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Identification of virulence factors genies (eaeA) and (bpfA) of Enteropathogenic Escherichia coli strains isolated from children with diarrhea in Kerbala city

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Abstract

This study aimed at assessing some of the microbial agents associated with childhood diarrhea in Karbala city and determine the prevalence of eaeA and bfpA genes in Enteropathogenic Escherichia coli (EPEC) are taken from stool specimens of children. One hundred fifty from diarrhea stool samples collected and cultured for isolation and identification morphological characterization, biochemical methods, and API 20E. The PCR technique was used to detect 25 microbial isolates of Diarrheagenic E.coli (DGEC). The present results showed that DGEC the cause of diarrhea, is not a single species, but rather a group of strains or pathotypes that differ in their pathogenic mechanisms and the symptoms they produce. The number of isolates 21 EPEC isolates, representing 84% of the isolates, respectively. Nine ETEC isolates, representing 36% of the isolates, recorded positive bacterial culture growth for EAEC only in normal children (16%).The number of EHEC isolates was 3 representing 12% respectively. The EIEC isolates did not show significant growth. Enteropathogenic Escherichia coli (EPEC) was the most frequent pathotype in this study.The results showed the PCR technique documented 84% isolates of (EPEC) harbored) bfpA) gene 16 isolates representing 64% of the total isolates and (eaeA)gene representing 64% also .

Keywords: Diarrheagenic E.coli (DGEC), Enteropathogenic Escherichia coli (EPEC), eaeA gene, bfpA gene, Polymerase Chain Reaction (PCR), Diarrhea, Children, Kerbala, Iraq.

1. Introduction

Diarrheagenic E.coli (DGEC) groups is one of the leading causes of gastrointestinal disorders worldwide and an important public health challenge and the most frequent of the numerous enteropathogenic organisms (Deneke Wolde., et al.,2024). Diarrhea-related mortality in children has been of increasing public concerns recent years. (DGEC) causes by microbial are genetically the most versatile and are the source of many phage-mediated genes and plasmid. while its organisms are typically nonpathogens but a part of the normal microflora of the intestinal tract, certain subsets of this microbial species have acquired genes that enable them to cause extraintestinal or intestinal disease. (Borodovich T., et al.,2024).

Diarrheagenic E.coli Several distinct divided into five main groups basis on clinical features, epidemiological and virulence determinants.(Homsi Ahmad.et al., 2023). Detection of Some which include the enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), enteroinvasive (EIEC) and enterohaemorrhagic E. coli (EHEC) for detection the virulence factors eaeA for the structural genes of intimin of EPEC bfpA for the structural gene of the bundle forming pilus of EPEC, hlyA for the plasmid encoded enterohemolysin of EHEC, elt and stla for the enterotoxins of ETEC, ial for the invasion associated locus of the invasion plasmid found in EIEC, CVD432 for the nucleotide sequence of the EcoR1 Pst DNA fragment of EAEC.The primers were selected on the basis of similar studies done earlier.(Sagolshem Priyokumar Singh, et al., 2022) by monoplex PCR. The multiplex PCR assays.

EPEC are significant microbial etiologies The term (EPEC) used in 1995 by Neter et al.,9 to explain a number of E. coli strains epidemiologically related to a series of outbreaks of infantile diarrhea in the 1940s and 1950s.(Ali Harb , et al., 2020, Al-Khafaji JKT, et al., 2020). EPEC pathogenicity is the formation of attaching and effacing (A/E) lesions on intestinal mucosa)Massiel Cepeda-Molero, et al .,2020).

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EPEC virulence encoded factors., encoded by the eaeA gene (E. coli attaching and effacing) located within the Locus of Enterocyte Effacement (LEE) pathogenicity island (Maniha, Syeda Muntaka, et al,.2020). Intimin An outer membrane adhesin essential for intimate attachment and A/E lesion formation. Another gene encodes bfpA gene for Bundle-Forming Pilus (BFP) EPEC Adherence Factor (EAF) mediating initial bacterial aggregation and localized adherence to epithelial cells, the major pilin subunit and is a key molecular marker for BFP (Oh JY , et al., 2024; Kaewsaeng et al., 2022). the presence (bfpA) EPEC lead to divided Adherence Factor (EAF) plasmid consider encoded by the bfp operon on the large EPEC to Typical EPEC (tEPEC)consiste eaeA , bfpA and Atypical EPEC (aEPEC) eaeA , bfpA- as a major cause of pathogenicity endemic diarrhea (Ledwaba SE, et al,.2022) The data on EPEC frequency as global and regional studies pathotype distribution associated with virulence genes still in limited in many Iraqi cities, including Kerbala.This study Determining the prevalence of E. coli in diarrheic children in Kerbala. Investigating the prevalence of key virulence genes (eaeA and bfpA) among isolates using PCR.

2. Materials and Methods

2.1. Sample collection: collected 150 of stool samples of children with diarrhea from in and out patients in Karbala Teaching Hospital for Children. collected stool specimens in sterile containers and Take a small amount of the specimen and place it on a microscope slide. If the stool specimen is still solid, add a drop or two of saline to the specimen and mix cultured the samples.

2.2. Isolation and Identification of E. coli: A total of 150 stool specimens from units diagnosed, cultered the Samples by streakin onto differential MacConkey agar and selective Eosin Methylene Blue agar - EMB) media. incubated Plates at 37°C for 18-24 hours. The colony appears Lactose-fermenting colonies (pink on MacConkey, metallic sheen on EMB) were selected. Presumptive E. coli isolates were confirmed biochemically using standard tests: Oxidase (-), fermentation profiles (lactose, glucose, sucrose), Indole, Methyl Red, Voges-Proskauer, Citrate utilization (Simmons), motility (SIM/MIU), and Urease (Mazumder R, et al,. 2022). to primarily identify bacterial isolates. Then identification 25 isolates were genetically engineered by molecular way by targeting a segment of the 16S rRNA gene with specific primers Table (1).

2.3. DNA extraction: The bacteria DNA was extracted using Easy pure by (IDT Coralville, USA). (Hegde et al., 2012). and the concentration and purity of extracted DNA were determined by nanodrop . The extracted DNA was kept at -20 until used.

2.4. Polymerase Chain Reaction (PCR): PCR was performed to detect Diarrheagenic E.coli(DGEC) grupes as EPEC eaeA and bfpA , ETEC elt , Stla genes ,Ial EIEC, EAEC CVD432 , EHEC HlyA genes using specific primers used in molecular diagnosis of resistance genes in DGEC bacteria Table (2).(Abdelwahab GE, et al,.2022).

2.5. Statistical analysis: All statistical analyses were performed using SPSS software version (27). Frequencies and percentages were calculated. Chi-square or Fisher's exact test was used for comparisons ($p<0.05$ considered significant).

Table 1. The sequences of primers, annealing temperature, amplicon size

Primer name	5- sequence-3		Amplicon size (bp)	Annealing temperature
16SrRNA E.coli	R	wl-14667 TTCTGGATACCTAAC GCAATACCC	919	55°C/ 30 sec
	F	wl-3110 AGAGTTTGATCMTG GCTCAG		

Table 2. Sequence of primers used in the diagnosis of resistance genes (A Hegde.et al.,2012)

Primer name	5- sequence-3		Amplicon size (bp)	Annealing temperature
EPEC *EaeA*	F	TGATAAGCTGCAGTCGAATCC	229	58°C/1 min
	R	CTGAACCAGATCGTAACGGC		
bfpA	F	CACCGTTACCGCAGGTGTGA	450	58°C/1 min
	R	GTTGCCGCTTCAGCAGGAGT		
ETEC *elt Stla*	F	CTCTATGTGCACACGGAGC	322	58°C/1 min
	R	CCATACTGATTGCCGCAAT		
	F	TCTTTCCCCTCTTTTAGTCAG TC	170	57°C/1 min
	R	CCGCACAGGCAGGATTAC		
EIEC *Ial*	F	CTGGTAGGTATGGTGAGG	320	~55°C/1 min
	R	CCAGGCCAACAATTATTTCC		
EAEC *VD432*	F	CTGGCGAAAGACTGTATCAT	630	55 °C / 1 min
	R	CAATGTATAGAAATCCGCTGT T		
EHEC *HlyA*	F	GCATCATCAAGCGTACGTTCC	534	57°C/ / 1 min
	R	AATGAGCCAAGCTGGTTAAG CT		

Results and Discussion

The results of the present study showed that Out of the 150 stool specimens took from children with diarrhea 25 isolates of DGEC from children aged from two months to 14 years of both sexes at Teaching Hospital for Children, Karbala. PCR assays showed 100% specificity in identifying 25 isolates by molecular way by targeting a segment of the 16S rRNA gene with specific primers Figure (1).

The current study showed that DGEC Escherichia coli, the cause of diarrhea, is not a single species, but rather a group of strains or pathotypes that differ in their pathogenic mechanisms and the symptoms they produce.

The number of isolates from both groups was 21 EPEC isolates, representing 84% of the isolates, respectively 9 ETEC isolates, representing 36% of the isolates, recorded positive bacterial culture growth for EAEC only in normal children (16%).

The number of EHEC isolates was 3 representing 12%, respectively. The EIEC isolates did not show significant growth E.coli isolated from children with diarrhea was subjected to the multiplex PCR assays described below to detect the presence of DGEC group. Table (3)Figure (2-6).

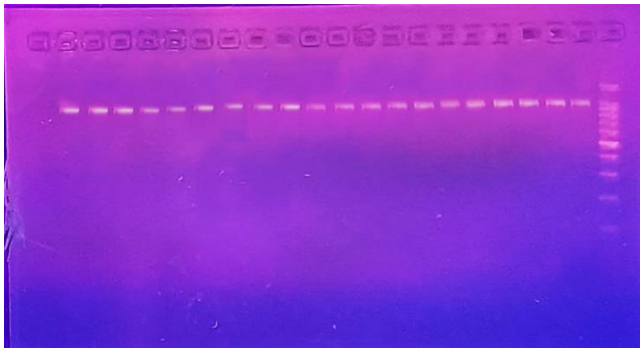

Figure 1. Identity of E. coli was confirmed by targeting a particular region of the 16S rRNA gene using conventional PCR.electrophoresed in agarose gel (1%) at a potential difference of 80 volts for an hour and a half after staining the gel with ethidium bromide and exposing it to ultraviolet light. The gene bands,The 16S rRNA gene bands in all the indicator isolates weighed 919

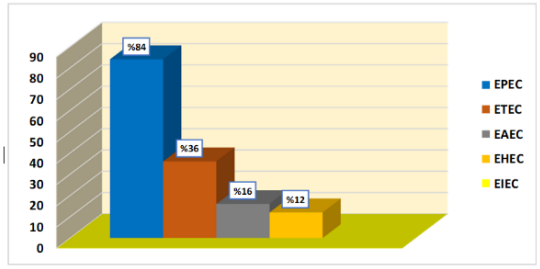

Figure 2. Percentage distribution of bacterial culture strains.

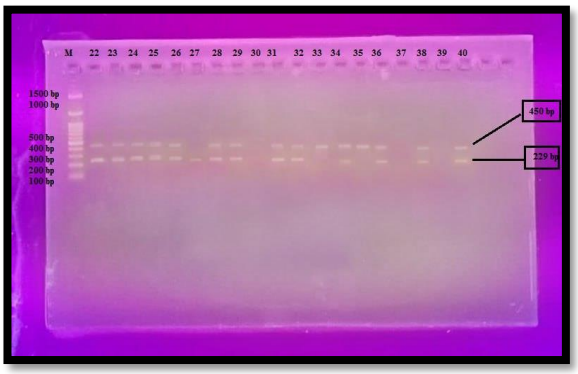

Figure 3. Electrophoresis of 1.5% agarose gel at 75 V for the smartwatch for the genes (eaeA) and (bpfA) using the EPEC multiplex polymerase chain reaction (PCR) technique after staining the gel with broad-spectrum ethidium bromide (BBR) for UV light.

- The eaeA gene appeared in 14 isolates (30, 33, 34, 37, 39), with a weight of 229 bp.

- The bpfA gene appeared in 15 isolates (27, 30, 37, 39), with a weight of 450 bp.

- The scale (M) represents the effective path for its weight (100-1500 bp).

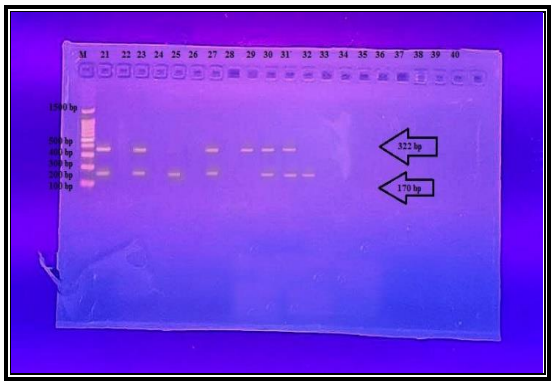

Figure 4. Electrophoresis of 1.5% agarose gel and 75% legumes for the smart watch for two genes (elt) and (Stla) using the multiplex polymerase chain reaction technique and attributed to the ETEC bacteria.

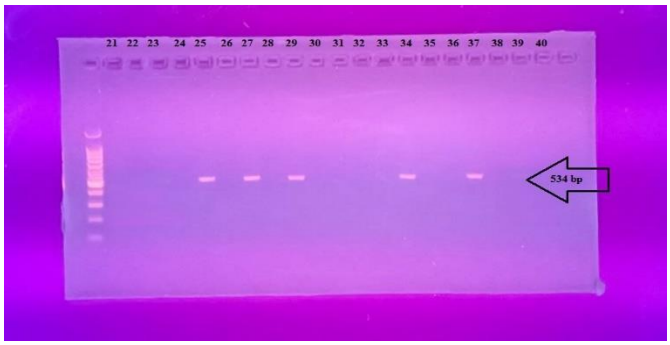

Figure 5. Electrophoresis of 1.5% agarose gel and 75% legumes for the smart watch for (hlyA) gene using the monoplex polymerase chain reaction technique and attributed to the EAEC bacteria.

Figure 6. Electrophoresis of 1.5% agarose gel and 75% legumes for the smart watch for (CVD43) using the monoplex polymerase chain reaction technique and attributed to the EAEC bacteria.

The results of the analysis of this type of strains carrying the bpfA gene were distributed: 16 isolates were isolated, representing 64% of the total isolates. A similar study by Cabrera-Sosa, L., & Ochoa (2020) indicated that the presence of EPEC was associated with an increased risk of acute diarrhea in children. Research suggests that pathogenic E. coli carrying the EPEC (bpfA) gene may be associated with the development of certain clinical symptoms, warranting further research to understand its role in autism and diarrhea. The bpfA gene is important for protein synthesis, as this protein is part of a structure called the bundle-forming pilus (BFP). The bpfA mutation indicates a version of the bacteria that contains a modified or missing bpfA gene. EPEC strains carrying the eaeA gene were also found 16 isolates carrying the same gene were found in children, representing 64% of children. EPEC strains carrying the eaeA gene are known to cause a specific type of diarrhea characterized by watery stools, often seen in infants and young children. A 1993 study by Donnenberg MS and others demonstrated that the eaeA gene in EPEC is essential for its close association with intestinal epithelial cells in animal models. A deletion/insertion mutation was introduced into the EPEC O127:H6 strain, and the results showed that the modified strain was unable to tightly associate with intestinal epithelial cells, highlighting the importance of the eaeA gene in causing infection. The study also demonstrated that the eaeA gene is an important virulence factor associated with enteropathogenic Escherichia coli (EPEC). It encodes a protein known as intimin, which plays a crucial role in the ability of bacteria to adhere to and colonize the intestinal lining. Intimin facilitates the formation of a strong bond between bacteria and host cells, which is critical for infection. EPEC strains that possess the eaeA gene are known to cause a specific type of diarrhea characterized by watery stools, often seen in infants and young children. The aforementioned differences in the distribution of diagnostic Escherichia coli genes isolated from patients with diarrheal infection were significant at the P≤0.05 level. It is believed that the antagonistic ability of one strain to prevail over the emergence of the virus is responsible. A 2021 study indicated that diarrheal infection is the second most common cause of death in children under the age of five in Iraq. This study showed that 21.3% of children under the age of six suffered from diarrheal infection. The results of a 2021 study in the Iraqi Journal of Medical Sciences also showed that 15% of diarrheal infections in children under the age of five were caused by EPEC. Another study conducted in 2022 in the Basra Journal of Medical Sciences found that 12% of childhood diarrhea cases in Basra Governorate were associated with EPEC.

In Saudi Arabia, a study published in 2023 in the Saudi Journal of Medical Research showed that 10% of childhood diarrhea cases in the Eastern Province were due to EPEC. In Jordan, a 2022 study in the Jordanian Journal of Medical Sciences showed that 8% of childhood diarrhea cases were associated with EPEC (Alghadeer S, et al,.2021).

The drug susceptibility of 25 DGEC isolates infected with diarrheal infection was tested by disc diffusion method to seven types of antibiotics used in the treatment of diarrheal infection according to the recommendations of the World Health Organization

(WHO, 2020). Antibiotic sensitivity testing was performed for Escherichia coli isolates against seven antibiotics, including Co-Trimoxazole (COT), Amoxyclav (AMC), Trimethoprim (TR), Levofloxacin (LE), Ceftriaxone (CTR), Meropenem (MRP), and Cefoxitin (CX). The isolates analyzed showed different sensitivity patterns, and most of the DGEC isolates were multidrug resistant. Table (4) shows the numbers and percentages of antibiotic sensitivity of the isolates. The tested isolates showed high resistance to most antibiotics, especially to Ceftriaxone (CTR), where the number of isolates reached 19 isolates, representing a percentage of 76%. The statistical results of the Chi-square test showed the presence of statistically significant differences at the probability level of P≤ 0.05). A study in India in 2020 showed results identical to the current study, as the resistance of E. coli strains to the antibiotic Cefoxitin (CX) reached 100%. Another study also conducted in India in 2017 showed similar results, as the percentage of E. coli resistance reached 93%. Antibiotic resistance was also observed in 89-93% of E. coli isolates from patients with diarrheal infection. Results conducted in Bangladesh in 2021 showed that resistance was detected at a rate of (91%) among E. coli strains isolated from stool samples of children with diarrhea, mainly due to the production of the enzyme AmpC β-lactamase.

The results of studies conducted in Egypt, Pakistan, and India in 2020, 2019, and 2017, respectively, were similar to the results of the current study on E. coli resistance to the antibiotic ceftriaxone, with rates of 89%, 88-92%, and 90% of bacterial isolates taken from children with diarrheal infections, respectively. This is related to the prevalence of isolates producing extended-spectrum beta-lactamase enzymes. Meanwhile, the results of the isolates showed 100% susceptibility patterns to the antibiotic meropenem (MRP), a carbapenem antibiotic. The statistical results of the chi-square test showed statistically significant differences at a probability level of P≤ 0.05. In a similar study conducted in India in 2022, the results of the E. coli susceptibility test to the antibiotic meropenem (MRP) were 92%. (Amin ET, et al 2018).

This antibiotic is effective in treating complicated infections such as gastroenteritis or bloodstream infections, demonstrating activity against multiple strains of Gram-negative bacteria (Yusuf E,et al.,2021) It is also used to treat a variety of bacterial infections, including those that produce beta-lactamase enzymes, specifically those caused by Escherichia coli (Husna A, et al., 2023). In some cases, using this antibiotic to treat diarrheal infections caused by E. coli may worsen the condition, especially in certain strains such as those that produce Shiga toxin (STEC) (Mühlen S, et al, 2020).

Table 4. Sensitivity of DGEC groups isolated from children with diarrheal infection

Type of antibiotic	Isolates		
	sensitive isolates	Moderately sensitive isolates	resistant isolates
Co-Trimoxazole (COT)	14(56)	0(0)	11(44)
Amoxyclav (AMC	5(20)	8(32)	12(48)
Trimethoprim (TR)	9(36)	1(4)	15(60)
Levofloxacin (LE)	12(44)	0(0)	13(52)
Ceftriaxone (CTR)	1(4)	5(20)	19(76)
Meropenem (MRP)	25(100)	0(0)	0(0)
Cefoxitin (CX)	14(56)	0(0)	11(44)
قيمة مربع كاي X^2	104.90		
قيمةP المحسوبة	<0.0001*		

Conclusion

DGEC strains contribute significantly to pediatric diarrhea in Kerbala City. The high prevalence of eaeA confirms the role of the attaching and effacing (A/E) lesion in pathogenesis. and bfpA These findings underscore the need for enhanced public health interventions and continuous surveillance of enteric pathogens in this region.

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