GENOTYPE, PHENOTYPE AND VIRULENCE GENES MARKERS IN *ESCHERICHIA COLI*: MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY ASSOCIATED WITH DIARRHOEA AMONG CHILDREN IN BABIL PROVINCE, IRAQ

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ABSTRACT: *Background: in Babil there are relatively few studies have been done to revealed and classify diarrheagenic Escherichia coli (DEC) strains among children with diarrhea. This study aimed at investigating DEC among children in Babil aged less than 2 years hospitalized. Methods and patients: A total of 200 children with diarrhea and 75 without diarrhea were their stools investigated using culture on MacConkey and EMB agar, and the E. coli isolates were examined for detection of diarrheagenic E. coli types, antimicrobial susceptibility pattern. virulence genes detecting 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 enterohemorghagic- Escherichia coli (EHEC). Results: The study has indicated that diarrheagenic E. coli isolates were found mostly in stools of children with diarrhea. DEC diarrhoeagenic E. coli were 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%; 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*

Conclusion: This study revealed the high incidence of diarrheagenic E. coli isolates and high prevalent 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 Babil.

KEY WORDS: Diarrheagenic, antimicrobial-resistance genes, Escherichia coli, Virulence genes, Phylogenetic grouping, Antibiotic susceptibility, Diarrhea, Babil, 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 50th to 70th of last century it was estimated that more than 4 million children died annually from diarrhea in developing world [2,3]. Mortality due to The major etiologic 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 is the most important etiologic agent of childhood diarrhea and represents 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- ssociated diarrhea in certain area [9,10].

DEC are commenly used as marker of fecal contamination of waterways in most areas, which was naturally habitat in the intestinal tracts of warm-blooded animals [11], 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 techniques (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 revealed 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.

METHODOLOGY

Clinical specimens

During the period from October 2013 and October 2014, 275 (200 as patient and 75 as control) stool samples from children were investigated in babylon 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 Mac Conkey 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.

DNA preparation

All colonies of E. coli which appears 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*105 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; stx1and 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] 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 MgCl2,400μM 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).

RESULTS

DEC diarrhoeagenic E. coli were 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 figure1. 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.

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; rimethoprim/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.

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 [26,25,22].

The results of this study showed high rates of antimicrobial resistance (1-91%) to many antimicrobial classes, including generation cephasporines, and MDR *E.coli* accounted for higly percent 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].

CONCLUSIONS

Prevalence for EPEC and ETEC in our study revealed to be unexpectedly elevated in babil, so we recommended that all patients with diarrhea should be routinely survey 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.

Table 1. primer sequences which used to detect various virulence genes in E. coli

No.	Group	Gene		Primer	bp.
1	EAEC	pCVD432	F	CTGGCGAAAGACTGTATCAT and	630
			R	CAATGTATAGAAATCCGCTGTT	
2	EPEC	eaeA	F	AAACAGGTGAAACTGTTGCC	454
			R	CTCTGCAGATTAACCCTCTGC	
		bfpA	F	AATGGTGCTTGCGCTTGCTGC	326
			R	GCCGCTTTATCCAACCTGGTA	
3	EHEC	stx1	F	CAACACTGGATGATCTCAG	350
			R	CCCCCTCAACTGCTAATA	
		stx2	F	ATCAGTCGTCACTCACTGGT	110
			R	CTGCTGTCACAGTGACAAA	
4	ETEC	estA1 heat-stable (ST)	F	ATGAAAAAGCTAATGTTGGCA	239
			R	TTAATAACATCCAGCACAGGCA	

		estA2-4 heat-stable (ST)	F	AATTGCTACTATTCATGCTTTC AGGAC	133
			R	TCT TTT TCA CCT TTC GCT CAG G	
5		eltB heat-labile (LT)	F	CATAATGAGTACTTCGATAGAG GAAC	402
			R	CCTGCTAATCTGTAACCATCC	

Figure 1 : Diarrheagenic Escherichia coli (DEC) related to age and gender
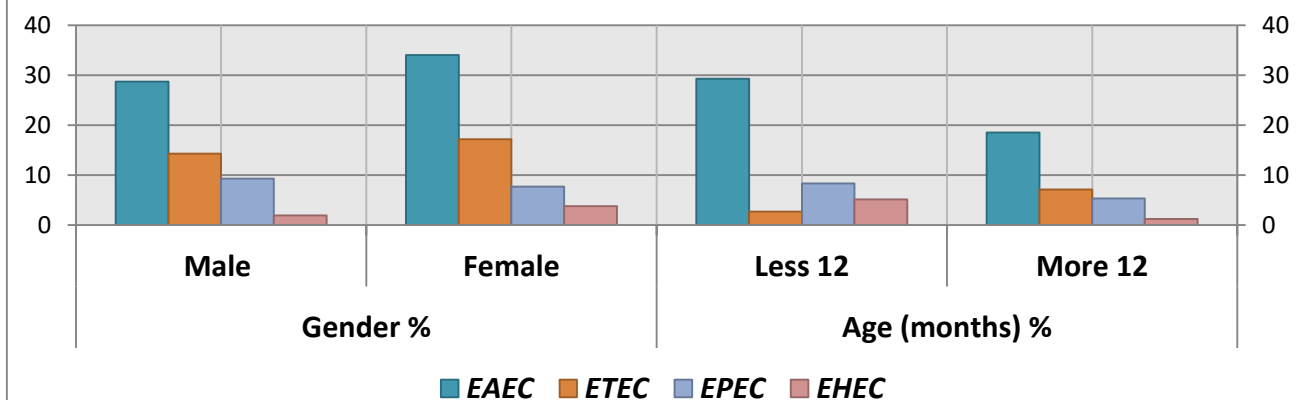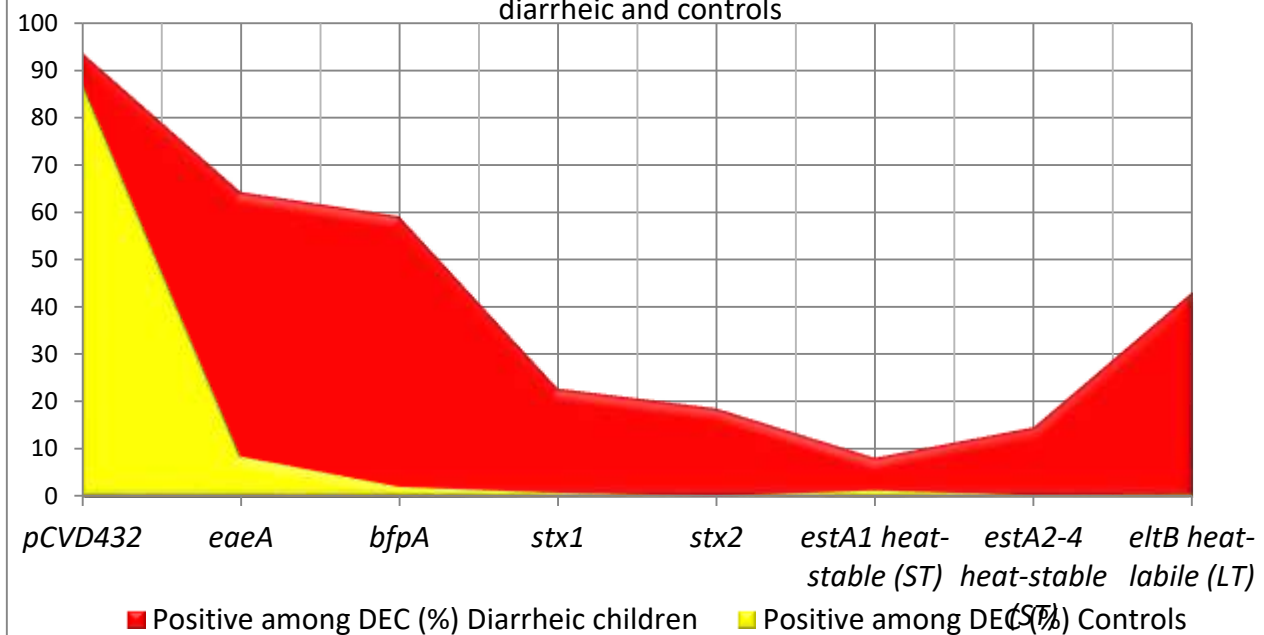

Figure 2: Virulence genes among diarrheagenic *E. coli* (DEC) isolated from diarrheic and controls
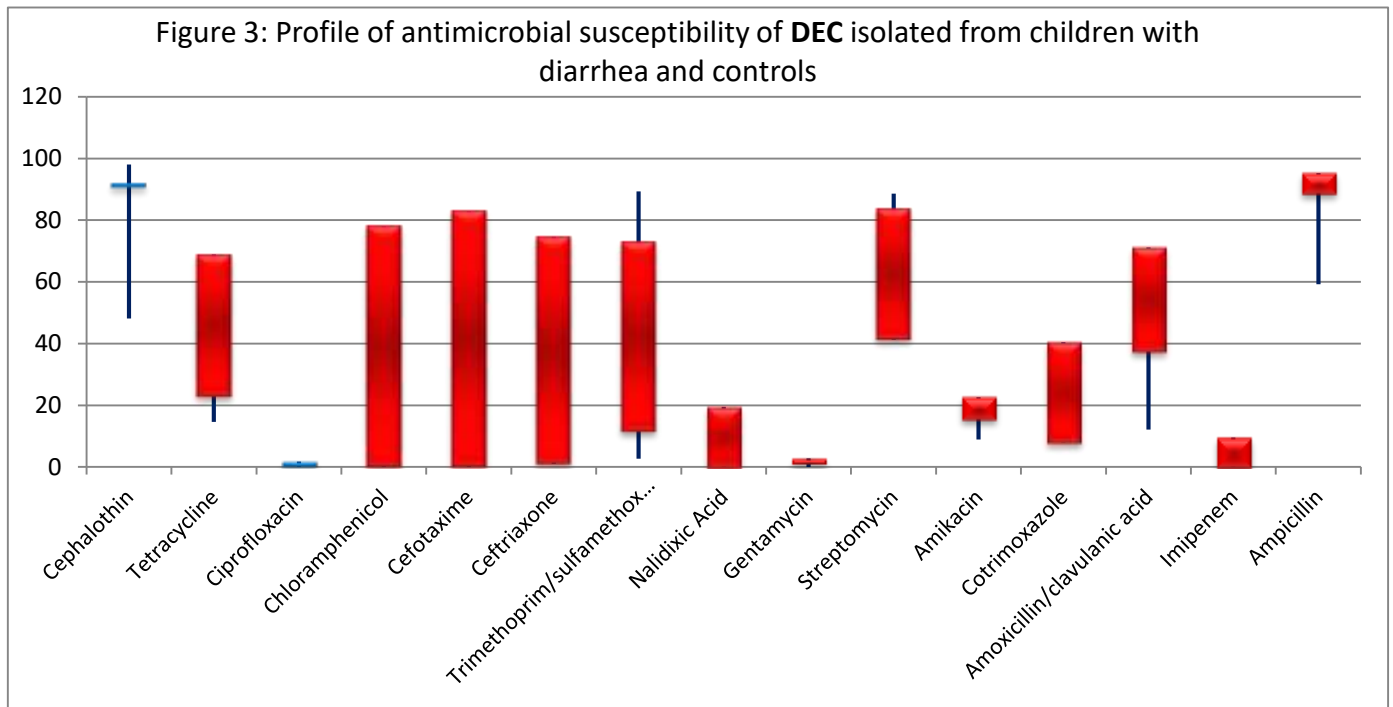
Figure 3: Profile of antimicrobial susceptibility of **DEC** isolated from children with diarrhea and controls

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

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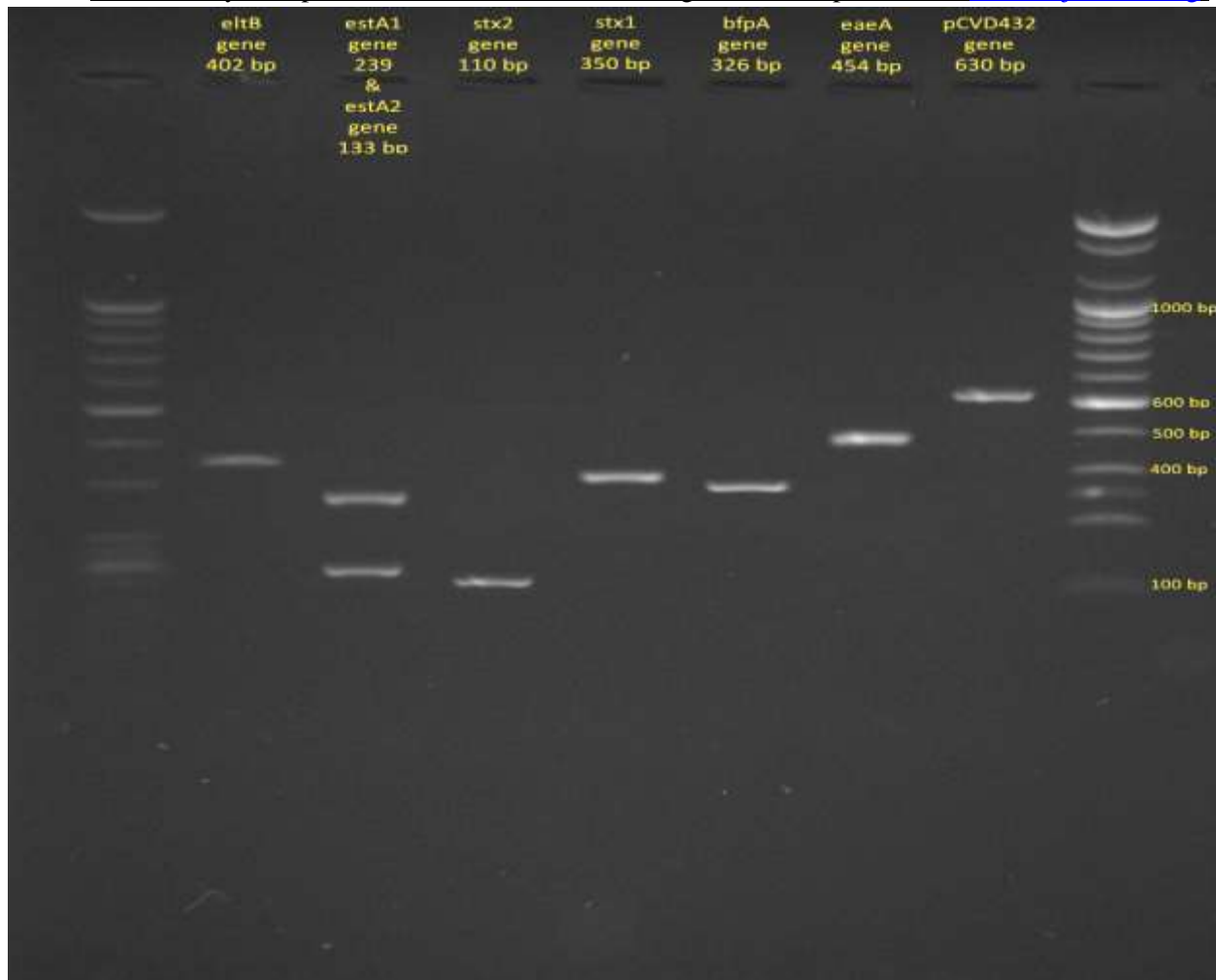
Figure 4: Ethidium bromide-stained Agarose Gel Electrophoresis of PCR-amplified products from extracted Diarrheagenic E. coli DNA amplified with primers, DNA molecular size marker (100-bp ladder)

References

1. Bern C, Martines J, de Zoysa I, Glass RI: The magnitude of the global problem of diarrhoeal disease: a ten-year update. Bull World Health Organ 1992, 70(6):705-714.

2. Snyder JD, Merson MH: The magnitude of the global problem of acute diarrhoeal disease: a review of active surveillance data. Bull World Health Organ 1982, 60(4):605-613.

3. Parashar U, Umesh D, Bresee JS, Joseph S, Glass RI, Roger I: The global burden of diarrhoeal disease in children. Bulletin of the World Health Organization 2003, 81(4):236.

4. Guerrant RL, Hughes JM, Lima NL, Crane J: Diarrhea in developed and developing countries: magnitude, special settings, and etiologies. Rev Infect Dis 1990, 12(Suppl 1):S41-50.

5. Harrington SM, Dudley EG, Nataro JP: Pathogenesis of enteroaggregative Escherichia coli infection. FEMS Microbiol Lett 2006, 254(1):12-18.

6. Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic Escherichia coli. Clin.Microbiol. Rev. 11:142–201.

7. Nessa K, Dilruba, A, Islam J, Kabir, Hossain L. Usefulness of a Multiplex PCR for Detection of Diarrheagenic Escherichia coli in diagnostic Microbiology Laboratory setting, Bangladesh, J Med Microbiol, 2007; 01:38-42.

8. 13. Toma C, Lu Y, Higa, N, Nakasone N, Rivas, Iwanaga M. Multiplex PCR assay for Identification of Human Diarrheagenic Escherichia coli.J Clin Microbiol 2003;41:2669-2671.

9. Shaheen HI, Khalil SB, Rao MR, Abu Elyazeed R, Wierzba TF, Peruski, Jr. LF, Putnam S, Navarro A, Morsy BZ, Cravioto A, Clemens JD, Svennerholm AM, Savarino SJ. Phenotypic profiles of enterotoxigenic Escherichia coli associated with early childhood diarrhea in rural Egypt. J Clin Microbiol. (2004).42: 5588-5595.

10. Shaheen HI, Abdel Messih IA, Klena JD, Mansour A, El-Wakkeel Z, Wierzba TF, Sanders JW, Khalil SB, Rockabrand DM, Monteville MR, Rozmajzl PJ, Svennerholm AM, Frenck RW. Genotypic analysis of enterotoxigenic Escherichia coli in samples obtained from Egyptian children presenting to referral hospitals. J Clin Microbiol. (2009). 47: 189-197.

11. Kaper JB, Nataro JPT, Mobley HL. Pathogenic Escherichia coli. Nat Rev Microbiol. (2004). 2: 123–140.

12. Ishii S, Ksoll WB, Hicks RE, Sadoswky MJ. Presence and growth of naturalized Escherichia coli in temperate soils from Lake Superior watersheds. Appl Environ Microbiol. (2006) 72: 612–621.

13. S, Meyer KP, Sadowsky MJ. Relationship between phylogenetic groups, genotypic clusters, and virulence gene profiles of Escherichia coli strains from diverse human and animal sources. Appl Environ Microbiol. (2007)73: 5703–5710.

14. Byappanahalli MN, Whitman RL, Shively DA, Sadowsky MJ, Ishii S. Population structure, persistence, and seasonality of autochthonous Escherichia coli in temperate, coastal forest soil from a Great Lakes watershed. Environ Microbiol. (2006) 8: 504–513.

15. Topp E, Welsh M, Tien YC, Dang A, Lazarovits G. Straindependent variability in growth and survival of Escherichia coli in agricultural soil. FEMS Microbiol Ecol. (2003)44: 303–308.

16. Aranda KR, Fagundes-Neto U, Scaletsky IC: Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic Escherichia coli and Shigella spp. J Clin Microbiol 2004, 42:5849-5853.

17. Vidal M, Kruger E, Duran C, Lagos R, Levine M, Prado V, Toro C, Vidal R: Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic Escherichia coli associated with enteric infections. J Clin Microbiol 2005, 43:5362-5365.

18. Pabst WL, Altwegg M, Kind C, Mirjanic S, Hardegger D, Nadal D: Prevalence of enteroaggregative Escherichia coli among children with and without diarrhea in Switzerland. J Clin Microbiol 2003, 41:2289-2293.

19. Nweze EI. Virulence Properties of Diarrheagenic E. coli and Etiology of Diarrhea in Infants, Young Children and Other Age Groups in Southeast, Nigeria. Am-Eurasian JSci Res 2009;4: 173-9.

20. Collee JG, Duguid JP, Fraser AG, Marmion BP (1989) Practical Medical Microbiology. Third edition. Edinburgh: Churchill Livingstone. pp. pp. 432 -455.

21. Nada RA, Shaheen HI, Touni I, Fahmy D, Armstrong AW, Weiner M, Klena JD. Design and validation of a multiplex polymerase chain reaction for the identification of enterotoxigenic Escherichia coli and associated colonization factor antigens. Diagn Microbiol Infect Dis. (2010) 67: 134-142.

22. Ali MM, Ahmed SF, Klena JD, Mohamed ZK, Moussa TA l. : Enteroaggregative Escherichia coli in diarrheic children in Egypt: molecular characterization and antimicrobial susceptibility. J Infect Dev Ctries 2014;8(5):589-96.

23. Dow AM, Toth I, Malik A , Herpay M , Nogrady N, et al. Phenotypic and genetic characterization of enteropathogenic Escherichia coli (EPEC) and enteroa ggregative E.coli (EAEC) from diarrhoeal and non diarrhoeal children in libya. comparative immunology, microbiology and infectious diseases 2006; 290 : 100-113.

24. Haghi F, Zeighami H, Hajiahmadi F, Khoshvaght H, Bayat M. Frequency and antimicrobial resistance of diarrhoeagenic Escherichia coli from young children in Iran. J Med Microbiol 2014; 63(3):427-32

25. Albert MJ, Rotimi VO, Dhar R, Silpikurian S, Pacsa AS, et al. Diarrhoeagenic Escherichia coli are not a significant cause of diarrhoea in hospitalised children in Kuwait. BMC Microbiol; 2009;9:62

26. Shehabi AA, Kuri-Bulos N, Hajjaj GK. Characterization of diarrhoeagenic Escherichia coli isolates in jordanian children. Scand J Infect Dis 2003; 35:368-371.

27. Abu Salah AM, Badran FE , Shehabi AA. High incidence of multidrug resistant Escherichia coli producing CTX-M-type ESBLs colonizing the intestine of Jordanian infants. IAJAA 2013; 3,4:3

28. Bindayna KM, Murtadha M. High prevalence of blaCTX-M in Enterobacteriaceae isolates from the Kingdom of Bahrain. Asian Pac J Trop Med 2011; 4(12):937-40.

29. Harajly M, Khairallah MT, Corkill JE, Araj GF, et al. Frequency of conjugative transfer of plasmid-encoded ISEcp1 - blaCTX-M-15 and aac(6')-lb-cr genes in Enterobacteriaceae at a tertiary care center in Lebanon - role of transferases. Ann Clin Microbiol Antimicrob 2010; 9:19.

30. Lo´pez-Cerero L, Egea P, Rodrı´guez-Ban J, Pascual A. Similarities between the genetic environments of bla CTX-M-15 in Escherichia coli from clinical and food samples from Spain and overseas travelers. Antimicrob Chemother 2011; 66(9):2177.

31. Baroud M, Araj GF, Matar GM. Spread of CTX-M-15 Extended Spectrum β-lactamases Encoding Genes Among Enterobacteriaceae in the Middle Eastern Region. IAJAA 2011; 1: 1-6.