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Distribution of Pathogenic *Escherichia coli* among Children with Severe Diarrhea in Al-Najaf Al-Ashraf City, Iraq

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Abstract: ***Introduction: Diarrhea is considered an abnormal fecal discharge characterized by a frequent and or fluid stool. E. coli is the most important bacterial cause of diarrhea in children, its remain harmlessly confined to the intestinal lumen. However, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even the normal "nonpathogenic" strains of E.coli can cause infection. Objective: This study aimed to investigate the distribution of pathogenic E.coli among children suffering from severe diarrhea by detection of some their virulence genes. Materials and methods: A total of 204 stool samples were collected from children with severe diarrhea under 5 years whom admitted to AL-ZahraaandAl-Hakeem hospital during a period from 3/9/2016 to 30/11/2016. All samples were cultured on Red and Green chrom agar for isolation of pathogenic E. coli. PCR technique has been carried out for detection ofeaeA,stx1, stx2 vtx1, vtx2 and Pcvd432 in 50 isolates that selective randomly. Results: One hundred and seventy six isolates were belonging to pathogenic E. coli.The results of the amplification of 50 DNA samples showed that 8(16%) isolates were possessed eaeA gene while 1 (2%) and 6(12%) isolates were carried stx1 and stx2 gene respectively whereas 7(14%) and 1(2%) isolates were possess pcvd432 and vtx2respectively. Conclusion: diarrhea and bloody diarrhea were widely distributed in children in Al-Najaf Al-Ashraf city and mainly associated with presence of Atypical E. coli, Shiga-Like ToxinE.coli and Vero-Like Toxin E. coli.***

Keywords: PECE, STEC, EHEC, Diarrhea, PCR

1. Introduction

Diarrhea is defined as a passage of three or more loss of liquid stool per day It constitutes a public health problem and agreat majority of cases occur among diarrhea under 5 years of age in the developing countries.(1)Several type of infection may lead to diarrhea such as viral, parasitic and bacterial causes. The Infections due to *E.coli* may be limited to the mucosal surfaces or can disseminate throughout the body (2). From its normal site in the human body, it is able to cause frequent opportunistic infection; it is often present in appendix, abscess, peritonitis, cholecystitis and septic wounds, which may cause bacteremia and endotoxic shock occasionally meningitis in neonates.

Several pathogenic and nonpathogenic factors are predisposed to calf diarrhea.(3)Single primary pathogen or co-infection can predispose to the development of diarrhea. Other factors such as nutritional factors, hygiene conditions and environmental factors could contribute to diarrhea. (4)

Based on the molecular and pathological criteria, the Diarrheagenic E.coli (DEC) are classified into several pathotypes such as: enterotoxigenic *E. coli*(ETEC), enteropathogenic *E. Coli*(EPEC), enterohemorrhagic *E.coli* (EHEC) enterinvasive *E. Coli*(EIEC), enteroaggregative *E. Coli*(EAEC), Diffusely-adherent *E.coli*(DAEC) and vero- or Shiga-like toxin producing *E.coli*(VTEC or STEC) (5). EPEC is a major cause of infantile diarrhea among children in developing countries.(6) The central mechanism of EPEC pathogenesis is a lesion called'attaching and effacing' (A/E), which is characterized by intimate adherence of bacteria to the intestinal epithelium.(7) The *eaeA*gene, which is located in the 'locus of enterocyte effacement' pathogenicity island (LEE), and the *bfpA*gene which located on a plasmid called the EPEC adherence factor (EAF) have been used for the identification of EPEC and for the subdivision of this group of bacteria into typical and atypical strains.(8)

In Iraq and even in other countries diarrhea is still a dangerous disease which may lead to death if it is not treated,So this study was aimed to investigate the prevalence of diarrheagenic *E.coli* among children in Al-Najaf Al-Ashraf city.

2. Materials and Methods

2.1. Sample Collection

A total of 204 stool sample were collected from children under 5years with severe diarrhea whom admitted to AL-Zahraa and Al-Hakeem hospital during a period from 3/9/2016 to 30/11/2016. All stool samples have been cultured directly on EMB, Green and red chromagar and incubated for 24 hr at 37˚C for isolation of pathogenic *E.coli*.

2.2. Identification of Pathogenic E. coli

It was carried out depending on convention methods (microscopic examination, cultural characteristic and biochemical tests) as mentioned previously.(9)

2.3. PCR technique

Fifty isolates were selected randomly for isolation of DNA by boiling method as described by Sambrook and Rosseel 2001. Amplification of *eaeA*, *stx1*, *stx2 vtx1*, *vtx2* and *Pcvd432* were carried out in PCR thermocycler (Biometra, SA) using a set of primers that mentioned in table (1). A reaction mixtureconsist of 5µl of Maxime PCR PreMix, 1.5µl for each F and R primer, 5µl of DNA template and

completed the volume to 20µl with nuclease free water. PCR conditions were explained in table (2).

2.4. Gel electrophoresis

Agarose gel electrophoresis was prepared with a final concentration of 1.5% agarose in 1X TBE buffer (BBL, USA) stained with 0.5mg/ml of ethidium bromide. The electrophoresis was carried out at 80 volt for 75 min. The gel was visualized by UV trans illuminator unit and photographed.

Table 1: Oligo synthesis nucleotides

Genes	Sequences 5' 3'	Molecular weight of amplicon (bp)	References
eaeA	GTGGCGAATACTGGCGAGACT	890	(10)
	CCCCATTCTTTTTCACCGTCG		
stx1	ACACTGGATGATCTCAGTGG	614	(11)
	CTGAATCCCCCTCCATTATG		
stx2	CCATGACAACGGACAGCAGTT	779	
	CCTGTCAACTGAGCAGCACTTTG		
vtx1	CATTGTCTGGTGACAGTAGCT	732	(10)
	CCCGTAATTTGCGCACTGAG3		
vtx2	CCATGACAACGGACAGCAGTT	779	
	CCTGTCAACTGAGCACTTTG		
Pcv D432	CTGGCGAAAGACTGTATCAT	630	
	CAATGTATAGAAATCCGCTGTT		

3. Results

3.1 Identification of Pathogenic E.coli

The result of the primary isolation of (PEC) from stool of severe diarrhea showed that out of 204 samples only 192 (96.5%) samples were positive for PEC on EMB agar, while the results of culturing on the Green and Red chrom agar showed that only 92(46.7%) and 80(40.6%) isolates were belong to PEC respectively (Table 3).

Table 2: The amplification condition of PCR technique

Genes	Initial denaturation°C/min	No. of cycle	Condition of one cycle			Final extension °C/min
			Denaturation °C/sec.	Annealing °C/sec	Extension °C/sec	
eaeA	94 /5	30	94 /15	65 /15	72 /75	72 /5
stx1,stx2	94 /5	35	94/60	58 /60	70 /120	70 /10
vtx1,vtx2	94 /5	35	94 /15	65 /15	72 /75	72 /5
pcvd432	94 /5	30	94 /30	55 /30	72 /30	72 /5

Table 3: The percentage of Pathogenic E. coli isolates on different media

No. of samples	NO.(%) of Pathogenic E. coli isolates on:		
	EMB agar	Chrom agar	Red Chrom agar
204	192(96.5)	92(46.7)	80(40.6)

3.2. Detection of *eaeA,vtx1, vtx2* genes:

The results of agarose gel electrophoresis of amplicon resulted from amplification of *eaeA* , *vtx1,vtx2* by multiplex PCR revealed that 8 isolates (16%) and one isolate (%2) were possess *eaeA* gene and *vtx2* by appearing of amplicon with molecular weight 890 bp and 779bp respectively. While none of isolates have shown positive results for *vtx1* amplification (Figure 1).

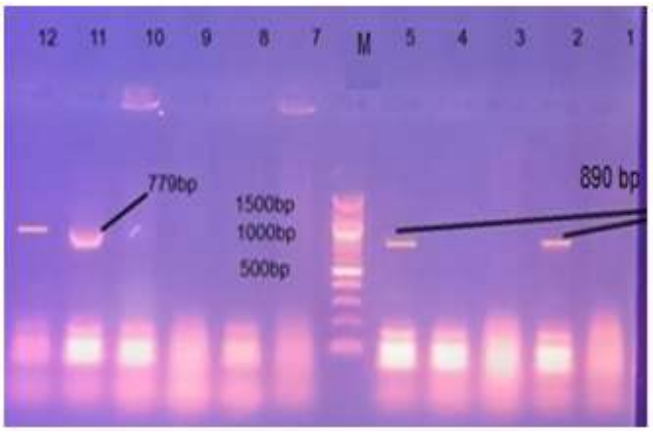

Figure 1: Agarose gel electrophoresis of amplicon belong to*eaeA* (890bp),*vtx1*(732bp), *vtx2*(779bp). M: DNA ladder (100bp).Lin 2,5,12: amplicon of PEC that possess *eaeA*, Line 11:positive result for amplification of *vtx2* Lin 1,3,4,7,8,9,10:negative results for amplification of *eaeA,vtx1,vtx2.*

3.3. Detection of *stx1*and *stx2*genes:

The results of agarose gel electrophoresis for amplicon resulted from amplification of *stx1* and *stx2* in EPEC using diplex PCR technique showed that 6(12%) isolates were carried *stx2* and one (2%)isolate was carried *Stx2* gene by the appearing of amplicon with molecular weight 779 bp and 614 bp respectively(Figure 2).

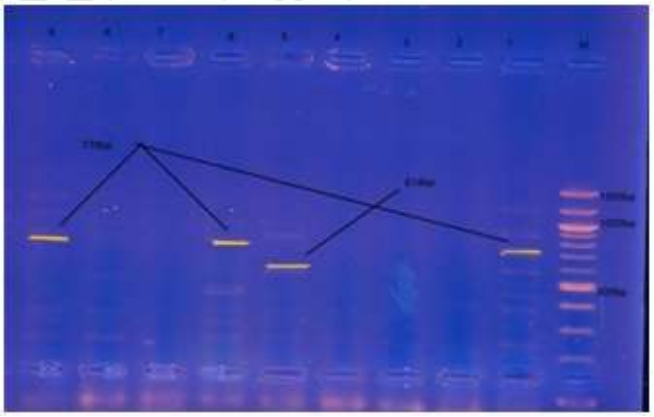

Figure 2: Agarose gel electrophoresis of ampliconbelong to*stx1* (614)bp and stx2(779)bp.Lin M: DNA ladder (100bp) Lin 1,6,9 : amplicon of PEC that possess *stx2*.Lin 5:positive results for amplification of *stx1*. Lin 2, 3, 4, 5, 7, 8: negative results for amplification of *stx1 and stx2.*

3.4. Detection of *pcvD*432 gene:

Figure (3) showed the results of agarose gel electrophoresis of amplicon resulted from the amplification of *pcvD*432, its revealed that 5 (10 %) isolates have *pcvD432* by the appearing of amplicon with molecular weight 630 bp.

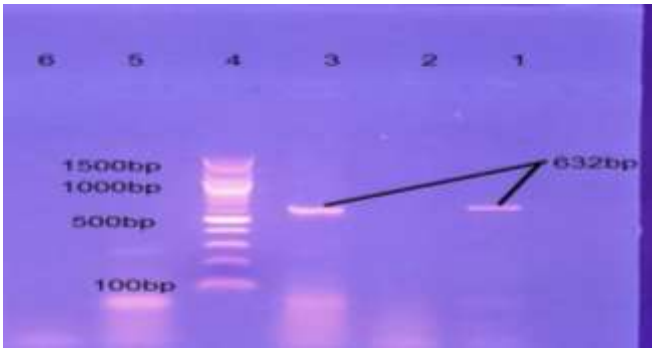

Figure 3: Agarose gel electrophoresis of ampliconbelong to *pcvD432*(630)bp.Lin M: DNA ladder (100bp) Lin 1,3: positive results for amplification of *pcvD432*. Lin 2,5,6:negative results for amplification of *pcvD432*.

4. Discussion

Pathogenic *E. coli*a Gram negative coccobacilli represented major causes of diarrhea disease in the world. The results of primary isolation on EMB referred to the ability of colonies to form metallic sheen colonies which may due to the ability of colonies to accumulate a precipitation that results from a chemical reaction between eosin and methylene blue around colonies which result in a metallic sheen[12],while a green and red colonies of PEC that appear on green and red chrom agar may due to the presence of chrom factor (one component of chrom agar) which responsible for inhibition of all other type of bacteria and also gave a specific color that correlated with type of chrom agar.

*eaeA*plays an important role in the intimate adhesion to the intestinal epithelial cells and in producing attaching and effacing *(A/E)* lesions, The first steps in colonization of EPEC intestinal epithelial cell involved adhesion of bacterial cell to the host surface and formation of lesion aprocess that mediated by producing of adhesion protein which encoded by *eaeA* gene.[14]

Several studies showed a high distribution of *eaeA* gene among EPEC. [15]Found that 39/225 isolates were possess eaeA gene and one isolates of them were belong to EPEC serotype O125. [16]found that all EPEC isolates have *eaeA*, while in Iran [17] showed that 45/111 (40.5%) isolates were possess *eaeA*. On the other hand, several researches confirmed a low percentage of distribution of *eaeA* among EPEC that isolated from patient suffering from diarrhea. [18]showed that all EPEC strains isolated from children with diarrhea did not react with *eaeA*specific primer in Japan, while[19] found none of 77 serological identified EPEC strain have *eaeA* .

Verotoxin 1 and verotoxin2 were produce from enterohemorrhagic *E.coli* group(EHEC) and cause bloody diarrhea which lead to dysentery. Only little of EHEC strain cause illness in children and considered to be a major cause of food borne illness. Also these strains often cause gastroenteritis, enterocolitis and bloody diarrhea and sometime cause the sever complication of hemolytic–uremic syndrome(HUS).[20]*Vtx1* and *Vtx2* located on chromosomeon Pathogenicity Island and play an important role in infected with bloody diarrhea. Many studies referred to awide distribution of EHEC which possessed vtx1 and vtx2 that correlated with bloody diarrhea in children.[10,21]

*stx1*and *stx2* a genes encoding shiga like toxin were important virulence factor which correlated with bloody diarrhea and it possess by EHEC.[22] showed that shiga-like toxin was very similar to shigelledysentria type 1 and the strains that possessed only stx1 and stx2 usually known as STEC. Enterohemorrhagic*E. coli* producing Shiga like toxin previously named as verotoxin-producing *E. coli* are responsible for bloody diarrhea, haemorrhagic colitis which may be complicated by hemolytic uremic syndrome (HUS) with sometimes severe neurological symptoms.[23] It was known that the *stx2* toxins which resulted in HUS in human was more frequently than *stx1*.

The current study showed that one isolates of PEC may be represented as EHEC due to the presence of *stx2* and *eaeA*. Many studies improved that the expression of *stx1* and *stx2* in human isolates may lead to kidney failure.[24] Many factors may play important role in distribution of STEC which lead to bloody diarrhea in children as well as in patient such as consumption of raw milk.[25] Showed that 10%of systemic STEC infection was correlated with consumption of raw milk while [26] recorded that infection with STEC O157 was result from consumption of pasteurization milk. On the other hand, zoonotic health hazard might be associated with contamination of environment.Interaction between several management and environmental factors could end up with diarrhea. Infectious diarrhea in calves is commonly associated with DEC. Persistence of the problem of NCD might associated with the poor environmental hygiene, failure to clearly understand the ecology of disease and biased epidemiological data.[27] Although medications, and herd management have been implemented to minimize the economic loss, the NCD economic impact is still significant [21]. *PcvD432* a gene encoded of EAEC has been considered as one of the bacterial causative agent of acute and persistent diarrhea in children in different studies.[28]Many researches that investigate the distribution of EAEC using PCR technique depending on *pcvD432* revealed avariable percentage of its occurrence[10,29] Using of *pcvD432* for identification of EAEC has more sensitivity and specificity.[30]Also many studies demonstrated a better correlation between *pcvD432* and clinical finding that referred to the presence of EAEC (symptoms of disease). [31] The results of the current study referred to a high distribution of *pcvD432* among *E.coli*whichmay due to:i-These strains may belong to EAEC (atravellorE.coli) which may due to a history of travel outside of AL-Najaf city (a data not in proved).[32]Founded that the percentage of patients suffering from diarrhea who had a history of travel was higher than patients without a history of travels. ii-Distribution of EAEC was usually associated with age group.[33]And[34] observed that the children younger than 5 years old significantly more affected with EHEC.iii-Transferring of *pcvD432* by one of genetic transferring mechanism to EPEC, and this suggestion was improved by the fact that the results of current study showed that 5 isolates were possess both *eaeA* and *pcvD432*. Many studies confirmed the role of EPEC as a major causative agent of diarrhea in children harboring EAEC.[10, 30, 31]

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