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Original Article

Prevalence of Enterohaemorrhagic and Enteroaggregative *E. coli* among Children with Diarrhea in Najaf, Iraq

Ali Mandeel, H[1], Shahid Jassim, A[2], Kadhum Naeem, A[3]*

1. Ministry of Education, General Directorate of Education in Al-Qadisiyah, Al-Qadisiyah, Iraq
2. Ministry of Education, General Directorate of Education in Najaf, Najaf, Iraq
3. Department of Biology, College of Education for Girls, University of Kufa, Kufa, Iraq

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Corresponding Author: ahlam.albdairi@uokufa.edu.iq

Abstract

Diarrhea is one of the leading causes of morbidity and mortality among children under 5 years old in developing countries. The classification of diarrheagenic *E. coli* (DEC) strains among children with diarrhea is still receiving low attention. The present study aimed at determining the prevalence of enterohaemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) among children under 5 years old suffering from acute diarrhea. Stool samples (n=100) were collected from children under 5 years old suffering from acute diarrhea for the molecular detection of EHEC (using *stx1* and *stx2*) and EAEC (using *aat*) by polymerase chain reaction technique. The results showed a high percentage of isolation of EHEC from stool samples, in compression to EAEC; accordingly, among 75 identified DEC isolates, 15.9% belonged to EHEC, while 5.3% belonged to EAEC. Among EHEC, *stx1* was highly prevalent among isolates (9.3%), in comparison to *stx2* (6.6%). A high frequency of EHEC was detected in males in the age group of 7-12 months, whereas EAEC was found in females in the age group of 13-19 months. In conclusion, EHEC and EAEC were associated with bloody and watery diarrhea among children under 5 years old. Genes associated with virulence factors (i.e., *stx1*, *stx2*, and *aat*) could be used as genetic markers for the detection of EHAE and EAEC.

Keywords: *aat*, Diarrhea, EAEC, EHEC, PCR, *stx*

1. Introduction

Diarrheagenic *Escherichia coli* (DEC) is an important intestinal pathogen causing a wide variety of gastrointestinal diseases, particularly among children in developing countries. There are several pathogenic strains associated with diarrhea, namely enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli*, and diffusely adherent *E. coli* (1).

Enterohaemorrhagic *E. coli* is a group of pathogenic *E. coli* that can cause diarrhea or hemorrhagic colitis in humans which occasionally progresses to hemolytic uremic syndrome (HUS), an important cause of acute renal failure in children and morbidity and mortality in adults (1). Enterohaemorrhagic *E. coli* is closely related to EPEC, with which it shares similar trends in terms of the disruption of intestinal barrier function. The main pathogenic feature of EHEC is the production of cytotoxins called Shiga toxins (i.e., *Stx1* and *Stx2*; closely related to the Shiga toxin of *Shigella dysenteriae*). Stx2 is a more virulent toxin and its action is characterized by inhibiting protein synthesis in colon epithelial cells, resulting in cell death (2). Such mucosal damage in the colon is similar to what is observed in *Clostridioides difficile* infections. The toxin is then absorbed from the gut into the circulation where it further damages vascular endothelial cells in organs,

such as the colon and kidneys, leading to the aggravation of hemorrhagic colitis (3).

Enteroaggregative *Escherichia coli* is defined by its distinctive aggregative or "stacked-brick" pattern of adherence to cultured human epithelial cells. This pathotype, termed "aggregative adherence" (AA), is associated with specific fimbriae encoded by plasmids (4). This pathotype is associated with (i) acute, prolonged, and persistent pediatric diarrhea in developing countries, most prominently in children under 2 years old and malnourished children; (ii) acute and persistent diarrhea in HIV-infected adults and children; and (iii) acute traveler's diarrhea (4). It has been reported that EAEC is the 2nd most common cause of traveler's diarrhea after ETEC (4).

Person-to-person transmission of EHEC and enteroaggregative hemorrhagic *E. coli* can contribute to disease spread during outbreaks, via the fecal-oral route. Enterohaemorrhagic *E. coli* can spread among animals by direct contact or via water troughs, shared feed, contaminated pastures, or other environmental sources (5).

The present research aimed at determining the distribution of EHEC and EAEC among children under 5 years old suffering from acute diarrhea.

2. Materials and Methods

2.1. Samples Collection and Bacterial Isolation

Stool samples (n=100) were collected from children under 5 years old suffering from acute diarrhea admitted to the Al-Zahraa Teaching Hospital, Najaf, Iraq. The primary isolation of diarrheagenic *E. coli* was carried out by culturing all samples on MacConkey agar and Eosin Methylene Blue (EMB). The confirmation of bacterial isolates identification was obtained by a biochemical test as previously described (6).

2.2. Polymerase Chain Reaction

The boiling method, which was described previously by Keir (10), was followed for the extraction of bacterial genomic DNA. The concentration and purity of extracted DNA were measured by a DNA/RNA spectrophotometer (Bio-Droop).

All primers (Table 1) were supplemented by Macrogen (Korea) and dissolved in ddH_2O following the recommended instructions, and then diluted to prepare a 10pmol/µl working solution. Polymerase chain reaction (PCR) mixture was prepared by adding 1.5 µl of each forward and reverse primer and 3 µl of DNA template to the PCR reaction mixture (PCR-Premix kit-i-Taq) and completed at the volume of 20 µl by adding PCR grade water. The PCR conditions for the amplification of Stx1 were: at 95°C for 2 min, 30 cycles at 95°C for 30 s, at 60°C for 30 s, at 72°C for 50 s, and a final extension at 72°C for 5 min. On the other hand, PCR conditions the for amplification of Stx2 were: at 94°C for 5 min, 30 cycles at 94°C for 45 s, at 58°C for 45 s, at 72°C for 45 s, and a final extension at 72°C for 7 min. The PCR protocol followed for the amplification of *aat* was: at 95°C for 2 min, 30 cycles at 95°C for 30 s, at 59.9°C for 30 s, at 72°C for 30 s, and a final extension at 72°C for 5 min. The amplicons were separated in 2% (wt/vol) agarose gel, stained with ethidium bromide (0.5 µg/µl) at 70 vol for 60 min, and visualized using a gel documentation system (Biometra, Germany).

Table 1. Oligo-synthesis nucleotide sequences

Patho- and serotypes	Primers	Sequences 5' → 3'	Amplicon size (bp)	References
EHEC	stx-1 F	AGTCGTACGGGGATGCAGATAAAT	418	(7)
	stx-1 R	CCGGACACATAGAAGGAAACTCAT		
	stx-2 F	GGCACTGTCTGAAACTGCCC	255	
	stx-2 R	TCGCCAGTTATCTGACATTCTG		
EAEC	aat F	AGGTTTGATAATGATGTCCTTGAGGA	152	
	aat R	TCAGCTAATAATGTATAGAAATCCGCTGTT		

EHEC: Enterohaemorrhagic *E. coli*; EAEC: Enteroaggregative *E. coli*

3. Results and Discussion

Considering the morphological characterization of bacterial colonies on EMB agar and MacConkey agar and the biochemical test used to confirm the identification of bacterial isolates, the results showed that only 75 isolates were identified as *E. coli*. The findings of agarose gel electrophoresis of amplicon resulted from the amplification of *stx1* and *stx2* revealed that 7 and 5 isolates possessed *stx1* and *stx2*, respectively, while *aat* was detected in only 4 isolates. The frequency of EHEC was higher (15.9%) than that of EAEC (5.3%), as shown in table 2. The highest frequencies of EHEC in males were found in the age groups of 7-12 and 20-26 months in descending order, accounting for the prevalence rates of 50% and 33.3%, respectively. Likewise, the highest frequencies of EHEC in females were found in the age groups 7-12 and 20-26 months by decreasing order, which were 33.3% in each group (Table 3). On the other hand, EAEC was detected in male subjects only at the age group of 7-12 months, while the highest frequency of EAEC was found in female cases at the age group of 13-19 months, followed by the age group 20-26 months (66.6% and 33.3%, respectively).

Table 2. Frequency of EHEC and EAEC among patients with diarrhea

Pathotype	Genes	Number	*Frequency of isolates %
EHEC	*stx1*	7	9.3
	stx2	5	6.6
EAEC	*aat*	4	5.3
Total		16	21.3

EHEC: Enterohaemorrhagic *E. coli*; EAEC: Enteroaggregative *E. coli*
*Frequency %: Number/total number of *E.coli*-positive isolates identified in the samples (n=75)

Table 3. Distribution of EHEC and EAEC among different age groups of both genders

Age groups	Total number of DEC	Infection n (%)			
		Male		Female	
		EHEC	EAEC	EHEC	EAEC
1-6months	18	1(16.6)	0	1(16.6)	0 (0.0)
7-12months	33	3(50)	1(100)	2(33.3)	0 (0.0)
13-19months	14	0(0)	0	1(16.6)	2(66.6)
20-26months	10	2(33.3)	0	2(33.3)	1(33.3)
Total	75	6	1	6	3

DEC: Diarrheagenic *E. coli*; EHEC: Enterohaemorrhagic *E. coli*; EAEC: Enteroaggregative *E. coli*

Enterohaemorrhagic *E. coli* is a human pathogen responsible for outbreaks of bloody diarrhea and HUS worldwide. The main virulence determinants of EHEC pathogenesis are Shiga toxins (Stx). The results of the present study showed a high frequency of EHEC among children with diarrhea which might be due to numerous reasons. Among the most important reasons are the usage of contaminated food and water and the consumption of raw and non-pasteurized milk, which may act as a vehicle for the transmission of bacteria and have been involved in major EHEC outbreaks (8, 9). The results of the current study indicated a wider distribution of *stx1* (9.3%) than *stx2* among EHEC isolates (Table 2). Variable percentages of *stx1* and *stx2* distribution among EHEC were reported in numerous previous studies (7, 10-12).

Another subgroup of DEC that has received increasing attention during the past decade as a cause of watery diarrhea is EAEC (13). The results of the present study showed a low frequency of EAEC among children with severe diarrhea (Table 2), while the findings of previous studies referred to a high percentage of isolation of EAEC (14, 15). This discrepancy in the percentage of isolation may be due to the virulence marker used for the identification of isolates in previous studies. Although the *astA* gene was considered characteristic of EAEC strains (16), the *aat* gene, an anti-aggregation protein transporter gene (previously called CVD432 or AA probe), has remained the most common target in molecular assays for the detection of EAEC (13, 17). Based on the results of the current study, EHEC and EAEC were associated with bloody and watery diarrhea among children under 5 years old. Genes associated with virulence factors (i.e., *stx1*, *stx2*, and *aat*) could be used as genetic markers for the detection of EHAE and EAEC.

Authors' Contribution

Study concept and design: A. S. J.
Acquisition of data: H. A. M.

Analysis and interpretation of data: A. K.N.
Drafting of the manuscript: A. K. N.
Critical revision of the manuscript for important intellectual content: H. A. M.
Statistical analysis: A. K. N.
Administrative, technical, and material support: A. K. N.

Ethics

The study protocol was approved by the ethics board of the University of Kufa, Kufa, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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