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 enterohemorrhagic- 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, Babylon, 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-associated diarrhea in certain area [9,10].

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], 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*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] were used to screen all E. coli isolates for EAEC-associated
virulence genes including: aggR, a transcriptional activator; aggA, fimbriae AAFI; aafA, fimbriae AAFII; 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 figure 1. 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; rifampicin/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 high 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

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Gene</th>
<th>Primer</th>
<th>bp.</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>EAEC</td>
<td>pCVD432</td>
<td>F: CTGCGGAAAGACTGTATCAT and R: CAATGTATAAGAAATCCGCTGTT</td>
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<tr>
<td>2</td>
<td>EPEC</td>
<td>eaeA</td>
<td>F: AAACAGGTGAAACTGTTGCC and R: CTCTCGAGATTAAACCCTCTGC</td>
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<td></td>
<td></td>
<td>bfpA</td>
<td>F: AATGGTGCTTGCCTTGCTG and R: GCCGCTTATCCAACCTGGTA</td>
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<tr>
<td>3</td>
<td>EHEC</td>
<td>stx1</td>
<td>F: CAACCTGGATGATCTCAG and R: CCCCTCAACTGCTAATA</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>stx2</td>
<td>F: ATCAGTCGCACCTCAGCTGCTG and R: CTGCTGTCAGTGACAAA</td>
<td>110</td>
</tr>
<tr>
<td>4</td>
<td>ETEC</td>
<td>estA1 heat-stable (ST)</td>
<td>F: ATGAAAAAGCTAATGGTTGGCA and R: TTAATAACATCCAGCACAGGC</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>AATTGCTACTATTCATGCTTT</td>
<td>AGGAC</td>
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</tr>
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<td></td>
<td></td>
<td>R</td>
<td>TCT TTT TCA CCT TTC GCT</td>
<td>CAG G</td>
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<tr>
<td>estA2-4 heat-stable (ST)</td>
<td>5</td>
<td></td>
<td></td>
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<tr>
<td>eltB heat-labile (LT)</td>
<td></td>
<td></td>
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<td>402</td>
</tr>
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</table>

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

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>EHEC</th>
<th>EAEC</th>
<th>EPEC</th>
<th>ETEC</th>
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<tr>
<td>Cephalothin</td>
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<td>98</td>
<td>48.1</td>
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<td>Tetracycline</td>
<td>22.9</td>
<td>14.6</td>
<td>59.1</td>
<td>68.9</td>
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<td>Chloramphenicol</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Ciprofloxacin</td>
<td>0</td>
<td>2.8</td>
<td>2.1</td>
<td>78</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>82.9</td>
</tr>
<tr>
<td>Trimethoprim/sulfamethoxazole</td>
<td>1</td>
<td>4</td>
<td>2.1</td>
<td>74.6</td>
</tr>
<tr>
<td>Amikacin</td>
<td>11.5</td>
<td>89.3</td>
<td>2.7</td>
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<tr>
<td>Nalidixic Acid</td>
<td>0</td>
<td>1.01</td>
<td>2.05</td>
<td>19.5</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>41.3</td>
<td>88.6</td>
<td>50.1</td>
<td>83.8</td>
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<td>Streptomycin</td>
<td>15.4</td>
<td>8.9</td>
<td>19.2</td>
<td>22.6</td>
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<td>Cotrimoxazole</td>
<td>8</td>
<td>12</td>
<td>28.2</td>
<td>40.5</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>37.6</td>
<td>61.02</td>
<td>12.1</td>
<td>71.2</td>
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<tr>
<td>Imipenem</td>
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<td>0.2</td>
<td>1</td>
<td>9.5</td>
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<tr>
<td>Ampicillin</td>
<td>88.6</td>
<td>91.4</td>
<td>59.2</td>
<td>95.2</td>
</tr>
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</table>

Figure 3: Profile of antimicrobial susceptibility of DEC isolated from children with diarrhea and controls
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


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