic discs were gently and firmly placed on the agar plates, which were then left at room temperature for 1 h to allow diffusion of the antibiotics into the agar medium. The plates were then incubated at 35 - 37°C for 24 h. Zones of growth inhibition were then measured to the nearest millimetre and recorded. The mean of triplicate results was taken as the zone diameter.

### **DNA Preparation**

All colonies of *E. coli* which appear on MacConkey Agar after incubated for 18 h at 37°C will made suspension from it by rinsing the entire plate with 2-3 ml of sterile normal saline. MacFarland Standard applied on bacterial suspension then heated at 95°C for 15 minutes. 2,5 µl (corresponding to approximately  $7,5 \times 10^5$  CFU) were applied directly to the multiplex PCR. The remainder of the sample was stored at -20°C.

**Genotypic Methods**

Screening for virulence factors of DEC was previously reported using PCR methods [21] were used to screen isolates of *E. coli* for genes associated with DEC. These included: pCVD432 encoding for EAEC; *eaeA* and *bfpA* for EPEC; *stx1* and *stx2* for Shiga-like toxin 1 (SLT-1) and Shiga-like toxin 2 (SLT-2), respectively, for STEC; *estA1* and *estA2-4* for heat-stable (ST) and *eltB* for heat-labile (LT) toxins of ETEC; and *ipaH* for EIEC.

Also, PCR techniques [14,21,33] were used to screen all *E. coli* isolates for EAEC-associated virulence genes including: *aggR*, a transcriptional activator; *aggA*, fimbriae AAFI; *aafA*, fimbriae AAF II; *agg3A*, fimbriae AAF III; *astA*, aggregative stable toxin 1 (EAST 1); *pet*, plasmid-encoded heat-labile toxin; *aap*, anti-aggregation protein; and *pic*, protein involved in colonization. In

addition, ETEC-positive *E. coli* isolates were examined for coli surface antigens (CS) by PCR.

Product sizes of PCR amplicons used for distinguishing the different virulence genes associated with DEC are shown in Table 1.

Cell lysates, obtained using Charge Switch® gDNA Mini Bacteria Kit provides a fast and reliable magnetic bead-based method for purifying genomic DNA (gDNA) from both gram-negative and gram-positive bacteria (Invetrogen, USA). Amplification was carried out in a 50 µL for multiplex and 20 µL for monoplex reaction mixture containing 3 µM MgCl<sub>2</sub>, 400µM each dNTP, 10 µL 5X Green Go Taq Flexi buffer (Bioneer, Korea), 2.5 U Go Taq DNA polymerase (Bioneer). Reactions were performed in a Viriti PCR System (Applied Biosystems Incorporated, USA).

**Table 1:** Primer sequences used to detect various virulence genes of *E. coli*

No.	Group	Gene		Primer	bp.
1	EAEC	<i>pCVD432</i>	F	CTGGCGAAAGACTGTATCAT and	630
			R	CAATGTATAGAAATCCGCTGTT	
2	EPEC	<i>eaeA</i>	F	AAACAGGTGAAACTGTTGCC	454
			R	CTCTGCAGATTAACCCTCTGC	
		<i>bfpA</i>	F	AATGGTGCTTGCGCTTGCTGC	326
			R	GCCGCTTTATCCAACCTGGTA	
3	EHEC	<i>stx1</i>	F	CAACACTGGATGATCTCAG	350
			R	CCCCCTCAACTGCTAATA	
		<i>stx2</i>	F	ATCAGTCGTCACACTCACTCACTGGT	110
			R	CTGCTGTCACAGTGACAAA	
4	ETEC	<i>estA1 heat-stable (ST)</i>	F	ATGAAAAAGCTAATGTTGGCA	239
			R	TTAATAACATCCAGCACAGGCA	
		<i>estA2-4 heat-stable (ST)</i>	F	AATTGCTACTATTCATGCTTTCAGGAC	133
			R	TCT TTT TCA CCT TTC GCT CAG G	
5	<i>eltB heat-labile (LT)</i>	F	CATAATGAGTACTTCGATAGAGGAAC	402	
		R	CCTGCTAATCTGTAACCATCC		

**Results**

DEC diarrhoeagenic *E. coli* was significantly detected among diarrheic children (44.8%) compared with control children (16.4%). Of the DEC pathotypes examined, EAEC was

found in (64.73%), ETEC in (19.5%), EPEC in (10.5%) and EHEC in (5.27%) of diarrheic children while EIEC were not detected as shown in figure 1 and 2.

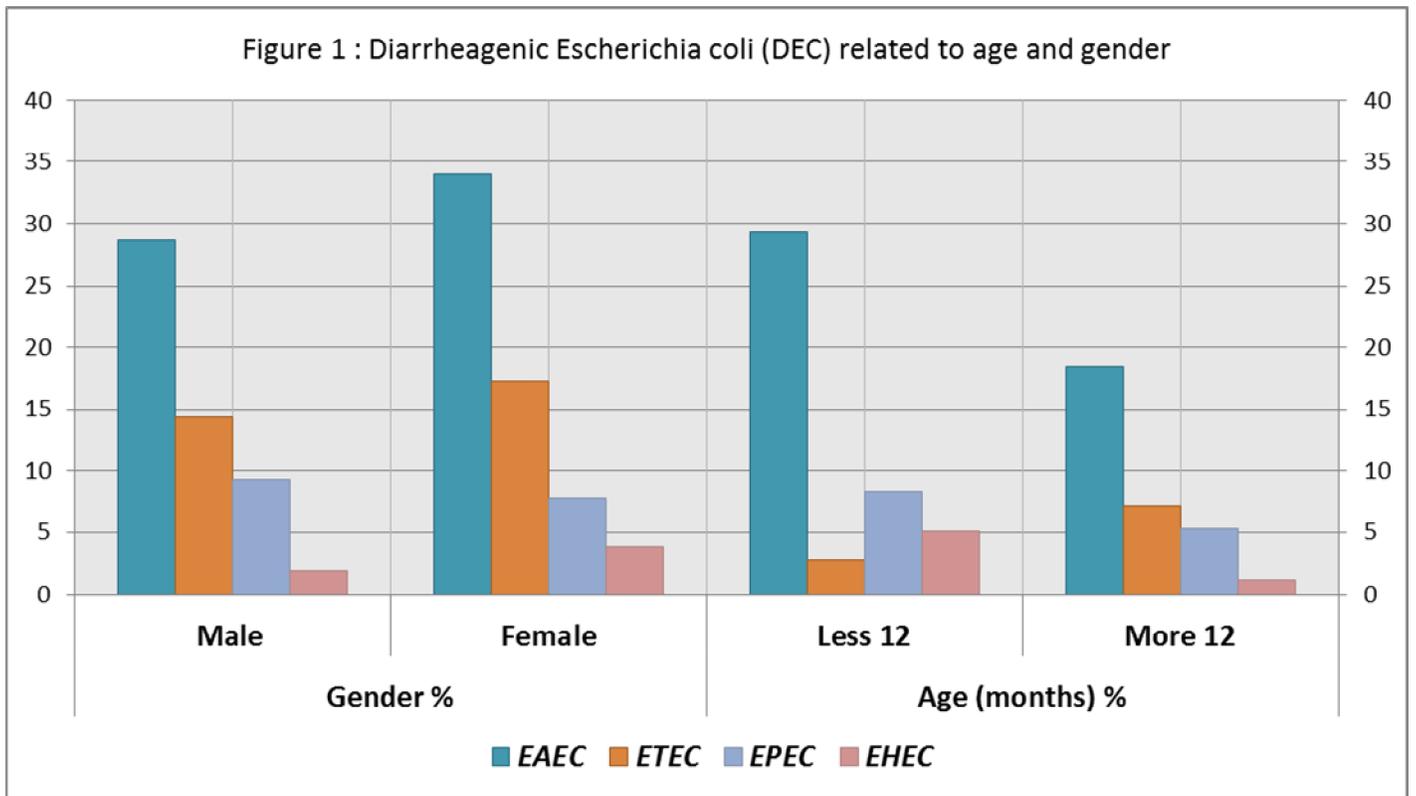
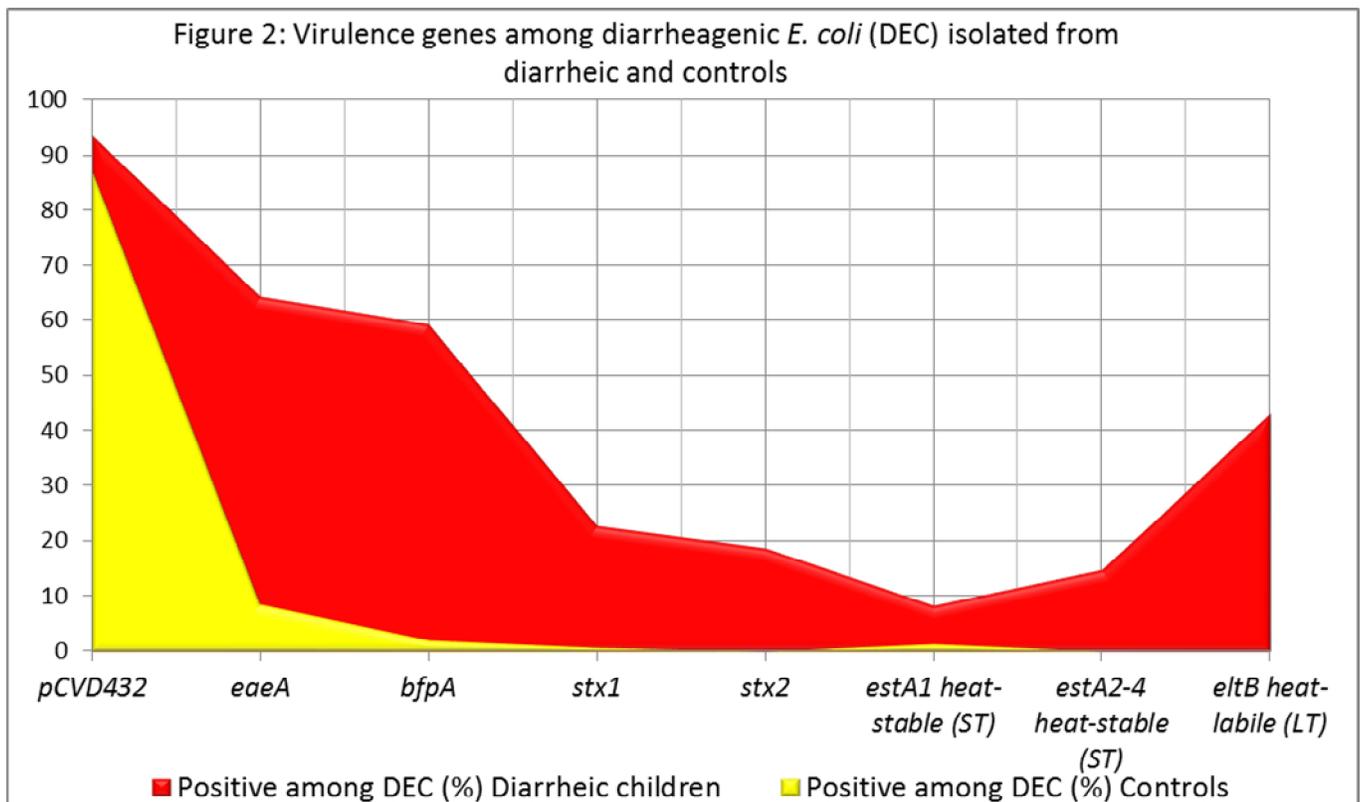


Figure 3 revealed the virulence-gene factors in DEC isolated from children with diarrhea

and from controls. The distribution of virulence gene was; pCVD432 93.6%,



87.3%; eaeA 64.2%, 8.5%; bfpA 59.1%, 2.05%; stx1 22.8%, 0.8%; stx2 18.6%, 0%; estA1 heat-stable (ST) 8.14%, 1.3%; estA2-4 heat-stable (ST) 14.6%, 0.2%; eltB heat-labile (LT) 43.1%, 0.4% in DEC and control children respectively as revealed in Figure 4. As shown in table 2; the antibiotic resistance results of the diarrheagenic *E. coli* isolates are shown in Figure 3. The results has shown that for ETEC, EPEC, EAEC, EHEC as follow: Cephalothin 90.3, 48.1, 98, 91.8; Tetracycline 68.9, 59.1, 14.6, 22.9;

Ciprofloxacin 0, 0, 0, 1.7; Chloramphenicol 78, 2.1, 2.8, 0; Cefotaxime, 82.9, 1.5, 0, 0; Ceftriaxone 74.6, 2.1, 4, 1; Trimethoprim/sulfamethoxazole 72.9, 2.7, 89.3, 11.5; Nalidixic Acid 19.5, 2.05, 1.01, 0; Gentamycin 2.8, 1.03, 0, 1; Streptomycin 83.8, 50.1, 88.6, 41.3; Amikacin 22.6, 19.2, 8.9, 15.4; Cotrimoxazole 40.5, 28.2, 12, 8; Amoxicillin/clavulanic acid 71.2, 12.1, 61.02, 37.6 Imipenem 9.5, 1, 0.2, 0; Ampicillin 95.2, 59.2, 91.4, 88.6 respectively.

**Table 2:** Profile of antimicrobial susceptibility of DEC isolated from children with diarrhea

No. (%) resistant				Antibiotic
EHEC	EAEC	EPEC	ETEC	
91.8	98	48.1	90.3	Cephalothin
22.9	14.6	59.1	68.9	Tetracycline
1.7	0	0	0	Ciprofloxacin
0	2.8	2.1	78	Chloramphenicol
0	0	1.5	82.9	Cefotaxime
1	4	2.1	74.6	Ceftriaxone
11.5	89.3	2.7	72.9	Trimethoprim/sulfamethoxazole
0	1.01	2.05	19.5	Nalidixic Acid
1	0	1.03	2.8	Gentamycin
41.3	88.6	50.1	83.8	Streptomycin
15.4	8.9	19.2	22.6	Amikacin
8	12	28.2	40.5	Cotrimoxazole
37.6	61.02	12.1	71.2	Amoxicillin/clavulanic acid
0	0.2	1	9.5	Imipenem
88.6	91.4	59.2	95.2	Ampicillin

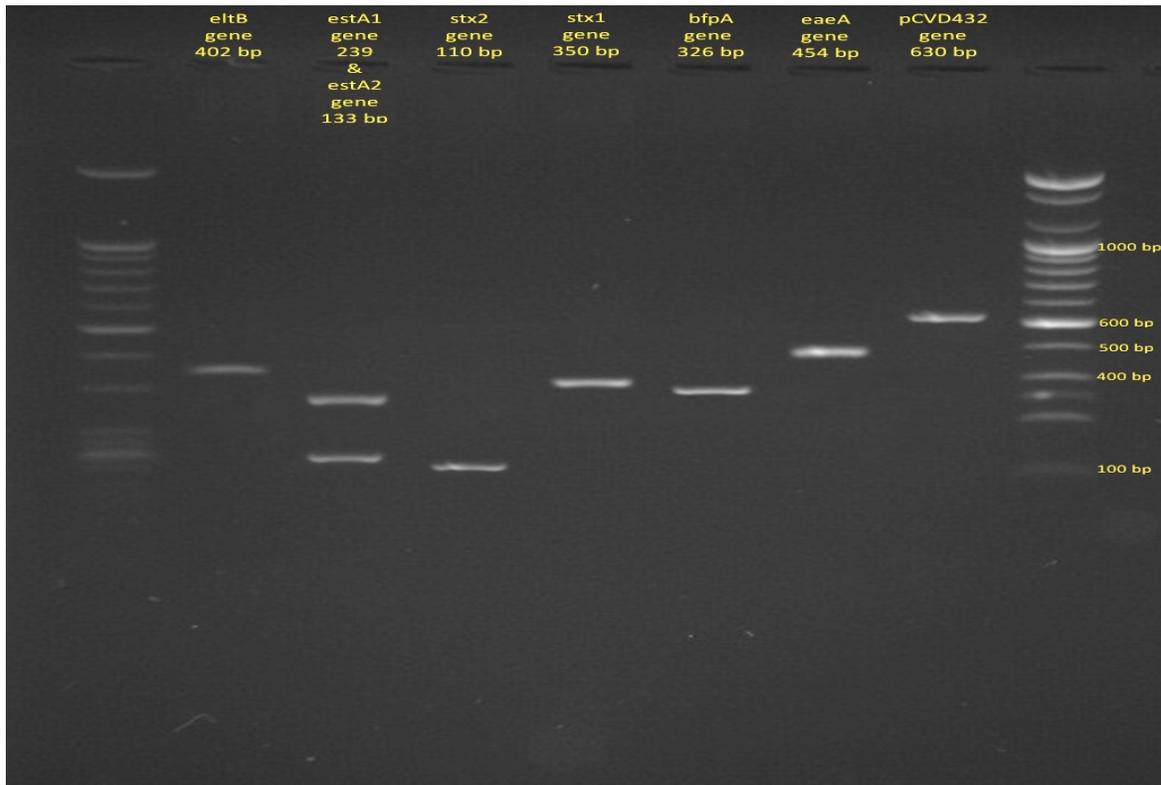
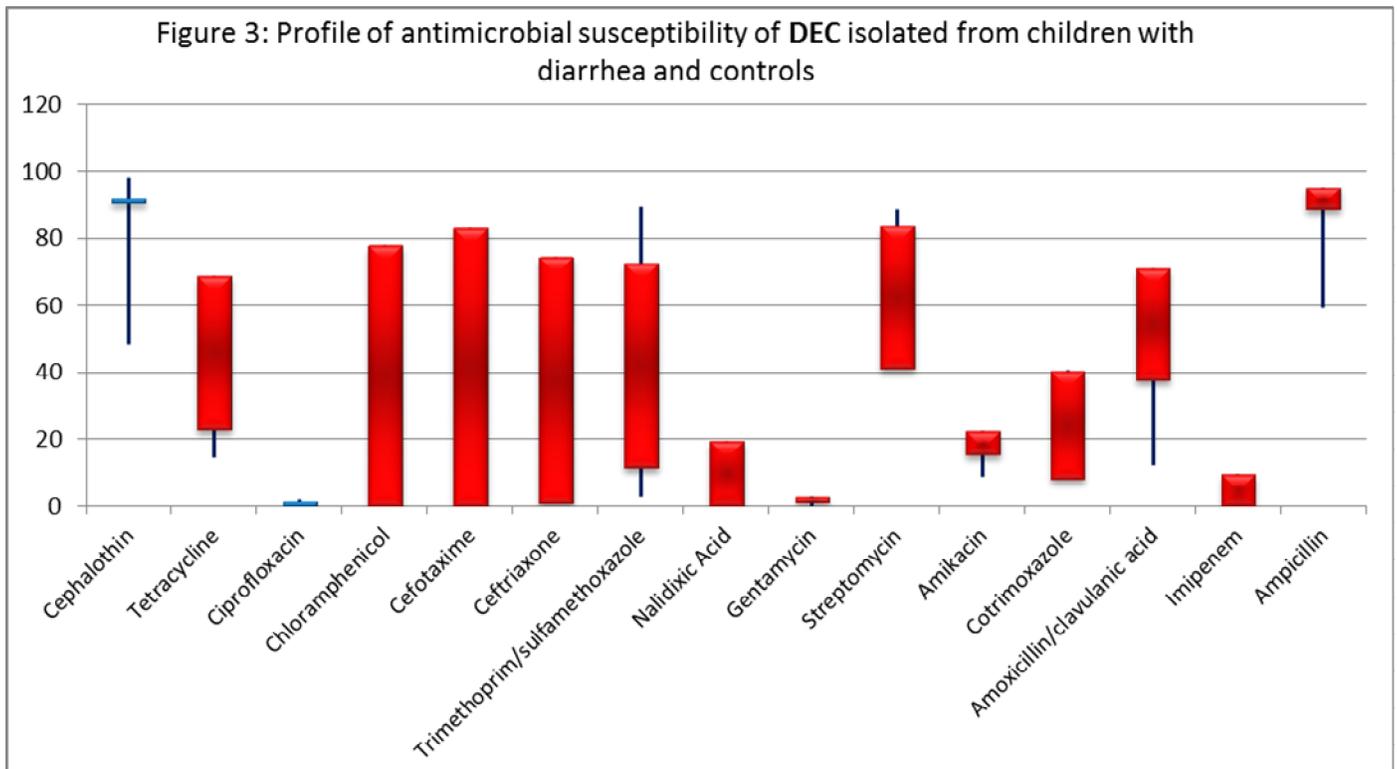


Figure 4: Ethidium bromide-stained Agarose Gel Electrophoresis of PCR-amplified products from extracted Diarrheagenic *E. coli* DNA amplified with primers, DNA molecular size marker (100-bp ladder)

## **Discussion**

Our study showed that diarrheagenic *E.coli* isolates were found only in of children with different ages and gender as shown in figure 1, and some diarrheagenic *E. coli* isolate was detected in the control children group. The study also indicated the presence of (ETEC), (EAEC), (EIEC) and (EHEC), whereas the two types.

The rate of EAEC and ETEC among diarrheal cases in our study is agreed to other similar studies recently reported from Iran, Kuwait and Egypt [22,23,24], in contrast to the other studies carried out in Kuwait and Jordan which had shown that EAEC was not a cause of diarrhea in their examined pediatric population [25,26]. However, the absence or low occurrence rate of EIEC in diarrhea cases and controls in this study is similar to the results of other reported studies from Egypt, Kuwait and Jordan [26, 25, and 22].

The results of this study showed high rates of antimicrobial resistance (1-91%) to many antimicrobial classes, including generation cephalosporines, and MDR *E. coli* accounted for highly rates of all isolates (Table 2). These results are similar to some extend to many recent studies carried out in other Arabic countries [27, 28]. Over the past decade, the bla (CTX-M) genes have become the most prevalent ESBLs among multidrug resistant organisms worldwide, started first in certain countries of Europe and South America, and later spread to India China, and other countries [29, 30, 31,32, 33].

## **Conclusions**

Prevalence of EPEC and ETEC in our study revealed to be unexpectedly elevated in Thi Qar, so we recommended that all patients with diarrhea should be routinely surveyed for causative pathogens, especially children below the age of two and in those specimens in which no other pathogen can be identified. We also demonstrate the use of a novel

multiplex PCR for the detection of diarrhoeagenic *E. coli* from stool specimens.

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